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DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

Annual Report

1975

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ARGONNE NATIONAL LABORATORY, ARGONNE, ILLINOIS

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ARGONNE NATIONAL LABORATORY
9700 South Cass Avenue
Argonne, Illinois 60439

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DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

ANNUAL REPORT
1975

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

Preceding Report
ANL-75-30, December 1974

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9. BIOPHYSICS

1. INTRODUCTION

Timothy E. O'Connor, Division Director

This report describes studies conducted during calendar year 1975, period when the Division of Biological and Medical Research was initiating its response to the challenge posed by the enlarged mission in the field of energy production of its principal sponsor, the U. S. Energy Research and Development Administration. The individual reports may therefore be read for their intrinsic interest, or as points of departure in the evolution of a more comprehensive research program in the toxicology of pollutants associated with both nuclear and nonnuclear modes of energy production. Some of the research highlights deserve particular comment.

The continuing Neutron and Gamma-Ray Toxicity Studies indicate that on single exposure of mice to fission neutrons, the neutron dose-response curve is nonlinear, with the life shortening effect decreasing from 3 to 4 day/rad to 1 day/rad, with increasing dose over the range of 20-240 rad. Thus linear extrapolations from high neutron doses to estimate life shortening at low doses would underestimate risk. The underestimation is even greater when the enhancement of life shortening that has been observed with fractionated neutron exposures is considered. These findings indicate the need for data on the effects of exposure to neutrons at doses lower than 20 rads.

Studies on Radiation Toxicity in Dogs have shown that on continuing exposure of beagle dogs to ^{60}Co gamma radiation, the morbidity responsible for life shortening is dose-rate dependent. Exposure rates in excess of 17 R/day destroy the blood-cell producing elements of bone marrow and cause death within 1 to 2 months. Exposure rates of 5-17 R/day produce a 50-75% incidence of anemia or myeloid leukemia. At exposure rates of 5 R/day or below, bone marrow function is essentially normal; from preliminary data, causes of death appear to be related to degenerative diseases and malignancies of other tissues. The capacity to produce myeloid leukemia in the beagle at intermediate gamma-radiation dosage would appear to be a unique resource for the study of the pathogenesis and therapy of canine myeloid leukemia as a model for human myeloid leukemia. In studies on the effects of gamma irradiation on the developing fetus, the observed results are also greatly dependent on dose rate. Interestingly, the beagle embryo and fetus can develop a functional hematopoietic system *de novo* at the same exposure rates, 10-17 R/day, that are just sublethal for continued bone marrow function in the adult. The major effect produced by continuous irradiation of the fetus is sterility and consequent reproductive failure in adult life, with the female gonad being more sensitive than the male. The female, exposed throughout fetal life at 5 R/day, develops anovular ovaries, accompanied by a highly abnormal profile of sex hormones in the circulating blood.

Studies on the effects of exposure to radiation of various qualities suggest that as the dose is decreased, the life shortening effects statistically vanish at doses that still produce morbidities that would be intolerable in man. Thus, future studies would appear to require increased emphasis on the definition and pathogenesis of these morbidities. Prudent extrapolation of such effects for risk estimates in man must be based on observations in more than one animal species.

The continuing findings in the Experimental Radiation Pathology and Oncology Program of association of oncornaviruses with spontaneous and radiation-induced tumors in rodents raise the question of the significance of such viruses. Are they essential for the development of radionuclide-induced osteogenic sarcomas in rodents and gamma irradiation-induced myeloid leukemias of the dog, or do they represent merely a concomitant event? Clearly the answers to these questions will be significant in our comprehension of the etiology and appropriate treatment of radiation-induced cancers in general. New experimental approaches based on nucleic acid hybridizations and competitive radioimmune assays would appear to provide promising technologies for the definitive elucidation of these questions.

Liposome encapsulation of cancer drugs appears to offer an improved method for delivery of these drugs to tumor sites, with decreased toxicity to the host through avoidance of drug uptake by normal proliferating tissues. Preclinical evaluation of the efficacy of a variety of liposome-cancer drug formulations is now receiving further intensive investigation.

Among the advances in several areas of the Biophysics Group, two topics deserve particular comment. Work of Dr. A. B. Edmundson and his associates in the Crystallography Section has yielded a wealth of structural information on the Mcg (Bence-Jones) and IgG1 myeloma proteins and attracted favorable comment in articles in Science and Nature. Studies by Dr. P. D. Klein and his associates resulted in further applications of stable isotopes as replacements of radiotracers in diagnostic procedures. A major advance of the past year was the development of simple, inexpensive $^{13}CO_2$ breath tests for routine clinical applications. ^{13}C-labeled substrates capable of releasing $^{13}CO_2$ on metabolism were synthesized, tested, and validated for use in testing regimens for hepatic microsomal function, bacterial overgrowth in the small intestine, and bile salt malabsorption. Dr. Klein organized the Second International Conference on Stable Isotopes held at Oak Brook, October 20-23, 1975, which provided a significant stimulus to the rapidly growing use of stable isotopes in a variety of diseases.

For nine months of the period during which the above studies were conducted Dr. J. F. Thomson was Acting Division Director. The Division owes Dr. Thomson its gratitude for his fine leadership. As the new Division Director, I deeply appreciate the continuing counsel afforded by Dr. Thomson in his capacity as Associate Division Director. I am also grateful for the fine cooperation given me by all the members of the staff, as together we seek optimal paths in meeting our responsibilities.

A few words on the perspectives for further advances may be appropriate. The Division has a commitment to the elucidation of the potential biological hazards associated with various options for energy production. The public acceptance of new energy technologies may well depend on demonstrated

occupational and environmental safety of these technologies. The energy needs of the nation are vital for maintenance of quality of life, national economic viability, and defense posture. The urgency of these needs requires that toxicology studies be conducted with a keen awareness of the scheduling of energy technology developments. This inevitably requires setting of priorities within available research budgets. At the same time our commitments to basic research, sound scholarship, and recruitment of first-rate minds to solve the problems of tomorrow must be maintained.

Decisions that seek the resolution of these priorities must surely be based on dialog at several levels. Within the Division, selection of new research topics and discarding of some of the old is being approached through intensive debate by the staff members. The Division is fortunate in its productive interactions with the Radiological and Environmental Research Division and the Environmental Impact Studies Division, under the overall leadership of Dr. W. K. Sinclair, Associate Laboratory Director, and also with other energy-technology divisions at Argonne National Laboratory. The Division also benefits from a continuous exchange of views with program leaders at ERDA headquarters and at other relevant government agencies. Of particular importance is a growing interaction with both The University of Chicago and the Argonne Universities Association. One manifestation of this interaction is the strong support tendered by both these institutions in organization of an AUA-ANL Bicentennial Conference on "Accomplishments and Challenges for American Life Sciences" to be held at Argonne National Laboratory, October 11-13, 1976. The conference will be an occasion of joy at past achievements and will also illustrate the role of the interaction of the individual investigator, private philanthropies, government agencies, and communications media in these achievements.

As a community of scholars and investigators dedicated to the solution of biological and medical problems associated with energy production, the Division will best meet its responsibilities through awareness of national needs and professional interaction with colleagues at the regional, national, and international levels.

ADMINISTRATIVE STAFF

Timothy E. O'Connor (Director)
John F. Thomson (Associate Director)
Ronald L. Breyne (Assistant Division Director)
J. William Harrison (Executive Assistant)
Robert J. Flynn (Assistant Director, Animal Facilities)
Marcia W. Rosenthal (Technical Editor)
Robert J. Robertson (Staff Assistant)

SUPPORT STAFF

Jeanne A. Blomquist (Programmer)
Thomas J. Doody (Glassblower)
William J. Eisler (Engineering Specialist)
Martin R. Kraimer (Computer Scientist)

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2. RADIATION TOXICITY IN DOGS

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GROUP LEADER'S OVERVIEW

William P. Norris, Group Leader

This study deals with the responses of beagle dogs to continuous irradiation of the whole body. The objectives are (1) to determine the relative importance of total gamma radiation dose and radiation dose rate to mammalian responses to ionizing radiations; (2) to provide data, under conditions of known dosimetry, that will assist in the interpretation of mammalian responses to internally deposited radionuclides--a situation that involves prolonged radiation exposures to portions of the body, but under conditions where meaningful estimates of radiation dose and dose rate are usually impossible; (3) to define, using an animal sufficiently large to make it possible, the details of the sequence of clinical changes that accompany continuous exposure to ionizing radiations and to assess the relative radiosensitivities of body organ and tissue systems under these conditions; (4) to make interspecies comparisons of responses to continuous irradiation using specifically defined end points, with the purpose of developing satisfactory models that will allow for prediction of the effects to be expected in exposed populations of humans; (5) to define the effects of continuous irradiation on the developing beagle fetus, and to make comparisons of these effects with those produced by tritiated water, administered throughout pregnancy under conditions that produce comparable doses of radiation to the fetus.

Three related, but separate, studies are in progress. In the first, young adult beagles of both sexes are placed in the γ-ray field, to be kept there 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 until they have accumulated predetermined amounts of total exposure ranging up to 4000 R, delivered at various daily exposure rates. They are then removed from the radiation field and kept for the rest of their lives to allow development of late effects attributable to radiation exposure. In the third study, pregnant beagles are irradiated, at one of four daily exposure rates, for all or part of their gestation periods, to produce an evaluation of the effects of continuous irradiation in the developing fetus.

All of these studies are done by arranging dogs at various distances from a calibrated ^{60}Co γ-ray source, where they are irradiated during 22 hours

of each day. The remaining 2 hours are used for animal care, maintenance, and clinical evaluation of the dogs.

The combined results demonstrate that the cellular and organ systems of the dog respond predictably, and in a differential manner, depending on exposure rate. Exposure rates in excess of 17 R/day destroy the blood-cell producing elements of bone marrow and cause death, therefore, within 1 to 2 months. Minimally sublethal exposure rates to bone marrow (5-17 R/day), however, produce a very high (50-75%) incidence of anemia or myeloid leukemia. Furthermore, at exposure rates of 5 R/day or below, bone marrow appears to function in an essentially normal fashion, and causes of death appear, from preliminary data, to be related to degenerative disease and malignancies of other tissues.

The effects of irradiation on the developing fetus are also shown to be greatly dependent on dose rate. Thus, the embryo and fetus can develop a functional hematopoietic system *de novo* at the same exposure rates (10-17 R/day) that are just sublethal for continued bone marrow function in the adult. Radiation-induced congenital abnormalities are not prominent under these conditions. The major effect produced by continuous irradiation of the fetus is sterility and consequent reproductive failure in adult life, with the female gonad being more sensitive than the male. The female fetus, exposed throughout fetal life at 5 R/day, develops ovaries that contain no ova in 100% of the fetuses so irradiated. This is a most sensitive indicator of radiation damage. Furthermore, such damage to the ovary results in a highly abnormal profile of hormones in the circulating blood. According to some current theories of the origins of cancer, this continuing endocrine imbalance should result in malignancies of related organs, such as the pituitary, thyroids, and ovary. These animals are under continuing observation to determine whether such expectations will prove true.

Our demonstrated abilities to produce leukemia in high incidence, as well as major hormonal imbalances in dogs irradiated *in utero*, provide highly important opportunities for future research in mechanisms of irradiation injury and in the mechanisms of induction of cancer.

We have recently completed our first comparisons of radiation-specific death rate in dogs and mice subjected to continuous, whole-body irradiation. (Data from mice were taken from earlier data published by Sacher and Grahn of ANL). The results demonstrate that the two species respond in a comparable manner, and support the model of irradiation-induced responses suggested by Sacher.

All of this work has indicated the advisability of acquiring data from dogs exposed continually at exposure rates below 5 R/day. A facility for this purpose is essentially complete, and is scheduled for operation in January, 1976. The exposure rates that will become available range down to 0.4 R/day--a dose rate that we expect to allow continued reproduction in the irradiated dogs. The objectives stated at the outset will be pursued at these lower exposure rates, with the ultimate extrapolation to effects in man remaining the prime target.

RADIATION TOXICITY IN DOGS 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)
*Pearson, Donald L. (Scientific Assistant)
Polk, Patrick H. (Scientific Assistant)
Seed, Thomas M. (Assistant Biologist)
Tolle, David V. (Scientific Associate)

PUBLICATIONS

Fritz, T. E., L. S. Lombard, S. A. Tyler, and W. P. Norris. Pathology and familial incidence of orchitis and its relation to thyroiditis in a closed beagle colony. Exp. Mol. Pathol., in press.

Norris, W. P., S. A. Tyler, and G. A. Sacher. An inter-species comparison of the response of mice and dogs to continuous ^{60}Co γ-irradiation. Proceedings of the IAEA International Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press.

Abstracts

Brennan, P. C., M. L. White, and T. E. Fritz. Cell-mediated immunity in beagle dogs: Effect of age, radiation and genetic background. Radiat. Res. 62, 604-605 (1975).

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*Deceased April 5, 1975.

ERDA RT-01-01
ANL 63100

RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. I. CHARACTERIZATION OF THE ANL BEAGLE

Thomas E. Fritz, Principal Investigator
Calvin M. Poole, Louise S. Lombard, William P. Norris, David V. Tolle, Donald E. Doyle, Lillian V. Kaspar, William G. Keenan,* Patrick H. Polk, and Patricia C. Brennan,† Participating Investigators*

OBJECTIVES

It is essential to characterize the normal, unirradiated, ANL beagle--in all stages of its development, growth, and aging processes--in order to interpret properly the findings from experiments dealing with the effects of exposure to ionizing radiations. This becomes increasingly important as the radiation doses and dose rates employed allow for survival approaching that of unirradiated controls.

EXPERIMENTAL METHODS

The ANL beagle colony is derived from a breeding nucleus of ∿ 40 animals. It has been a closed colony (i.e., no new stock has been introduced) since its inception 15 years ago. The main body of the colony remains outbred (inbreeding coefficients < 10%), but there has been a limited amount of deliberate inbreeding. As pups are weaned, every 15th animal is assigned the status of "colony control." This procedure has produced a longitudinal, random sample from the colony that has a relatively stable population of ∿ 100 dogs. These animals are followed by the same procedures used for the experimental groups--clinical, hematological, and biochemical assays during life, followed by a complete postmortem examination and histological work-up of tissues to determine the cause of death.

BACKGROUND AND PREVIOUS FINDINGS

The living population of beagles at ANL numbers ∿ 650. The total number, including stillborn pups, that is identified in our record system is 3224. Fifty "colony controls" have died. Four inherited abnormalities--cryptorchidism, epilepsy, lymphocytic thyroiditis, lymphocytic orchitis--have been identified among dogs in the colony. Selective breeding procedures have nearly eliminated cryptorchidism and epilepsy, while carriers of lymphocytic throiditis and orchitis are largely identified. None of these inherited traits are unique to the ANL colony.

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*Animal Facilities Staff.

†Neutron and Gamma-Ray Toxicity Group.

Computer programs are used to evaluate prospectively the inbreeding coefficients of pups from possible matings.

MAJOR NEW FINDINGS

Enough of the "colony controls" have died to allow preliminary estimates of the findings that may be expected. The mean life expectancy of the ANL beagle is projected to be 12.3 years. The dominant tumor types are those of epithelial origin. The most common sites of tumors in the control dogs have been mammary glands, genitals, digestive tract, skin, and endocrine glands. Although mammary tumors were the most numerous, very few ($\sim$ 15%) were malignant. [In contrast, tumors occurring in our cesium-137 injected dogs were of mesenchymal origin (connective tissue) and were of a type that appear in very low frequency in control dogs.] There have been no cases of myelogenous leukemia.

There are no sex differences in the hematologic parameters of beagles, except that thrombocytes consistently average 75-150 x 10^3 cells/mm^3 higher in females. Erythrocyte levels rise from 4-5 x 10^6 cells/mm^3 in 1-2 month old pups to a maximum value of 8 x 10^6 cells/mm^3 at 18 months of age. Thereafter, there is a slow decline with age and a compensatory increase in the hemoglobin content per cell.

There are statistically significant sex differences in serum concentrations of iron and cholesterol. There are also statistically significant linear regressions with age in serum levels of glutamic-pyruvic transaminase, glutamic oxalacetic transaminase, creatinine phosphokinase, iron, and cholesterol.

A fifth inherited characteristic was identified within a subgroup of the colony during the past year, and was traced back for several generations. This is a macrocytic, hypochromic anemia characterized by mean red cell corpuscular volumes ranging from 75 to 107 cubic microns (control range: 68 to 73) and mean corpuscular hemoglobin concentrations ranging from 22 to 28 g/dl (control range: 32.5 to 35.5). In peripheral blood there are increased numbers of nucleated red cells and reticulocytes, and misshapen leptocytic red cells. Bone marrow evaluation shows erythroid hyperplasia with some megaloblastic features. The serum iron level is normal, and the Coombs test for possible autoimmune hemolytic etiology is negative.

Cellular immune competence, as assayed by blast transformation of lymphocytes using concanavalin A and phytohemagglutinin, is not measurable at birth, begins to rise by 5 weeks of age, and approaches adult levels by 6 months of age.

SIGNIFICANCE

The detailed characterization of the normal, control population is essential. The identification of unusual inherited traits is potentially important in relating experimental findings to other outbred populations, including the human.

PROPOSED COURSE OF THE PROJECT

We will continue to observe and further characterize the ANL "colony control" dogs, using the methods described above.

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RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. II. RESPONSE OF YOUNG-ADULT BEAGLES TO CONTINUOUS EXPOSURE TO ^{60}Co γ-RAYS

William P. Norris, Principal Investigator
Thomas E. Fritz, Louise S. Lombard, Patrick H. Polk, David V. Tolle, Donald E. Doyle, Norbert D. Kretz, and Lillian V. Kaspar, Participating Investigators

OBJECTIVES

The objective is to characterize the responses of the young-adult beagle dog subjected, for duration of life, to continuous, whole-body, ^{60}Co γ-irradiation delivered at exposure rates ranging from those that are acutely lethal to those that allow for survival approaching a normal life-span. In addition to providing needed information on the relationships between exposure rate and the type of induced pathology, the mortality data are critically needed to extend our current interspecies comparisons of the responses of mice and dogs exposed in this manner. We anticipate that the data will allow important extensions of models of radiation-induced injury.

EXPERIMENTAL METHODS

Young-adult (13-month-old) beagle dogs of both sexes are housed in specially constructed fiberglass cages located at various distances from a calibrated ^{60}Co γ-ray source. They are irradiated during 22 hours of each day, with the remaining 2 hours being used for animal care, maintenance, and observation. Each dog is evaluated regularly by clinical examinations and hematology. Semen of the males is collected periodically for sperm counts. Biochemical assays of serum and bacteriologic assays are performed as required. All dogs becoming moribund or dying are necropsied. All major organs and tissues are weighed and specimens are collected for routine and special techniques of microscopic examination. Tissue and cell impressions are also collected for cytologic examination, particularly specimens from hemato-poietic tissues, tumors, and any exudates. Examinations are conducted on tissues and exudates to define the bacteriologic flora.

BACKGROUND AND PREVIOUS FINDINGS

This study covers the exposure-rate range from 5 to 300 R/day. The majority of the animals are dead, and the causes of their deaths have been determined. Major interest centers on the effects observed at 5-35 R/day where, with decreasing daily dose rate, there were systematic, consistent, highly dose-rate-dependent responses and causes of death limited primarily to the hematopoietic system, along with suggestions of reduced immune competence. Septicemia was the sole cause of death at exposure rates of 35 R/day and above, while anemia became prominent at 10-17 R/day. Septicemia did not occur at dose rates lower than 17 R/day. At 10 and 5 R/day, myelogenous leukemia became the most important cause of death, occuring in an incidence of $\sim$ 50%.

Until this year, 8 of 24 dogs irradiated at 5 R/day were still alive after 8 years of continuous exposure.

MAJOR NEW FINDINGS

During the past year, another six dogs have died at the 5 R/day exposure rate, leaving only two survivors in this experiment. The causes of death included three malignancies (osteogenic sarcoma, mammary carcinoma, ovarian carcinoma), one chronic anemia, one endometritis and secondary peritonitis, and one advanced case of degeneration of the liver. There have been no new cases of leukemia in the last 900 days and none in the last nine decedents exposed at 5 R/day, whereas the previous 11 dogs all died of leukemia. The osteosarcoma in the vertebrae of one of the dogs dying this year is the first to occur in any of our dogs.

A retrospective evaluation of the hematological data from the dogs exposed to either 5 or 10 R/day showed that the impending onset of myelogenous leukemia can be reliably predicted, well in advance of clinically obvious signs, from the steady rise in the thrombocyte count that occurs after the animal has accumulated a total exposure of $\sim$ 2000 R.

The radiation-specific excess mortality rate in continuously irradiated beagles was compared to that of similarly irradiated mice, using the data of Sacher and Grahn. As in the mouse, mortality in the beagle increases as the square of dose rate, with the dog being 2.5 times as radiosensitive as the mouse over the dose-rate range of 20 to 100 rad/day. At lower daily dose rates, the dog becomes increasingly more radioresistant as compared to the mouse.

SIGNIFICANCE

These studies have defined the effect of radiation exposure rate on the survival of dogs irradiated continuously to death. The results show that the hematopoietic system (bone marrow) is the most sensitive target at risk down to exposures as low as 5 R/day. There is a progressive lengthening of survival time and a progression of causes of death--namely, septicemia, anemia, and leukemia--that are related to the exposure rates. At exposure rates of 35 R/day and above, the bone marrow ceases to function and dogs die acutely of septicemia due to a deficiency of leukocytes. At exposure rates of

17 and 10 R/day, where damage occurs more slowly, the marrow of some dogs ceases to function in the production of erythrocytes, resulting in anemia, while in other dogs the marrow proliferates in an abnormal manner, resulting in leukemia. At exposures of 5 R/day, leukemia occurs in the earlier half of the decedents whereas among those dogs that survive longer, death is due to other malignancies and degenerative and inflammatory processes.

A malignancy of the bone (osteosarcoma) in one of these dogs is singularly significant because such tumors commonly occur after ingestion of radioactive isotopes, such as ^{226}Ra or ^{90}Sr, that selectively deposit in bone.

Responses at various exposure rates represent a pattern of competing risks, and it is clear that hematopoietic injury will obscure the appearance of osteogenic sarcoma. The occurrence of this tumor, not found in untreated beagle dogs, in our γ-irradiated beagle represents, therefore, a significant estimate of the maximum dose rate (5 R/day) expected to result in a significant incidence of bone tumors.

The data have identified several dose-rate-dependent, physiological end points (mentioned above) that are produced by ionizing radiations. These end points are expected to become useful in the further elaboration of models of radiation injury that use interspecies comparisons of responses to extrapolate to effects expected in human populations. The demonstration of the utility of such a model would benefit every area of toxicology.

In addition, these responses, developed under conditions of known dosimetry, will assist in the interpretation of mammalian responses to internally deposited radionuclides--a situation that involves prolonged radiation exposures to portions of the body, but under conditions where meaningful estimates of radiation dose and dose rate are usually impossible. An important extension of this example is toward the data derived from the intensively studied group of humans suffering from the effects of skeletally deposited ^{226}Ra.

Our demonstrated ability to produce a high incidence of leukemia in the dog provides an important scientific opportunity to investigate the genesis of this malignancy. Initial efforts in this direction are described in section IV of this report.

PROPOSED COURSE OF PROJECT

The work described above has indicated the advisability of acquiring data from dogs exposed continuously at exposure rates below 5 R/day. A facility for this purpose is essentially complete, and should become operational early in 1976. The facility 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 will enter the radiation field as they reach 400 days of age. The littermates will be randomized among the three exposure rates and a control group that will contain 50 animals. The dogs will be housed in fiberglass cages, constructed especially for this study, and will be kept there for duration of life.

A portion of the animals and/or space in the facility (∿ 15-20%) will be devoted to determining the effect of dose rate on the development and function of the reproductive organs irradiated in the developing fetus and in the young growing beagle. This work is described in more detail in Section VI of this report.

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RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. III. INFLUENCE OF DOSE RATE AND TOTAL DOSE ON LATE EFFECTS INDUCED BY TERMINATED EXPOSURES OF YOUNG-ADULT BEAGLES TO ^{60}Co γ-RAYS

William P. Norris, Principal Investigator
Thomas E. Fritz, Louise S. Lombard, David V. Tolle, Donald E. Doyle, Patrick H. Polk, Calvin M. Poole, William G. Keenan,* Lillian V. Kaspar, Patricia C. Brennan,† and Maria E. Ortiz,‡ Participating Investigators*

OBJECTIVES

When continuous, whole-body γ-irradiation of dogs is terminated at predetermined levels of total exposure, the resulting clinical signs and pathology are generally quite different from those seen when the exposures are continued until death. In the latter case, the causes of death are highly correlated with dose rate, and bone marrow has been the critical tissue at all the dose rates studied so far. With terminated exposure, the relative importance of total dose and dose rate is essentially unknown.

These studies are aimed at determining (1) the responses and rates of recovery of the hematopoietic and cellular immune systems and the effect of total dose and dose rate on such recovery, and (2) the influences of total dose and dose rate on the late effects that are produced. Appropriate comparisons of the effects produced by terminated exposures with those of exposures lasting for duration of life are expected to provide important information regarding the responses of separate cell and organ systems to ionizing radiations.

EXPERIMENTAL METHODS

To define the relative importance of total dose and dose rate to the delayed effects of whole-body γ-irradiation, young-adult beagles of both

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*Animal Facilities Staff.

†Neutron and Gamma-Ray Toxicity Group.

‡Faculty Research Participant, California Polytechnic Institute.

sexes are exposed in the γ field until the total exposure reaches either 600 or 1400 R at exposure rates of either 35, 17, 10, or 5 R/day. Other groups of dogs are given 2000 R at rates of either 17, 10, or 5 R/day, and another group is being given 4000 R at 10 R/day. The limitations on total dose are high mortality at exposures above 2000 and 1400 R at 17 R/day and 35 R/day, respectively, and the extremely long times required for exposure above 2000 R at 5 R/day.

Exposures are terminated at these values of total exposure because (1) 1400 and 2000 R are the LD_{50} at 35 and 17 R/day, respectively; (2) 1000-1400 rad is the integrated dose delivered by 50% lethal doses of ^{137}Cs in dogs of similar age; and (3) 2000 R is about the minimal total exposure that produces either anemia or leukemia at these exposure rates.

Each dog is evaluated regularly by clinical examinations and hematology. Biochemical assays of serum and bacteriologic assays are performed as required. All dogs becoming moribund or dying are necropsied. All major organs and tissues are weighed, and specimens are collected for routine and special techniques of microscopic examination. Tissue and cell impressions are also collected for cytologic examination, particularly specimens from hematopoietic tissues, tumors, and any exudates. Antemortem examinations on blood and postmortem examinations on tissues are conducted to define the bacteriologic flora.

Lymphocytes are separated from peripheral blood by the method of Thorsby and Bratlie (Histocompatability Testing 1970, Ed. P. I. Terasaki. Williams & Wilkins Co., Baltimore, Md., 1970, p. 655). The lymphocytes are washed and suspended in a culture medium supplemented with glutamine, fetal calf serum, and antibiotics. Viable lymphocytes (1×10^6 cells) are cultured with the mitogens concanavalin A (Con A) or phytohemagglutinin (PHA) for 48 hours at 37°C in 5% CO_2. The cells are then pulsed with 1 μCi tritiated thymidine (specific activity 6 Ci/mM) and reincubated for 18 hours. The cells are collected on millipore filters, and the DNA is precipitated with cold trichloroacetic acid. The amount of tritiated thymidine incorporation, measured in a liquid scintillation counter, is proportional to the number of cells responding to the stimulating mitogen, and provides a measure of cell-mediated immune function.

BACKGROUND AND PREVIOUS FINDINGS

A total of 283 dogs have been given protracted whole-body exposures terminated at fixed values of total exposure between 600-4000 R. The groups of dogs (20 dogs per group) scheduled to receive total exposures of 1400 R at rates of either 5, 10, 17, or 35 R/day have now been completed, as have those scheduled to receive 2000 R at 5, 10, and 17 R/day and 4000 R at 10 R/day. Ninety-three of these died, of either septicemia or anemia, during the irradiation period or within 100 days after exposure was terminated. Three other dogs died with myeloproliferative disease at 250, 407, and 576 days, respectively, after the exposures were terminated, and another of lymphatic leukemia at 611 days. All four of the latter were irradiated for a total of 4000 R at 10 R/day.

Dogs irradiated at exposure rates of 35 R/day and above for total doses of 1400 R or more uniformly die of septicemia at an early time, while dogs irradiated at lower exposure rates survive total doses many times as great and survive much longer before developing anemia and leukemia.

Since Con A and PHA specifically stimulate dog peripheral T cells to proliferate, they can be used to measure dog cell-mediated immune function. There is an age-associated decline in responsiveness to these mitogens that is augmented by protracted gamma irradiation at low dose rates.

MAJOR NEW FINDINGS

Because of the schedule on which dogs were staged into the γ field, survival time to date varies considerably. Some of these dogs have been observed for more than 3000 days postexposure. Subsequent to the early wave of myeloproliferative disease noted above, there were no further deaths that appear related to the irradiation except for two dogs dying at 1825 and 2901 days, of splenic sarcomas (neurofibrosarcoma and hemangiosarcoma). Similar neoplasms have been seen in several instances in dogs that survived doses of ^{137}Cs that were near the acutely lethal dose.

The minimum terminated exposure to produce myeloproliferative disease, so far, is 1700 R, and the mean induction time is $\sim$ 400 days. Apparently, the incidence of myeloproliferative disease decreases rapidly in dogs that survive beyond this time. This finding is similar to the data on the incidence of leukemia in Japanese populations exposed to radiation from atomic bombs.

Hematologic data were collected to evaluate the recovery pattern as a function of total dose and dose rate. Comparing the various groups of dogs, one observes large differences in the rates at which the cellular elements in peripheral blood return toward normal values after the exposures are terminated. Recovery rates become progressively slower as the total time of exposure is increased; that is, as the same total exposure is delivered at lower exposure rates over longer periods of time.

Four dogs that received 1400 R at a dose rate of 35 R/day died within 20 days after completion of irradiation, and all were septicemic, as expected. Surprisingly, the PHA and Con A responses of lymphocytes from these dogs rose dramatically with increasing total dose, reaching levels 10-20 times pre-exposure values shortly before death. At the same time, the total peripheral lymphocyte count fell to less than 10% of normal. These results indicate that the radioresistant lymphocytes are T cells, probably long-lived, non-dividing memory cells. The dramatic increase in mitogen responsiveness may be partially explained by enrichment of the responding population as a result of removal of the B-cell population and the more radiosensitive fraction of the T-cell population. Data from mice also indicate that B cells are more radiosensitive than are T cells. If this is true in dogs, then the septicemia that develops in dogs at a dose rate of 35 R/day can be partially explained by the lack of cells able to mount a humoral antibody response to invading bacteria. Dogs exposed at the same rate (35 R/day), but to a total dose of only 1015 R, showed similar increased responses to PHA and Con A while in the

gamma field. These high levels persisted in three dogs for 30 days after removal from the field, but by 65 days, the values for all dogs had returned to pre-exposure levels. Dogs exposed to 1400 R at 10 R/day had moderately elevated responses in the irradiation field. These higher levels were still evident 60 days after irradiation.

SIGNIFICANCE

Results of evaluations of the hematopoietic and the cellular immune systems, during continuous irradiation and postirradiation recovery, both suggest that lower dose rates increase the extent of nonrecoverable damage associated with a given total dose of radiation. This concurs with Casarett's observations on the effects of dose rate on spermatogenesis (Dose Rate in Mammalian Radiation Biology, USAEC CONF-680410). Such information is crucial to further development of mathematical models of radiation injury, and can also supply helpful guidance in treatment of human malignancies by radiation therapy.

The data already in hand strongly indicate that the hematopoietic system has an enormous potential to recover from radiation-induced injury, and that the risk of malignancy in hematopoietic tissues decreases rapidly following termination of exposure.

PROPOSED COURSE OF THE PROJECT

The exposures to the last of the groups of dogs proposed for this study should be completed during the coming year. The hematopoietic and cellular immune systems will be evaluated at regular intervals, as described previously. We will continue to seek additional, nondestructive procedures whereby we may derive useful information from these dogs.

All the dogs will be kept for duration of life to determine the nature of their clinical problems and eventual causes of death.

ERDA RT-01-01
ANL 63100

RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. IV. CELLULAR MECHANISMS OF RESPONSES TO CONTINUOUS ^{60}Co γ-IRRADIATION THAT RESULT IN MYELOGENOUS LEUKEMIA

Thomas M. Seed, Principal Investigator
William P. Norris, Thomas E. Fritz, Calvin M. Poole, David V. Tolle, and Rosemarie L. Devine,† Participating Investigators*

OBJECTIVES

The previous two sections of this report have demonstrated that the high incidence of myelogenous leukemia in beagles subjected to continuous ^{60}Co γ-irradiation at rates of 5-10 R/day provides an ideal model system for longitudinal study of the genesis of this disease. As a prelude to the design of more comprehensive studies of the genesis of leukemia, we have surveyed, using the electron microscope, the collection of specimens taken from dogs that developed leukemia in the experiments just described.

Our primary objective is to identify those cellular and subcellular changes that occur within the bone marrow during the onset and progression of leukemia--with particular attention given to: (1) identification of abnormalities in maturation sequences of hematopoietic elements, and (2) the search for viral inclusions which may be implicated in induction of leukemia.

EXPERIMENTAL METHODS

Standard procedures were used for collecting and preparing tissue for electron microscopy. Samples were examined with a Siemens 101 transmission electron microscope.

BACKGROUND AND PREVIOUS FINDINGS

Preliminary ultrastructural observations on the radiation-induced leukemia in the ANL beagle have been made by Tahmisian et al. (Tahmisian, T. N., et al., ANL-7870, 1971, p. 143) and Tolle et al. (Tolle, D. V., et al., ANL-7970, 1972, p. 201). These observations, which we have verified, include: (1) an asynchrony of granule maturation and differentiation within granulocytes; (2) asynchrony in the nuclear-cytoplasmic maturation sequence; and (3) a coalescence of cytoplasmic granules. All of these suggest that the normally rigid sequence of granulocyte maturation is grossly disturbed, in terms of both the nuclear and cytoplasm phases and the synchrony between the two.

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*Animal Facilities Staff.

†Carcinogenesis Group.

MAJOR NEW FINDINGS

We have not yet found evidence of virus-like inclusions within these marrow preparations. However, since only a limited number of leukemic marrows have been examined, all from the terminal stage of the disease, this important negative finding is not yet finally established.

Ultrastructural observations have indicated that the degree of tissue pathology is directly related to the extent to which myeloid infiltration alters normal tissue architecture through free-cell replacement and modification of the supportive structures. The leukemic cell population is, in most cases, predominated by the younger, less differentiated myeloid cells. The late myeloblastic-early promyelocytic cells have unique ultrastructural features, including a characteristic bizarre arrangement of rough endoplasmic reticulum, that distinguish them from stages within normal healthy animals. The presence of these characterizing features of leukemic cells in aleukemic tissues may be prognostic of developing disease.

SIGNIFICANCE

This canine model system, which allows for longitudinal observation of developing leukemia, presents unique opportunities for answering a number of questions about the genesis of leukemia and for understanding the mechanisms of induction of cancer--an essential first step toward improved methods for cancer prevention and therapy.

PROPOSED COURSE OF THE PROJECT

The design of future experiments envisages a comprehensive, multiphasic intergroup examination of morphological, hematological, immunological, and virological variables that can be altered by the continuous insult of low daily doses of gamma irradiation. These variables will be analyzed in a sequential fashion in individual animals throughout the course of irradiation and into the period of patent disease.

We plan to irradiate dogs continuously at 10 R/day in order to develop, for study, the two major pathological consequences of low dose ^{60}Co γ-irradiation, namely, aplastic anemias and myeloid leukemia.

The study will involve the following:

1) A morphological assessment of the architectural changes within sequentially sampled bone marrow will be made at the level of both light and electron microscopy. Attention will be given to supportive structure, the vasculature, and the ultrastructure of the free hematopoietic cells. Our major intent is to localize, in time, fine changes in marrow cell structure that occur concomitantly with leukemic transformation.

2) The size of hematopoietic cell compartments will be studied throughout the course of treatment. Specifically, a quantitation of hematopoietic stem cell numbers will be determined, and the titers of regulatory humoral factors will be assayed by *in vitro* culture techniques.

3) The functional integrity of the immune systems of irradiated dogs will be assessed by determining the numbers of immunologically competent cells and their responsiveness to specific stimuli.

4) Attempts will be made to detect and isolate oncornaviruses in order to elucidate the role of these agents in γ-radiation-induced leukemogenesis. Virus presence will be assessed by electron microscopic examination as well as by various classic virologic techniques (cocultivation, virus rescue, etc.) and molecular biological approaches (nucleic acid hybridization, radioimmunoassay, etc.).

5) Bone marrow grafting and its obligate correlate, histocompatibility typing, are other planned studies.

ERDA RT-01-01
ANL 63100

RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. V. LATE TOXICITY AND PATHOLOGY OF SINGLE, NEAR-LETHAL DOSES OF ^{137}Cs

Thomas E. Fritz, Principal Investigator
Louise S. Lombard, William P. Norris, David V. Tolle, and Patrick H. Polk, Participating Investigators

OBJECTIVES

The study was undertaken originally because ^{137}Cs is an important, long-lived fission product that presents a "biologically soluble" radioisotope. This work, which is nearly complete, is reported here because all the results suggest that injected ^{137}Cs irradiates the whole body in an essentially uniform pattern. The data are therefore directly comparable to those obtained with continuous external ^{60}Co irradiation. The dose rate decreases following administration, as the ^{137}Cs is excreted witha biological half-time of $\sim$ 30 days.

EXPERIMENTAL METHODS

A total of 73 dogs in three age groups received single intravenous injections of doses of ^{137}Cs near those found to be lethal within 30 days. The health status of each dog was monitored through standard clinical laboratory methods, including blood chemistry analyses and hematologic examinations. All dogs that died were necropsied, and tissues and organs were weighed and processed for microscopic examination.

BACKGROUND AND PREVIOUS FINDINGS

Forty dogs survived more than 100 days, while 33 dogs died earlier. All deaths within the first 100 days were caused by damage to the hematopoietic system, resulting in either septicemia or anemia. Hematologic recovery, in

the survivors, became apparent as the computed dose rate fell below ∿ 15 rad/day. The average life-span of the 40 dogs that survived more than 100 days was appreciably shortened and the incidence of cancer and degenerative diseases was higher than in control dogs. In these animals, the integrated radiation dose ranged from ∿ 600 to 1400 rad. Only five were still alive last year.

MAJOR NEW FINDINGS

During the past year, another two dogs died, leaving only three survivors in the experiment. Both dogs were over 13 years of age and had a multitude of changes commonly associated with old age. The most significant change in one (4356 days of age at death) was widespread chronic debilitating seborrheic dermatitis. The second (4830 days of age) had two malignancies: a sarcoma of the intestine, and a carcinoma that had metastasized to the lung and mesenteric lymph nodes.

A summary of the tumors found among the 37 decedents shows that the number of mesenchymal tumors (malignant or benign tumors derived from the connective tissue) is much higher than the occurrence of similar tumors in our untreated control population. In the control population, 25/117 (21%) of the tumors were mesenchymal in origin, whereas in the ^{137}Cs-injected chronic survivors 26/61 (43%) were mesenchymal. These data suggest that radiation-induced mesenchymal tumors occur more frequently than tumors derived from epithelium. Malignancies that we have previously classified as neurofibrosarcomas (cancers of the nerve sheath) are particularly noteworthy, since 11 have occurred in the ^{137}Cs group and only one among all the much larger population of control dogs.

A transient increase in platelet counts (thrombocytosis) following splenectomy in the dog and human is a predictable finding. In dogs it is first observed 2 to 10 days following splenectomy, reaches a peak in 4 to 6 weeks, and decreases thereafter to presplenectomy levels. In a number of dogs that received ^{137}Cs, splenectomy was performed years later because of splenic hematomas. The majority of these dogs have shown a persistent post-splenectomy thrombocytosis lasting for years, with platelet counts as high as 2×10^{12} per liter compared to the normal level of $2\text{-}5 \times 10^{11}$. This persistent thrombocytosis, in most dogs, resembles autonomous thrombocytosis, in which platelet production is apparently unresponsive to the normal regulatory processes. In a few dogs, megakaryocytic hyperplasia is observed on bone marrow evaluation. Animals with persistent thrombocytosis offer a possible model for the study of both thrombokinetics and thrombopoietin.

SIGNIFICANCE

The data on lesions seen in dogs exposed to near-lethal doses of ^{137}Cs show that the earliest lethal effects are reflected solely in the hematopoietic system, specifically the bone marrow. Dogs surviving these early insults to the bone marrow pass into a phase of relatively normal health, and only after periods approaching 10 years do degenerative and neoplastic diseases become important. The combination of total dose and dose rate from injected ^{137}Cs, based on results from whole-body ^{60}Co irradiation, is not expected to produce leukemias and none were observed.

PROPOSED COURSE OF THE PROJECT

No further laboratory work with ^{137}Cs is anticipated, following completion of this study. There will, however, be a continuing responsiveness toward problems associated with the dose-effect relationships that exist with internally deposited radionuclides, and the rationalization of these observations through the results from continuous exposures to γ-rays from calibrated sources external to the body.

ERDA RT-01-01
ANL 63100

RESPONSES OF BEAGLE DOGS TO CONTINUOUS IRRADIATION OF THE WHOLE BODY. VI. EFFECT OF DOSE RATE ON THE DEVELOPMENT OF THE REPRODUCTIVE ORGANS OF FETAL AND YOUNG, GROWING BEAGLES

William P. Norris, Principal Investigator
Thomas E. Fritz, Thomas M. Seed, David V. Tolle, Calvin M. Poole, William G. Keenan,* Lillian V. Kaspar, and Patrick H. Polk, Participating Investigators*

OBJECTIVES

The developing fetus is regarded as being exquisitely sensitive to ionizing radiations, but it has not been studied adequately to determine the influence of dose rate on the damage that may be produced. The objective of this work is to determine the relative importance of total radiation dose and dose rate to the developing beagle fetus, as well as to identify the dose rate that, when administered continuously, allows for continued reproduction of the species *Canis familiaris*.

EXPERIMENTAL METHODS

Virgin beagle bitches are bred to proven sires and irradiated in the ^{60}Co γ field at rates of either 5, 10, 17, or 35 R/day for their entire period of gestation, or selected portions thereof. Their young are evaluated at regular intervals after birth by clinical and hematological examinations. After the age of puberty, their reproductive potential is estimated from sperm counts in the males, and by microscopic examination of unilaterally biopsied ovaries from the females. The pups are weighed at regular intervals, and will be kept for duration of life to determine whether late effects caused by radiation exposure *in utero* can be detected.

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*Animal Facilities Staff.

BACKGROUND AND PREVIOUS FINDINGS

Previous work in this laboratory has shown that female beagles, irradiated continuously with ^{60}Co γ-rays at exposure rates of 5-17 R/day from conception to parturition, produce apparently normal litters. The data are strongly suggestive that exposure to 17 R/day may interfere in the stage of pregnancy prior to implantation of the ova, resulting in the loss of entire litters. Exposure to γ-rays during the period of organogenesis (20-40 days post-conception) at rates as high as 35 R/day was also followed by birth of apparently normal, viable litters.

Sixty-eight pups, irradiated while *in utero*, are under observation. There is a tendency in these pups to attain somewhat less than normal size in adult life. Much more striking, however, is the effect on the reproductive systems of these animals irradiated while *in utero*. At the lowest daily exposure rate (5 R/day) that has been available for study thus far, all females irradiated *in utero* had anovular ovaries and were sterile. Males, similarly irradiated at 5 R/day, had viable sperm and were probably fertile. At higher exposure rates (10 R/day) both sexes were sterile. Sterility in the female was associated with demonstrable endocrine imbalance.

MAJOR NEW FINDINGS

During the past year, among the dogs that were irradiated during fetal life and that reached more than 100 days of age, three have died. Except for one dying of chronic nephritis at 918 days of age, the deaths were apparently not related to the radiation exposures. Although we have seen cases of nephritis in our control dogs, the occurrence of such a severe lesion at less than three years of age is unusual, and suggests there may be some relation to the irradiation given during fetal life.

Also during the past year, four additional litters were born that received irradiation during fetal life. The most recent litter had two pups born with severe congenital abnormalities. Previously only one pup irradiated during gestation had abnormalities. There have now been three pups with birth defects among 118 born following *in utero* irradiation. The significance of the defects can be determined only by making appropriate comparisons to the defects among the untreated newborn puppies. For example, 4 untreated pups out of 41 neonatal deaths this year also had significant abnormalities.

SIGNIFICANCE

The response of the developing beagle fetus to ionizing radiations is shown to be highly dose-rate dependent. Congenital abnormalities have not been identified in unusually high incidence even at exposure rates as high as 35 R/day. The developing gonads, and especially the ovary, are among the most highly radiosensitive tissues, and provide an excellent early indicator of radiation injury.

Adult bitches, irradiated while *in utero*, come into estrus and mate even though their ovaries contain no ova. Their abnormal hormonal responses, associated with this condition, provide for important new insights into mechanisms of endocrine function.

PROPOSED COURSE OF THE PROJECT

Fifteen estrus bitches will be bred to proven males on two successive days and placed in the ^{60}Co γ-ray field. These will be separated into three groups of five dogs each and exposed to either 2.5, 1.0, or 0.4 R/day throughout their periods of gestation. Following parturition, the puppies will be removed from the irradiation field. Examination of their reproductive organs by serial biopsy and/or at the time they are sacrificed will determine whether any of these exposure rates will allow for effective reproductive activity in adult life.

To the extent that reproduction appears possible, as determined by histological examination of the gonads, the dogs will be mated. Their young, if any, will be examined to determine whether they possess anomalies that can be ascribed to the *in utero* irradiation of the parents. (This study relates closely to a comparable experiment, described in the following section of this report, in which puppies will be exposed to tritiated water while *in utero*).

If the species *Canis familiaris* can survive for at least one generation under continuous irradiation as high as 0.4 R/day, this fact can be determined rather rapidly. Identification of such a value will provide yet another parameter that will be useful for interspecies comparisons of responses to continuous irradiation.

ERDA RT-03-01
ANL 60600

A COMPARATIVE APPROACH TO THE DESIGN OF A LOW-LEVEL RADIATION EXPERIMENT

Sylvanus A. Tyler, Principal Investigator*
George A. Sacher and William P. Norris, Participating Investigators*

OBJECTIVES

The primary objective of this work is the development of an experimental plan for the estimation of the effects of low dose rates of ^{60}Co gamma rays

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*Aging Research Group.

on young adult beagle dogs that would yield a maximum of statistically significant information using a minimum number of animals.

BACKGROUND AND PREVIOUS FINDINGS

G. A. Sacher (Radiol. Clin. North Am. 3, 227, 1965) and D. Grahn (Late Effects of Radiation, Taylor and Francis, Ltd., London, 1960, pp. 101-136) have shown in two strains of mice ($B6CF_1$ and LAF1) continuously irradiated with ^{60}Co gamma rays that: (1) for dose rates between 18 and 70 R/day, specific mortality rates vary as the square of the dose rate, as in the dog; and (2) for dose rates from 1 to 18 R/day, the specific mortality rate varies in proportion to dose rate.

The specific mortality rate in young adult beagles under continuous exposure to ^{60}Co gamma rays at dose rates of 5 to 72 Roentgens per day varies as the square of the daily dose. However, the trend of mortality rates at lower doses is not known, and estimates are needed to decide whether it continues downward with the same trend as at higher daily doses or changes to a first-power trend at some point.

EXPERIMENTAL METHODS AND MAJOR NEW FINDINGS

The available information obtained from dogs given higher levels of continuous ^{60}Co irradiation, and from mice given both high and low levels of irradiation, was analyzed.

1) A scaling factor was established by least squares that superimposed the specific mortality rates of the dogs at dose rates within the second power range on the second power trend for the mouse.

2) The assumption was accepted that with the appropriate scaling factor the dog would follow both the linear and quadratic branches of the mouse response.

3) The dose rate, predicted by the mouse model, at which the dog should change from a second power trend to a linear trend was estimated as 5 R/day.

PROPOSED COURSE OF PROJECT

To determine experimentally if a change in trend is significantly effected, a single experiment with 100 dogs irradiated at 2.5 R/day is planned.

Dose rates of 2.5, 1.0, and 0.4 R/day have been selected for experimental use, and a ^{60}Co gamma irradiation facility is being constructed on this basis. (See Section II of this report.) The first two dose rates will determine whether the trend in mortality rates changes significantly below 5 R/day. At the lowest dose rate no significant data regarding radiation specific death rates are expected. At all dose rates, but particularly at the lowest, induced physiological and pathological end points are expected to be of great interest.

ERDA RT-01-01
ANL 63101

EVALUATION OF RADIATION HAZARDS ASSOCIATED WITH EXPOSURE TO TRITIATED WATER (HTO)

William P. Norris, Principal Investigator
Thomas E. Fritz, Thomas M. Seed, and Norbert D. Kretz, Participating Investigators

OBJECTIVES

Tritium, in small amounts, is an inevitable, uncontrollable environmental contaminant from conventional nuclear reactors, since it arises from neutron activation of deuterium in the cooling water. This, together with its appreciably long physical half-life (12.3 years), suggests that it is important to attempt to answer three questions, largely unanswered: (1) is the distribution of exchangeable body water sufficiently uniform to allow the low energy electrons from tritium to irradiate the body uniformly; (2) are there special biochemical, physiological, or developmental situations in which the incorporation of tritium from body water HTO into important bioorganic compounds will produce significant increases above current estimates of tritium toxicity; and (3) what is the correct value of the quality factor (Q) for tritium electrons?

EXPERIMENTAL METHODS

We propose to study the effects of tritium on the developing beagle fetus (especially the female) under conditions where the body water of the dam is maintained, from conception to parturition, at concentrations of HTO calculated to deliver the same radiation doses used in our present and projected studies of the effects of continuous exposure to ^{60}Co γ-irradiation (5.0, 2.5, 1.0, and 0.4 R/day).

In the first series of experiments the following protocol will be observed:

1) Virgin beagle bitches will be bred to proven sires and placed immediately into one of the three following experimental categories:

a) Maintained throughout gestation at HTO body water concentrations estimated to deliver radiation doses equivalent to γ-ray exposures to either 5.0, 2.5, 1.0, or 0.4 R/day. This procedure will be monitored by measurements of the specific activity of ^{3}H in plasma water. Pups will be taken from the dam at birth and bottle fed to avoid continued exposure to ^{3}H in breast milk.

b) Maintained throughout gestation in a ^{60}Co γ-ray field at exposure rates of either 5.0, 2.5, 1.0, or 0.4 R/day.

c) Nonirradiated controls under comparable conditions of caging and treatment.

Because the time course of differentiation and maturation of the germinal cells in the two sexes is markedly different, correspondingly different methods will be used to evaluate radiation-induced changes in the gonads of the two sexes.

2) Evaluation of Ovarian Damage: The primordial ova are completely differentiated after the first meiotic division which, in the beagle, occurs within about 5 days after birth. Thereafter, there is no apparent change in the primordial ova except for the follicular growth and maturation that occurs just prior to ovulation. Primordial ova from puppies exposed to HTO *in utero* should contain organically bound tritium that will continue to irradiate the ova until they are shed from the ovary. Procedures for evaluation of ovarian damage in females irradiated *in utero* with either HTO or ^{60}Co γ-rays will be as follows:

a) Two female pups from each original group of 10 will be killed at ages of 7 days, 5 months, and 1 year. Their ovaries will be evaluated by histologic examination, determination of total numbers of ova from serial sections, autoradiography to ascertain the distribution of ^{3}H (in those dogs exposed to HTO), and direct measurements of ^{3}H content of the ovaries.

b) The remaining 4 females from each original group of 10 will be bred to proven sires at first estrus following 1 year of age. If they conceive, they will be killed when they wean their litters; if they do not, they will be killed at 35 days postbreeding, a time at which failure to conceive can be reliably determined. In either case, the ovaries will be assayed as described in (a) above.

c) Pups derived from matings in (b) will be held for 9 months to allow identification of any developmental anomalies, killed, and examined at autopsy.

d) The original groups of 10 will be followed to time of sacrifice by our regular clinical and hematological procedures. In addition, plasma levels of leuteinizing hormone (LH), follicle stimulating hormone (FSH), estrogens, and progesterone will be determined by radioimmunoassay procedures. All matings will be made at times indicated by the rise in LH that coincides with ovulation.

e) The data will be used to determine 1) the similarities and/or differences between the effects of continuous γ-radiation and radiation from HTO; 2) the exposure rates delivered *in utero* that produce minimal, or no, reduction in ovarian function; 3) the effects of organically bound ^{3}H in the postnatal behavior of the ovary.

3) Evaluation of Testicular Damage: Spermatogonia, the basic male germ cells, can be identified, late in organogenesis, in the fetal testicle. They multiply slowly in fetal and juvenile life and do not differentiate further until they come under the influence of pituitary FSH at time of puberty. Fetal and juvenile spermatogonia are less radiosensitive, when exposed to 5 R/day, than the differentiating spermatogonia in the adult. (Males exposed to 5 R/day *in utero* produce normal numbers of sperm as adults.) Spermatogonia in the newborn are also expected to contain organically bound ^{3}H, when HTO is given the dam. Procedures for evaluation of testicular damage in males irradiated *in utero* with either HTO or ^{60}Co γ-rays will be as follows:

a) All male pups irradiated *in utero* with ^{60}Co γ-rays will be kept in the γ field, at the same exposure rate used while they were *in utero*, until evaluation procedures are completed. From 6 of the 10 males expected in each dose-rate group, testicles will be removed, at regular intervals, for histologic evaluation and estimation of numbers of spermatogonia in the seminiferous tubules. This procedure will cover the period from birth to ∿ 8 months of age--a time when the control testes will have reached full reproductive function.

If they appear fertile at puberty, the remaining 4 intact males in each dose rate group will be maintained for an extended period in the γ-ray field to determine how long spermatogenesis will continue at these exposure rates. These males will be allowed to breed at intervals to ascertain whether they are capable of reproduction.

b) Testicles of the male pups exposed to HTO *in utero* will be sampled on a schedule identical to that described above (a). They will also be evaluated by similar histologic procedures, and autoradiographs will be made to determine the distribution of bound tritium in the various cellular elements. Specimens of these testes will be assayed for total ^{3}H content.

c) In addition to routine clinical and hematologic evaluation, blood samples from both the γ-irradiated and the HTO-treated male pups will be assayed, at regular intervals, for their contents of LH, FSH, and testosterone.

d) The data will be analyzed to make the best possible comparisons between the effects of continuous exposure of the male fetus to HTO and continuous γ-irradiation.

BACKGROUND AND PREVIOUS FINDINGS

The short residence time of water in the body (the biological half-time of exchangeable water in the dog is estimated as 7.3 days) presents substantial problems in determining experimentally the hazards associated with prolonged exposures to tritiated water. Our current studies of the effects of continuous, whole-body γ-irradiation in the beagle dog, however, have shown that exposure of the bred bitch to 5 R/day from conception to parturition produces severe, histologically uniform, damage in the developing ovaries of her female pups. This is the only effect of such irradiation that has become apparent so far, and demonstrates that the developing fetal ovary is easily one of the most radiosensitive of all tissue systems.

This system offers, therefore, an opportunity to study mammalian responses to constant concentrations of tritium in body water under conditions that: (1) are experimentally feasible (HTO levels in the dam must be maintained for only ∿ 60 days), (2) are biologically meaningful (reproduction in the dog is impossible at continuous exposure rates of 5 R/day), (3) allow direct comparison with results of current studies of the effects of continuous γ-irradiation, (4) allow for isolation of the effects of continuous irradiation of the developing fetal ovary and subsequent postnatal damage to the ovary from organically bound tritium, and (5) employ a highly sensitive indicator of radiation damage.

MAJOR NEW DEVELOPMENTS

Appropriately ventilated space was identified in which to conduct the exposures of the pregnant bitches to tritiated water. The space required extensive clearing and renovation, most of which has been done. The ANL Division of Industrial Hygiene and Safety and the ANL Committee on Disposal of Radioactive Wastes were consulted regarding proper handling and disposition of the tritium to be excreted by the dogs. Resolution of these questions is still pending and will determine the ultimate design of appropriate isolators for the tritium-bearing dogs.

FUTURE COURSE OF WORK

We expect to pursue the objectives of this study as outlined above.

03078

3. THERAPY OF METAL POISONING

ERDA RT-01-02
ANL 64100
ANL 64200
EPA-IAG-D6-E681

GROUP LEADER'S OVERVIEW

Arthur Lindenbaum, Group Leader

Three new developments have influenced the work being carried out under this program during the past year, and probably will markedly influence the character of future work. First is the beginning of a new study entitled "Studies on the metabolism of plutonium." In this work we seek answers of direct relevance to questions regarding the effects of different levels of radioactive pollutants on the individual and, by extension, on populations. Most of the experimental work on plutonium is done with the most ubiquitous isotope ^{239}Pu, which, for ease of measurement, is administered to experimental animals at levels far exceeding permissible body burdens set for man by radiation protection agencies. To establish the validity of these findings under realistic conditions, therefore, we are examining the effect of plutonium mass on its ultimate deposition pattern in living tissues, particularly as the mass is reduced to levels in the range of the maximum permissible body burden for man. In addition to our use of a variety of plutonium isotopes for this purpose, we shall test the feasibility of utilizing the gamma emissions from other plutonium isotopes for whole body organ scanning procedures. Such a development could open up the possibility of carrying out kinetic studies on living animals (and man?), with several beneficial results. The combined expertise available for this work in two Argonne divisions, BIM and CHM, provides an example of the forces that a national laboratory like Argonne can summon to attack important problems.

The second new development is the start of a long-anticipated nonnuclear phase of our work with initial studies being directed toward lead decorporation. Early in our work on actinide metabolism and therapy, it became clear that plutonium is prototypical of a large class of polyvalent metals whose compounds exhibit variable tendencies to undergo hydrolysis and aggregation under physiological conditions, and to become concentrated not only in specific animal organs but in (or associated with) specialized cells of the body. Our selection of lead as the first nonradioactive toxic metal to be compared with plutonium was dictated by the availability of an easy and sensitive analytical method for lead quantitation, involving the radioactive isotope ^{210}Pb. Furthermore, our initial studies comparing the metabolism of lead acetate and lead citrate were greatly facilitated by techniques already developed for

plutonium. Likewise, for future therapeutic work on lead removal, we plan to utilize esters of DTPA (diethylenetriaminepentaacetic acid) prepared originally for use in studies of plutonium localization and decorporation.

The third new development includes a variety of cooperative projects now under way, or projected, in which our group will combine forces with others within or outside BIM. Our work on plutonium isotopes (with A. M. Friedman and J. C. Sullivan of the Chemistry Division) is one such effort. Another is a combined cytogenetic, autoradiographic, and radiochemical study (with D. Grahn, BIM) of the nonuniformity of plutonium deposition in the mouse testis and the consequences of variable alpha irradiation on sperm counts, sperm abnormalities, and expressions of genetic damage in F_1 progeny.

Of special mention with respect to our continuing work on plutonium metabolism and therapy are two recent developments. First, we have found condensation products of acrylic and itaconic acids that, like the pyran copolymers, are effective, with the chelating agent DTPA, in removing liver plutonium otherwise long retained. Second is the finding that the main route of excretion of monomeric plutonium from the liver is probably *via* the parenchymal cells.

The work being carried out under this program has culminated, in addition to several papers published in the open literature or in press, in four oral (and to be published) presentations at three national or international symposia.

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 1975

Guilmette, Raymond A. (Postdoctoral Appointee)
*Parks, John E. (Postdoctoral Appointee)

PUBLICATIONS

Baxter, D. W., N. G. Doan, and A. Lindenbaum. Differences in early retention of lead acetate and lead citrate in mouse tissues. Proceedings of the Symposium on Biological Implications of Metals in the Environment, Richland, Wash., Sept. 29-Oct. 1, 1975, in press.

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*Terminated during 1975.

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Stevens, W., D. R. Atherton, W. S. S. Jee, D. S. Buster, B. J. Grube, F. W. Bruenger, and A. Lindenbaum. The induction of osteogenic sarcoma by polymeric plutonium ($^{239}PuIV$) in beagles. The Health Effects of Plutonium and Radium, Ed. W. S. S. Jee. The J. W. Press, Salt Lake City, in press.

Abstracts

Bhattacharyya, M. H., and A. Lindenbaum. The association of monomeric plutonium with mouse liver parenchymal cells. Radiat. Res. 62, 575-576 (1975).

Guilmette, R. A., and A. Lindenbaum. Effectiveness of interferon-inducing agents for removal of polymeric plutonium from the mouse liver. Health Phys. 29, 921-922 (1975).

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ERDA RT-01-02
ANL 64100
EPA-IAG-D6-E681

PHYSICAL CHARACTER OF TOXIC METAL COMPOUNDS AS RELATED TO TISSUE DISTRIBUTION, TOXICITY, AND THERAPEUTIC REMOVAL

Arthur Lindenbaum, Principal Investigator
David W. Baxter and Nancy G. Doan,† Participating Investigators*

OBJECTIVES

The objectives of this program are (1) to define the particulate nature of toxic metal compounds in the environment and in animal tissues, (2) to relate differences in particulate nature to differences in tissue distribution and toxicity, and (3) to devise effective means for decorporation of these toxic metals.

BACKGROUND AND PREVIOUS FINDINGS

Results from our previous metabolic experiments with plutonium demonstrated the importance of the physicochemical form of an easily hydrolyzed metal compound on its tissue distribution and on selection of optimal decorporation procedures. Our previous findings and techniques with plutonium, therefore, were utilized in initiating studies with other toxic metals.

EXPERIMENTAL METHODS

The physical nature of the more commonly occurring metal compounds is determined by a simple ultrafiltration technique. These characterized compounds are injected into mice and the distribution of the metal in various tissues is analyzed by radiotracer techniques. Differences in patterns of deposition are then evaluated with respect to physicochemical characteristics of the injection solution and with respect to decorporation therapy. In addition, selected tissue sections are examined for pathological changes.

MAJOR NEW FINDINGS

In comparing two compounds of lead injected intravenously into mice, i.e., lead acetate and lead citrate, we found that, as expected, the lower solubility of lead acetate resulted in higher initial deposition in liver and spleen; however, more than 90% of the initial organ deposits had cleared by 14 days after injection. Significantly, the levels of both lead salts in the brain, while relatively low, remained constant over the duration of the experiment (2 weeks).

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*Visiting Research Associate, Chicago College of Osteopathic Medicine.

†Student Aide; present address: Washington University School of Medicine, St. Louis, Mo.

SIGNIFICANCE

These initial findings for lead indicate that many of the results obtained in our laboratory with the actinide radioelements probably have application for toxic metal compounds of environmental concern.

PROPOSED COURSE OF THE PROJECT

We shall examine the gross and microscopic tissue localization of lead compounds such as lead acetate and lead citrate in organs such as the liver and brain (the organ most affected in children suffering from lead poisoning). Some therapeutic removal techniques, developed in our work on plutonium therapy, will be tested for effectiveness in decorporation of lead.

ERDA RT-01-02
ANL 64100

INTERACTIONS OF MONOMERIC PLUTONIUM WITH SPECIFIC COMPONENTS OF MOUSE LIVER AND SKELETON

Maryka Bhattacharyya, Principal Investigator
Arthur Lindenbaum and David Peterson, Participating Investigators

OBJECTIVES

The objectives are (1) development and application of a technique of liver parenchymal cell isolation to measure the uptake, retention, and excretion of plutonium by specific cells of the liver; and (2) development of techniques for inducing loss of bone mineral and matrix as related to possible concomitant removal of plutonium deposited in the skeleton.

BACKGROUND AND PREVIOUS FINDINGS

Previous studies of microlocalization of plutonium in different liver cell types have employed light microscope autoradiography (Lindenbaum, A., et al., Diagnosis and Treatment of Deposited Radionuclides, Eds. A. Kornberg and W. D. Norwood. Excerpta Medica Foundation, Amsterdam, 1968, pp. 56-64; Taylor, G. N., et al., Radiobiology of Plutonium, Eds. B. J. Stover and W. S. S. Jee. The J. W. Press, Salt Lake City, 1972, pp. 105-127). Exact location and quantitation of plutonium alpha tracks by this method, however, is tedious and subject to error because the alpha tracks are long in relation to liver cell sizes. The measurement of plutonium in isolated liver cells is a direct and rapid measurement which lends itself to further subcellular localization studies, should such information be required.

High intramuscular doses of vitamin D have been shown to induce the removal of bone mineral with increased excretion of bone mineral into the urine (Frankel, S., and S. Yasumura, Endocrinology 88, 267, 1971) for about one week, starting 4 days after vitamin D injection. The question can be asked, does a high dose of vitamin D_3 also reduce the burden of skeletal plutonium?

EXPERIMENTAL METHODS

Intact parenchymal cells were isolated from livers from 1 hour to 6 days after intravenous injection of monomeric plutonium citrate into mice. In addition, the amount of plutonium removed from parenchymal cells was measured 6 and 24 hours after injection of the chelating agent DTPA (diethylenetriaminepentaacetic acid).

Three days after injection of monomeric plutonium, mice were given a high dose of vitamin D_3 intramuscularly. Serum and urinary calcium levels were monitored to trace the effect of vitamin D_3 on bone mineral removal. Urinary plutonium levels were also measured, along with femur plutonium levels, 10 days after vitamin D_3 treatment.

MAJOR NEW FINDINGS

By 6 days after monomeric plutonium injection into mice, 70% of the total liver plutonium was located in the parenchymal cells. In contrast to the long retention of monomeric plutonium in the dog liver, the mouse liver rapidly loses monomeric plutonium. The present finding suggests, therefore, that the route of excretion of monomeric plutonium from the mouse liver is *via* the parenchymal cells. For DTPA-induced removal of liver plutonium, this definitely appears to be the case. By 24 hours after DTPA injection, there was a 70% reduction in the amount of plutonium associated with liver parenchymal cells; in contrast, the reduction in plutonium from the remainder of the liver was only 30%, and this reduction occurred also in the control animals.

In spite of high serum and urinary calcium levels found after administration of vitamin D_3 to plutonium-injected mice, no increase in urinary plutonium excretion was observed. Increased bone mineral excretion under these conditions did not result in increased urinary excretion of plutonium. Femur plutonium levels also were not reduced by vitamin D_3 treatment.

SIGNIFICANCE

Basic studies of this nature not only provide information regarding the association of toxic metals such as plutonium with specific tissue components, but also may be expected to provide a rational basis for the development of effective techniques for plutonium decorporation.

PROPOSED COURSE OF THE PROJECT

Liver studies: (1) ^{241}Pu, a low energy, β-emitting isotope obtainable from the Chemistry Division of Argonne, will be used for liver autoradiography several hours after monomeric plutonium injection into mice. The very short β-track produced by ^{241}Pu should allow more precise cellular and subcellular localization of plutonium in liver sections, in particular shortly after plutonium administration when assay of isolated parenchymal cells indicates that plutonium is in the liver but not yet tightly bound to these cells. (2) The bile ducts of plutonium-injected rats will be cannulated to determine the effect of DTPA on plutonium excretion into the bile. (Differences in biliary excretion between rodents and dogs could account for the long retention time of monomeric plutonium in the dog liver.) We plan to use ^{14}C-DTPA, previously synthesized in this laboratory, in these studies. (3) We will continue kinetic studies of the effect of DTPA on the release of plutonium from isolated mouse liver parenchymal cells. This work is aimed at helping to understand the mechanism of action of DTPA, particularly its prolonged action.

Skeletal studies: Plutonium-injected mice will be subjected to a low phosphate diet to observe the effects of this diet on skeletally deposited plutonium. It has been demonstrated that by 2 weeks on this diet, the cortex of the rat bone is decreased to one-third its original thickness. The bone resorption rate at the endosteal surface, where plutonium is deposited at the highest concentration, is increased approximately 5-fold (Baylink, D., et al., J. Clin. Invest. 50, 2519 (1971).

ERDA RT-01-02
ANL 64100

METABOLISM AND THERAPEUTIC DECORPORATION OF PLUTONIUM IN THE MOUSE AND DOG

Raymond A. Guilmette, Principal Investigator
Arthur Lindenbaum and Elizabeth Moretti, Participating Investigators

OBJECTIVES

In this program, we (1) study the uptake and retention of different physicochemical forms of plutonium in the mouse and dog; (2) develop therapeutic means of accelerating the removal of persistent deposits of polymeric plutonium, in particular from the liver; (3) extend our knowledge of the effectiveness of DTPA (diethylenetriaminepentaacetic acid) in removing monomeric (minimally polymeric) plutonium from the dog, as related to the distribution of the plutonium at the beginning of therapy; (4) investigate the effect of mass of plutonium on its distribution and retention; and (5) investigate the role of chemical toxicity of plutonium on its carcinogenic potential.

BACKGROUND AND PREVIOUS FINDINGS

Previous studies have shown the dependence of the tissue distribution and retention of plutonium on its physicochemical state at injection (Schubert, J., et al., Radiat. Res. 15, 220, 1961). Since it was also demonstrated earlier that DTPA, the treatment of choice to date for incorporated plutonium, was not very effective in removing aggregated or particulate forms of the nuclide from reticuloendothelial organs like the liver, several experimental approaches have been attempted to alter the functional state of the reticuloendothelial system and therefore affect the metabolism of plutonium. The results have recently been summarized (Lindenbaum, A., et al., Proceedings of an IAEA Seminar on the Diagnosis and Treatment of Internally Deposited Radionuclides, Vienna, 1975, in press).

An alternative approach for increasing the removal of polymeric plutonium (Pu-P) has been the use of esterified DTPA (Markley, J. F., Int. J. Radiat. Biol. 7, 405, 1963). Increased amounts of Pu-P could be decorporated if the pentaethyl ester of DTPA was given with $CaNa_3DTPA$, and the effects of the two chemical forms were additive. However, since the pentaethyl ester was toxic, we have used other less toxic n-alkyl esters of DTPA for decorporation therapy.

DTPA is effective for the removal of intravenously administered monomeric plutonium (Pu-M). However, the efficacy depends on the promptness with which one initiates DTPA treatment. When twice weekly DTPA treatment in the dog was begun 6 days after Pu-M injection, 96% of the liver burden and 50% of the skeletal burden were removed in 3 months, amounts in excess of normal metabolic clearance (Baxter, D. W., et al., Radiat. Res. 55, 144, 1973).

EXPERIMENTAL METHODS

Mice, injected intravenously with Pu-P, are treated with therapeutic substances designed to remove plutonium not affected by DTPA treatment. Experimental protocols are illustrated by A. Lindenbaum et al. (Proceedings of an IAEA Seminar on the Diagnosis and Treatment of Internally Deposited Radionuclides, 1975, Vienna, in press). Decorporation of Pu-M in the dog by early administration of DTPA (6 hours after plutonium injection) followed our established procedures as previously published (Baxter, D. W., et al., Radiat. Res. 55, 144, 1973). The study of the mass effect of plutonium on its metabolism is being studied in the mouse using isotopes of plutonium, ^{236}Pu, ^{239}Pu, ^{242}Pu, ^{244}Pu, whose specific activities range from 0.02 to 532,000 mCi/g. This allows us to inject, for equal radioactivities, masses differing by 10^7. Initial studies involve evaluation of tissue distribution and retention as relates to mass.

MAJOR NEW FINDINGS

Of the compounds tested to date for removal of Pu-P from the mouse liver, the most successful have been three polyanionic copolymers: two pyrans (copolymers of divinyl ether and maleic anhydride) and another, designated EMH-227 (copolymer of acrylic acid and itaconic acid). These compounds, when used with DTPA, removed 30 to 60% of the hepatic plutonium that could not be removed by DTPA alone. Experiments with analogs of these copolymers (i.e.,

differing in molecular weight, charge density, structure, etc.) indicate the need for a fairly large molecule (M > 20,000) with a high charge density.

Efforts aimed at increasing Pu-P removal through the use of di-n-alkyl esters (< 9 carbon chain length) of DTPA have not produced significant therapeutic results, perhaps because of the remaining carboxyl groups, the charge density of which did not allow as great a degree of membrane permeability as originally sought. The toxicity of the materials further suggests that pursuit of research along these lines might not be productive.

The use of $CaNa_3DTPA$ in removing plutonium from the dog when therapy was begun 6 hours after injection of Pu-M was very effective in removing plutonium from the body, in particular from the soft tissue and the circulation. Treatment at the DTPA dose suggested for man, i.e., 0.036 mmole/kg, resulted in excretion of 40% of the injected dose (ID) on the first day, compared to < 3% in untreated dogs. Treatment at a somewhat higher dose, i.e., 0.25 mmoles/kg, resulted in an excretion of 61% ID in 1 day and about 74% ID at the end of 1 week of treatment. Virtually all plutonium removed by therapy was excreted via the urine, very little via the feces. It appears that 0.036 mmoles/kg, given 6 hours after Pu injection, is less than optimal therapy.

SIGNIFICANCE

The information cited above, regarding an optimal regimen of DTPA treatment, is of immediate usefulness to clinicians dealing with accidents involving contamination of workers with plutonium and other actinides. Likewise, in removing DTPA-resistant plutonium, positive results in experimental animals may be expected to lead to eventual human applications. Furthermore, the therapeutic procedures developed for actinide decontamination may very well find application against nonradioactive, polyvalent, toxic metals whose compounds undergo physicochemical reactions similar to those of plutonium.

PROPOSED COURSE OF THE PROJECT

Extension to the dog of treatment with pyran copolymers deemed successful in the mouse is an obvious next step, since the metabolism of plutonium in the dog is considered more like that in man. Continuing studies with mice will focus on some problems of pyran toxicity and translocation of plutonium, as well as definition of structural parameters of the copolymer relevant to its therapeutic efficacy.

Efforts directed at determining the effect of mass of plutonium on its distribution, retention, and toxicity will also be continued (see following report). These studies must be limited to mice for the present, because of scarcity of the pure isotopes, particularly ^{244}Pu.

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ANL 64200

COMPARATIVE STUDIES OF THE TISSUE DISTRIBUTION OF PLUTONIUM ISOTOPES

Arthur Lindenbaum and Raymond A. Guilmette, Principal Investigators
Arnold M. Friedman, James C. Sullivan,* and John J. Russell, Participating Investigators*

OBJECTIVES

In this work, we compare the tissue distribution of plutonium isotopes of widely differing specific activities, investigate the role of oxidation state of plutonium *in vivo* using Mössbauer spectrometry, and measure the kinetics of uptake and retention of gamma-emitting isotopes of plutonium by external *in vivo* gamma scanning techniques.

BACKGROUND AND PREVIOUS FINDINGS

It is usually assumed that the chemical behavior of plutonium *in vivo* is independent of concentration. This assumption, however, has not been verified experimentally. The chemical behavior of plutonium, which has a variety of oxidation states, shows a marked dependence on initial concentration of plutonium in chemical systems *in vitro*. Such behavior could be magnified in complex biological systems. Since analytical limitations make it experimentally necessary to use 1 to 100 μg/kg of ^{239}Pu, the isotope most commonly used in animal studies, little information is available on the behavior of plutonium at levels approximating the maximum permissible body burden for man. Some preliminary studies elsewhere, comparing the distribution of ^{239}Pu with that obtained with ^{238}Pu or ^{237}Pu, indicate that there may be a mass effect, but evaluation of the data is complicated by dosimetric considerations.

It is generally assumed that the chemical form of the plutonium in living tissue is the form characteristic of the compounds after chemical separation *in vitro*, rather than under the physiological conditions encountered in animal tissues (low concentrations, presence of organic metabolites, etc.). Generally, relatively simple characteristics of the plutonium species *in vivo*, such as the oxidation state or the coordination characteristics, are known only by *a posteriori* inference. The work of J. Carritt et al. (J. Biol. Chem. 171, 273, 1947) demonstrates that the oxidation state of plutonium is important. After injection of Pu(VI), the urinary excretion was 7.5% of the injected dose on the first day, compared to 0.3-0.7% for either Pu(III) or Pu(IV).

EXPERIMENTAL METHODS

To study the effect of mass of plutonium on its uptake and distribution, four isotopes of plutonium, 236, 239, 242, 244, whose specific activities differ by 10^7, will be injected into mice using equal activity amounts of

*Chemistry Division, Argonne National Laboratory.

each isotope. Tissue distribution measurements will then be performed using standard radiochemical methods. In addition, autoradiography will be used to define the microdistribution patterns in liver and bone. Mössbauer spectroscopic analysis of tissue samples containing the isotope ^{237}Pu will be performed to study the *in vivo* oxidation state of plutonium. Using established γ-scintillation scanning methods, two short-lived gamma-emitting isotopes of plutonium, ^{237}Pu ($t_{1/2}$ = 4.5 days) and ^{245}Pu ($t_{1/2}$ = 10 hours), will be used for studying the short-term *in vivo* kinetics of plutonium metabolism.

MAJOR NEW FINDINGS

This new program has been under way for only a few months.

SIGNIFICANCE

Extension of our knowledge of the metabolism of plutonium to the mass range most likely to occur in cases of human accidental contamination is essential to understanding the problems associated with plutonium poisoning, and is also relevant to considerations of appropriate decorporation therapy. Studies involving the use of γ-emitting plutonium isotopes will aid in defining the early kinetics of plutonium in mammals, as well as allowing evaluation of the possible use of the short-lived non-alpha-emitting isotope ^{245}Pu for future possible studies in human volunteers.

ERDA RT-01-02
ANL 64100

MICRODISTRIBUTION STUDIES OF MONOMERIC AND POLYMERIC PLUTONIUM IN BEAGLE DOG LIVER AND BONE

John J. Russell, Principal Investigator
Arthur Lindenbaum, Participating Investigator

OBJECTIVES

The purpose of this work is (1) to define and quantify the cellular locations of plutonium deposition, their changes with time, and the possible pathological and histochemical changes induced by such deposits in liver and bone; and (2) to evaluate, by autoradiographic techniques, the cytogenetic abnormalities induced by plutonium distributed in mouse and dog gonads in hopes of establishing a risk factor for man.

BACKGROUND AND PREVIOUS FINDINGS

Earlier studies reported that the fraction of alpha tracks associated with different cell populations in the liver did not change significantly up to 1 year. A varying degree of association of hemosiderin deposits with plutonium was noted (Russell, J. J., and A. Lindenbaum, Health Phys. 29, 921, 1975).

D. Green et al. (Nature 255, 77, 1975) described the chromosomal aberrations induced in mouse testis stem cells by the nonhomogenous distribution of monomeric plutonium as demonstrated by a nonquantitative autoradiographic technique. Our technique, which is quantitative, is being used to refine measurements of the microdistribution of plutonium in mouse and dog gonads.

EXPERIMENTAL METHODS

A quantitative autoradiographic technique is used to measure the microscopic distribution of highly polymeric plutonium in mouse and dog liver and bone at 6, 90, 360, and 720 days after intravenous administration. This technique is also used to assay changes in the microdistribution of plutonium in gonadal tissue. Histochemical techniques are used to identify tissue changes of pathological concern that are often associated with plutonium deposits in liver, spleen, and bone marrow.

MAJOR NEW FINDINGS

From 6 days through 1 year, no significant change was found in the fraction of plutonium (as measured by alpha tracks) associated with liver parenchymal and littoral cells. The fraction of liver tissue that was devoid of alpha activity--we define this as the "discreteness index"--increased from 69% to 87% over this time period. Thus, we show that a large volume of the liver is not undergoing direct alpha irradiation. Such information indicates the need for a more rational approach to the problem of calculating the radiation dose delivered to the tissues by radionuclides like plutonium.

By 6 days following intravenous injection of monomeric plutonium into mice, approximately 99% of the plutonium burden in the testes is in the interstitial and tubular tissue; thus some of the stem cells that become sperm are within range of the alpha emissions. This work is a joint effort, with chromosomal abnormalities being evaluated by D. Grahn (see report by D. Grahn et al. in Section 11).

SIGNIFICANCE

The translocation of internally deposited plutonium, a known carcinogen, results in the irradiation of an increased fraction of cells in the animal organism. Microdistribution studies permit us to monitor the extent of translocation with time, to relate this process to delayed toxic effects, and to assess the effectiveness of experimental therapeutic procedures being devised for eventual human application.

PROPOSED COURSE OF THE PROJECT

(1) The extent of translocation of polymeric plutonium after 2 years in the dog liver, as well as any pathological effects, will be determined. (2) Autoradiography will be used to help elucidate the mechanism of DTPA action in liver, using ^{14}C-DTPA. (3) A short-term autoradiographic study of differences in microdistribution of plutonium isotopes differing widely in specific activity will be carried out as part of a cooperative effort with A. M. Friedman and J. C. Sullivan of the Chemistry Division (see preceding report). (4) In cooperation with D. Grahn, BIM, a combined cytogenetic, radiochemical, and autoradiographic study of the differential distribution of ^{239}Pu in mouse gonads will be continued. (5) The LR-115 technique, to be placed on a quantitative basis, will be used to locate the sites of deposition of both monomeric and polymeric plutonium in dog bones. This technique will be used also to assess the extent of removal or translocation of liver or skeletal plutonium induced by new therapeutic techniques under evaluation as part of the overall effort defined by this program. (6) Additional studies on the tissue distribution and tumor affinities of injected compounds of transition metals such as samarium-153 (Friedman, A. M., et al., J. Nucl. Med. 16, 528, 1975) are needed to determine the potential clinical usefulness of these compounds for tumor localization in man. (This work is being done in cooperation with A. M. Friedman and J. C. Sullivan, Chemistry Division; also E. W. Fordham, P. H. Shirazi, and G. V. S. Rayudu, Presbyterian-St. Luke's Medical Center, Chicago).

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4. NEUTRON AND GAMMA-RAY TOXICITY STUDIES

ERDA RT-03-01
ANL 60300

GROUP LEADER'S OVERVIEW

E. John Ainsworth, Group Leader

The focus of our Program is on late effects of neutron and gamma radiation and assessment of risk. Our principal research activities are in two complementary areas: (1) life-span experiments with large populations of laboratory mice to compare the effectiveness of single or protracted doses of neutron or gamma radiation for life shortening due to cancer and other debilitating noncancerous diseases; and (2) basic research on cellular injury and recovery for the evaluation of potential contributions of latent injury in the mouse circulatory, immune, and hematopoietic systems to life shortening, and for the comparison of late radiation effects in proliferating tissues. The data are used to test existing models and to formulate new models for prediction of radiation hazards and the relative biological effectiveness (RBE) of fission neutrons, particularly at low radiation doses.

In 1975 the last mice succumbed in our first late effects experiment, JM-2, and the initial analysis of mortality and pathology data was completed. Considerable effort was devoted to the irradiation of animals and collection of mortality and pathology data from ongoing experiments in the JM-3 series which, when completed, will permit accurate definition of the shapes of dose-response curves and RBE for life shortening and neoplastic and nonneoplastic diseases after single or protracted exposures to neutron or gamma radiation. Current experiments focus specifically on doses expected to produce 2-15% life shortening.

The shapes of the curves relating percent life shortening to radiation dose provide the following new information: (1) The neutron dose-response curve is nonlinear, with the life shortening effect decreasing from 3-4 day/rad to 1 day/rad with increasing dose over the range of 20-240 rad. Clearly, linear extrapolations from high neutron doses to estimate life shortening at low doses would underestimate risk; the underestimation is even greater when the enhancement of life shortening produced by fractionated neutron exposure, described previously by us, is also considered. These results from single neutron doses deviate from predictions of total dose dependency based on the predictive model of Kellerer and Rossi. (2) The shape of the gamma radiation dose-response curve is linear over the range of 90 to 788 rad; linear dose-response curves for gamma radiation have been described previously by others, but a quadratic function has been considered by some to be most applicable. We have developed a mathematical model that predicts that the shape of the

gamma dose-response curve may be linear or quadratic, depending on dose rate, whereas the neutron dose-response curve is curvilinear, and enhancement results from neutron dose fractionation.

New results on Harderian gland tumors in mice suggest this system is an excellent model for determination of dose-response relationship at neutron doses possibly as low as 5-10 neutron rad. Based on total incidence, the RBE at a single dose of 20 neutron rad is estimated at 16. The incidence, the time of appearance, and the degree of malignancy of Harderian gland tumors are dependent on dose and radiation quality; neutron irradiation increases both the incidence and the degree of malignancy of the tumors more markedly than gamma irradiation. Irradiation of mice with pituitary isografts, which raise the level of prolactin, produces a synergistic effect on incidence of this tumor. Experiments in progress will determine whether the hormone increases susceptibility to neoplastic transformation or acts as a promoter of tumor growth.

Damage to blood vessels may contribute significantly to degenerative changes in many tissues. Studies of late damage to the vascular system emphasize relationships between functional deterioration assessed by clearance of injected ^{133}Xe and morphologic changes observed by light or electron microscopy. Based on qualitative morphologic indices, neutron dose fractionation produces greater late vascular damage than does a single exposure to the same total dose. The RBE for vascular damage after single doses is greater than 3, and preliminary results indicate an RBE of approximately 10 under conditions of dose fractionation. Different ^{133}Xe clearance patterns are observed in long-term survivors of neutron or gamma radiation; measurement of blood pressure changes, soon to be initiated, may facilitate interpretation of the ^{133}Xe results. Ultrastructural changes in the mouse heart muscle and associated blood vessels show that some of the muscle degeneration observed at 3 months after neutron irradiation is repaired by 12 months. Also, damage to coronary arteries and arterioles is more severe at 12 than at 6 months and is associated with marked fibrotic changes. Other degenerative changes in mitochondria point to an involvement of enzymatic or metabolic processes. A collaborative study of the repair of blood vessel damage in the rabbit ear shows that undifferentiated cells are the principal cell type found in interstitial repair sites after X-irradiation.

Although radiation effects on humoral immunity have been studied extensively, less is known about late effects of radiation on cell-mediated immunity, especially in relation to carcinogenesis. Cellular immune competence in mice has been evaluated at various times after neutron or gamma irradiation by: (1) mitogenic response, i.e., the ability of immunocompetent cells to divide in response to a proliferative stimulus; (2) the capacity of lymph node cells to produce the graft-versus-host reaction; and (3) the susceptibility to respiratory infection with *Pasteurella pneumotropica*. New results from each test system show a significant deficiency in cellular immune competence, which persists for 200-400 days after single doses of 240 neutron or 790 gamma rad. Fractionation of the neutron dose produces greater damage to cellular immune competence than does the same single dose. In contrast, fractionation of the gamma dose produces less damage than does the same single dose.

Late effects of radiation on the hematopoietic stem cell (HSC) compartment in femur and spleen are assayed by the spleen colony technique. New results show that single or fractionated doses of 240 neutron or 855 gamma rad produce

a sustained depression of the stem cell population in femur and spleen, which probably persists for life. After graded single doses of neutron or gamma radiation, the late depression of the HSC compartment appears related to gamma dose but independent of neutron dose over the range studied. Late damage to the HSC compartment is reduced by neutron or gamma dose fractionation for interfraction intervals of 4 or 24 hours; thus cellular repair or proliferative state of hematopoietic tissues at the time of irradiation may play a role in late damage. Experiments in progress are attempting to establish whether the late depression of HSC population is due to residual injury to stem cells or to the stroma.

The mechanism of enhancement produced by neutron dose fractionation is a principal question on which ongoing and future studies are focused. Although relationships in any tissue between cell killing and potential for neoplasia or other late manifestations of injury are not known at this time, enhancement could result from increased cell killing following radiation-induced cell proliferation. This is one of several hypotheses that future studies of cell and tissue injury, as well as life shortening, will test. Since enhancement may also be produced by other high-LET radiations from internal emitters and from external radiation sources (some of which may be used in radiation therapy), an improved understanding of enhancement is important for radiation risk assessment and definition of the relationships among cell killing, tissue injury, and cancer.

Essential supportive dosimetry continues, and during this year analysis of our results from the International Neutron Dosimetry Intercomparison was completed. Other dosimetric studies concerned the correlation of measured depth-dose distributions in a hydrogenous phantom with dose distributions expected from Monte Carlo calculations. Short-term experiments evaluated the radiosensitivity of a transmissible murine leukemia line and determined the role played by the radiosensitivity and repair capability of clonogenic cells of the intestinal crypts in $LD_{50/6}$ differences in two mouse strains.

Much of our Program's scope and experimental capability has direct relevance to nonnuclear toxicology. In anticipation of future studies in this area in which pulmonary damage and respiratory disease will be important, an assay procedure for lung lymphocytes has been developed. Preliminary results indicate that most of the lymphocytes found in mouse lung originate from bone marrow (B cells).

NEUTRON AND GAMMA-RAY TOXICITY STUDIES STAFF

REGULAR STAFF

Ainsworth, E. John (Biologist)
Allen, Katherine H. (Scientific Associate)
Brennan, Patricia C. (Biologist)
Christian, Emily J. B. (Scientific Associate)
Cooke, Eugenia M. (Scientific Assistant)
Hulesch, Jane L. (Scientific Assistant)
Jordan, Donn L. (Scientific Associate)
Kickels, Wayne T. (Scientific Assistant)
Miller, Marietta (Scientific Associate)
Stearner, S. Phyllis (Biologist)

DOSIMETRY AND DATA MANAGEMENT STAFF

Borak, Thomas B. (Assistant Physicist)
Holmblad, Gordon L. (Scientific Associate)
Johnson, Emil G. (Engineering Assistant)
Trier, Joseph E. (Engineering Assistant)
Williamson, Frank S. (Physicist)

TEMPORARY STAFF DURING 1975

Crouse, David A. (Postdoctoral Appointee)
Scott, Bobby R. (Postdoctoral Appointee)
Yang, Vivian V. (Postdoctoral Appointee)

PUBLICATIONS

Ainsworth, E. J., R. J. M. Fry, P. C. Brennan, S. P. Stearner, J. H. Rust, and F. S. Williamson. Life shortening, neoplasia and systemic injuries in mice after single or fractionated doses of neutron or gamma radiation. Proceedings of the IAEA International Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press.

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ANL 60300

LATE EFFECTS OF NEUTRON OR GAMMA RADIATION

E. John Ainsworth, Principal Investigator
R. J. Michael Fry, [*] *Louise S. Lombard,* [†] *John H. Rust,* [‡] *Bobby R. Scott, and Frank S. Williamson, Participating Investigators*

OBJECTIVES

The objective of this Program is to obtain needed dose-response data on life shortening, cancer, late-occurring degenerative diseases, and cellular injury after neutron and gamma radiation. This information contributes to the development of models that assist radiation risk estimation for humans and that improve our understanding of how deleterious late effects of radiation, and/or other environmental substances, relate to cellular and tissue damage. The deleterious effects are appraised as a function of dose, dose rate, radiation type, duration of exposure, and age at exposure. We compare quantitatively the late effects produced by single and multiple exposures to fission neutrons from the JANUS reactor or to ^{60}Co gamma radiation to determine the relative biological effectiveness (RBE) for neutrons and the mechanism(s) that account for the increased effect of neutron radiation relative to gamma irradiation. The need is to identify any excess risk that results from occupational, therapeutic, or inadvertent exposure of populations at low radiation doses. The information obtained for fission neutrons could also contribute to risk assessments for other densely ionizing radiations such as alpha particles, pions, and heavy ions.

BACKGROUND AND PREVIOUS FINDINGS

Previous comparisons in other laboratories of the late effects of fission neutrons with those of gamma radiation in mice revealed: (1) the greater hazard (i.e., more life shortening and, in some cases, higher cancer rates) after neutron irradiation, and (2) the need for more complete information on the effects of dose, dose rate, and the fraction of the life-span over which the irradiation is given on the radiation hazard and the relative biological effectiveness for fission neutrons. The contribution of radiation-induced (or promoted) cancer to life shortening, particularly in the case of neutron-irradiated animals, was unclear from previous work. Accordingly, the highest priority in the present program was assigned to obtaining risk estimates for low doses of neutron or gamma radiation administered over long periods of time; the effects of single doses also required a more complete assessment. The last of the 10,000 animals involved in the first late effects experiment, initiated in 1971 and designated JM-2, died in November 1975. Several interim

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[*] Carcinogenesis Group.

[†] Carcinogenesis and Radiation Toxicity in Dogs Groups.

[‡] Consultant, University of Chicago.

reports of the mortality results have been published (Ainsworth, E. J., et al., Proceedings of the 2nd European Symposium on Late Effects of Radiation (ESLER TWO), Casaccia, Rome, 1972, in press; Ainsworth, E. J., et al., Proceedings of an IAEA Seminar on the Biological Effects of Neutron Irradiation, Vienna, IAEA-SM-179/1, 1974, pp. 359-379; Ainsworth, E. J., et al., Proceedings of the International Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press).

Briefly, the results showed that: (1) Under conditions of fractionated exposures administered over 6 months, the neutron RBE for life shortening is approximately 10; 24 weekly doses of 3.3 neutron and 36 gamma rad produce similar life shortening. (2) In the case of single doses, RBE for life shortening is dose dependent and is greater than 4.5 at 20 rad and is less than 3.6 at 240 neutron rad. (3) When a gamma radiation dose is given in many small fractions rather than as a large single dose, the result is a sparing effect (reduction) on life shortening; the extent of this sparing effect is approximately 3-fold. (4) When a neutron dose is given in many small fractions, rather than as a large single dose, life shortening and cancer incidence are increased, not decreased as was the case for gamma radiation. (This enhancing effect is statistically significant in both sexes at a total dose of 240 rad and in males, but not females, at a total dose of 80 rad). (5) A high incidence of lethal lung tumors occurs in our irradiated animals. We were concerned that the enhancing effect of neutron dose fractionation might be due to the response of this single type of tumor; since analyses of our results show that the enhancement is still observed when mice dying from lung tumors are removed from the test sample, we conclude that causes of death other than lung tumors are also enhanced by neutron dose fractionation.

EXPERIMENTAL METHODS

Male and female hybrid B6CF$_1$/An1 mice, bred in this Laboratory, are exposed to ^{60}Co gamma radiation, or to fission spectrum neutrons from the JANUS reactor. Young adult animals 110-120 days of age receive either a single exposure or a sequence of fractionated radiation doses lasting up to 6 months. Age at the time of exposure is one variable in our experiments, so some groups receive a single dose at approximately 180 or 270 days of age. When programmed irradiations are completed, the animals are placed in a "geriatric room" where they are observed 12 times per week, and animals with visible or probable tumors, or showing signs of other diseases, are isolated. Mice are killed and autopsied when considered terminally ill, or are autopsied after natural death. Computer systems described elsewhere (Williamson, F. S., ANL-7870, 1971, p. 13) direct the loading of experimental animals and other irradiation procedures. Dosimetry information is stored in computer files, as are mortality, autopsy, and histopathology data. Various computer programs are also used for reduction, analysis, and evaluation of the various kinds of data generated by this program.

MAJOR NEW FINDINGS

New findings emerge slowly and sequentially from radiation toxicology studies. Mortality data become available first, then information on gross pathology and microscopic pathology.

Based on life shortening results from three single doses of neutron or gamma radiation, RBE is dose dependent and is markedly higher at low doses than at high doses; the range in RBE is from 7.6 at 20 neutron rad to 2.0 at 240 neutron rad. Older animals are more susceptible to the life shortening effects of neutron than to gamma radiation.

Results from these experiments are not sufficient to determine the relationship between dose and RBE for life shortening under conditions of long-term irradiation. This information will become available from ongoing experiments, but at a weekly dose of 3.3 neutron rad, we have found that the neutron RBE is 13 and 10, respectively, for males and females. In the past, straight lines have been fitted to curves which related neutron or gamma dose to percent life shortening, i.e., linear dose-response curves. A linear relationship appears adequate for the gamma radiation results, but the shape of the neutron dose-response curve is clearly nonlinear with effectiveness decreasing with increasing dose.

New information on cancer responses shows that lung tumors are not the only cancers that show an increased incidence after neutron dose fractionation. In the Harderian gland, a small secretory gland located behind the eye, neutron dose fractionation produces a higher tumor incidence and greater malignancy than does the same total dose given in a single exposure. In mice receiving a single neutron dose, the percent lethal Harderian gland tumors increased with increasing dose; this dose-dependent response did not occur after gamma radiation. Not all tumors respond with an enhanced incidence to neutron dose fractionation; the incidence of mammary gland tumors is not increased by fractionation. Comparisons between neutron- and gamma-irradiated animals suggest: (1) the time between irradiation and appearance of thymic, lung, and Harderian gland tumors may be shorter after neutron irradiation, (2) a higher percentage of lung and Harderian gland tumors are lethal after neutron irradiation, and (3) neutrons are approximately 17 times as effective as gamma radiation in producing Harderian gland tumors.

MODELLING

Modelling is an important component of our Program, and a new model has been developed to account for radiation-induced life shortening in mice at low and moderate doses. The kinetics of production of reversible and irreversible injury after neutron or gamma radiation is considered in the model. Radiation-induced life shortening is assumed to be a consequence of the irreversible injury. Predictions of the model are consistent with existing neutron data, including enhancement of life shortening after neutron dose fractionation and curvilinear relationship between (single) neutron dose and percent life shortening. The model also considered an effect of gamma radiation dose rate on life shortening; consequently, RBE values at various gamma dose rates may be estimated. Other calculations indicate that the shape of the curve relating gamma dose to life shortening is influenced by dose rate.

SIGNIFICANCE

The major thrust of our research is in two areas: (1) the testing of theoretical and predictive models useful for the assessment of human radiation

risks at low doses (since the present data on life shortening and cancer are not explainable by existing models, the development of new models is now necessary); and (2) the obtaining of new information to explain the high RBE for fission neutrons in producing life shortening.

Results of our work will have impact on (1) estimation of cancer risk, life shortening, or other diseases after low doses of neutron or gamma radiation sustained at low dose rates; (2) assignment of the Quality Factor to be used in risk assessment for fission neutrons (and perhaps other related radiations); (3) estimation of late damage to normal tissues that may be produced during radiation therapy; and (4) understanding relationships between tissue injury and induction or promotion of cancer.

Our results show that the dose-response curve for life shortening produced by fission neutrons is not linear. Consequently, a linear prediction of neutron hazard would underestimate risk at low doses and overestimate risk at high doses. In addition, if greater hazard results from dose fractionation or low neutron dose rates at low doses, the use of single-dose risk estimates will underestimate the radiation hazard.

In terms of an enhanced hazard associated with neutron dose fractionation, the present findings are qualitatively similar to observations in humans and mice irradiated internally with alpha particles from injected radioisotopes. More bone tumors result from many injected low doses of alpha-emitting isotopes than from one or a few injections. The evidence suggests enhancement is a general phenomenon when fission neutron or related radiations are involved. Our findings have implications relating to cell injury and carcinogenesis, hazard evaluation at low radiation doses, and radiation therapy of cancers with neutrons or related radiation such as pions.

PROPOSED COURSE OF THE PROJECT

The priorities for future research are: (1) to continue analysis of the mortality and pathology data from the 10,000 mice in our initial experiment, JM-2 (histopathological diagnosis is completed on 3000 mice, and tissues from an additional 3000 are ready for evaluation); (2) to continue four experiments in progress which will provide the needed additional dose-response information on life shortening and cancer rates in $\sim$ 15,000 mice so that curve shapes can be described accurately and used for radiation hazard prediction at low doses by means of mathematical models; (3) to initiate new experiments at lower single and protracted doses of neutron and gamma radiation (data from ongoing experiments show more life shortening than was originally anticipated, and predictive models will benefit from new information at doses that produce less than the 5-9% life shortening we observe at the lowest doses used currently); (4) to initiate new experiments with neutron-irradiated mice to evaluate independently the role of dose per fraction and time between radiation fractions on life shortening, cancer, and tissue damage; this is necessary since no existing model for life shortening or cancer predicts the effects of neutron dose fraction observed in our initial experiments; (5) to initiate studies with a much longer-lived mouse, *Peromyscus leucopus*, to determine the extent to which predictive models derived from experiments with conventional laboratory mice have relevance to other species and, it is hoped, to man.

The results of the experiments outlined above will be definitive; no additional large-scale mouse experiments will be needed solely for radiation risk assessment. Any further new thrusts would focus on additional interspecies comparisons and could involve *P. leucopus* and the beagle. The experience gained in long-term radiation toxicity studies will facilitate future toxicological studies with other energy-related pollutants.

Time goals for completion of our radiation experiments are: (1) analysis of tumor data from JM-2, 1977; (2) interim evaluation of mortality data after single or fractionated doses of neutron or gamma radiation (experiments JM-3 and JM-4) administered over 23 weeks, 1977; (3) interim evaluation of tumor data from JM-3 and JM-4, 1978, and complete evaluation, 1979; (4) interim evaluation of mortality data from mice irradiated for 60 weeks (JM-7) and for duration of life (JM-8), 1978; (5) completion of mortality data from JM-7 and JM-8, 1980; (6) complete evaluation of all tumor data from JM-7 and JM-8 by 1981. Time goals are difficult to assign for the two experiments which have not begun: (1) experiments with $\sim$ 2500 $B6CF_1$ mice to evaluate neutron dose per fraction and interfraction time, and (2) studies with 1000 *P. leucopus* for interspecies comparison; we estimate that all mortality and tumor data should be analyzed from the experiment with $B6CF_1$ mice by 1980; and by 1982, most mortality and tumor data from the *P. leucopus* experiment should be reduced.

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PROGRESS IN DATA MANAGEMENT AND ANALYSIS

Frank S. Williamson, Principal Investigator
Martin R. Kraimer, Participating Investigator*

OBJECTIVES

Our objective is to provide means of storing all data relevant to the JANUS Program, and of data retrieval and analysis.

BACKGROUND AND PREVIOUS FINDINGS

Previous work has resulted in the creation of files, and computer programs, to record assignments and other vital data, and irradiation parameters pertaining to repeated exposures of each cage of mice, in experiments JM-2 to JM-8. The status of 11 animal rooms is maintained in direct-access storage, and programs permit the reservation and confirmation of space, relocation of cages, and subtraction of dead animals.

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*Computer Scientist, Administrative Staff.

Programs to calculate survival times and plot cumulative mortality use fixed-interval methods. Mean survival times are calculated using 1-day intervals, with results which are indistinguishable from those obtained by the Kaplan-Meir or Hoel-Walburg methods (Hoel, D. G., and H. E. Walburg, Jr., J. Natl. Cancer Inst. 49, 361, 1972).

EXPERIMENTAL METHODS

Assignment of animals to living space, and to locations in irradiation facilities for exposure, are at random and are made by computer. Histories of these operations are maintained without human data input except dates or dosimetry monitor parameters. Data that must be entered manually are entered by a secretary whenever possible, using a computer terminal similar to the standard Selectric typewriter. Computer programs are written in the PL/I programming language and use the BIMFILE file management system (see Williamson, F. S., et al., in Section 13 of this report).

MAJOR NEW DEVELOPMENTS

Approximately 250 computer runs to calculate and plot mortality parameters were made during the year. A new program was written to experiment with exponential spline fits to cumulative mortality data, with calculations of the first derivative (death rate) for plotting. A program variant allows selected life table data to be saved in a form that can be retrieved by ANL's SPEAKEZ language for experiments with trial algorithms.

Interactive procedures were developed to streamline file backup and maintenance and also irradiation scheduling, animal assignments, and mortality calculations. These procedures make it practicable for persons with minimal computer experience, but with knowledge of the experimental goals and protocols, to handle the data.

Autopsy data for the JM-2 experiment are now being built into a file, and programs have been written to handle histological data. Histological coding has been started.

SIGNIFICANCE

Several innovative techniques have been developed for JANUS data management, and have withstood 5 years of use with gratifyingly little need for change. These techniques are designed to minimize the need for human record keeping, and to detect errors through the use of redundancy. They will be equally valuable in the management of any long-term, large-scale, animal toxicology experiments.

PROPOSED COURSE OF THE PROJECT

Data files will be converted to the BIMFILE-2 format to permit use of the enhanced facilities now available. Batch entry of autopsy and histology data from computer terminals will be supported, and work will be done toward on-line interactive data entry of these data at the bench.

An analysis program system is under development. This is flexible and open-ended, and will allow specifications of complex logical selections of data from multiple files with the specification of the analysis program to be used. At this point it will be a quick and simple matter to add new analytical algorithms as they are devised.

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RADIATION EFFECTS ON HOST IMMUNOLOGICAL FUNCTION

Patricia C. Brennan, Principal Investigator
David A. Crouse, Donn L. Jordan, Wayne T. Kickels, and
Richard C. Simkins, Participating Investigators*

OBJECTIVES

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

BACKGROUND AND PREVIOUS FINDINGS

Relationships between alterations in the functional status of the immune system and either susceptibility to cancer induction or proliferation of neoplastic cells are subjects of intense interest and speculation (Prehn, R. T., Clinical Immunobiology, Vol. 2. Eds. F. H. Bach and R. A. Good. Academic Press, New York, 1974, p. 191). Radiation effects on humoral immunity, i.e., circulating antibodies, have been studied extensively (Jaroslow, B. N., Medical Radiation Biology, Eds. G. V. Dalrymple, et al. Saunders Co., Philadelphia, 1973, pp. 198-202), but much less is known about cell-mediated immune competence, specifically the ability of subpopulations of T cells to destroy foreign cells, in relation to radiation carcinogenesis. The few published reports on the effects of radiation on cell-mediated immune function relate to the *in vitro* radiosensitivity of lymphocytes to gamma or X-irradiation or to the very early *in vivo* effects of gamma irradiation. Virtually nothing is known about the late effects of chronic low-level exposure to gamma radiation or about the acute or chronic exposure to neutron irradiation.

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*Animal Facilities Staff.

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 some preliminary observations on the proliferative response of T cells and B cells (bone marrow-derived) (Ainsworth, E. J., et al., Proceedings of the IAEA Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press). Briefly, we found that both the number and the proliferative ability of T cells are substantially below normal for as long as 44 weeks after gamma irradiation and 60 weeks after neutron irradiation. We observed a transient recovery in T-cell responsiveness at 21-28 weeks after irradiation and in B-cell responsiveness at 8-10 weeks after irradiation. T cells from gamma-irradiated animals were more severely affected at earlier time points, but were more responsive than cells from neutron-irradiated mice at later times. B cells from gamma-irradiated mice were consistently less responsive to the mitogen than cells from neutron-irradiated mice.

Enhanced susceptibility to experimental respiratory infection following chronic exposure to low-level gamma radiation has been reported, but no similar data exist for neutron-irradiated animals. Consequently, the study of altered host susceptibility to respiratory infection constitutes an important aspect of the JANUS Program. Previously (Ainsworth, E. J., et al., Proceedings of an IAEA Seminar on the Biological Effects of Neutron Irradiation, Vienna, IAEA-SM-1791/1, 1974, pp. 359-379; Brennan, P. C., et al., ANL-75-30, 1974, p. 62), we reported that: (1) respiratory challenge with *Mycoplasma pulmonis* 5-21 days and 6 weeks after a single exposure of neutron or gamma radiation, and 12-42 weeks after fractionated exposure, reveals that neutron-irradiated mice are more susceptible to infection at all time intervals tested, and that the effect is more pronounced at later times; and (2) the clearance of a challenge dose of *Pasteurella pneumotropica* is more impaired in neutron-irradiated than in gamma-irradiated mice; immunofluorescence data suggest that whereas pulmonary macrophages in the irradiated host are capable of engulfing invading *P. pneumotropica* cells, the ability to kill them is impaired.

The lymphocytic leukemia (L1-V) originated in an aged, irradiated $B6CF_1$ mouse. The history of passage, morphology, and cytological studies has been described earlier. Aged, irradiated $B6CF_1$ mice are even more susceptible to transplantation and lethality of the tumor than are aged, unirradiated controls [Ainsworth, E. J., et al., Proceedings of the 2nd European Symposium on Late Effects of Radiation (ESLER TWO), Casaccia, Rome, 1972, in press]. Preliminary studies on the radiosensitivity have also been reported (Evans, T. C., et al., Radiat. Res. 62, 550, 1975).

EXPERIMENTAL METHODS

The T-cell population of lymphocytes is primarily involved in cell-mediated immunity, whereas the B-cell population is involved in the production of humoral antibodies. Several experimental approaches are used to detect changes in these lymphocyte populations: (1) enumeration of the number of spleen T and B cells (Brennan, P. C., and B. N. Jaroslow, Cell. Immunol. 15, 51, 1975); (2) measurement of the proliferative response to the T-cell mitogens, concanavalin A (Con A) and phytohemagglutinin (PHA), and the B-cell mitogen, bacterial lipopolysaccharide (LPS), by standard techniques; and (3) a modification of the technique described by Bennet (Transplantation 11, 158, 1971) whereby the ability of lymph node cells to evoke an *in vivo* graft-versus-host (GVH) reaction is quantitated.

Host response to respiratory challenge with *M. pulmonis* and *P. pneumotropica* is determined using standard techniques. During this reporting period construction was completed of a modified Henderson aerosol generator apparatus (Henderson, D. W., J. Hyg. 50, 53, 1952) embodying novel features; this system was utilized in preliminary experiments to characterize the aerosol dose of *P. pneumotropica*.

The radiosensitivity of the radiation-induced lymphocytic leukemia was determined using standard spleen colony techniques with *in vitro* exposure of the cells or split-dose exposure of the host and cells. Techniques for the *in vitro* culture of the cell line, frozen storage of the cells, and clarification of the viral etiology of the tumor were also evaluated.

MAJOR NEW FINDINGS

Following a single exposure of 80 neutron or 269 gamma rad, there was no significant loss of GVH reactivity for about 7 days; however a steep decline then occurred with a nadir between 11 and 21 days. By 45 days after irradiation, the gamma-irradiated mice responded at near control levels, whereas the neutron-irradiated mice responded significantly better than controls. In contrast to the allograft reactivity of lymph node cells, the responsiveness of spleen cells to mitogens was depressed 1 day after exposure and remained significantly below normal for 11 days, with a gradual return to normal by 18 days after irradiation. After single high doses (240 neutron and 807 gamma rad), there was an immediate and complete loss of GVH reactivity and mitogen responsiveness which persisted for 2 weeks. GVH reactivity was impaired until 10 weeks after exposure, to a greater extent in gamma than in neutron-irradiated mice. Three weeks after exposure, there was a return to normal in the spleen B-cell response to LPS but not in the T-cell response. In contrast, the response of cells from gamma-irradiated mice was depressed for all mitogens 3 weeks after exposure.

Preliminary results from a comparison of fractionated versus single exposure indicate that allograft reactivity and spleen cell responsiveness are more severely impaired by fractionation of the neutron dose. These results are apparent between 1 day and 24 weeks after the last fraction. On the other hand, a significant sparing effect is observed when the gamma dose is fractionated.

In preliminary experiments to characterize the aerosol dose of *P. pneumotropica* delivered by the modified Henderson apparatus we found that: (1) 70% relative humidity during aerosolizing is optimum for *P. pneumotropica* survival, (2) 90-100% of challenged mice can clear a dose of 10^5-10^6 cells in 4 days, and (3) mice must be examined individually because the occasional mouse that does not clear the challenge influences dramatically the results of pooled lung samples. Using these findings as a basis, we then assessed the ability of mice to clear an aerosolized challenge of *P. pneumotropica* following single or fractionated doses of neutron or gamma radiation. The bacterial challenge was administered 2 weeks after the last fraction, and 26 weeks after a single dose. Mice irradiated with single doses of either neutron or gamma radiation cleared the organism satisfactorily (i.e., in 4 days), whereas mice that received the same total dose in 24 fractions did not.

During this reporting period we have developed methods to obtain bronchial-associated lymphocytes from the murine lung. Immunofluorescence staining shows that ∿ 15% of these lymphocytes are T cells and the remainder are B cells.

We have determined that neutrons are twice as effective as X-rays in killing leukemic L1-V cells. Normal spleen cells are about twice as sensitive as L1-V cells to either X-ray or neutron exposure. The tumor has been adapted to *in vitro* culture conditions and retains its neoplastic charactistics after *in vitro* passages. Sonicated preparations of tumor cells that contain no viable cells are also neoplastic, indicating the possible presence of leukemia virus.

SIGNIFICANCE

One of the goals of the JANUS Program is to improve our understanding of the cellular perturbations that contribute to late effects. We consistently demonstrated late effects of both neutron and gamma radiation on several characteristics of cell-mediated immunity, at times later than 300 days after irradiation. The relationship between these changes in immune competence and either sensitivity to tumor induction or tumor progression is presently unclear. However, it seems reasonable to assume that the late effects we observe have a direct bearing on radiation carcinogenesis, as well as on neoplastic processes induced or promoted by other environmental insults.

The results of respiratory challenge provide new information on the late effects of neutron or gamma radiation on another host defense mechanism. Furthermore, the techniques developed are directly applicable to the study of early and late effects of air-borne pollutants on this end point.

PROPOSED COURSE OF THE PROJECT

A major effort will be made to characterize the radiation-induced deficiency in immune competence by determining which cell subpopulations are primarily responsible. Homing techniques, specific lysis of subpopulations, and irradiation during the development of immune competence will be used to delineate these subpopulations. Attempts will also be made to restore cellular immune function by transplants, in order to evaluate the role of the immune system in radiation carcinogenesis.

Because high priority is being given to studies of immune competence, less effort will be devoted to developing a model system to evaluate the factors affecting pulmonary defense mechanisms.

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CARDIOVASCULAR RESEARCH

S. Phyllis Stearner, Principal Investigator
Vivian V. Yang, Emily J. B. Christian, Rosemarie L. Devine, and Thomas B. Borak, Participating Investigators*

OBJECTIVES

The objectives of these studies are to determine long-term (late) radiation effects on the cardiovascular system and to compare these effects with normal aging changes. Late radiation damage to this system is an important contributing factor to the degenerative changes in all tissues of an animal. Our investigations are designed to determine changes in the microvasculature at intervals in the living mouse, to compare these with electron microscopic findings, and with functional changes in capillary efficiency. Studies are also in progress to determine concomitant changes in blood pressure. The repair capacity of the vascular wall following radiation injury is of concern in estimations of radiosensitivity of the microvasculature. It has received attention in a collaborative study with the Department of Radiology, University of Chicago, Chicago, Illinois (M. L. Griem and G. E. Dimitrievich). We have initiated electron microscopic studies of the microvasculature in rabbit ear chambers in order to identify the cell aggregates that appear along vessel walls within a few days after irradiation.

As an extension of radiation studies on the peripheral vasculature in the mouse, electron microscopic observations of the irradiated heart are in progress. Effects on myocardial blood vessels and on cardiac muscle are evaluated with respect to age and radiation treatments.

BACKGROUND AND PREVIOUS FINDINGS

The microvasculature of loose connective tissue has been used by Lindop and co-workers as a model system for the study of radiation effects on non-specialized blood vessels. Our *in vivo* observations of the pinna indicate thickened vessel walls and irregular diameters with some nonfunctioning capillaries at 6-12 months after irradiation. At later times, some vessels were tortuous and capillary networks appeared decreased. Electron microscopic examination revealed degenerative changes in small arteries and veins 20 months after either single or fractionated neutron exposures of 240 rad (at this time there was about 50% mortality in the group). Fibrosis in neutron-irradiated mice was apparent with both light and electron microscopy.

Functional decreases in capillary efficiency (blood flow) were observed at 24-30 months after irradiation; but after 30 months, when control mortality exceeded 50%, capillary efficiency increased. Long-term observations of

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*Carcinogenesis Group.

clearance rates showed a decreased capillary efficiency in gamma-irradiated mice, with no clear dose dependence (269 and 780 rad), but no decrease in capillary efficiency after 240 neutron rad. In all irradiated groups, as in controls, an increase in capillary efficiency was observed toward the end of the life-span when group mortality exceeded 50%.

The rabbit ear chamber has been used as a site for *in vivo* study of repair of radiation damage to the microvasculature in the living animal (Dimitrievich, G. E., et al., Radiat. Res. 62, 610, 1975). Electron microscopic study of such aggregates was initiated in order to elucidate the nature of the cell type.

In general the heart is considered to be fairly resistant to irradiation and has received little attention in connection with studies of relatively low-level, total-body radiation exposures. However, radiation effects on the heart are recognized as a possible cause of complications associated with various procedures in radiotherapy, and late cardiac muscle damage has been described after experimental irradiations of mouse and chicken. Previous electron microscopic studies have been limited to partial body exposures in the mouse and rabbit over periods of less than 6 months.

EXPERIMENTAL METHODS

Animals, irradiation procedures, and schedules were those used in all experiments in the JANUS program. The methods in our cardiovascular studies include (1) direct microscopic observations of the circulation, (2) electron microscopic study of the microvasculature of the mouse pinna, and (3) clearance studies with radioactive xenon, ^{133}Xe, to determine alterations in capillary function.

For electron microscopic studies of radiation damage to the microvasculature in the rabbit ear chamber, transparent chambers, into which a network of blood vessels penetrates, were prepared and X-irradiated in the Department of Radiology, University of Chicago, by G. S. Dimitrievich. Tissue samples were taken from unirradiated chambers and from chambers exposed to 400 or 700 rad of 250 kV X-rays and were prepared for electron microscopic study. Entire blood vessels were serially cut in cross section.

For electron microscopic study of long-term radiation effects on the heart, treatment groups included single doses of 780 gamma rad or 240 neutron rad and 24-week fractionated doses of 855 or 2850 gamma rad, or 240 neutron rad.

MAJOR NEW FINDINGS

In vivo observations after single and fractionated neutron doses suggested an enhancement of damage with fractionated treatments. Although present studies are not sufficiently extensive to permit quantitative evaluation of changes, greater injury after fractionated neutron dose is consistent with the enhancement of life shortening and tumor incidence in similarly treated mice in other experiments in the JANUS program. At 20 months after neutron treatment, the endothelium was relatively unaltered in surviving functioning blood

vessels, when compared to controls of the same age. The minimal endothelial cell changes may reflect earlier repair of injury, but no evidence of excess proliferation was seen. Smooth muscle degeneration, especially of arterioles, was more severe in neutron-treated animals. In contrast to the vascular damage seen in neutron-treated mice at 20 months, a lesser amount of damage was seen after gamma-ray treatment. Electron microscopic study of small blood vessels in the pinna showed less prominent changes, especially in the walls of small arteries. Of special interest was the apparent endothelial proliferation in arterioles, to the extent of obstructing blood vessels in some instances. These electron microscopic findings are consistent with the increased vascular tortuosities observed with the light microscope.

Evaluation of changes in capillary efficiency in neutron-irradiated groups revealed evidence of an irregular dose relationship. In groups exposed to 20 rad single dose, there was no apparent difference from controls through about 24 months. Thereafter, however, there was a more rapid increase in capillary efficiency which can be correlated with the shorter life expectancy resulting from this radiation exposure. After exposure to 80 rad, however, there was a decreased capillary efficiency similar to that observed in gamma-irradiated mice. After the highest neutron exposure (240 rad), there was no decrease in capillary efficiency; instead an increased capillary efficiency was noted throughout the period from 12 months to the end of the life-span at 24 months.

Electron microscopic study of tissue from rabbit ear chambers showed that the cell aggregates in irradiated tissue were located in the interstitial region subjacent to the endothelium lining the blood vessel. The aggregated cells had morphological characteristics of relatively undifferentiated mesenchymal-type cells. Such cell aggregates were less frequently observed in controls; they may represent postirradiation proliferation that results in the initial intraluminal bulges observed by light microscopy.

Ultrastructural changes in heart muscle and associated blood vessels in the mouse were noted from 4 days to 1 year after exposure. Treatment groups studied to date include those that received single-dose, total-body exposure to 240 neutron rad or 780 gamma rad. Heart muscle showed areas of degeneration at 4 days. Damage was most severe at 1-3 months, and was less prominent at 6-12 months. Small blood vessels also showed the greatest amount of damage at 1-3 months, but microvascular damage was still abnormally high at 6-12 months. Larger coronary vessels, especially arteries and arterioles, showed more degenerative changes at 12 months. Qualitative differences between neutron- and gamma-treated groups were limited to some increase in fibrosis after neutron exposure. Quantitative estimation of muscle and capillary damage revealed that injury was not significantly different in the two irradiated groups, but that both were different from the control. Degenerative changes in mitochondria, as well as changes in their size and number, suggest that enzymatic differences may be present in the irradiated heart.

SIGNIFICANCE

Cardiovascular changes in the irradiated mouse may contribute to tissue damage and life shortening. Moreover, information on cardiovascular injury has relevance to normal tissue damage sustained during radiation therapy. In

studies of the microvasculature in the pinna at 18-20 months after irradiation, the endothelium is largely intact, but smooth muscle degeneration is prominent. This difference may be the result of a greater degree of endothelial repair. Prominent smooth muscle damage to small arteries, especially after fractionated neutron doses, are significant in long-term risk assessment.

Degenerative changes in the heart within the first 3 months after 240 neutron or 780 gamma rad indicate that this organ is more radiosensitive than previously recognized. Prominent coronary artery degeneration, noted at 12 months, may be expected to increase in severity at later times. The possible relation of early damage to later degenerative diseases clearly requires special effort. Differences in arteriole sensitivity to injury can influence long-term radiation effects and will receive attention in these investigations.

Suggestions of qualitative differences between late effects of gamma and neutron irradiation are indicated not only from results of capillary efficiency estimates but from structural studies as well. The increased capillary efficiency observed at later times after irradiation, especially in neutron-treated animals, suggests a hypertensive condition may be present.

PROPOSED COURSE OF THE PROJECT

Comparison of ultrastructural changes in the microvasculature of the pinna after neutron and gamma irradiation will be extended, with samples taken at earlier times after exposure to determine the sequence of development of late lesions. Information about the development of late ultrastructural changes will permit correlations to be made with microscopic observations in living animals and also with the result of vascular functional studies (both capillary efficiency and blood pressure estimations). Electron microscopic studies of the heart will be extended to fractionated dose groups, and all treatment groups (single and fractionated) will continue to be sampled as long as survivors remain. Special interest centers around the comparison of radiation and aging changes in the heart and the major vessels during the latter part of the life-span. Morphological evidence of damage to mitochondrial membranes suggests that attention be given to the assay of various mitochondrial enzymes in order to see whether there are biochemical lesions that can be correlated with the morphological changes. Tissue from the irradiated rabbit ear chamber will be evaluated as to fine structural characteristics of the cell type associated with what appears to be repair sites surrounding the microvasculature following radiation exposures. The interrelations of these cell aggregates (repair sites) with other cells and structures in the vessel wall, the basement membrane in particular, will be studied.

ERDA RT-03-01
ANL 60300

LATE RADIATION DAMAGE TO THE HEMATOPOIETIC SYSTEM

E. John Ainsworth, Principal Investigator
David A. Crouse, Eugenia M. Cooke, Jane L. Hulesch, Marietta Miller, and Roy M. Vigneulle, Participating Investigators*

OBJECTIVES

This project proposes: (1) to evaluate the contribution of late injury in the hematopoietic system to life shortening, leukemia, and deficiences in immune responses; and (2) to study fundamental processes of radiation injury and repair and cell repopulation using the response of the hematopoietic stem cell as the model system.

BACKGROUND AND PREVIOUS FINDINGS

Previous work (Upton, A. C., et al., Radiat. Res. 41, 469, 1970) showed that at some radiation doses as much life shortening was observed in animals without tumors as in animals with tumors that could have contributed to death. Late damage in tissues or organs concerned with blood cell formation, i.e., the hematopoietic system, could contribute significantly to illness or death in various ways. Deficiences in blood cell production could result in anemia and increased susceptibility to infections. The immune system could also be compromised, since the most progenitive (mother) cell in the bone marrow, the hematopoietic stem cell (HSC), produces daughter cells essential for immune responses. Also, it is the HSC which, in response to virus or other events, becomes transformed into a leukemic stem cell; consequently, studies of the late effects of radiation on the HSC population have relevance to leukemia.

Previously reported results concerned with both the early (1-3 months) and late (1-2.5 years) damage to the hematopoietic system after neutron or gamma radiation are: (1) Based on early damage and repopulation of both HSC and circulating blood cells, no single RBE holds for all indices of hematopoietic injury (Miller, M., E. M. Cooke, and E. J. Ainsworth, Radiat. Res. 51, 473, 1972). (2) Multifractionation of a neutron or gamma dose (over 3 weeks) reduces hematopoietic injury during the first month after irradiation; the reduction of damage is less for neutron than for gamma radiation; and dose fractionation does not result in an earlier return to "normal" blood counts after either type of radiation (Miller, M., E. M. Cooke, and E. J. Ainsworth, Radiat. Res. 59, 50, 1974). (3) Neutron irradiation at a low dose rate results in an early increase in blood counts during the first 2 weeks after irradiation in comparison with the responses of mice irradiated at a high dose rate. (4) A significant deficiency ($\sim$ 50%) in the HSC population is observed in both neutron- and gamma-irradiated animals, and at certain ages a greater reduction results from dose fractionation with either radiation in comparison with the effect of a single dose. (5) In spite of the deficiency in HSC, blood counts are normal, with exception of platelets which are reduced significantly.

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*Laboratory Graduate Program Participant, University of Illinois, Urbana.

EXPERIMENTAL METHODS

B6CF$_1$/Anl mice were irradiated with fission neutrons from the JANUS reactor or with ^{60}Co gamma radiation using methods and dose measurements described elsewhere (Ainsworth, E. J., et al., Proceedings of an IAEA Seminar on the Biological Effects of Neutron Irradiation, Vienna, IAEA-SM-179/1, 1974, pp. 359-379). Blood counts or cell counts of marrow or of spleen samples were made with a Coulter Counter by conventional methods (Ainsworth, E. J., et al., ANL-8070, 1973, p. 13). The stem cell content in bone marrow, spleen, or blood was assayed by tissue transplantation procedures (Ainsworth, E. J., and R. M. Larsen, Radiat. Res. 40, 149, 1969).

MAJOR NEW FINDINGS

After fractionated neutron irradiation (24 doses of 3.3 rad administered over 23 weeks), greater injury results in the HSC population in the bone marrow than in the spleen. Migration of stem cells via the blood may influence the HSC content of either the marrow or spleen, and the blood content of stem cells is now being assayed to understand better the redistribution phenomena. After a higher weekly dose, 10 rad per week for 23 weeks, both the spleen and the femur HSC populations are reduced at 1 or 2 days after the last radiation dose, and very little recovery occurs thereafter. In female mice no significant increase in the bone marrow HSC population occurs between 1 day after the last radiation dose and the subsequent 30 days; in males a small increase in HSC content occurs. The relationship between the absence of HSC repopulation in females and their greater life shortening in comparison with males is unknown at this time.

The late damage to the HSC population produced by a single dose of neutron or gamma radiation differs quantitatively. In neutron-irradiated mice the HSC repopulation observed late in life is not influenced by the number of HSC killed by the initial radiation dose, but some dose-dependence is observed after gamma irradiation. The explanation for this phenomenon is not known, and the needed information concerns the possible contribution of damage to connective tissue and blood vessels as compared with injury to the stem cells or the control systems that influence the size of the HSC population.

Split-dose experiments have been conducted to evaluate the effect of repair and the proliferative state of the marrow on late damage to the HSC compartment. In comparison with the late effect of the same single dose, less damage was observed when the doses were separated by 4 or 24 hours. When the interval between fractions was 14 days, the femur HSC content was lower than was observed after the same single dose. Both cellular repair processes and the number of HSC in cell division at the time of irradiation may influence late damage to the stem cell compartment.

Peripheral blood counts fail to convey fully the residual damage to the hematopoietic system, since "adjustments" are made whereby nearly normal blood counts are maintained by aged, irradiated animals. We find normal white blood cell counts at long times after a single dose of neutron or gamma radiation, but we now have evidence for anemia and problems in hemoglobin synthesis. These "adjustments," perhaps involving more cell divisions in the stem cell compartment, may be related to an increased risk of cancer to the blood-forming tissues.

SIGNIFICANCE

The relationship between early radiation damage, such as the killing of dividing cells, and late damage or cancer risk is unknown. The study of tissues concerned with blood cell formation provides a model system for evaluation of these interrelationships, since both the number of HSC and their ability to divide and produce normally functioning mature cells can be measured accurately. These results will be related to information on cancer of blood cell-forming tissues that is forthcoming from our studies of life shortening and cancer rates. An improved understanding of tissue injury and cell division in relation to cancer is fundamental to risk estimation for either radiation or other environmental agents.

PROPOSED COURSE OF THE PROJECT

Studies are designed to determine if the stem cell deficiency observed in aged, irradiated animals is extrinsic or intrinsic to the stem cell. Light and electron microscope studies of bone marrow and spleen will contribute to evaluation of the effects of extrinsic factors, such as damage to supporting tissues and blood vessels, and intrinsic factors will be evaluated by determining how effectively stem cells divide and their daughter cells produce red and white blood cells and platelets. We hope to devise a means to measure the susceptibility of stem cells from aged, irradiated animals to transformation to leukemic condition by a mouse leukemia virus. A small effort will be devoted to assessment of the HSC damage in mice that results from alpha radiation from plutonium.

ERDA RT-03-01
ANL 60300

COMPARISON OF THE RADIOSENSITIVITY OF THE SMALL INTESTINE OF $B6CF_1$ AND BALB/c MICE TO GAMMA AND JANUS FISSION NEUTRON RADIATIONS

R. J. Michael Fry, Principal Investigator*
Anthony R. Sallese and Wayne R. Hanson,* Participating Investigators*

OBJECTIVES

The aim of these experiments is to establish the cellular and tissue differences that underlie the differences in whole body radiosensitivity between $B6CF_1$/Anl (C57BL/6 x BALB/c) and BALB/c mice.

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*Carcinogenesis Group.

BACKGROUND AND PREVIOUS FINDINGS

The findings of a preliminary investigation of some of the differences in radiation response between $B6CF_1$ and the parent strains have been reported (Fry, R. J. M., et al., Radiat. Res. 51, 534, 1972). Although these investigations indicated a difference in the radiosensitivity of the crypt cells between $B6CF_1$ and BALB/c mice, the results were ambiguous. We have therefore confirmed and extended the previous investigations.

EXPERIMENTAL METHODS

The following methods have been used in various experiments: (1) microcolony assay (Withers, H. R., and M. M. Elkind, Int. J. Radiat. Biol. 17, 261, 1970), (2) study of repopulation of proliferative cells after a single exposure of 1200 R ^{60}Co γ-radiation, (3) assay of recovery with the split-dose techniques, (4) determination of $LD_{50/6}$ for JANUS fission neutrons and ^{60}Co γ-radiation, (5) determination of cell cycle times of epithelial cells in the proliferative zone in the intestinal crypt, and (6) determination of crypt sections per circumference and labeled cells per circumference.

MAJOR NEW FINDINGS

We have confirmed that the D_o for clonogenic cells of the crypts of Lieberkühn is about 80 R and 150 R (^{60}Co gamma radiation) for BALB/c and $B6CF_1$ mice, respectively, and that the rate of repopulation is slower in BALB/c than in $B6CF_1$ mice. Results from the experiment to assay the amount of recovery between two radiation exposures suggest somewhat less recovery between the split doses in the crypt cells in the BALB/c than in the $B6CF_1$ mouse. For example, the survival ratio, using 730 R as the first dose, was 13 and 18 for a 2-hr interval, and 18 and 28 for a 5-hr interval in BALB/c and $B6CF_1$, respectively.

Caution is necessary in the interpretation of comparisons of recovery because the degree of recovery is dependent on the size of the first dose and therefore the cellular effects of the first dose.

A similar value for D_o of the crypt clonogenic cells was found in $B6CF_1$ and BALB/c mice for radiation with JANUS reactor fission neutrons, which suggests that differences in the number of hypoxic cells may be important.

SIGNIFICANCE

The factors that influence radiosensitivity are generally known; however, strain- and species-dependent differences in the cellular, tissue, and whole animal response to radiation are still poorly understood. This is the first demonstration of the occurrence of strain dependent differences in crypt cell radiosensitivity. Previous results (Fry, R. J. M., et al., Radiat. Res. 51, 534, 1972) have shown that the $LD_{50/30}$ is markedly lower in BALB/c mice than in $B6CF_1$ which cannot be explained on differences in the radiosensitivity of bone marrow colony-forming units. Thus the influence of gut death on the results for the $LD_{50/30}$ assay are considerable, although $LD_{50/30}$ is considered a reliable measure of marrow stem cell survival and repopulation.

PROPOSED COURSE OF THE PROJECT

We are investigating further the strain-dependent differences in (1) split dose recovery, (2) the cell cycle time of crypt clonogenic cells, (3) the cell kinetics of the crypt cells, and (4) the response to multifractionation regimes.

ERDA RT-03-01
ANL 60400
NIH CA 18431-01
NIH CA 18081-01

INVESTIGATIONS OF INTESTINAL CLONOGENIC CELLS

Wayne R. Hanson, Participating Investigator*
R. J. Michael Fry and Anthony R. Sallese,* Participating Investigators*

OBJECTIVES

Two main questions remain about the proliferative characteristics of intestinal crypt cells, namely: (1) What fraction of the crypt population is clonogenic? (2) Is there a distinct stem cell compartment, and if so, what is the cycle time of the cells? The aim of these studies is to answer these questions.

BACKGROUND AND PREVIOUS FINDINGS

The microcolonies seen in the regenerating intestine following irradiation are believed to come from single surviving crypt cells. It was generally believed that surviving epithelial cells with a short cell cycle, undergoing amplification divisions could contribute to the observed microcolonies. This view was questioned by Hendry and Potten (Hendry, J. H., and C. S. Potten, Int. J. Radiat. Biol. 25, 583, 1974), as well as by others. Despite their studies, which implicated a subpopulation of cells with a long cell cycle time, the cells responsible for the microcolonies still remain in doubt.

EXPERIMENTAL METHODS

B6CF$_1$/An1 mice were injected with 0.75 mCi of high specific activity ^{3}H-thymidine (60 Ci/mmol) or with four injections of colcemid (2 μg/g body) weight), at 3-hour intervals. The purpose of both procedures was to reduce the number of cells in cycle in the proliferative zone of the intestinal

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* Carcinogenesis Group.

crypt. The mice that received either the colcemid or ^{3}H-thymidine were irradiated with various doses of ^{60}Co gamma rays in order to perform the microcolony assay (Withers, H. R., and M. M. Elkind, Int. J. Radiat. Biol. 17, 261, 1970).

MAJOR NEW FINDINGS

The injection of the high specific activity ^{3}H-thymidine reduced the number of cells per crypt column from 21 to 13. The injections of colcemid reduced the number of cells per crypt from 254 ± 9 to 156 ± 8.0 (mean ± SE) and the number of cells in DNA synthesis (S) from 90 ± 5 to 48 ± 4. Despite the marked reduction in the number of proliferative cells, there was no change in the survival curve.

SIGNIFICANCE

These results suggest that not all of the proliferative cells of the crypt are clonogenic, and that the intestinal stem cell must have a long cell cycle.

PROPOSED COURSE OF THE PROJECT

We propose (1) to estimate the duration of the cell cycle of the putative stem cells, and (2) to estimate the number of stem cells in the crypts of the small intestine. The methods of investigation will include continuous labeling with ^{3}H-thymidine and the methods used in the current experiments.

ERDA RT-03-01
ANL 60300

NEUTRON AND GAMMA RAY DEPTH-DOSE DISTRIBUTIONS IN HOMOGENEOUS PHANTOMS

Thomas B. Borak and Frank S. Williamson, Principal Investigators
Gordon L. Holmblad, Participating Investigator

OBJECTIVES

The principal objectives are: (1) to determine the neutron and gamma ray dose contributions in animal species of diverse sizes irradiated in the JANUS facility; this information will be used to guide the planning of future external radiation toxicity studies; and (2) to provide for storage of all details of radiation treatment, with means of retrieval and analysis. As our knowledge of mechanisms of radiation injury advances, we will have the capability to reevaluate physical radiation doses with whatever degree of refinement is desirable.

BACKGROUND AND PREVIOUS FINDINGS

The High Flux Room of the JANUS reactor is a unique radiation facility. It can produce a 1000:1 range of dose rates to typical loadings of 400 mice, with negligible dose contamination by thermal neutrons and less than 3 percent dose contamination from gamma rays (Williamson, F. S., et al., Proceedings of the First Symposium on Neutron Dosimetry in Biology and Medicine, Neuherberg/München, 1972, pp. 743-755).

Monte Carlo calculations, in our dosimetry application, involve random sampling of neutron histories as they collide with atoms in a phantom under study. The program used now has a capacity for up to 45 regions of different atomic composition (Frigerio, N. A., et al., Phys. Med. Biol. 18, 53, 1973).

In setting up a computer study of this degree of complexity we must have confidence in the basic nuclear data (cross sections), and the correct definition of the problem geometry. We have documented, and verified against nationally accepted compilations, cross sections for neutrons and gamma rays of less than 8 MeV energy. We also developed a utility program which prepares line drawings of the problem geometry as interpreted by the computer program (Borak, T. B., ANL-75-30, 1974, p. 71).

Dosimetry data for the JANUS High Flux Room is stored in direct-access computer files. Computer programs calculate the locations of load frames, each containing 50 mice, to give a desired mean dose. Neutron and gamma ray doses at each mouse location are stored in direct-access storage and will be used to determine the total dose received by each individual mouse in each experiment.

EXPERIMENTAL METHODS

Our approach to dosimetry involves both computation and measurement. We measure the characteristics of a radiation field, then use Monte Caro calculation to obtain the internal distribution of dose components in an irradiated subject. With this approach it is important to verify calculations by contriving test cases that lend themselves to unambiguous measurement.

The International Neutron Dosimetry Intercomparison (INDI), held at Brookhaven National Laboratory in 1973, provided a good test (Goodman, L. J., et al., Second Symposium on Neutron Dosimetry in Biology and Medicine, Neuherberg/München, 1974, in press). Our participation in INDI gives us the relationship between our own measurements with tissue-equivalent and argon-filled magnesium ionization chambers and those of the other participants, but no insight into their correctness. The Monte Carlo calculation was made for the case of 5.5 MeV neutrons incident on a water cube of 30-cm side.

In the JANUS facility a polyethylene cylinder, 60 cm long and 12 cm diameter, was used to simulate a beagle dog. Transverse holes along the cylinder axis could accommodate either a radiation detector or a polyethylene plug. Detectors used were: (1) gold foils, bare or cadmium covered; (2) 0.5-cc magnesium ionization chamber with argon gas flow; and (3) 0.5-cc tissue-equivalent plastic ionization chamber with tissue-equivalent gas flow. From the measurements made with these detectors, values were obtained for fast neutron dose, thermal neutron flux, and gamma ray dose.

MAJOR NEW DEVELOPMENTS

For the INDI case of 5.5 MeV neutrons, our measured neutron doses in air, and at 5-, 10-, and 20-cm depth in water, fall within -0.2 to +4 percent of the mean value from all participants. Our Monte Carlo calculations yield neutron doses within the extremes reported for all participants, but were significantly lower at 5- and 10-cm depths than our measured values. We believe this difference is due to the displacement of water by the measuring device.

The Monte Carlo calculation yields gamma doses, resulting from neutron interactions in the phantom, which are much smaller than those measured. However, if the gamma background that we measured in air (with no phantom present) is assumed to originate from the source, the resulting combinations of air-measured and phantom-produced gamma doses fall within the INDI limits. Our measured gamma doses are approximately 7 percent greater than the INDI mean, but well within the reported range of ± 49 percent about the mean.

In the JANUS High Flux Room, position (a) has the polyethylene cylinder oriented so that one circular end faces the main source of neutrons. The neutron dose falls off rapidly along the cylinder axis, to 30 percent at 5.1 cm, 14 percent at 10.2 cm, and 10 percent at 55.9 cm. It then rises to 27 percent at the other end because some neutrons, scattered from the walls of the room, enter the cylinder from all directions. The gamma dose over a large central volume is 50-90 percent of the corresponding neutron dose. With the cylinder placed at right angles to position (a), neutrons from the main source are entering via the cylindrical surface. For this case the neutron dose has a more uniform distribution, with a large central volume receiving less than 30 percent of the dose at entry and about 45-55 percent gamma dose.

SIGNIFICANCE

The diversity of values for the International Neutron Dosimetry Intercomparison necessitates additional study on our part, with a view to evaluating the adequacy of our own dosimetry methods. The calculations explain apparent anomalies in gamma distribution and strengthen confidence in our existing measurement techniques. For the case of the large cylindrical phantom in JANUS, the rapid falloff of neutron dose in the first few centimeters of tissue, in conjunction with a gamma dose greater than 50 percent of the neutron dose which is produced within the tissue by neutron interactions, shows already that size of the biological specimen is a critical factor. For physical reasons alone, it will be necessary to treat the radiobiology of larger species, and especially man, as something more complex than simple extensions of mouse studies.

PROPOSED COURSE OF THE PROJECT

We plan to proceed further with a program to incorporate nuclear cross sections from the national archive (ENDF/B IV). This will add quality assurance to complex dosimetry calculations, and the radiobiology which is dependent on them.

We propose to develop the Monte Carlo program further: (1) to include isotropic sources and nonuniform cylindrical sources needed to model the JANUS High Flux Room; and (2) to follow recoil particle histories in interfaces between regions of different atomic composition (e.g., bone/marrow cavities).

The cylindrical polyethylene phantom case will be calculated and compared with the measured data.

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5. CARCINOGENESIS

ERDA RT-03-01
ANL 60400
ANL 60401
NIH CA 18434-01
NIH CA 18081-01
EPA-IAG-D6-E681

GROUP LEADER'S OVERVIEW

R. J. Michael Fry, Group Leader

The long-term aims of the studies in this program are concerned with: (1) various aspects of the natural history and biology of cancer, (2) the mechanism of induction and of the advancement of time of appearance of tumors, (3) the development of systems suitable for the assay of oncogenesis and co-carcinogenesis, and (4) the elucidation of some of the factors important to the problem of extrapolation of estimates of risk made in experimental systems to the estimate of risk in man.

It is clear that one of the most difficult problems in the determination of oncogenic risks of chemical and physical agents is the identification and assay of cocarcinogens. Until there is a greater understanding of the mechanisms of cocarcinogenesis, the methods of identification will be empirical.

It is necessary to have a number of test systems in order to study the various factors related to cocarcinogenesis; some of these are clearly tissue specific. The liver tumor system that we have developed is clearly useful for certain compounds, and the liver is an excellent tissue for the study of the mechanisms of cocarcinogenesis. This year we report on the relatively rapid induction of what appears histologically to be carcinoma of the thyroid by aminotriazole. The thyroid should provide another test system for investigating certain other interactions.

In a collaborative study with the Neutron and Gamma-Ray Toxicity Group, we have established a new example of synergism in carcinogenesis, namely between radiation and pituitary hormone(s) in the production of Harderian gland tumors. Not only does a synergistic effect on incidence occur, but also on the degree of malignancy of the tumor induced. This finding opens up the possibility of examining some of the underlying factors in the induction of malignancy.

We thus have three different model systems for the study of various aspects of cocarcinogenesis: (1) various chemicals, including nononcogenic polycyclic hydrocarbons, in liver tumorigenesis; (2) ionizing radiation and

aminotriazole in thyroid tumorigenesis; and (3) in conjunction with the JANUS Program, the interaction of radiation and hormones in the production of Harderian gland, mammary gland, and other tumors.

The steady evolution of the program has continued, and we are now studying various aspects of oncogenesis at the molecular, cellular, tissue, and whole animal level. The following individual reports are organized into three broad categories: modulation of tumorigenesis; mechanism of tumorigenesis; and skin and pulmonary carcinogenesis, a new program directed toward the effects of energy-related pollutants on living organisms. This year we are introducing an important aspect, new to our studies of chemical oncogenesis, namely, cytochrome P-450 and its hydroxylase activity.

Our studies are basic for the understanding of tissue and species susceptibility to proximate oncogens that are enzymatically activated and deactivated. Such studies are essential in the elucidation of the oncogenic effects of environmental pollutants such as polycyclic hydrocarbons.

CARCINOGENESIS STAFF

REGULAR STAFF

Buess, Evelyn M. (Scientific Assistant)
Cameron, Erma C. (Scientific Assistant)
Chubb, G. Theodore (Engineering Specialist)
Devine, Rosemarie L. (Scientific Associate)
Feinstein, Robert N. (Senior Biochemist)
Fry, R. J. Michael (Senior Physiologist)
Grube, Donald D. (Scientific Associate)
Haugen, David A. (Assistant Biochemist)
Kisieleski, Walter E. (Chemist)
Lombard, Louise S. (Veterinary Pathologist)
Ludeman, V. Ann (Scientific Assistant)
Peraino, Carl (Biochemist)
Prapuolenis, Aldona M. (Scientific Assistant)
Sallese, Anthony R. (Scientific Assistant)
Staffeldt, Everett F. (Scientific Associate)

TEMPORARY STAFF DURING 1975

Hanson, Wayne R. (Research Associate)
*Lindahl, Ronald G. (Postdoctoral Appointee)
Shenoy, Surendra T. (Postdoctoral Appointee)

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*Terminated during 1975.

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MODULATION OF TUMORIGENESIS: EFFECTS OF PHENOBARBITAL, BUTYLATED HYDROXYTOLUENE, AND BENZ[A]ANTHRACENE ON AAF-INDUCED HEPATIC TUMORIGENESIS

Carl Peraino, Principal Investigator
R. J. Michael Fry, Everett Staffeldt, John P. Christopher, [*] *and David Haugen, Participating Investigators*

OBJECTIVES

The aim of this work is to analyze the mechanism of tumorigenesis using liver as the model system. Our general approach involves the attempt to modify the onset of neoplasia, using treatments that exert known effects on the structure and metabolism of the target cell. Determining which of the molecular effects of the modifying agent are responsible for the perturbation of tumorigenesis should provide information on the critical molecular events associated with neoplasia.

Additional experiments involve the development of a system whereby putative enhancers can be tested for their effects on nucleocytoplasmic transport *in vitro*.

BACKGROUND AND PREVIOUS FINDINGS

The initial studies in this laboratory showed that phenobarbital feeding enhanced hepatic tumorigenesis in rats previously fed the liver carcinogen 2-acetylaminofluorene (AAF) (Peraino, C., et al., Cancer Res. 31, 1506, 1971). Subsequently we reported that exposure to phenobarbital for more than 20 days was required to produce enhancement, and that enhancement was retained when phenobarbital feeding was delayed for 30 days after the cessation of AAF feeding (Peraino, C., et al., Cancer Res. 33, 2701, 1973). Recently we assessed the relative tumorigenic enhancing ability of amobarbital, diphenylhydantoin, and DDT, agents that resemble phenobarbital to varying degrees in their effects on liver structure and metabolism. These comparisons showed that amobarbital and diphenylhydantoin had no enhancing activity, whereas the enhancing effect of DDT was similar to that of phenobarbital (Peraino, C., et al., Cancer Res. 35, 2884, 1975).

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[*] Laboratory Graduate Program Participant, Oregon State University.

On the basis of the results described above, we are engaged in studies designed to define more rigorously the exposure conditions under which phenobarbital causes enhancement. These experiments should provide information on whether low doses of AAF produce precancerous changes that are irreversible, and also whether the enhancing effect of phenobarbital is similarly irreversible.

In addition, we are continuing to test other substances for relative enhancing ability, with special emphasis on energy-related pollutants. The comparative biochemical effects of these substances in the liver can then be correlated with their relative enhancing abilities to provide information on the molecular events specifically associated with enhancement.

Benz[a]anthracene has been chosen as the model polycyclic hydrocarbon for use in our initial studies, because its apparent lack of inherent carcinogenicity simplifies the interpretation of its effects on AAF-induced hepatic tumorigenesis. In addition, it is important to know whether hydrocarbons that are believed to produce little direct carcinogenic risk may, in fact, be tumor enhancers and thus present a more subtle hazard. Benz[a]anthracene, like phenobarbital and other polycyclic hydrocarbons such as 3-methylcholanthrene and benzo[a]pyrene, increases liver size and the levels of microsomal cytochromes and microsomal enzyme activity. In addition, benz[a]anthracene protects against hepatic tumorigenesis in rats when fed simultaneously with a liver carcinogen, a characteristic shared by the known enhancer, phenobarbital. Thus, benz[a]anthracene appears to exert metabolic effects relevant to the question of enhancement without possessing the carcinogenic properties of methylcholanthrene and benzo[a]pyrene.

EXPERIMENTAL METHODS

Rats receive a brief dietary exposure to AAF in an amount designed to produce a low incidence of liver tumors. After this treatment the rats are fed a diet containing the putative modifying agent (sequential feeding procedure). Rats are then killed at intervals throughout the experiment and examined for liver tumors.

MAJOR NEW FINDINGS

The present studies are still in their initial stages, and the conclusions may change as additional data are accumulated, but the results obtained thus far suggest the following:

1) The feeding of phenobarbital for 150 days after the cessation of AAF feeding produces a tumor yield that is intermediate between that in rats given AAF followed by a control diet and those given AAF followed by phenobarbital for the duration of the experiment. Thus, continual exposure to phenobarbital may be required for maximal tumorigenic enhancement.

2) If a 60-day interval is interposed between the end of AAF feeding and the beginning of phenobarbital feeding, tumorigenic enhancement is still nearly maximal, but if the treatment-free interval is extended to 120 days,

enhancement does not occur. These data indicate that the tumorigenic effect of AAF that is subject to enhancement by phenobarbital is reversible between 60 and 120 days after the AAF exposure, or that the time for the enhancement to be expressed is increased greatly.

3) Butylated hydroxytoluene (BHT), a commonly used food additive that resembles phenobarbital in its effects on the liver, is being tested for enhancing activity for the reasons indicated in the "Background" section. Thus far no enhancing activity has been detected.

4) The nucleocytoplasmic transport studies indicated that such transport is not stimulated in phenobarbital-treated rats. Therefore modification of this process is evidently not part of the tumorigenic enhancement mechanism.

5) Preliminary acute exposure studies with benz[a]anthracene suggest that this compound resembles phenobarbital in its effect on liver size and DNA synthesis, and that it induces liver microsomal hydroxylases. Benz[a]anthracene and phenobarbital induce two distinctly different microsomal cytochromes and related enzyme activities.

SIGNIFICANCE

The present study will define more rigorously the characteristics of the enhancing effect of phenobarbital on AAF-induced liver tumorigenesis. This should improve our understanding of the enhancement process and thereby help to provide insight into the mechanism of tumorigenesis. The studies of BHT and benz[a]anthracene will determine whether these compounds are enhancers, or not, and will add them to the list of compounds with different enhancing abilities that we can compare biochemically. In addition, our findings will have practical significance in terms of the human risk assesment for such compounds.

PROPOSED COURSE OF THE PROJECT

The necessity for additional experiments on the conditions of phenobarbital exposure will be assessed at the conclusion of the present study. Depending on these results, further studies may be carried out to determine accurately: (1) the minimum phenobarbital exposure that produces maximum enhancement, and (2) the maximum interval between the AAF and phenobarbital exposures that still allows phenobarbital to exert its full enhancing effect. Knowledge of the first will narrow the range of possible phenobarbital effects that are relevant to enhancement to the effects occurring within a given time period, and will also establish whether a finite exposure to phenobarbital (which does not produce irreversible changes in liver when given alone) leads to the irreversible expression of tumorigenic changes in the livers of rats previously exposed to AAF. Knowledge of point (2) will provide information on the reversibility of the AAF-induced effects prior to the administration of phenobarbital, as well as the time interval during which AAF-induced preneoplastic events might be detectable as biochemical changes in the liver.

Comparisons of the tumorigenic enhancing activities of environmental contaminants will continue. In addition to the direct identification of

agents that may represent a human health hazard, these studies will generate a group of compounds, with differing enhancing abilities, which can then be compared biochemically.

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MECHANISM OF TUMORIGENESIS: CONTROL OF GENE EXPRESSION--MECHANISMS OF REGULATION OF ORNITHINE AMINOTRANSFERASE AND SERINE DEHYDRATASE IN RAT LIVER CELLS

Surendra Shenoy, Principal Investigator
Carl Peraino and Aldona Prapuolenis, Participating Investigators

OBJECTIVES

The adaptive responses of serine dehydratase and ornithine aminotransferase in rat liver are studied as a model system for exploring the mechanism of gene expression in normal liver. The ultimate objectives are to define the primary regulatory effectors *in vivo*, and to determine the stage of protein synthesis at which these effectors act.

BACKGROUND AND PREVIOUS FINDINGS

Using specific antibodies for each enzyme, we measured the rate of incorporation of labeled amino acid into enzyme protein *in vivo*, and found that in rats on high protein diet the glucocorticoid treatment simultaneously stimulated the synthesis of serine dehydratase and depressed the synthesis of ornithine aminotransferase (Peraino, C., et al., ANL-8070, 1974, p. 87). An important question is whether these divergent regulatory effects are exerted at the level of gene transcription or messenger RNA (mRNA) translation.

EXPERIMENTAL METHODS

Messenger RNA was isolated from rat liver by a phenol extraction procedure and was purified by affinity chromatography on oligo d(T) cellulose. The activity of the isolated message was tested in a cell-free protein synthesizing system derived from wheat germ, and the relative amounts of labeled amino acid incorporated into the two enzymes were determined by immunoprecipitation with specific antibodies and acrylamide gel electrophoresis of the immunoprecipitates.

MAJOR NEW FINDINGS

The protein synthetic assay was standardized with respect to concentrations of various substrates and cofactors. When the products of the assay system were immunoprecipitated and analyzed electrophoretically on SDS gels, radioactive protein peaks were obtained that did not have the mobility of either of the native enzymes. Further work is in progress to determine whether these peaks are related to serine dehydratase and ornithine aminotransferase.

SIGNIFICANCE

The present work is directed toward developing techniques for studying the control of gene expression in the liver cell with the expectation that such techniques can be ultimately used to detect essential molecular differences between normal and neoplastic liver.

PROPOSED COURSE OF THE PROJECT

It is proposed that mRNA will be isolated from rats exposed to high protein diet with or without glucocorticoid treatment and the resultant changes in the mRNA activities for serine dehydratase and ornithine aminotransferase will be determined by the techniques described above.

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MECHANISM OF TUMORIGENESIS: ISOZYMES AND CANCER

Robert N. Feinstein, Principal Investigator
Erma C. Cameron and Ronald Lindahl, Participating Investigators

OBJECTIVES

Our objectives are to detect and measure changes in molecular forms of enzymes ("isozymes") due to the carcinogenic change, to elucidate the mechanism whereby the carcinogenic transformation brings these changes about, and to evaluate the significance these changes may have in tumor metabolism.

BACKGROUND AND PREVIOUS FINDINGS

The study of isozyme changes in tumors has become very widespread (Schapira, F., Adv. Cancer Res. 18, 77, 1973). Most frequently, the isozyme pattern of the tumor, while differing from that of the normal adult

tissue, resembles that of the corresponding fetal tissue. We have particularly investigated the enzyme aldehyde dehydrogenase in normal rat liver and in the liver tumor produced by feeding rats the carcinogenic chemical 2-acetylaminofluorene (AAF). In this case, the tumor enzyme differs markedly from that of normal liver, but it does not resemble that of fetal liver either. The tumor change is both qualitative (new forms of the enzyme) and quantitative (much greater activity than normal liver).

EXPERIMENTAL METHODS

Electrophoresis, isoelectric focusing, and standard quantitative enzyme assays are employed. Tumors are induced in mice by including carcinogenic agents in the diet, by introducing tumor virus, or by irradiation.

MAJOR NEW FINDINGS

Although the administration of carcinogens, such as AAF or dimethylaminoazobenzene, induces liver tumors that invariably exhibit the characteristic new forms of aldehyde dehydrogenase, liver tumors induced in rats by the carcinogenic agent ethionine do not show these characteristic new forms. Specifically, only one tumor out of 29 induced by ethionine did show these isozymes; the other 28 tumors not only showed no new forms, but the aldehyde dehydrogenase activity was actually less than that of normal liver.

We have also found some changes in the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in mouse lung tumors as compared to normal mouse lungs, and we have observed an interesting decrease from normal in the ability of a liver tumor to degrade the coenzyme nicotinamide adenine dinucleotide (NAD).

SIGNIFICANCE

The significance of the isozyme change in the liver tumor aldehyde dehydrogenase lies in the fact that it is a very convenient tool for studying the mechanism whereby a normal tissue, upon undergoing the carcinogenic transformation, produces new forms of proteins. The exact chemical nature of the modification is not yet known. In the case of the mouse lung tumors, we hope to use the listed enzyme changes to determine whether radiation-induced lung tumors are simply speeded-up spontaneously occurring tumors, or represent a distinct, different cancer entity. The significance of NAD degradation lies in the fact that this coenzyme may well be a limiting factor in certain areas of the biochemical economy of both normal and tumor tissues, and a greater or lesser degradation may be of importance.

PROPOSED COURSE OF THE PROJECT

Further aldehyde dehydrogenase studies are being continued by Dr. Lindahl, now at the University of Alabama, in consultation with Dr. Feinstein and Dr. R. J. M. Fry, and studies in this Laboratory will be reduced. The lung tumor project requires the accumulation of data on enzyme activity in

the lungs of normal mice of various ages, and in lung tumors that have arisen spontaneously, as well as those observed following radiation. The project on NAD degradation is only beginning; data must be obtained on the extent of the phenomenon, its importance, and its exact biochemistry.

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MECHANISM OF TUMORIGENESIS: ROLE OF HYDROGEN PEROXIDE IN TUMOR PRODUCTION AND THE EFFECT OF AMINOTRIAZOLE

Robert N. Feinstein, Principal Investigator
Zoilo Gonzalez-Lama, Participating Investigator*

OBJECTIVES

Our objectives are to define a possible role of hydrogen peroxide in tumor production, to elucidate the mechanism whereby dietary aminotriazole induces thyroid hyperplasia and carcinoma, and to elucidate the mechanism whereby dietary aminotriazole exhibits differential lethality to certain mouse strains.

BACKGROUND AND PREVIOUS FINDINGS

O. Warburg long ago theorized that, no matter what the original insult (chemical or physical), the actual carcinogenic transformation was brought about by a molecule of hydrogen peroxide (Warburg, O., et al., Z. Naturforsch. 12b, 393, 1957). This theory was impossible to test experimentally in rodents *in vivo*, because of the peroxide-degrading catalase activity of their blood and tissues. The development by the present principal investigator of a mutant mouse with essentially no blood catalase, and an unstable tissue catalase, now permits the experiment. To reduce tissue catalase, and hence whole body catalase, to the lowest possible level, the catalase inhibitor 3-amino-1,2,4-triazole is incorporated in the diet. Because aminotriazole has itself been established as a carcinogen, its use necessitates extra control groups in the experiment.

EXPERIMENTAL METHODS

Mammary tumors are induced in C3H female mice by introduction of the Bittner milk factor (mouse mammary tumor virus, MMTV). Male C3H mice tend

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*Visiting Resident Associate, University of Granada, Spain.

to develop other spontaneous tumors as they age. Thymic lymphomas are induced in C57BL mice by repeated irradiation. Aminotriazole is incorporated into a pelletized diet. Death and tumor incidence are recorded. Tissues are examined biochemically, for enzyme activity and for isozyme patterns, and histologically.

MAJOR NEW FINDINGS

As observed by others, mice on a diet containing aminotriazole exhibit thyroid hyperplasia (20-30 times normal weight) and thyroid carcinoma (Innes, J. R. M., et al., J. Nat. Cancer Inst. 42, 1101, 1969). Although this is observed equally in acatalasemic mice (developed at ANL) and mice with normal catalase, the death rate of the normal mice is considerably greater than that of the acatalasemic mutants. No significant difference has yet been observed between the two strains as to the development of mammary or other tumors.

SIGNIFICANCE

The significance of this work lies in three directions: first, as a major test of the Warburg theory of carcinogenesis; second, as an opportunity to study the mechanism of the effects of aminotriazole on the thyroid (including both hyperplasia and tumor induction); and third, as a possible experimental model for the role of catalase and peroxidase in atherosclerosis, a role that has been postulated by others (Goldfischer, S., et al., Science 173, 65, 1971).

PROPOSED COURSE OF THE PROJECT

This is a long-term project, in that some of the expected tumors will not appear for a year or more. In the main, the experiment will consist simply of observing, measuring, and recording tumor development. However, in the case of thymic lymphoma in the irradiated C57BL mice, we plan to observe catalase isozyme patterns, because changes in catalase isozymes have been reported in human leukemic leukocytes (Nishimura, E., et al., Cancer Res. 32, 2353, 1972). In addition, we plan to study the mechanisms of the differential death rate observed upon feeding aminotriazole to normal and acatalasemic mice; we plan particularly to examine blood lipid levels, and to search for possible atherosclerotic differences between the two strains.

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MECHANISM OF TUMORIGENESIS: THE EFFECT OF PITUITARY ISOGRAFTS ON RADIATION CARCINOGENESIS IN THE MAMMARY AND HARDERIAN GLANDS OF MICE

R. J. Michael Fry, Principal Investigator
Alfred G. Garcia, Everett Staffeldt, Katherine H. Allen,† Anthony R. Sallese, Rosemarie L. Devine, Louise S. Lombard, Theodore N. Tahmisian,‡ and E. J. Ainsworth,† Participating Investigators*

OBJECTIVES

The aims of these experiments are to determine the tumorigenic response of $B6CF_1$ mice irradiated with low and high linear energy transfer (LET) radiation, and to establish the influence of pituitary hormones on tumorigenesis. The long-term aims are: (1) to establish the dose-response relationships for fission neutron (fn) and ^{60}Co gamma irradiation, and (2) to determine the mechanisms of interaction of pituitary hormones and oncogenic agents, in particular radiation.

BACKGROUND AND PREVIOUS FINDINGS

In mice the incidence of tumors in any one strain is influenced by a complex interaction of genetic, endocrine, and viral factors (Russfield, A. B., PHS Publication No. 1332, 1966). We have previously found that the natural incidence of mammary tumors in female $B6CF_1$ mice was about 1% and that protracted irradiation at dose rates ranging from 0.3 R to 6.0 R per day did not increase the incidence (Fry, R. J. M., et al., Proceedings of the IAEA Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press). It was not clear whether this lack of response was due to an inherent low susceptibility, or to the low dose rate of the irradiation. Furthermore, it was not known whether the susceptibility of the $B6CF_1$ mouse could be altered with an increase in pituitary hormone levels. It became clear in the course of this and related experiments that the response of the Harderian glands was of particular interest.

EXPERIMENTAL METHODS

Exposure to fission spectrum neutrons was carried out in the JANUS reactor. An increased level of pituitary hormones, in particular prolactin, was obtained by grafting two pituitaries from isogenic mice into the spleens

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* Laboratory Graduate Program Participant, Northern Illinois University.

† Neutron and Gamma-Ray Toxicity Group.

‡ Consultant, Division of Biological and Medical Research.

of hosts. The hormonal activity of the isograft was assessed from: (1) the estrous cycle, assayed from vaginal smears; and (2) the proliferative activity of the mammary and Harderian glands, determined on autoradiographs.

MAJOR NEW FINDINGS

The greatest increase in mammary gland tumors occurred after single exposures to 80 rad fn and 269 rad γ; with both types of radiation, fractionation reduced the response. The incidence of Harderian gland tumors rose from the control value of 2.5% to 14.4% and 18.6% after 20 and 80 rad fn, respectively; and to 5.1% and 10.8% after 90 and 288 rad γ, respectively. These results indicate that an incidence of 10% Harderian gland tumors would be produced by 15 rad fn compared to 260 rad γ-radiation, a relative biological effectiveness (RBE) of about 16.

In unirradiated mice, the pituitary isografts increased the incidence of mammary gland tumors from less than 1% in controls to about 19%, but had little or no effect on the incidence of Harderian gland tumors. However, irradiation of mice bearing isografts resulted in an increase in Harderian gland tumors to an incidence of 20% and 37% after 20 and 80 rad fn, respectively. These results suggest a synergistic effect of radiation and the hormone(s) secreted by the pituitary isograft. Furthermore, we found that the degree of malignancy of the Harderian gland tumors, judged by metastases and infiltration, was both dose dependent and LET dependent and was increased by the combination or irradiation and a pituitary isograft.

SIGNIFICANCE

The results revealed an unexpected hormone responsiveness of the Harderian gland and that the gland has potential as a tumor test system. The finding that the degree of malignancy is dependent on the dose of irradiation and LET is the first quantitative demonstration of this phenomenon.

PROPOSED COURSE OF THE PROJECT

With the collaboration of J. Furth, we hope to establish which hormone receptors are present in the cells of the Harderian gland. We hope to answer the question of whether the pituitary hormones act as a promoting agent or alter the susceptibility of the cells to radiation induced carcinogenesis. The shape of the dose-response curve for tumorigenesis after neutron irradiation will be more fully characterized. It is clear that the effectiveness per rad for induction of the Harderian gland tumors decreases with dose; the reasons for this will be sought. The underlying factors that determine the malignancy of these tumors will be investigated.

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SKIN AND PULMONARY CARCINOGENESIS: PHOTOSENSITIZING EFFECTS OF 8-METHOXYPSORALEN IN THE SKIN OF HAIRLESS MICE; SPECTRAL DEPENDENCE FOR THE INDUCTION OF DNA INTERSTRAND CROSS-LINKAGES

Ronald D. Ley, Principal Investigator*
Donald D. Grube and R. J. Michael Fry, Participating Investigators

OBJECTIVES

The main objective is to measure accurately the efficiencies for cross-link induction in epidermal DNA of two strains of hairless mice treated with 8-methoxypsoralen (8-MOP) and exposed to one of three different wavelength spectra which have been studied for their tumorigenic effect.

BACKGROUND AND PREVIOUS FINDINGS

It has been observed at this Laboratory and elsewhere that combined treatment with 8-MOP and near ultraviolet (UV) light induces skin tumors in hairless mice. However, the use of exposure levels thought to equalize the induction of DNA cross-links by three different wavelength spectra resulted in quite different tumor induction responses. Thus it was necessary to measure accurately the efficiencies for DNA cross-link induction with the three wavelength spectra to determine whether small differences in cross-link induction could be correlated with differences in tumor induction responses.

EXPERIMENTAL METHODS

Renaturation properties of DNA extracted from the epidermis of mice treated with 8-MOP and UV were used to measure the induction of DNA interstrand cross-linkages. The light sources were: (1) the Magnaflux mercury vapor lamp, the spectral emission of which consists of a high intensity line at 365 nm ± 3 nm, with less than 1% of the total fluence emitted at 334 and 405 nm, with lower emission rates observed as a continuum between 334 and 405 nm; (2) unfiltered Westinghouse F40BLB fluorescent lamp which emits a continuous spectrum between 300-400 nm with approximately 1% of the fluence below 320 nm; and (3) a filtered BLB fluorescent source which emits a continuous spectrum from 320 nm to 400 nm.

MAJOR NEW FINDINGS

The efficiencies for DNA cross-link induction we have measured for the three wavelength spectra indicate that small differences in levels of cross-link induction cannot account for the spectral dependency observed for tumor

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*Mammalian Cell Biology Group.

induction. However, as tumor induction studies required fractionated exposures to 8-MOP and UV protracted over 24 weeks, it could not be assumed that spectral differences for cross-linking efficiencies remained constant, as the early exposures alter epidermal thickness. Therefore additional cross-linking efficiencies were determined with groups of mice that had been subjected to multiple treatments with 8-MOP and UV. These experiments showed that although prior exposure to 8-MOP plus UV resulted in a decrease in observed efficiencies for cross-link induction, the decrease was comparable for the different wavelength spectra.

SIGNIFICANCE

These findings indicate that the DNA cross-link induced by treatment with 8-MOP and UV is not, by itself, the lesion responsible for tumorigenesis.

PROPOSED COURSE OF THE PROJECT

Studies will continue to determine whether the wavelength dependency for tumor induction can be correlated with other photoproducts, e.g., psoralen monofunctional adducts, photoproducts induced by UV alone, or an interaction between two or more photoproducts.

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SKIN AND PULMONARY CARCINOGENESIS: STUDIES ON THE CUTANEOUS EFFECTS OF CHEMICAL PHOTOSENSITIZATION AND MODIFYING FACTORS IN ONCOGENESIS

Donald D. Grube, Principal Investigator
Ronald D. Ley, and R. J. Michael Fry, Participating Investigators*

OBJECTIVES

The aims are (1) to investigate strain-dependent differences in susceptibility for tumor induction in two strains of hairless mice, and (2) to investigate the effect of combining exposures of ultraviolet light (UV), namely 280-400 nm, and exposure to psoralen and 365-nm light.

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*Mammalian Cell Biology Group.

BACKGROUND AND PREVIOUS FINDINGS

Topical administration of 8-methoxypsoralen (8-MOP) photosensitizes skin to UV and extends the action spectrum for erythema, tissue damage, and tumorigenesis in mice beyond 320 nm. In earlier experiments we have characterized the dose-response relationships for the acute cutaneous effects of such treatments in two strains of hairless mice using different wavelength spectra. Cytokinetic studies demonstrated that combined treatment with each spectrum induced an ordered sequence of cytological and proliferative changes, and the dose responses were similar for the two strains of mice. Furthermore, in each strain, the spectral-dependent differences for cytotoxic effects were paralleled by the same relative differences for the induction of psoralen cross-links in epidermal DNA.

In an initial series of experiments each of the wavelength spectra induced epidermal carcinomas in the photosensitized skin of SKH:hr-1 mice (Grube, D. D., et al., ANL-75-30, 1974, p.88). However, when the exposures were adjusted to induce similar psoralen cross-link and cutaneous damage, the rate of appearance of tumors was markedly increased in the 300-400 nm and 320-400 nm exposure groups as compared to emission at principally 365 nm. This finding suggested a possible interaction of wavelength-dependent lesions (Grube, D. D., et al., Proc. Am. Assoc. Cancer Res. 16, 117, 1975). We chose to investigate the effect of combining exposures to light of 280-400 nm (UVB + UVA) with the 8-MOP + 365-nm light treatment used previously. The lower two doses used, at 280-400 nm, were known not to be tumorigenic, and the rate of tumor induction with the selected dose of 8-MOP plus 365-nm light was known and was low. Therefore the results of the experiment should indicate clearly whether the combined treatments were additive, synergistic, or not interactive.

EXPERIMENTAL METHODS

In the first series of experiments comparative tumor studies were conducted using two strains of hairless mice, SKH:hr-1 and HRS/J/An1, the latter exhibiting a reduced susceptibility to UV-induced skin tumors (Forbes, D. P., Personal Communication). In these studies mice were treated topically with 8-MOP in combination with different fluences and wavelength spectra of UV. Conventional autoradiographic and histologic techniques were used to measure cytokinetic responses in the epidermis of each strain after single and fractionated exposures to combined treatment.

In a second series of experiments groups of female SKH:hr-1 hairless mice were exposed to 20, 100, or 500 J/m^2 UV (280-400 nm, FS40 T12 lamp), 3/week for 24 weeks, either alone or immediately prior to exposure to 8-MOP and 365-nm light (Magnaflux lamp). In another group, the exposure to 8-MOP and 365-nm light was made prior to exposure to the 280-400 nm light. Mice were examined weekly for tumors. All tumors were confirmed histologically. The results for each group were expressed as a cumulative incidence as a function of time.

MAJOR NEW FINDINGS

1) Under the same exposure conditions, the tumor response in HRS/J/Anl mice differed markedly from that previously observed in the SKH:hr-1 strain. Whereas treatment with 8-MOP and exposures to broad wavelength emissions (300-400 nm) resulted in the earliest onset of tumors in SKH:hr-1 mice, the same treatments were ineffective in the HRS/J/Anl strain. Similarly, under conditions in which tumors were induced in both strains of mice, the rate of appearance for tumors was consistently less in the HRS/J/Anl strain.

2) In the groups of SKH:hr-1 mice exposed to the combination of 280-400 nm light and 8-MOP plus 365-nm light, the latent period was shorter than in the group exposed to 8-MOP plus 365-nm light (50% tumor incidence at 41-48 weeks and 80 weeks, respectively). It is clear that there is a synergistic effect on the rate of tumor appearance with combined exposures of 280-400 nm light and 8-MOP plus 365-nm light. The sequence of treatments, i.e., exposure to 280-400 nm light prior to or after exposure to 8-MOP plus 365-nm light does not appreciably alter the synergism. This finding suggests that the lesions responsible for the synergistic effect on tumor response are present for a time at least equal to the time taken to carry out the exposures. From our previous results with different spectra of light, we believe the synergistic effect is possibly due to 280-320 nm light (UVB).

SIGNIFICANCE

1) The genetic basis of strain-dependent differences in the natural incidence and susceptibility for tumor induction are known for a number of murine tumor systems. However, such differences have not been fully documented for the skin of mice. The parallel studies on induction of skin tumors in two strains of mice offer possibilities for the investigation of species-dependent differences in physiological and biochemical functions related to carcinogenesis. An understanding of the factors that determine susceptibility is essential for the estimates of risk.

2) The observed synergism on the rate of tumor appearance with combined exposures of 280-400 nm light and 8-MOP plus 365-nm light provides a new approach to the investigation of lesions causally related to tumorigenesis. Furthermore the use of combined treatments will allow correlative studies between mutagenesis and carcinogenesis, and between repair and carcinogenesis.

PROPOSED COURSE OF THE PROJECT

We propose to determine the kinetics of the interaction between UVB light and psoralen plus 365 nm. It should be possible to establish if the number of induced lesions is reduced with time and whether or not a reduction correlates with any change in the interaction for tumor induction. We will also investigate whether the effect of the UVB light in the interaction is on the initiation events or whether UVB acts a promoter.

We will determine whether the strain-dependent difference in susceptibility is independent of the carcinogen by a study of tumor induction with benzo[a]pyrene. We will also study the strain-dependent difference in repair capability and cell kinetics.

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SKIN AND PULMONARY CARCINOGENESIS: ULTRAVIOLET LIGHT INDUCED DAMAGE IN EPIDERMAL DNA OF HAIRLESS MICE

Ronald D. Ley, Principal Investigator*
Beverly A. Sedita and Donald D. Grube, Participating Investigators*

OBJECTIVES

We plan to adapt techniques, currently used with procaryotes and mammalian cells grown *in vitro*, to measure the induction and repair of damage to DNA in mouse epithelial cells *in vivo*.

BACKGROUND AND PREVIOUS FINDINGS

We have found that two strains of hairless mice, HRS/J/An1 and SKH:hr-1, exhibit large differences in sensitivity to the induction of skin tumors by exposures to combined treatment of psoralen and near ultraviolet light (Grube, D. D., et al., Photochem. Photobiol., in press). As sensitivity to tumor induction may reflect the ability of a cell population to repair or circumvent damage to its genetic material, we have initiated *in vivo* studies to characterize the repair capabilities of these two strains of mice.

EXPERIMENTAL METHODS

Damage-specific nucleases were used, in conjunction with DNA sedimentation techniques, to measure the induction and persistence of lesions in epidermal DNA after exposure to ultraviolet (UV) light.

MAJOR NEW FINDINGS

Hairless mice exposed to UV (280-400 nm) accumulate lesions in epidermal DNA, which are possibly pyrimidine dimers, as a function of time. The rate of lesion induction is similar for both strains of mice. At the exposure levels used, the lesions appear to persist in the DNA for up to 24 hours after their induction. The persistence of the DNA lesions indicates that mouse epithelial cells *in vivo* have little capacity for excision repair.

SIGNIFICANCE

These findings support observations made *in vitro* that rodent cells exhibit little or no excision repair of UV-induced damage (Trosko, J. E., et al., Radiat. Res. 24, 667, 1965). In addition, it would appear that the different susceptibility of the two strains of mice to tumor induction cannot be explained in terms of different capacities for excision repair.

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*Mammalian Cell Biology Group.

PROPOSED COURSE OF THE PROJECT

We intend to determine whether the UV-induced lesions we have observed in mouse epidermal DNA are pyrimidine dimers, or some other type of photoproduct. In addition, other parameters, e.g., repair replication and unscheduled DNA synthesis, will be measured to characterize further the *in vivo* repair capabilities of mouse epithelial cells in the two strains of mice.

ERDA RT-03-01
ANL 60401

SKIN AND PULMONARY CARCINOGENESIS: 8-METHOXYPSORALEN AND ULTRAVIOLET LIGHT INDUCED GENETIC EFFECTS IN THE MITOCHONDRIAL AND NUCLEAR GENOMES OF SACCHAROMYCES CEREVISIAE

Gunnard K. Jacobson, Principal Investigator*
Ronald D. Ley, Participating Investigator*

OBJECTIVES

The aim is to investigate whether treatment by 8-methoxypsoralen (8-MOP) and ultraviolet (UV) light has genetic consequences in the yeast nuclear genome via mutation, mitotic gene conversion, or mitotic crossing-over, and in the mitochondrial genome, via the cytoplasmic petite mutation. Estimates of DNA damage and repair can then be used to ascertain the possible relationship between DNA interstrand cross-links and pyrimidine dimers to mutagenesis. A successful demonstration will establish that a known carcinogenic induction procedure in higher eucaryotes can induce genetic damage in a lower eucaryote.

BACKGROUND AND PREVIOUS FINDINGS

In addition to 254-nm light alone, it has been shown that cytoplasmic petites can be induced by treatment with 8-MOP and 365-nm light (Auerbeck, D., and E. Moustacchi, Biochim. Biophys. Acta 395, 393, 1975).

Our research has indicated that exposure to short wavelength UV enhances the tumorigenic effects of 8-MOP and 365-nm light. This suggested that the combination of two different lesions in DNA enhances the probability of tumorigenesis in mice.

To date, the possibility of synergistically inducing mutations with combined 8-MOP-365 nm plus short wavelength UV has not been investigated.

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*Mammalian Cell Biology Group.

EXPERIMENTAL METHODS

Standard genetic analysis of forward mutation rates, mitotic gene conversion, mitotic crossing-over, and cytoplasmic petite induction are used.

MAJOR NEW FINDINGS

Preliminary experiments with a diploid yeast in stationary phase suggest that pretreatment with 10 µg/ml 8-MOP and 15,000 ergs of 365-nm light enhances the lethal effects of 254-nm UV. In addition, an apparent synergistic induction of petites was observed.

SIGNIFICANCE

The significance of these data lies in the possible correlation between mutagenesis and carcinogenesis. However, the data represent initial pilot experiments and more extensive investigation is needed before drawing any conclusions with respect to this system. In addition, one must exercise extreme caution in extrapolating mutagenesis data from the labile yeast mitochondrial genome to possible mutagenic behavior in the nuclear genome of higher eucaryotes. Nevertheless, one must also realize the possible value of the yeast mitochondrial genome as a sensitive assay system for compounds which might interact with DNA.

PROPOSED COURSE OF THE PROJECT

The induction of petites by 8-MOP and 365-nm light plus 254-nm UV will be more extensively studied.

Experiments are being planned to investigate the effects of 8-MOP and UV on the nuclear genome of yeast. These experiments will study the rates of forward mutation, mitotic gene conversion, and mitotic crossing-over. Molecular studies on DNA can be performed to correlate DNA damage and repair with survival and mutation rate. The results from these experiments will be more suitable for extrapolation to mutation in higher cells.

The possibility also exists for the study of various known carcinogens or carcinogenic induction procedures on genetic recombination occurring during meiosis. Such information may be of possible teratological importance.

ERDA RT-03-01
ANL 60401

SKIN AND PULMONARY CARCINOGENESIS: SPECIES AND AGE-DEPENDENT DIFFERENCES IN SUSCEPTIBILITY TO ONCOGENIC AGENTS

Walter E. Kisieleski, Principal Investigator
Evelyn M. Buess and R. J. Michael Fry, Participating Investigators

OBJECTIVES

The aim is to determine the factors, and their relative role, that determine species and age-dependent susceptibility to chemical oncogenesis.

BACKGROUND AND PREVIOUS FINDINGS

A considerable change in the susceptibility to chemically induced tumorigenesis occurs over the first few weeks after birth. One or more of the changes with age may contribute to the change in tumor susceptibility: (1) number of cells at risk, which varies with growth and with changes in the distribution of cells in the cell cycle stages; (2) changes in immunocompetence; and (3) uptake, distribution, activation, and deactivation of the oncogen.

EXPERIMENTAL METHODS

Mice of various strains and ages, as well as deer mice (*Peromyscus leucopus*), have been used to investigate (1) susceptibility to lung tumorigenesis, (2) catabolism of urethan, and (3) proliferative activity in the lung tissue. The uptake, tissue distribution, cellular binding, and excretion of urethan, labeled with either tritium or carbon-14, were measured by combustion and liquid scintillation counting.

MAJOR NEW FINDINGS

The catabolism of urethan, which was used as a representative of carcinogens that increase the incidence of murine lung tumors, was found to occur in newborn mice at about 1/10th the rate found in adults. The rate of catabolism increased slowly over the first 10 days of life and increased sharply between 15 and 20 days of age. These results correlate in general with the cancerogenic effects of urethan in newborn and young adult mice. However, similar age-dependent changes in catabolism in urethan were found in *P. leucopus*, which is a species that appears, so far, to be resistant to induction of tumors by urethan.

SIGNIFICANCE

These studies will help to delineate the factors that determine susceptibility and therefore help in determining appropriate methods of assessment of risk. Furthermore, the results will help in the choice of *in vivo* assay systems.

PROPOSED COURSE OF THE PROJECT

We hope to (1) determine the age and strain dependent differences in the enzyme metabolic activity related to the metabolism of the carcinogens, and (2) investigate the factors that influence the number of cells at risk.

ERDA RT-03-01
ANL 60401

SKIN AND PULMONARY CARCINOGENESIS: THE RELATIONSHIP OF LIFE-SPAN TO THE LATENT PERIOD IN TUMORIGENESIS

R. J. Michael Fry, Principal Investigator
Donald D. Grube, Participating Investigator

OBJECTIVES

We wish to determine whether the latent period and susceptibility for tumorigenesis is correlated with life-span. An understanding of the factors that determine tumor susceptibility and expression are necessary for a satisfactory basis of extrapolation of risk estimates across species.

BACKGROUND AND PREVIOUS FINDINGS

Various hypotheses have been advanced to explain the apparent increase of the latent period for tumor development with the life-span of the species. However, a critical examination of these hypotheses, and a demonstration that the latent period is indeed proportional to life-span, are needed before latent period can be used with assurance to extrapolate tumor risk from, for example, mouse to man. Experiments with the rat and dog using nonradioactive implants and radioactive discs (Brues, A. M., et al., ANL-7635, 1969, p. 115) provide data on latent periods for tumor induction in long- and short-lived species of experimental animals. We are using this technique in the white-footed deer mouse, *Peromyscus leucopus*, which has a life-span intermediate between the rat and dog.

EXPERIMENTAL METHODS

Radioactive discs, consisting of laminated Mylar with 2.5-μCi sources of $^{90}Sr/^{90}Y$ (Minnesota Mining and Manufacturing Company), were placed in the subcutaneous tissue of *P. leucopus*. The animals were weighed at various ages to assess the growth rate. Tumors were examined histologically, and the cumulative percent of animals with tumors was plotted as a function of time.

MAJOR NEW FINDINGS

From as yet incomplete tumor data, it is apparent that sarcomas appeared in the deer mouse at least as early as in the rat, despite the fact that the life-span of the deer mouse is significantly greater than in the rat. No sarcomas have yet been found in the dogs. It is clear that species-dependent differences in susceptibility as well as species-dependent differences in latent period are involved.

SIGNIFICANCE

Our results clearly indicate that no general correlation between life-span and latent period for tumorigenesis can be made. This conclusion is important because of the renewed interest in the use of latent period as an assay of tumorigenic effect.

PROPOSED COURSE OF THE PROJECT

In order to examine the relationship between life-span and susceptibility to sarcomagenesis, as well as to latent period, the latent period for induction and the incidence of sarcomas is being determined in dog, mouse, and deer mouse using nonradioactive discs. This experiment will include study of such aspects as comparative proliferative activity in the connective tissue.

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6. EXPERIMENTAL RADIATION PATHOLOGY AND ONCOLOGY

ERDA RT-03-01
ANL 60500

GROUP LEADER'S OVERVIEW

Miriam P. Finkel, Group Leader

The program goal is to provide basic data for evaluating the hazard to man from radioactive materials deposited within the body. The original objective, to obtain dose-response information and to provide data from several species for extrapolating animal data to man, is receiving less attention at present as effort is being put into determining how radiation causes bone cancer and whether viruses play a role.

The program began with the very early radiotoxicologic investigations of materials important in the development of the atomic bomb and the necessity to establish maximum permissible levels of exposure to these materials. With the demonstration that bone cancer is the most sensitive indicator of damage from transuranic elements and some of the fission products, bone pathology became the focus of attention.

When it became evident that questions of human hazard cannot be answered unequivocally on the basis of dose-response relationships, different approaches were considered, and one based on knowledge of mechanisms of cancer induction seemed most likely to be successful. The detection of viruses in both radiation-induced and spontaneous bone cancer of mice, and the present evidence for a similar virus in bone cancer of man, support the hypothesis that radiation causes cancer by activating endogenous neoplastic information, which can also be expressed as oncornavirus. Present emphases therefore concern (1) understanding the biological, biochemical, and physical attributes of the five murine oncornaviruses that have now been isolated in the course of the program, (2) demonstrating the existence of a comparable human oncornavirus, and (3) discovering how radiation and virus interact in the induction of bone cancer.

Significant contributions to radionuclide hazard evaluation and to cancer etiology have already been made by this program, but more information is still needed. If it can be shown that the cancer-inducing action of radiation is mediated through triggering viral information, problems of radiation dose necessary for cancer induction will be simplified, and, when the dose and mechanism are known, there will be a firm basis for estimating the risk to man from environmental contamination with radioactive materials. In addition, if it is demonstrated that human cancer also is associated with oncornaviruses, advances can be expected in the prevention, diagnosis, and treatment of the human disease.

The following individual reports describe separate current or planned components of this broad, but unified, program.

EXPERIMENTAL RADIATION PATHOLOGY AND ONCOLOGY STAFF

REGULAR STAFF

Dale, Phylis J. (Scientific Assistant)
Finkel, Miriam P. (Senior Biologist)
Greco, Isabel L. (Scientific Assistant)
Pahnke, Vernon A. (Scientific Assistant)
Reilly, Christopher A., Jr. (Microbiologist)
Rockus, Gabriele (Scientific Assistant)

TEMPORARY STAFF DURING 1975

Gutzeit, Diane L. (Postdoctoral Appointee)
Lee, Chung K. (Postdoctoral Appointee)

PUBLICATIONS

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., in press.

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Reilly, C. A., Jr., and M. P. Finkel. *In vivo* interference of virus-induced osteosarcomas by a benign bone tumor virus. VIIth International Symposium of Comparative Leukemia Research, in press.

ERDA RT-03-01
ANL 60500

RADIOELEMENT TOXICITY

Miriam P. Finkel, Principal Investigator
Diane L. Gutzeit, Isabel L. Greco, and Gabriele Rockus, Participating Investigators

OBJECTIVES

The objectives are to provide basic data for evaluating the hazard to man from incorporated radionuclides by (1) obtaining dose-response information, (2) determining the most sensitive criterion of effect, (3) providing data from several species as a basis for extrapolating animal data to man, and (4) determining the absorbed energy necessary for bone cancer induction.

BACKGROUND AND PREVIOUS FINDINGS

The project was undertaken initially to determine the biological effects of materials encountered during the development of the atomic bomb. When cancer proved to be one of the most serious hazards, particular attention was given to that disease. The early work, summarized by Finkel, M. P., and B. O. Biskis (Progress in Experimental Tumor Research, Vol. 10, Ed. F. Hamburger. S. Karger, Basel, 1968, pp. 72-111), forms a basis for the continuing program. A few beagles are still alive in an experiment designed to test the influence of (1) duration of exposure to ^{90}Sr, and (2) age at the time of first exposure.

EXPERIMENTAL METHODS

Animals are exposed to a range of dose levels of a given radionuclide and watched throughout their lives for evidence of any difference from control animals in disease incidence and longevity. There are (1) standardized mouse experiments for determining relative effectiveness of different radionuclides, (2) experiments with the same radionuclides in different species, and (3) experiments in which tumor response is measured as the physical, biological, and temporal parameters of dosage are varied.

MAJOR NEW FINDINGS

Four control beagles, 14 to 15-1/2 years of age, died during the past year with varying degrees of osteoarthritis; there was no evidence of other bone pathology. Three of the dogs had tumors of the stomach, mammary gland, and thyroid gland, respectively.

SIGNIFICANCE

Malignant bone tumors continue to show an extremely low spontaneous incidence in beagles, none having been reported in the literature or observed either here or in other ERDA laboratories. This finding increases the reliability of those cases that have occurred at the lower exposures.

PROPOSED COURSE OF THE PROJECT

The ^{90}Sr beagle experiment will be carried to conclusion; only nine controls remain alive.

Experiments with the following objectives should be given serious consideration:

1) Determine whether all or part of the latent period to bone cancer appearance after treatment of mice with ^{90}Sr is occupied with immune reactions against the developing cancer. If immunity does play a role, it should be possible to bypass immunity and develop a rapid system for detecting tumor initiation. Rapid detection would greatly facilitate radionuclide experiments aimed at determining both relative hazards and minimum effective doses.

2) Determine the amount of energy from ^{90}Y or X-ray required for bone cancer initiation and growth in mice. These two agents provide excellent opportunity to manipulate dosage pattern.

3) Generate more data on the toxicity in mice of uranium isotopes (as requested by NCRP) and of ^{239}Pu (in view of present need).

ERDA RT-03-01
ANL 60500

ROLE OF VIRUSES IN BONE CANCER OF ANIMALS AND MAN

Miriam P. Finkel, Principal Investigator
Christopher A. Reilly, Jr., Diane L. Gutzeit, Isabel L. Greco, Gabriele Rockus, Phylis J. Dale, and Vernon A. Pahnke, Jr., Participating Investigators

OBJECTIVES

Our aim is to determine whether bone tumors of animals and man are caused by viruses.

BACKGROUND AND PREVIOUS FINDINGS

A search for virus in spontaneous and radiation-induced bone cancer of mice was undertaken because of the demonstration in other laboratories that viruses were involved in some mouse tumors and because of the desire to know whether they were also involved in the bone tumors induced by radionuclides. At the present time this search has produced five oncornaviruses. Two cause bone cancer (FBJ in CF#1 mice and FBR in X/Gf mice), one causes benign bone tumors called osteomas (RFB in CF#1 mice), and two cause bone tumors of the reticular tissues, or "leukemia" (CF#1-RTTV and X/Gf-RTTV).

With the successful isolation of the first murine bone cancer virus, the search was extended to dogs and man. Strong evidence has been accumulated for such an agent in man: (1) mesenchymal tumors appear in greater number in Syrian hamsters inoculated with cell-free extracts of human bone cancer than in control hamsters, (2) virus-like particles appear occasionally in the human tumors and frequently in the hamster tumors, and (3) immunologic relationships have been demonstrated between bone cancer patients and the hamster tumors.

EXPERIMENTAL METHODS

Cell-free extracts of bone tumors arising spontaneously in mice and human patients and in response to radionuclide exposure in experimental animals are injected into newborn animals of the same strain, except in the case of human cancers, in which cell-free extracts are injected into newborn Syrian hamsters. Tumors appearing in recipients of these extracts are extracted for injection into new hosts, and this process is continued in order to increase the amount and potency of the causative agent. Some tumors are grown in tissue culture and as tumor-transplant lines for the production of materials for electron microscopy, antigen purification, and molecular studies. Primary human bone cancers also are grown in culture.

MAJOR NEW FINDINGS

One of the two cell-free passage lines of X/Gf-RTTV appears to be contaminated with an agent that causes bone pathology (increased density and thickness) very similar to that produced by RFB-osteoma virus in CF#1 mice. Cell-free extracts of the abnormal bones have been injected into newborn X/Gf mice, and, in only three successive passages, the time to roentgenographic appearance of bone pathology has been reduced from ten months to one.

Cell-free extracts of materials originally derived from 110 patients with bone cancer have been injected into approximately 5000 hamsters, and 73 (1.5%) have developed mesenchymal tumors. The incidence in 400 control animals is two (0.5%). Thirty-five percent of the patients are responsible for all of the hamster tumors.

SIGNIFICANCE

The new agent now being isolated from X/Gf bones is extremely interesting:

osteoma has never been reported in X/Gf mice, these benign bone tumors are not induced by radionuclides, and the history of this RTTV line starts with a cell-free extract of a ^{90}Sr-induced bone cancer. The parallel with the situation in CF#1 mice is striking. In that strain the osteoma virus has been isolated seven times, once from a combined extract of an osteoma and an FBJ osteosarcoma from the same mouse, and six times from extract lines starting with ^{90}Sr-induced bone cancer. The importance of having both malignant and benign bone tumor viruses in the same strain is discussed in the following report dealing with the biology of natural oncornaviruses.

PROPOSED COURSE OF THE PROJECT

Isolation of the suspected X/Gf-osteoma virus will be completed. Experiments now in progress will continue. New hamsters will receive human cancer extracts when methods have been devised to make the Syrian hamsters more susceptible to cancer induction by the suspected human agent.

ERDA RT-03-01
ANL 60500

BIOLOGY OF NATIVE MURINE ONCORNAVIRUSES

Christopher A. Reilly, Jr., Principal Investigator
Miriam P. Finkel, Diane L. Gutzeit, Phylis J. Dale, and Isabel L. Greco, Participating Investigators

OBJECTIVES

Our objective is to determine for the five murine oncornaviruses previously isolated by this group the host range (oncogenic potential in a variety of mouse strains), immunologic similarities, and biologic interactions.

BACKGROUND AND PREVIOUS FINDINGS

These oncornaviruses arose after the injection into mice of cell-free extracts of spontaneous or radiation-induced murine tumors. The two bone cancer viruses, FBJ from CF#1 mice and FBR from X/Gf mice, are very potent oncogenic agents in their respective strains of origin, but each is only weakly oncogenic in the other strain. The benign bone tumor virus, RFB from CF#1 mice, readily induces tumors in CF#1 and CBA mice; it is moderately oncogenic in NIH Swiss, and weakly oncogenic in X/Gf mice. Antibody is specific for each virus except that antibody directed against RFB virus has some degree of neutralizing capacity for FBJ virus. Interference studies indicate interaction and competition between the native CF#1 viruses, FBJ and RFB (Reilly, C. A., Jr., and M. P. Finkel, Bibl. Haematol., No. 43. Eds.

J. Clemmesen and D. S. Yohn. Karger, Basel, 1976, pp. 441-444). The two other viruses that have been isolated induce tumors of the reticular tissues: CF#1-RTTV and X/Gf-RTTV.

EXPERIMENTAL METHODS

Three oncornaviruses from CF#1 mice and two from X/Gf mice are tested for tumor induction in four mouse strains: (1) CF#1, the standard mouse for radiotoxicity studies; (2) X/Gf, a tumor-resistant mouse; (3) NIH Swiss, a virus-free strain except for an endogenous xenotropic virus; and (4) CBA, a commonly used strain with a low incidence of spontaneous tumors. To determine the immunologic relatedness of the viruses, serum samples prepared from mice infected with each virus are tested for neutralizing ability against the other viruses. Inactivated but immunologically reactive virus and interferon, an endogenous cell substance with antiviral activity, are used to examine natural tumor expression. Biological interactions of these viruses are determined through dual infections.

MAJOR NEW FINDINGS

Interference of RFB virus against FBJ tumor induction was complete in some instances when CF#1 pups were foster-nursed by NIH Swiss dams. Interference occurred even when RFB virus had been inactivated.

Preinoculation with herpes simplex virus type II delayed the appearance of FBJ bone cancers but did not decrease their number. Interferon, on the other hand, both delayed cancer appearance and decreased cancer incidence, although it had little effect on established tumors. Interferon had no effect on RFB osteomas.

FBJ genome was rescued from a non-virus-producing FBJ-induced hamster sarcoma cell line by Rauscher leukemia virus (in collaboration with J. Levy, University of California, San Francisco). The Rauscher leukemia virus pseudotype RLV-MSV (FBJ) induced typical FBJ bone cancer in CF#1 and Swiss mice.

CF#1-RTTV is equally oncogenic in CF#1 and CBA mice. NIH Swiss are less susceptible, and X/Gf mice are totally refractory.

SIGNIFICANCE

The interference studies suggest that differences in natural incidence of spontaneous malignant and benign bone tumors might be due to viral interactions. Mechanisms of this type could explain why extraction procedures with radionuclide-induced CF#1 bone cancer yield osteoma virus instead of bone cancer virus. With benign bone tumors being twice as frequent as malignant tumors in man, similar interference might also have a bearing on the fact that no malignant virus has as yet been isolated from human cancer.

PROPOSED COURSE OF THE PROJECT

(1) Interference studies will continue and be extended to the other native murine oncornaviruses isolated in this laboratory. (2) The presence of RFB virus in radionuclide-induced bone cancer and its subsequent effect on malignant virus expression will be examined. (3) The observation that a herpes virus commonly found in man can interfere in the mouse system will be verified and other viruses tested. (4) Interferon action will be examined critically. (5) Host-range determinations for X/Gf-RTTV will be completed.

ERDA RT-03-01
ANL 60500

ACTION AND PRODUCTION OF BONE TUMOR VIRUSES IN CELL CULTURE

Chung Keel Lee, Principal Investigator
C. A. Reilly, Jr., Gabriele Rockus, Isabel L. Greco, and Miriam P. Finkel, Participating Investigators

OBJECTIVES

Our aims are (1) to compare cellular transformation in culture with tumor production in animals by three murine bone tumor viruses, (2) to investigate interference between the viruses in culture, and (3) to activate virus production by stimulation of tumor cells in culture with 5-iododeoxyuridine (IUdR).

BACKGROUND AND PREVIOUS FINDINGS

FBJ osteosarcoma virus and RFB osteoma virus are highly oncogenic in CF#1, CBA, and NIH Swiss mice and weakly oncogenic in X/Gf mice; FBR osteosarcoma virus is oncogenic in X/Gf, but only weakly oncogenic in CF#1 and NIH Swiss. Recent work has shown that RFB virus interferes with the induction of bone cancer by FBJ virus (Reilly, C. A., Jr., and M. P. Finkel, Bibl. Haematol., No. 43. Eds. J. Clemmesen and D. S. Yohn. Karger, Basel, 1976, pp. 441-444). Our preliminary experiments with the hamster tumor cell line, RV 50/3, suggested that virus could be activated by IUdR.

EXPERIMENTAL METHODS

Cellular transformation of different target cells was determined by formation of foci of morphologically altered cells in culture and subsequent recovery of ^{3}H-uridine-labeled virus. Tissue culture lines of a human bone cancer and a hamster sarcoma, induced by a cell-free extract of human bone cancer, were used for virus activation studies. Electron microscopic observation of C-type particles and recovery of ^{3}H-uridine-labeled virus were used as criteria of activation.

MAJOR NEW FINDINGS

RFB virus produced no foci in embryo tissue culture cells of any strain, whereas FBJ virus formed foci in all of the cells tested. Although the cells of X/Gf had a very low incidence of focus formation, FBR virus gave a high incidence of focus formation in X/Gf cells, but a low incidence in CF#1 and CBA cells, and no foci in NIH Swiss cells. On a sucrose gradient, a peak of radioactivity was observed at a density of 1.16-1.17 g/cm^3, corresponding to the density of most animal RNA tumor viruses, whenever foci were present. In the case of RFB-treated cultures, there was a similar peak of radioactivity although there had been no foci. Typical C-type virus particles were observed whenever the radioactivity peak was obtained. Interference between RFB and FBJ viruses in cell cultures followed the same pattern as in animals, i.e., RFB benign bone tumor virus interferes with focus formation by FBJ virus. In both the hamster and the human cancer cell lines, IUdR activated virus production, but in low yield.

SIGNIFICANCE

The advantages of cell culture systems can be exploited with this demonstration that the three bone tumor viruses reflect the same behavior in cell culture as in the animal. This should allow better understanding of the mechanism of bone tumor induction. The interference observed between the CF#1 bone tumor viruses in cell cultures suggests involvement of a factor or factors other than immune mechanism in the interference phenomenon.

PROPOSED COURSE OF THE PROJECT

Cell lines from murine virus-transformed cells will be established to provide large quantities of pure virus. Interference studies will be continued, and attempts will be made by chemical activation to increase the yield of virus from human and hamster cancers.

ERDA RT-03-01
ANL 60500

EXPERIMENTAL APPROACHES TO THE IDENTIFICATION AND GROWTH OF A PUTATIVE HUMAN CANCER VIRUS

Diane L. Gutzeit, Principal Investigator
Miriam P. Finkel, Christopher A. Reilly, Jr., Phylis J. Dale, Vernon A. Pahnke, Jr., and Gabriele Rockus, Participating Investigators

OBJECTIVES

Our objectives are: (1) to provide immunologic evidence of a viral etiology for human bone cancer, and (2) to increase the susceptibility of Syrian hamsters to the putative human cancer virus in order to provide definitive proof of its existence.

BACKGROUND AND PREVIOUS FINDINGS

Sera from bone cancer patients were found to react with human bone cancer antigens, as demonstrated by fluorescence. These sera also reacted with six of seven mesenchymal tumors from hamsters inoculated with cell-free extracts of human sarcomas (Reilly, C. A., Jr., et al., Cancer 30, 603, 1972).

Mesenchymal tumors have developed in 1.5% of the 4850 Syrian hamsters inoculated at birth with cell-free extracts of human bone cancer, but in only 0.5% of the 400 control animals. Previous attempts to increase susceptibility by pretreatment with immunosuppressants or by urethan used as a cocarcinogen were not successful.

EXPERIMENTAL METHODS

Standard indirect fluorescent antibody procedures (U.S. Dept. of H.E.W., No. 729, 1960) are carried out with sera from bone cancer patients and cryostat sections or tissue-culture-cell slides of bone cancer and other mesenchymal tumors from hamsters that were inoculated at birth with cell-free extracts of human bone cancer.

In an attempt to increase the mesenchymal-tumor response of hamsters to the inoculation of extracts of human material, we are testing their susceptibility to FBJ virus oncogenesis under three different situations, because hamsters seem to be equally susceptible to cancer induction by the putative human agent as by this murine bone cancer virus:

1) Urinary bladder, when placed subcutaneously, forms osseous elements in some species. Therefore, fragments of hamster urinary bladder saturated with FBJ virus were transplanted subcutaneously in the inguinal area of 2 to 3-day-old hamsters.

2) FBJ virus was placed in direct contact with susceptible cells by injecting it into the femoral marrow cavity of weanling hamsters.

3) To bypass the immunological defense imparted to the newborn by the ingestion of maternal antibody in the milk, FBJ virus was injected into the fetus 1 to 3 days before birth.

MAJOR NEW FINDINGS

Cryostat sections of two human mesenchymal tumors and seven hamster tumors arising in animals given cell-free extracts of human bone cancer gave positive immunofluorescent reactions, a result suggesting a common antigen in the human and hamster tumors.

At the present stage of the *in utero* and urinary bladder transplant experiments, there is little improvement in growth response over previous trials.

SIGNIFICANCE

Identification of an antigen in tumors of hamsters inoculated with cell-free extracts of human bone cancer that reacts specifically with antibody in the serum of bone cancer patients suggests that the hamster tumors carry a human agent, and implies that these tumors were caused by the agent. By increasing the susceptibility of hamsters to bone cancer virus, we should be able to obtain more material for testing for the presence of a human agent. Proof of a human oncornavirus will have great influence on cancer research and has important implications for cancer induction through viral activation by radiation and other environmental pollutants.

PROPOSED COURSE OF THE PROJECT

The immunofluorescent technique will be refined to assure specificity and to avoid false positives. Other hamster tumors suspected of carrying a human agent will be examined as they become available.

Any successful procedures with FBJ virus will be applied to cell-free extracts of human cancer and of hamster mesenchymal tumors suspected of carrying a human virus. In this way material will be generated for the immunofluorescent and other studies.

ERDA RT-03-01
ANL 60500

ROLE OF ONCORNAVIRUSES IN TUMOR INDUCTION BY RADIATION

Miriam P. Finkel, Principal Investigator
Christopher A. Reilly, Jr., Diane L. Gutzeit, Isabel L. Greco, Gabriele Rockus, Phylis J. Dale, and Vernon A. Pahnke, Jr., Participating Investigators

OBJECTIVES

We wish to determine whether radiation causes bone cancer by activating an endogenous oncornavirus.

BACKGROUND AND PREVIOUS FINDINGS

The discovery of FBJ virus made it possible to induce bone cancer in CF#1 mice with virus as well as radionuclides. When ^{90}Sr and FBJ virus were given to the same animals, very complex enhancing and inhibiting interactions occurred, and the hypothesis was advanced that radiation causes cancer by inactivating an inhibitor of an endogenous virus. (Finkel, M. P., and B. O. Biskis, Delayed Effects of Bone-Seeking Radionuclides, Ed. C. Mays, et al., University of Utah Press, Salt Lake City, 1969, pp. 417-435; and Finkel, M. P., et al., Oncology, Vol. 1, Cellular and Molecular Mechanisms of Carcinogenesis, Ed. R. L. Clark. Year Book Medical Publishers, Chicago, 1971, pp. 422-434.)

EXPERIMENTAL METHODS

Evidence of FBJ and FBR bone cancer viruses is sought in radiation-induced bone cancer of CF#1 and X/Gf mice, respectively, by immunologic means: (1) neutralization of virus by sera of mice bearing radiation-induced bone cancer, (2) immunization against radiation-induced bone cancer by specific antibody and by virus vaccines, and (3) detection of viral components with techniques such as radioimmunoassay and molecular hybridization in tissues undergoing very early cancerous changes.

NEW FINDINGS

None.

SIGNIFICANCE

If the cancer-inducing action of radiation can be shown to be mediated through the triggering of oncogenic information normally present in the genetic library of the cell, the target for radiation induction of cancer will be known, and problems of radiation dose will then be able to be approached

properly. Knowledge of target and dose will provide a firmer basis for estimating risks to man than dose-response curves. Demonstration of viral etiology for ionizing radiation also would have important influence on investigations of cancer induction by biological and chemical agents.

PROPOSED COURSE OF THE PROJECT

Experiments will be undertaken to answer the following questions: (1) Are the effects of ^{90}Sr and FBJ virus additive, synergistic, or inhibitory (a repetition and expansion of a previous study)? (2) Are components of FBJ virus present in bone tissues irradiated by ^{90}Sr and destined to become cancerous? (3) Can antibody against FBJ virus or bone tumor virus vaccines prevent bone cancer induction by ^{90}Sr? (4) Can the benign bone tumor virus (RFB) interfere with bone cancer induction by ^{90}Sr?

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7. AGING RESEARCH

ERDA RT-03-01
ANL 60600
ANL 60601
EPA-IAG-D6-E681

GROUP LEADER'S OVERVIEW

George A. Sacher, Group Leader

The research program of the Aging Research Group, Effects of Energy-Related Pollutants on Aging, has three major components: (1) the interaction of genotype and environment in the aging processes of mammals; (2) the comparative analysis of the differences in longevity and aging among mammalian species; and (3) analysis of immunological factors in the aging process, in the two aspects of (a) aging of the immune system as a primary aging process, and (b) the role of the immune system in the defense of the organism against cancer. This information is essential for ERDA missions and for the formulation of public policy about aging.

The program on genotype-environment interaction with aging is based on the premise that aging is an aspect of reproductive fitness, and hence is the outcome of a number of independent genetic factors that are distributed in populations. Since this genetic variance is maintained by variations in the environment, it is highly likely that there is significant genetic variance in the long-term response to low levels of environmental pollutants, and that these effects are interactive. If this is so, then the most cost-effective and rapid procedure for evaluating the effects of pollutants is by means of multivariate designs in which several pollutants are simultaneously evaluated on sample populations with appropriate genetic structure. Our research on this topic, which began several years ago with ionizing radiation as the environmental perturbation, is currently beginning to examine the effect of low environmental temperature. Experiments employing selected chemical pollutants in the same genetic design will follow shortly.

The program on the comparative and evolutionary analysis of longevity and aging has had significant influence on gerontological thought, for it has established that mammalian longevity is almost completely determined (multiple correlation of 0.9 or higher) by a small set of constitutional parameters--brain weight, body weight, metabolic rate, and body temperature. Our laboratory research has shown that all these parameters play the same role within the family of small mouselike rodents. This work is providing a rational basis for interspecies extrapolation of the life-shortening effects of ionizing radiations. In the near future work will begin on the life-shortening effects of chemical agents in rodent species with different life-spans.

Elucidation of the autoimmune functions of the immune system, both functional and dysfunctional, is important for our understanding of the aging process. Also, because of its role in protecting the organism from biotic invasion from within as well as without, the immune system is a key factor in defense against neoplasia induced by environmental agents. One important outcome of our work in immunology is the evidence that the aging lesion in the immune system is not a diffuse deterioration, but rather a highly specific defect or defects. This leads to the presumption that the defect is genetically determined, and to the possibility--for which we already have some evidence--that it can be reversed or overcome. The current work on the suppression and enhancement of immune response by factors in mouse lymphoma cells gives new evidence for the existence of interactions among immune cells at the cell population level, of a kind that is especially susceptible to deterioration with age. The important role of cell-mediated immunity in the defense against cancer gives research on the effects of energy-related pollutants on the immune system special importance, and the new project on the *in vitro* assay of cell-mediated immunity may provide a valuable contribution to the toxicological evaluation of energy-related pollutants.

A program was initiated on the biometric analysis of the complete blood counts of a selected sample of 1154 Argonne Laboratory employees from whom blood cell counts were made annually from 1944 through 1962.

Research and development in this program overall saw reasonable progress during the year. A disappointing outcome was in the application of the Cytofluorograf (Bio/Physics, Inc.) to the counting of immunofluorescent-labeled lymphocytes. The instrument lacks sensitivity and specificity, and does not yield consistent correlations with optical scoring. On the other hand, our system for monitoring metabolism, which provides continuous monitoring of oxygen consumption, body temperature, and motor activity, has been highly reliable and productive. A Ph.D. dissertation (H. Braham) which was completed while the system was under development required considerable effort in manual transcription of the data. In its recently completed state, the system provides automatic data acquisition in digitized, computer-compatible form, and is a powerful instrument for the analysis of patterns of metabolism and activity, and their degradation with age or exposure to toxic environments.

B. N. Jaroslow's work on the immune suppressant and stimulatory factors in mouse lymphoma cells received editorial recognition (BioScience 25, 59, 1975), and he became a Fellow of the Gerontological Society in 1975.

G. A. Sacher was a consultant to the Futures Group on an NSF-sponsored project to evaluate the social impact of possible future developments in life prolongation research.

AGING RESEARCH STAFF

REGULAR STAFF

*Cerny, Elizabeth A. (Scientific Assistant)
Dornfeld, Suzanne S. (Scientific Assistant)
Duffy, Peter H. (Scientific Assistant)
Jaroslow, Bernard N. (Immunologist)
*Rahman, Yueh-Erh (Biologist)
Sacher, George A. (Senior Biologist)
Sanderson, Margaret (Scientific Associate)
Suhrbier, Katherine M. (Scientific Assistant)
Svihla, George (Biologist)
Tyler, Sylvanus A. (Mathematician)
*Wright, Betty Jean (Scientific Assistant)

TEMPORARY STAFF DURING 1975

†Cater, Jerome (Research Associate)
*Jonah, Margaret M. (Research Associate)

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*Now in Biochemistry Group.

†Terminated during 1975.

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ERDA RT-03-01
ANL 60600

COMPARATIVE MORPHOLOGY OF SHORT- AND LONG-LIVED RODENT SPECIES

Peter H. Duffy, Principal Investigator
George A. Sacher, Participating Investigator

OBJECTIVES

This is a project to measure and compare rates of adult growth (growth after sexual maturity) for body weight and linear body dimensions of the short-lived house mouse (*Mus musculus*) and the longer-lived white-footed mouse (*Peromyscus leucopus*).

BACKGROUND AND PREVIOUS FINDINGS

It is known that the laboratory rat has an indeterminate growth curve, with skeletal dimensions and body weight continuing to grow throughout the greater part of adult life (Berg, B. N., and C. R. Harmison, J. Gerontol. 12, 370, 1957). Laboratory mice grow in body weight during adult life, and Silberberg (Silberberg, R., Gerontologia 17, 236, 1971) showed that the epiphyses of the vertebrae and long bones of two inbred laboratory mouse strains do not close until near the end of life, but no studies of linear growth of adult mice have been reported. No data on any aspect of adult growth in *Peromyscus leucopus* were available in the literature. In the course of our research on age changes in energy metabolism of *Mus* and *Peromyscus* (Sacher, G. A., et al., ANL-75-30, 1975, p. 111) we saw indications that these two species have different growth trends during adult life, with *Mus* showing continued growth, as had been noted in previous studies, and *Peromyscus* attaining a constant body weight early in adult life. Since this finding was unexpected, and is important for our research on the comparative biology of aging, a study of lifetime trends in body weight and linear dimensions of the two species was planned and executed.

EXPERIMENTAL METHODS

Measurements were taken of head plus body length (HBL), tail length (TL), and body weight (BW) for *M. musculus* from 5 to 30 months of age. The same measurements were taken on *P. leucopus* from 6 to 60 months of age. Their maximum life-spans are 42 and 100 months, respectively.

MAJOR NEW FINDINGS

Mus (both sexes) grows about 15% in HBL and 45% in BW from 5 to 20 months of age. Thereafter, growth in HBL ceases, and BW decreases at age 30 months. TL for *Mus* males increases about 14% from 4.6 to 21 months of age, and TL for *Mus* females increases about 11% from 4.5 to 30.5 months of age.

There was no significant increase with age in HBL for *Peromyscus* of either sex from 6 to 60 months of age. However, there is a significant decrease of BW by about 16% in both sexes, and a significant increase of TL by about 6% in both sexes over the same time period.

The decrease of body weight in *Mus* after 20 months of age is a uniform aging characteristic and not a consequence of terminal disease.

SIGNIFICANCE

The striking differences in adult growth pattern of these two related rodent species raise questions about the relation of growth pattern to aging and longevity, and about the relation of growth regulation and cancer. In adult growth pattern, *Mus* resembles the laboratory rat, whereas *Peromyscus* resembles man. The growth characteristics of *Peromyscus* gain added interest from the observation that the histopathology of some tumors in *Peromyscus* shows striking resemblance to human tumors (L. Lombard, personal communication). Such findings give us reason to consider the hypothesis that there are fundamental differences of organization between "short-lived" and "long-lived" species of mammals. This insight may open new directions in the search for appropriate models of human aging and neoplastic diseases.

PROPOSED COURSE OF THE PROJECT

Further study of adult growth patterns in the order Rodentia is contemplated to determine the factors responsible for indeterminate and determinate growth.

Tracer studies of cellular turnover in *Mus* and *Peromyscus* are also under consideration.

ERDA RT-03-01
ANL 60600
ANL 60601

COMPARATIVE BIOLOGY OF AGING

George A. Sacher, Principal Investigator
Peter H. Duffy, Sylvanus A. Tyler, Howard W. Braham, William F. Blakely,† and William D. Wickart,‡ Participating Investigators*

OBJECTIVES

The program on comparative biology of aging is concentrated in three major areas: (1) comparison of aging changes in metabolism of two myomorph rodent species, *Mus musculus* and *Peromyscus leucopus*, which are morphologically and physiologically similar, but differ in longevity by a factor of 2.5; (2) genetics of metabolism of inbred and hybrid mice, studied with a 5 x 5 diallel design; and (3) metabolism, pathology, and survival of thermally and chemically stressed rodents. The investigation is focused on determining age-related changes in activity and metabolism, their significance for age-decrements in health and viability, and their modifiability by environmental and genetic factors.

BACKGROUND AND PREVIOUS FINDINGS

Our research has established that there is a twofold variation in life expectation among the small mouselike rodents, and that more than 80% of the variance is accounted for by regression on brain weight, body weight, metabolic rate, and body temperature (Sacher, G. A., Interdisciplinary Topics in Gerontology, Vol. 9. Ed. R. G. Cutler. Karger, Basel, 1976, pp. 69-82).

Although length of life is inversely related to metabolic rate between species (Sacher, ibid.), longevity within species, among mouse strains, has a small positive correlation with metabolic rate (Storer, J. B., Exp. Gerontol. 2, 173, 1967). On the other hand, increase of metabolic rate of rats in a cold environment is associated with a reciprocal decrease of life expectation (Carlson, L. D., et al., Radiat. Res. 7, 190, 1957). The effect of a cold environment on the life-shortening effects of ionizing radiations was examined by Carlson et al. (ibid.), but this has not been studied for tumor induction by chemical carcinogens, which can be expected to have strong interactions with metabolic rate.

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*Thesis Parts Student, Ohio State University.

†Participant in the 1975 Summer Graduate Student Program in Biology, University of Illinois, Urbana.

‡Fall 1975 participant in the Undergraduate Honors Research Participation Program, Knox College.

Age-decrement of resting metabolic rate, and of the maximum rate of metabolism, occurs in mouse, rat, and man. However, age changes in the metabolism of activity, both spontaneous and in response to stress, are on the whole poorly known and understood.

Decrease of the length of the circadian period has been reported for *Peromyscus* (Pittendrigh, C. S., and S. Daan, Science 186, 548, 1974), but little is known about decreases in the integrity or amplitude of the diel activity cycle, or about their significance for health, or for capacity to resist stress. An excitatory neuropharmacologic agent (L-dopa) has been reported to prolong life (Cotzias, G., et al., Proc. Nat. Acad. Sci. USA 71, 2466, 1974). The effects of such agents on spontaneous activity and metabolism are not known, nor is it known whether an increase in activity and metabolism accompanies the paradoxical prolongation of life that is sometimes observed in populations subject to mild stress (Sacher, G. A., Handbook of Biological Aging, Eds. J. Birren et al. Van Nostrand Reinhold, New York, 1976, in press).

EXPERIMENTAL METHODS

Age-dependent metabolism is monitored with a system consisting of (1) an automated continuous-flow oxygen utilization apparatus (Beckman G2), (2) telemetered body temperature using an implanted temperature transducer-transmitter, (3) spring-loaded activity sensors of the velocity transducer type, and (4) platinum resistance digital ambient temperature thermometers. Sixteen channels of information, four channels for each of four animals, are processed by an automatic-sequencing data acquisition system and recorded on punched paper tape for subsequent computer analysis.

A cold environment room has been developed that regulates temperature and humidity for long-term exposures. All animal populations are carefully monitored for disease morbidity, to maximize the yield of high-quality histopathology.

MAJOR NEW FINDINGS

An experiment has been completed in which resting, average, and maximum oxygen utilization was monitored as a function of age for *M. musculus* and *P. leucopus*. The age dependence of the three variables for each species seems to be closely related to the maximum life-span for the species. The initial values and slopes for all three variables are higher for *Mus* than for *Peromyscus* at equivalent fractions of the life-span, with the three regression lines intersecting at 64 months and 139 months, respectively, for the two species. A dramatic decrease in the amplitude and coherence of the diel metabolic cycle with age is observed in both species.

An experiment was performed to compare the ability of young and old house mice to increase energy production in a cold environment. The experimental procedure was to decrease temperature as a linear function of time, and observe the increase of oxygen consumption. Under these experimental conditions, 24-month-old mice performed as well as young mice down to -15°C, the limit of the equipment. It will be necessary to repeat the experiment using a helium-oxygen atmosphere to increase the rate of heat loss.

The metabolic patterns of *Mus* and *Peromyscus* were investigated in a 4-day sequence of temperature and light-dark cycles. The experiment was a 2 x 2 design in which 20 young male mice of the two species were divided into control and irradiated groups. The radiation doses were two-thirds of the LD_{50} of gamma rays for each species. The main finding is that irradiated *Mus* and *Peromyscus* are less efficient in the control of metabolic processes for thermoregulation than their unirradiated controls.

SIGNIFICANCE

These studies tell us that (1) the metabolism of activity for both species diminishes more rapidly with age than resting metabolism, and may be a significant life-limiting factor; and (2) irradiation impairs the efficiency of temperature regulation, but there are indications that the impairment is not identical with the age-related decrement in thermoregulation.

PROPOSED COURSE OF THE PROJECT

Intensive studies of metabolic patterns as a function of age, genotype, and environment will begin. The disintegration of diel patterns of metabolism and activity with age will be investigated in relation to the metabolic and behavioral demands of the cage environment, and to the influence of toxic substances and neuropharmacologic agents. In particular, an effort will be made to reestablish strong diel cycles in old mice. Time series analysis of the three continuously monitored variables will be initiated, with particular reference to their dynamic interrelations. We intend to examine the hypothesis that factors tending to increase or maintain the availability of energy in old animals will conduce to improved vigor, health, and longevity.

ERDA RT-03-01
ANL 60600

IMMUNOLOGIC CHANGES IN AGING ANIMALS: EXTRINSIC AND INTRINSIC FACTORS OF AGING THAT AFFECT IMMUNOCOMPETENT CELLS

Bernard N. Jaroslow, Principal Investigator
Katherine M. Suhrbier, Participating Investigator

OBJECTIVES

Our objectives are (1) to describe changes in the immune response of aging animals and to differentiate age-associated intrinsic changes at the cellular level from host environmental effects, such as a neoplastic disease; (2) to discover methods to restore age-associated losses in immunologic

activity; and (3) to determine which changes with age are programmed and to describe their place in the overall picture of development and senescence. These results are used to create a model that describes aging of the immune response, and that can be tested experimentally.

BACKGROUND AND PREVIOUS FINDINGS

Decline of antibody-forming capacity with age results from intrinsic factors, such as less proliferative activity of fewer responder cells, and undefined extrinsic factors in the internal environment of healthy, aging animals (Price, G. B., and T. Makinodan, J. Immunol. 108, 403, 1972). The ability of antibody-forming cells, in dogs, to make the usual switch from IgM-antibody to IgG-antibody formation was shown to decline with age, but this decline was overcome by giving the dogs multiple injections of antigen (Jaroslow, B. N., et al., J. Immunol. 112, 1467, 1974). An immunosuppressive extrinsic factor was found in mice. It is produced by spontaneous lymphomas, which frequently appear in 2-year-old mice (Jaroslow, B. N., et al., J. Nat. Cancer Inst. 54, 1427, 1975).

The development of immunologic competence follows a prescribed pattern (Solomon, J. B., Foetal and Neonatal Immunology, American Elsevier Co., New York, 1971), but no association has been made between this pattern and the decline of the immune response. Our studies of the development and senescence of cell-mediated immunity (Menon, M., et al., J. Gerontol. 29, 499, 1974) and of macrophage function (Jaroslow, B. N., and M. Miller, Advances in Radiation Research, Vol. 3. Gordon and Breach, New York, 1973, pp. 1381-1393), as well as those concerned with the decline of antibody production (see preceding paragraph), suggest a programmed pattern of the decline of immunologic competence with age that is related to its developmental sequence.

EXPERIMENTAL METHODS

Immune responsiveness is measured after immunizing the recipient animal, or after initiating the immune response in cultures of spleen cells.

Cultures are made with spleen cells from mice of different ages. The antigen, sheep red cells, is added to initiate the immune response. Agents that affect different cellular events during development of the immune response are tested to either suppress or enhance the immune response. The assay for production of antibody-forming cells is carried out by the "limited hemolysis in agar" technique.

MAJOR NEW FINDINGS

L-1V cells from a transplantable mouse lymphoma suppress the immune response only when they are added to a culture of immunocompetent mouse spleen cells during the induction period. In the same test system, an extract of a concentrated suspension of L-1V cells, added during induction, enhances the immune response about fivefold by stimulating the proliferative activity of the induced cells. The extract does not overcome the inhibition by live cells of the induction of the immune response.

Spleen cells from 10-day-old and from 600-day-old mice inhibit the immune response of 180-day-old mouse spleen cells in culture. The inhibition, which is associated with the proliferative phase of the immune response, is overcome by the lymphoma extract.

SIGNIFICANCE

Immunosuppression by tumor cells is associated with induction of the immune response, whereas inhibition by very young and old spleen cells is associated with the proliferative phase of the immune response. Attempts to restore immunocompetence in old animals must therefore be directed to the specific stage of the response that is affected, according to its intrinsic or extrinsic origins. Immunosuppression by very young and old cells indicates that there is an intrinsic immunosuppressive factor produced in the course of normal developmental processes.

PROPOSED COURSE OF THE PROJECT

We will continue to characterize the immunologically active factors in lymphoma extract and use them in our studies to investigate the suppressive factors found in spleen cells from young and old mice. The information from these experiments will then be incorporated into building a model that will describe the cellular changes that take place during development and senescence.

Parallel *in vivo* studies will be carried out to determine the significance of the *in vitro* studies for the organism.

ERDA RT-03-01
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EPA-IAG-D6-E681

EFFECTS OF ENVIRONMENTAL CARCINOGENS ON DEVELOPMENT OF THE IMMUNE RESPONSE: DEVELOPMENT OF QUANTITATIVE MEASURES OF CELL-MEDIATED IMMUNITY

Bernard N. Jaroslow, Principal Investigator
Suzanne S. Dornfeld, Participating Investigator

OBJECTIVES

The major objective of this program is to determine effects of an environmental carcinogen on the development and senescence of the immune response. Our first step toward this goal is to develop a quantitative assay of cell-mediated immunity.

BACKGROUND AND PREVIOUS FINDINGS

Because some carcinogens are known to be immunodepressant (Stjernswärd, J., J. Nat. Cancer Inst. 37, 505, 1966), and because cell-mediated immunity is a major component in immunity to cancer (Teller, M. N., Adv. Gerontol. Res. 4, 25, 1972; and Kersey, J. H., et al., Adv. Cancer Res. 18, 211, 1973), we are investigating the effects of environmental carcinogens on cell-mediated immunity.

EXPERIMENTAL METHODS

Mice (strain C57BL/6An1), or cultures of spleen cells from mice, are immunized against foreign target cells. The cytotoxic capacity of spleen cells is assayed by adding them to a suspension of target cells containing radioactive chromium. Target cells that are killed by immunocytes release radioactivity, and the amount released is related to the cytotoxic capacity of the immunocytes (Brunner, K. J., et al., Immunology 14, 181, 1968). We shall use an improved method (Dunkley, M., et al., J. Immunol. Methods 6, 39, 1974) for the development of our quantitative assay.

MAJOR NEW FINDINGS

The assay procedure has been adapted for our purposes, and the data show a consistent relationship over the titration range of the test cell suspension. Agents that enhance or suppress the immune response change the shape of the titration curve. The titration curves are being analyzed to determine the characteristics of the curve that are linked to the cytotoxic capacity of the spleen cell suspension.

SIGNIFICANCE

It is essential to develop a quantitative assay in order to determine effects of low-level, slow-acting agents (such as environmental carcinogens) on the developmental succession and capacity of cell-mediated immunity.

PROPOSED COURSE OF THE PROJECT

We shall describe the rise and fall of cell-mediated immunity with age in control animals and in experimental animals exposed to: (1) a low-dose, chronic exposure of an environmental carcinogen, to simulate projected environmental levels of exposure; (2) a single dose of carcinogen at a threshold level for tumor development; and (3) a single dose at a level to give 30% incidence of tumors. Exposure will be initiated in newborn, 2-month-old (age of peak responsiveness) and 400-day-old (age just prior to onset of spontaneous lymphomas) C57BL/6 mice.

ERDA RT-03-01
60600

COMPARATIVE STUDIES OF IMMUNOLOGY AND AGING: CYTOFLUOROMETRIC METHODS FOR COUNTING SPECIFIC LYMPHOCYTE POPULATIONS

George A. Sacher, Principal Investigator
Bernard N. Jaroslow, George Svihla, and Margaret M. Sanderson, Participating Investigators

OBJECTIVES

The primary objective of this project was to determine the number and proportion of thymus-derived cells in the spleen of the wild mouse (*Mus musculus*) and of the white-footed deer mouse (*Peromyscus leucopus*) at different ages throughout their life-spans. The goal was to compare the rise and fall of thymus-derived cells in the spleens of these two related species of rodents, which differ in longevity by a factor of 2.5, and to determine the relationship, if any, between the findings and the species longevity.

EXPERIMENTAL METHODS

This experiment was designed to make use of the high speed and fluorescence sensitivity of the Cytograf-Cytofluorograf (Bio/Physics, Inc.), as the only practical method for accomplishing the experimental objective. Thymus-derived spleen cells were labeled with specific fluorescent antibodies and counted in the Cytofluorograf.

MAJOR NEW FINDINGS

We have not obtained acceptable results either by methods described in the literature, or by modifications. Specificity of the Cytofluorograf was unacceptably low, and the results were not reproducible. Because the Cytofluorograf instrumentation is not likely to provide the data we require, the approach has been discarded.

ERDA RT-03-01
ANL 60600

TIME STUDY OF HEMATOLOGICAL VARIABLES IN A SELECTED GROUP OF EMPLOYEES

Sylvanus A. Tyler, Principal Investigator
Asher J. Finkel,[*] Austin M. Brues,[†] Ruth M. Hillard,[‡] Betty M. Vandolah,[‡] and Carol A. Fox,[**] Participating Investigators

OBJECTIVES

The objectives of this program are (1) to describe the blood count variables measured at least annually by the Health Division from 1944 through 1962 on a selected segment of the work force of Argonne National Laboratory; (2) to determine suitable variable transformations that render the distribution of each blood element approximately normal and homoscedastic; (3) to compare the characterizing statistics among major subgroups of employees; and (4) to ascertain whether there are any correlations between hematologic changes and other variables such as environmental radiation levels and aging of individuals.

BACKGROUND AND PREVIOUS FINDINGS

The records of the Argonne Health Division are a rich repository of data, collected under standard conditions and in many instances by the same technicians for a period over two decades. Similar opportunities are rare for the study of long-term biological trends in humans. The present program is based on an interest in correlating the hematological variables with variables in the intrinsic and extrinsic environment. For example, the period of study spans a period of fluctuating environmental radiation levels, marked by the peak of radioactive fallout during 1957-1959.

ENVIRONMENTAL METHODS

Only persons who were full-time employees of the laboratory in 1954 and were employed by the Laboratory through 1959 are included in the study, which is based on a total of 1154 employees.

The following blood elements were measured: hemoglobin, hematocrit, red blood count, white blood count, polymorphonuclear neutrophils, lymphocytes, and monocytes. Derived red cell parameters computed were mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration. For each year, frequency distributions based on age categories (18-22, 23-27, 28-32, etc.) are constructed by subgroups and the sample statistics are computed. Plots of the variance versus mean and standard deviation versus mean are made for each variable and derived parameter.

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[*] American Medical Association, Chicago, Illinois.

[†] Division of Radiological and Environmental Research.

[‡] Health Division.

[**] Computer Applications Staff.

MAJOR NEW FINDINGS

A scan of the analysis so far completed reveals a linear relationship of the standard deviation to the associated mean value for the major white blood elements, whereas a curvilinear relationship is evident with variance versus the mean value. These results suggest that the logarithmic transform of counts of white blood elements provides a suitable transform for eliminating the dependence of the standard deviation on the mean, thus making possible the use of normal statistical theory for purposes of estimation and tests of hypotheses.

SIGNIFICANCE

The assessment of hematological trends in a stable population carefully followed for over two decades will provide valuable information about the correlations between the human hematopoietic system and environmental variables.

PROPOSED COURSE OF THE PROJECT

Computer algorithms are now being constructed to test relationships between the increase in environmental radiation content during the period 1957-1959 and major blood variables.

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8. BIOCHEMISTRY

ERDA RT-03-02 and RT-01-02
ANL 61100
ANL 64101
NASA-P-7142-C

GROUP LEADER'S OVERVIEW

John F. Thomson, Group Leader

The Biochemistry group consists of several diverse activities representing two ERDA categories, plus NASA support. Two important changes have taken place in this group since the last report: (1) Dr. Y. E. Rahman's project on liposome-encapsulated drugs has been transferred from the Aging group to Biochemistry, and (2) Dr. F. Schlenk retired in November 1974, but is still affiliated with the Division as a Resident Research Associate.

The contributions of the group consist of six reports. The first is concerned with recent developments in the isolation and characterization of subcellular components of mammalian cells: the inhibition by imipramine of digitonin-induced lysis of mitochondrial membranes; age-dependent changes in mitochondrial sedimentability; peroxisomal enzymes; and collaborative studies on (1) near-UV effects on bacterial respiration, (2) radiation effects on mouse heart mitochondria, and (3) toxicity and distribution of liposome-encapsulated drugs.

Plant physiology is the theme of the next two reports. The first describes progress in a NASA-supported program on the involvement of organelles, especially dictyosomes, in the georesponse of roots, and the second covers work principally supported by ERDA on the interaction of light and gravity on differential growth of corn roots.

Progress in liposome encapsulation of drugs is presented in three contributions. The first deals with studies on the toxicity, distribution, therapeutic action, and mechanism of encapsulated cancer chemotherapeutic agents; the second with morphologic studies, based principally on electron microscopy; and the third with alteration of liposomal surface properties by varying the lipid composition, in order to modify tissue distribution.

Although these programs differ widely as far as test objects and experimental approaches are concerned, there is one underlying theme in common, the search for mechanisms of metabolic control. Knowledge of these controls in normal systems is essential for the understanding of the effects of any environmental contaminants, including radiation, that may perturb these systems; conversely, the effects of selective perturbation of these systems by chemicals or radiation are of immense value in establishing the normal state.

Most of the programs will continue along the same directions indicated in the progress reports. An exception is the plant physiology program, which will undergo some change of emphasis and direction.

BIOCHEMISTRY STAFF

REGULAR STAFF

Cerny, Elizabeth A. (Scientific Assistant)
Dainko, Julia L. (Scientific Assistant)
Nance, Sharron L. (Scientific Associate)
Rahman, Yueh-Erh (Biologist)
Shen-Miller, Jane (Botanist)
Thomson, John F. (Senior Biologist)
Tollaksen, Sandra L. (Scientific Assistant)
Wright, Betty J. (Scientific Associate)

TEMPORARY STAFF DURING 1975

Jonah, Margaret M. (Postdoctoral Appointee)
*McNitt, Rand E. (Postdoctoral Appointee)

PUBLICATIONS

Elliott, W. M., and J. Shen-Miller. Similarity in dose responses, action spectra and red light responses between phototropism and photoinhibition of growth. Photochem. Photobiol., in press.

Jonah, M. M., E. A. Cerny, and Y. E. Rahman. Tissue distribution of EDTA encapsulated within liposomes of varying surface properties. Biochem. Biophys. Acta 401, 336-348 (1975).

Rahman, Y. E., W. E. Kisieleski, E. M. Buess, and E. A. Cerny. Liposomes containing ^{3}H-actinomycin D. Differential tissue distribution by varying the mode of drug incorporation. Eur. J. Cancer 11, 883-889 (1975).

Rahman, Y. E., and C. Peraino. Effects of age on patterns of enzyme adaptation in male and female rats. Enzyme Induction in Aging and Protein Synthesis. MSS Information Corporation, N. Y., 1974, pp. 126-132.

Rahman, Y. E., and B. J. Wright. Liposomes containing chelating agents. Cellular penetration and a possible mechanism of metal removal. J. Cell. Biol. 65, 112-122 (1975).

Rosenthal, M. W., Y. E. Rahman, E. S. Moretti, and E. A. Cerny. Removal of polymeric plutonium by DTPA directed into cells by liposome encapsulation. Radiat. Res. 63, 262-274 (1975).

*Terminated during 1975.

Schlenk, F. Transmethylation - Past, present, and future aspects in biology and medicine. Atti dell-Accademia di Scienze Mediche e Chirurgiche 128, 242-253 (1975).

Schlenk, F., and J. L. Dainko. The S-*n*-propyl analogue of S-adenosylmethionine. Biochim. Biophys. Acta 385, 312-323 (1975).

Shen-Miller, J. Report of a NATO Institute on plant genetic manipulations. BioScience 25, 389-391 (1975).

Shen-Miller, J. Spectral sensitivity on corn-root geotropism. Plant Growth Substances 1973. Hirokawa Publishing Co., Inc., Tokyo, 1974, pp. 1095-1103.

Swick, R. W., S. L. Tollaksen, S. L. Nance, and J. F. Thomson. Distribution patterns of membrane-bound and soluble enzymes in mitochondrial populations. Arch. Biochem. Biophys. 168, 281-288 (1975).

Abstracts

Harsch, H. H., and Y. E. Rahman. Drug depression of CNS membrane transport. Tex. Rep. Biol. Med. 32, 800-801 (1974).

McNitt, R. E., and J. Shen-Miller. Organelle distribution in geotropism of corn roots. Plant Physiol. 56(s), 51 (1975).

ERDA RT-03-02
ANL 61100

ISOLATION OF CELLS AND SUBCELLULAR COMPONENTS BY CENTRIFUGATION TECHNIQUES

John F. Thomson, Principal Investigator
Sharron L. Nance, Sandra L. Tollaksen, and Bradford B. Smith, Participating Investigators*

OBJECTIVES

The goal of this program is the development and application of centrifugation techniques for separation, isolation, and characterization of cells and subcellular particles, primarily from mammalian tissues. We attempt to relate the concentration of cellular components to the size (as deduced from sedimentation behavior) of the particulates with which they are associated, rather than to morphologic labels of doubtful meaning. The procedures developed in this laboratory have been valuable in the comparison of tissues from normal animals and those treated in various ways.

BACKGROUND AND PREVIOUS FINDINGS

The procedures described below, which were designed originally to answer some questions concerning biochemical changes in tissues of animals exposed to total-body X-radiation, have proved to be an extremely sensitive system for detecting small changes in sedimentation characteristics of mitochondria and other cytoplasmic organelles isolated from animals treated in a variety of ways: dietary manipulation (high protein or fatty acid deficient dicts), drug treatment (corticosteroids, radioprotective aminothiols, aflatoxin), subtotal hepatectomy, hypoxia, etc. They have also proved to be useful as preparative techniques for isolation of mitochondria and peroxisomes.

EXPERIMENTAL METHODS

The basic technique has been to layer a preparation (e.g., a tissue homogenate or a suspension of cells) over a density gradient, constructed with varying concentrations of solute (sucrose, albumin, Ficoll) or solvent (deuterium oxide). After centrifugation, successive fractions are collected and analyzed by appropriate methods: enzyme assays, microscopic examination, cell counts, chemical composition, 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 obtained; we have written computer programs to facilitate the mathematical and statistical analyses of our data. Currently we are carrying out tissue fractionations in zonal centrifuges, which provide much finer resolution than can be obtained by other centrifugation procedures.

An important ancillary activity has been the development of enzymatic assays--spectrophotometric, colorimetric, polarographic--and chemical analyses for characterization of cells and their components.

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*Thesis Parts Student, San Diego State University.

MAJOR NEW FINDINGS

The antidepressant drug imipramine hydrochloride (N-dimethylaminopropyl-dihydrodibenzazepine hydrochloride), U.S.P., which has been shown (Wattiaux-DeConinck, S., F. DuBois, and R. Wattiaux, Eur. J. Biochem. 48, 407, 1974) to increase the resistance of mitochondrial membranes *in vitro* to elevated hydrostatic and osmotic pressure, has been shown to increase their resistance to lysis by digitonin. As the concentration of digitonin in the suspending medium is increased, first there is loss of inter-membrane enzymes, then solubilization of outer membrane enzymes, and finally release of matrix enzymes and fragmentation of the inner membrane. The presence of imipramine in the suspending medium increases the quantity of digitonin required to produce these changes. The stoichiometry among digitonin, imipramine, and membrane cholesterol remains to be established.

In connection with these experiments, we developed a modified assay for monoamine oxidase (MAO), an enzyme associated with the outer mitochondrial membrane. One of the conventional assays employs the spectrophotometric monitoring of the oxidative deamination of benzylamine to benzaldehyde; however, the measurement is complicated by endogenous reactions and high blanks. Treatment of the mitochondrial suspension with digitonin quantitatively solubilizes MAO, and eliminates the endogenous reactions.

Preliminary observations suggested that the sedimentability of mitochondria isolated from mouse liver increased with the age of the animal. Unequivocal confirmation of this finding, and establishment of the changes in chemical composition that would account for the differences in sedimentation characteristics require more work.

Recent studies on peroxisomes have been concerned primarily with the development of assay procedures that could be used easily and accurately with large numbers of samples. In addition to further work on a spectrophotometric assay for catalase previously described (Thomson, J. F., S. L. Nance, and S. L. Tollaksen, ANL-75-30, 1974, p. 144), we have also tried to improve the assay procedures for L-α-hydroxy acid oxidase and D-amino acid oxidase.

We have also been engaged in several collaborative projects, the results of which are described in greater detail in other sections of this Report. (1) With Dr. H. E. Kubitschek, our measurements of bacterial oxygen consumption showed that after exposure to near-UV, glucose transport rates are reduced more severely and recover more slowly than does oxidative metabolism. (2) Because Drs. V. V. Yang and S. P. Stearner have observed morphologic changes in the mitochondria of hearts of mice exposed to neutron and gamma radiation, we have begun a survey of mitochondrial enzymes prepared from the hearts of similarly irradiated animals, to see whether there are biochemical changes that can be correlated with electron-microscopic observations.

SIGNIFICANCE

The experimental procedures employed in this program have wide applicability to a variety of problems. In addition to increasing our knowledge of the fundamental physiology and biochemistry of normal cells and their components, they are valuable techniques for the study of any noxious agent that

may alter, directly or indirectly, the chemical composition (and thus the physicochemical properties) of subcellular constituents.

PROPOSED COURSE OF THE PROJECT

Most of the items mentioned above under "Major New Findings" will continue to be pursued. The work with imipramine will be expanded to survey other compounds with similar structure but diverse pharmacologic action. Studies on various properties of rodent liver mitochondria and peroxisomes will continue, with emphasis on age-, strain-, and sex-related changes in sedimentation characteristics. The survey of mitochondrial enzymes from hearts of irradiated mice will be completed during the year.

NASA P-7142-C

GROWTH AND DEVELOPMENT OF PLANTS IN COMPENSATED AND NORMAL EARTH FIELDS: INVOLVEMENT OF CELLULAR ORGANELLES IN GEORESPONDING ROOTS

Jane Shen-Miller, Principal Investigator
Rand E. McNitt, Participating Investigator

OBJECTIVES

Planning of experiments in simulated weightlessness for the clinostat (and ultimately for the free-fall state) is designed to resolve two major problems: the nature of the geosensor, and the bridge between the physical processes of perception and the hormonal implementation of sensor activation. Accordingly, our program has been concerned with the identification of the gravitational sensor in the higher plants. We undertook to examine the possible role of various cellular organelles in sensing, and in the physiological implementation of the plant response to gravity. The present study was designed to tabulate, quantitatively, the changes of organelle number with the onset of geotropism in different regions and different tissues of roots.

BACKGROUND AND PREVIOUS FINDINGS

The amyloplasts (starch grains) have been accepted as the statoliths involved in the geotropism of plants. However, our earlier study showed that, although the starch grain formed a gradient within cells of a geostimulated coleoptile, this gradient was observed in both the upper and lower half of a geostimulated coleoptile (Shen-Miller, J., and C. Miller, Plant Physiol. 49, 634, 1972). Further, a dictyosome gradient was also observed. This gradient was found only in the lower epidermal cells where cell expansion was greatest.

In addition, a dictyosome activity gradient was also observed in these cells. The activity of dictyosomes is controlled by the plant hormone indoleacetic acid (Gawlik, S. R., and J. Shen-Miller, Plant Physiol. 54, 217, 1974). The geotropic curvature direction of roots and shoots is directly opposite; the roots curve downward and shoots upward. In the present study we investigated organelle distribution within the geosensing cells of the root cap, and in the cells that undergo the curvature response in the root proper.

EXPERIMENTAL METHODS

Primary roots of corn, after irradiation by red light (660 nm) or without irradiation (dark control), were harvested at 15-minute intervals for a total of 150 minutes. (The roots were kept horizontal before and after irradiations. Only the red-irradiated roots showed geotropism, whereas the dark roots did not.) The harvested roots were fixed in glutaraldehyde-acrolein in sodium cacodylate buffer and processed for electron microscopy. Cellular organelle numbers were tabulated in the top and bottom of cells of the upper and lower outer cortex, and in central cells of the root cap.

MAJOR NEW FINDINGS

In the root cap cells, a significantly greater number of dictyosomes and nuclei were found toward the top of the cell in the red-exposed roots. The mitochondria did not show a preference. The amyloplasts were found exclusively in the bottom of both the red and the dark control cells. In the curving zone, dictyosomes and mitochondria show an outer peripheral distribution within the cells of upper and lower tissue of the control. This trend of dictyosome distribution was not observed in the upper tissue of the red-exposed roots. The cells in the upper tissue are the sites of greatest elongation. In the more basal region of the roots (beyond the curving zone), no difference in organelle distribution was noted between the dark and the red-exposed roots.

The distribution of dictyosomes and nuclei toward the top of cap cells as a result of red-light exposure could cause a differential membrane permeability and a differential transport of growth substances from the cap to the curving zone, and hence, a differential growth and a curvature.

SIGNIFICANCE

This research shows the interaction of light and gravity on the distribution of cellular organelles in the geosensing cells of the roots. The preferential distribution of dictyosomes and nuclei in the red-exposed root cap is not a passive displacement by the amyloplasts. (Amyloplasts settled to the bottom in both the red and dark cap cells.) The preferential organelle distribution is an active process that seemed to be controlled by the pigment phytochrome (the red-light effect). The changes of these organelles in the cap cells (the sensor cells) are direct clues to the involvement of dictyosomes and nuclei in the process of gravity sensing.

PROPOSED COURSE OF THE PROJECT

This project will be terminated upon preparation of a manuscript. We plan to initiate a study on organelle involvement associated with the epinastic response (leaf movement) of a plant. This response occurred soon after the plants were placed in a biosatellite. Such a study will yield further information on the role of organelles under the influence of gravity and the weightless environment.

ERDA RT-03-02
ANL 61100

HORMONAL AND METABOLIC BASES FOR RESPONSES TO ENVIRONMENTAL FACTORS: INTERACTION OF LIGHT AND GRAVITY ON THE DIFFERENTIAL GROWTH, AND LOCALIZATION OF THIS GROWTH IN ROOTS OF CORN

Jane Shen-Miller, Principal Investigator
Paul Chris Chalmers and Martin Wojciechowsky,† Participating Investigators*

OBJECTIVES

The strategy of a stationary green plant for efficient capture of light quanta from the sun requires a firm anchor for positioning and holding erect the above-ground stem. This study was undertaken to investigate the anchoring mechanism of the plant roots as affected by light and gravity. A time-sequence measurement was taken to determine (1) the occurrence of the peak response, and (2) the cells and tissues that are involved in this response, as a basis for studies on the elucidation of the site of hormone action within the plant roots.

BACKGROUND AND PREVIOUS FINDINGS

We have found that the downward growth of corn roots (geotropism) requires light (Shen-Miller, J., et al., ANL-75-30, 1974, p. 161). An action spectrum of root geotropism that shows the red light (660 nm) of the visible light to be most effective indicates the involvement of the pigment phytochrome (a chromoprotein whose structure is yet unclear). The downward growth of the

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*Spring, 1975 participant in the Undergraduate Honors Research Program, University of Bridgeport, Conn.

†Summer, 1975 participant in the Undergraduate Honors Research Program, Northern Michigan University.

root is a result of a differential elongation of cells across the diameter of the root. The perception mechanism of this response is located in the root cap (Juniper, B. E., et al., Nature 209, 93, 1966). It was in the cap region where phytochrome was found to be abundant in most of the grass roots (Pratt, L., and R. Coleman, Am. J. Bot. 61, 195, 1974). The differential expansion growth is under hormonal control (Scott, T. K., Annu. Rev. Plant Physiol. 23, 235, 1972).

EXPERIMENTAL METHODS

Primary roots of corn (Wisconsin hybrid, 64A x 22R) were used for the experiments. The roots extended horizontally when the seeds were germinated in complete darkness. The horizontal roots (under gravity stimulation) were exposed to red light (660 nm) at a dose of 1000 erg$\cdot$cm^{-2}. The total period of exposure was 60 seconds (not a saturating dose). One hour after irradiation the roots were shadowgraphed on photographic papers, at 15-minute intervals, for a period of 4 hours to record the curvature response of the roots. Beginning at the time of irradiation and continuing at 15-minute intervals for 4 hours, roots were sampled for histological examination. The roots were cut 5 mm from the apex and fixed in glutaraldehyde with cacodylate buffer. The tissues were then processed, embedded in resin, and sectioned. Cell lengths in each region and within each tissue were determined between the upper and lower half of horizontal roots, using a light microscope.

MAJOR NEW FINDINGS

The downward curvature of a horizontal root began at about 105 minutes after red light irradiation. The response reached a peak at about 150 minutes. From the cell length study we found the curvature (differential growth) to begin at 1.5 mm from the apical meristem of the roots. The differential growth localized within the 1.5- to 2.5-mm region. Of the four tissue layers, the outer cortex (cells beneath the epidermis) showed the greatest difference in cell length between the upper and lower half of a horizontal root. Cell length differences began at 60 minutes after the light exposure, before the onset of the visible curvature response as found in the first part of the study. The greatest difference in length occurred at 180 minutes.

SIGNIFICANCE

These findings pinpoint the response time and location of the response, on the cellular level, of the anchoring phenomenon of the root. The histological study defines the regions of the root where the plant hormone exerts an effect, and provides some bases for the prediction of the localization and translocation path of the hormone pool within the roots. Further, it points out the specific cells where cellular organelles can be studied in response to plant hormone action.

Roots are one of the sinks for the photosynthetic products from the leaves. The unloading of food products from the leaves has an effect in regulating the rate of photosynthesis. Plant hormones have a role in the mobilization of photosynthates. The understanding of hormonal regulation in

root growth bears directly on the productivity of the plant, which can be a source material for fuel and fiber (Szego, G. C., and C. C. Kemp, Chem. Technol. 3, 275, 1973).

PROPOSED COURSE OF THE PROJECT

This study will be discontinued after preparing a manuscript for publication.

ERDA RT-01-02
ANL 64101

THERAPEUTIC APPLICATIONS OF LIPOSOME-ENCAPSULATED DRUGS

Y. E. Rahman, Principal Investigator
E. A. Cerny, B. J. Wright, J. L. Dainko, M. M. Jonah, K. M. Strathy,[*]
J. F. Thomson, S. L. Tollaksen, S. L. Nance, W. E. Kisieleski,[†]
E. M. Buess,[†] *E. J. Ainsworth,*[‡] *J. L. Hulesch,*[‡] *and M. Miller,*[‡]
Participating Investigators

OBJECTIVES

A challenging problem in the field of chemotherapy is to deliver a given drug specifically to a target tissue. We use liposomes as carriers for two classes of drugs--chelating agents and antitumor drugs. Our objectives are: (1) to deliver the chelating agents inside of cells where toxic metals are located; (2) to alleviate the usual systemic toxicity of antitumor drugs, and thus improve their therapeutic indices; and (3) to deliver antitumor drugs directly to tumor cells.

BACKGROUND AND PREVIOUS FINDINGS

Liposomes, lipid spherules prepared with various phospholipids, were first introduced by Bangham in 1965 (Bangham, A. D., et al., J. Mol. Biol. 13, 238, 1965). In subsequent years, they have been extensively used as a cell model for studies of membrane transport and membrane interactions with polyene antibiotics, hormones, anaesthetics, and proteins.

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[*] Fall, 1975 participant in the Undergraduate Honors Research Program, St. Olaf College.

[†] Carcinogenesis Group.

[‡] Neutron and Gamma-Ray Toxicity Group.

Recently, we have used liposomes to encapsulate metal-chelating drugs, specifically EDTA and DTPA. Although intracellularly deposited toxic metals are not readily removable by the nonencapsulated DTPA, which cannot cross cellular membranes, liposome-encapsulated DTPA has been successful in removing various intracellularly deposited toxic metals in mice. For example, delayed injection of liposome-encapsulated chelating agent, given 24 days after plutonium, showed: (1) a greater advantage over nonencapsulated chelating agent in removal of plutonium from mouse liver, and (2) removal of over 20% of the plutonium from the femurs compared to no removal by the nonencapsulated form. Intraperitoneal or intravenous injections of liposome-encapsulated chelating agents were equally effective in removal of plutonium from mouse tissues. Liposome-encapsulated chelating agents were also more effective in removing lead burden from mouse skeleton. The liposome-encapsulated chelating drugs also have a remarkably different tissue disposition compared to that of the nonencapsulated form.

We have also successfully encapsulated within liposomes an antitumor drug, namely actinomycin D; and the following results were found: (1) encapsulated actinomycin D was less toxic to mice than the nonencapsulated form; (2) a single dose, or multiple doses, significantly increased the mean survival time of mice inoculated with Ehrlich ascites tumor cells; (3) liposomes containing actinomycin D were found within Ehrlich ascites tumor cells within minutes after the injection; and (4) tumor cell degeneration and death were subsequently observed.

EXPERIMENTAL METHODS

Radioactive drugs are encapsulated within liposomes; after they are injected into mice, their tissue distribution is determined by radioactivity analysis. Cellular uptake is investigated either quantitatively, after cell fractionation, or qualitatively by electron microscopic examination and the use of autoradiography. Comparative toxicity studies between liposome-encapsulated and nonencapsulated drugs are based on the following: LD_{50}, blood cell counts, stem cell counts, nucleic acid synthesis, as well as biochemical and morphological damage at the cellular and subcellular levels.

MAJOR NEW FINDINGS

In addition to actinomycin D, other antitumor drugs, belonging to different classes, have now been successfully encapsulated both in the aqueous or in the lipid phase of the liposomes. They are: nitrogen mustard, cyclophosphamide, methotrexate, puromycin, cytarabine, daunomycin, adriamycin, vinblastine, and vincristine. Two other drugs, namely mitomycin C and cis-dichlorodiammine platinum (II), can be incorporated only in the aqueous phase of the liposomes. When the tissue distribution of liposome-encapsulated adriamycin, either in aqueous or in lipid phase, was compared to that of nonencapsulated adriamycin in mice, there was significantly lower concentration in the heart and the intestine, and higher concentration in the lungs. These results suggest that the known toxicity of adriamycin to the cardiac muscle and the intestinal epithelium could be alleviated by liposome encapsulation.

Electron microscopic studies show that not only phagocytic cells, but also other tissue cells such as hepatocytes, lung alveolar cells, epithelial cells, etc. can readily take up liposomes.

Selective drug delivery was partially achieved by varying the phospholipids of the liposomes, or the mode of drug incorporation. For example, actinomycin D incorporated in the lipid phase of liposomes was found to have significantly higher concentration in the lungs (up to 40% of the total injected dose).

SIGNIFICANCE

The liposome approach to delivering drugs is a new method which is potentially useful for clinical application in metal poisoning and in cancer chemotherapy. In addition, basic knowledge on the cellular uptake of liposomes varying in their surface properties will lead us to understand further the biochemical nature of tissue specificity.

PROPOSED COURSE OF THE PROJECT

Considerable effort will be made toward achieving selective drug delivery to target cells. This will be approached by inserting various glycolipids and glycoproteins in the lipid bilayers of liposomes.

Various lung tumors will be developed as tumor models for testing the therapeutic advantages of liposome-encapsulated antitumor drugs. The emphasis on lung tumors is based on the fact that we can deliver a significantly higher dose of antitumor drugs to the lungs by liposome encapsulation.

Morphological studies will be continued with the following aims in mind: (1) identification of cells capable of liposome uptake, (2) understanding of the mechanism of intracellular drug release from liposomes, (3) comparison of cellular toxicity between liposome-encapsulated and nonencapsulated drugs.

ERDA RT-01-02
ANL 64101

CELLULAR UPTAKE OF LIPOSOMES CONTAINING CHELATING AGENTS AND ANTITUMOR DRUGS: MORPHOLOGICAL STUDIES

Betty Jean Wright, Principal Investigator
Yueh-Erh Rahman, Elizabeth A. Cerny, and Marion Bakula, Participating Investigators*

OBJECTIVES

Morphological studies on the cellular uptake of liposomes make up one of the aspects of the liposome project. The purpose of these studies is twofold: (1) to identify precisely which cell types within tissues contain liposomes, and (2) to use morphological techniques to provide visual evidence at the cellular level for the mechanisms of liposome uptake and subsequent intracellular drug release and action. Whole animal studies have shown that the liposome-encapsulated chelating agents and antitumor drugs are more effective than the nonencapsulated form. Identification of the cells containing liposomes provides a rationale for the selection and administration of appropriate drugs to specific cells for the most marked therapeutic effects.

BACKGROUND AND PREVIOUS FINDINGS

Early work with liposome-encapsulated chelating agents showed that the cytoplasm of both liver parenchymal cells and Kupffer cells contained liposomes (Rahman, Y. E., and B. J. Wright, J. Cell. Biol. 65, 112, 1975). Liposomes containing actinomycin D were also found in the cytoplasm of Ehrlich ascites tumor cells (Rahman, Y. E., et al., Proc. Soc. Exp. Biol. Med. 146, 1173, 1974).

EXPERIMENTAL METHODS

Liposomes with chelating agents or antitumor drugs incorporated in either the aqueous phase (APL) or lipid phase (LPL) were injected into mice, and tissue samples were obtained at chosen time intervals. Standard electron microscope preparations of these tissues were made and examined. In addition, autoradiography and cytochemical determination of acid phosphatase for electron microscopic examination were carried out.

MAJOR NEW FINDINGS

Electron microscopic examination of the liver, lungs, spleen, and bone marrow reveals that liposomes can be identified within cells in these tissues. No qualitative difference in the cell uptake is observed between liposomes containing EDTA or actinomycin D. Nor is there a difference in uptake between

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*Faculty Research Participant, St. Louis University.

APL and LPL. The spleen exhibits a great number of cell types containing liposomes: free and fixed reticuloendothelial cells, lymphocytes, granulocytes, megakaryocytes, and plasma cells. In the lung, liposomes are found in both epithelial and endothelial cells, macrophages, alveolar cells, and transient white blood cells. Bone marrow, an active complex tissue composed of many different cell types, reveals liposomes in the cytoplasm of both the young erythrocytic and granulocytic cells, and in the macrophages.

Electron microscopic autoradiographic studies of Ehrlich ascites tumor cells treated with LPL-encapsulated ^{3}H-actinomycin D revealed an earlier liposome uptake and a greater number of labeled cell nuclei than did cells treated with free ^{3}H-actinomycin D. These labeling experiments provide additional evidence for the delivery of the drug to its site of action.

Electron microscopic cytochemical tests for increased acid phosphatase activity in the Ehrlich ascites tumor cells treated with LPL-encapsulated actinomycin D were not conclusive. It was hoped that an increased lysosomal activity could be visualized in the tumor cell in order to confirm the proposed mechanism for drug release from the liposome.

SIGNIFICANCE

Liposome encapsulation of chelating agents and antitumor drugs is a new therapeutic approach to two medical problems of great importance--heavy metal poisoning and cancer. The morphological studies on the cellular uptake of liposomes are essential in order to establish the capability of different types of tissue cells to take up liposomes containing drugs, and to elucidate mechanisms of liposomal drug action.

PROPOSED COURSE OF THE PROJECT

We plan (1) to extend the electron microscopic tissue study to bone, as this tissue is particularly important in chelation therapy of heavy metal poisoning; (2) to study the cellular toxicity of adriamycin and liposome-encapsulated adriamycin on cardiac muscle; and (3) to elucidate the mechanism of intracellular drug release from liposomes by autoradiographic and cytochemical techniques.

ERDA RT-01-02
ANL 64101

SELECTIVE DELIVERY OF DRUGS BY ALTERATION OF LIPOSOMAL SURFACE PROPERTIES

Margaret M. Jonah, Principal Investigator
Yueh-Erh Rahman, Elizabeth A. Cerny, and Carol Hoenich, Participating Investigators*

OBJECTIVES

The objective of this research is to develop methods of selectively directing drugs to different tissue cells by encapsulating the drugs within lipid spherules (liposomes) having different surface properties.

BACKGROUND AND PREVIOUS FINDINGS

Liposome encapsulation of the metal chelating agent ethylenediaminetetraacetic acid (EDTA), and of the antitumor drug actinomycin D (Act. D), before injection of the drug into mice resulted in increased cellular penetration, prolonged retention in tissues, reduced toxicity, and increased therapeutic action of these drugs (Rahman, Y. E., et al., Science 180, 300, 1973; Rahman, Y. E., et al., J. Lab. Clin. Med. 83, 640, 1974; Rahman, Y. E., et al., Proc. Soc. Exp. Biol. Med. 146, 1173, 1974; and Rahman, Y. E., et al., Europ. J. Cancer, in press).

We have also found selective uptake of liposome-encapsulated drugs by certain mouse tissues; for example, spleen and marrow preferentially accumulated EDTA from liposomes with negative surface charge, while lungs and brain retained EDTA from liposomes with positive surface charge (Jonah, M. M., et al., Biochim. Biophys. Acta 401, 336, 1975). When Act. D was incorporated in the liposomal membrane, there was preferential accumulation of the drug in the lungs, but little retention in intestines; this selectivity was not observed when Act. D was incorporated in the aqueous phase of the liposomes (Rahman, Y. E., et al., Europ. J. Cancer, in press).

EXPERIMENTAL METHODS

Surface properties of liposomes were altered by use of different lipid components of biological membranes. The drugs used were either chelating agents or antitumor drugs. A water solution of EDTA was incorporated in the inner compartments (aqueous phase) of liposomes; Act. D was generally incorporated into the liposomal surface membranes (lipid phase), rather than in the aqueous phase. Distributions in mouse tissues of liposome-encapsulated Act. D and EDTA preparations were measured by use of radioactive forms of these drugs.

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*Participant in the 1975 Summer Institute in Biology, University of Colorado.

MAJOR NEW FINDINGS

Selective uptake of EDTA in lungs, with very low uptake in all other tissues was accomplished by encapsulation of EDTA in liposomes prepared from dipalmitoyl lecithin, a completely saturated form of the natural membrane phospholipid, lecithin. Lung membranes themselves contain large proportions of dipalmitoyl lecithin and may have had an affinity for these liposomal membranes.

Accumulation of liposome-encapsulated EDTA by lungs was also enhanced by inclusion of sphingomyelin in liposomal membranes. In contrast, Act. D encapsulated in liposomes made of sphingomyelin was not accumulated by lungs, possibly due to interactions of sphingomyelin with Act. D and consequent alteration of the liposomal surfaces.

A mixture of phospholipids extracted from mouse brain and partially purified to remove glycolipids has been used to prepare liposome-encapsulated Act. D or EDTA; however, these liposomes did not significantly improve uptake of either drug by the brain. Similarly, cerebrosides (brain glycolipids) included in liposomes did not increase brain uptake of encapsulated Act. D or EDTA.

Use of cerebrosides to prepare liposome-encapsulated EDTA did increase the drug uptake by liver. Liver cells may have surface receptor sites with affinity for the sugar moieties of cerebrosides.

SIGNIFICANCE

Continuing improvement in our capabilities to direct liposome-encapsulated drugs selectively to certain tissues through modification of the liposomal surface properties will increase efficiency of drug therapy, reduce systemic toxicity of the drug, and will expand the usefulness of this new drug delivery system in the treatment of heavy metal poisoning and in cancer chemotherapy, as well as in investigation of fundamental problems in cell biology.

PROPOSED COURSE OF THE PROJECT

Binding of drugs to liposomal lipids will be measured, particularly for those drugs which can be incorporated into liposomal membranes. Preliminary results indicate that surface charges of liposomes and distributions in tissues of liposome-encapsulated drugs are altered by binding of Act. D to some lipids.

Specificities of interactions of cell surfaces with particular liposomal membrane lipids will be tested using liposomes made of glycolipids. Different chemical forms of glycolipids can be isolated from many biological sources and used in measurements of liposome uptake by normal and tumor cells.

Methods of incorporation of glycopeptides and glycoproteins will be developed, and possible binding of these substances to the inner or the outer surface regions of liposomes will be measured. The association of liposomes and specific glycoproteins, including antibodies, can be used to direct the liposomes containing drugs to target cells.

Techniques for penetration of the blood-brain barrier by liposome-encapsulated drugs, such as Act. D and EDTA, will also be developed.

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9. BIOPHYSICS

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GROUP LEADER'S OVERVIEW

Steven S. Danyluk, Group Leader

Research activities of the Biophysics group are centered in four main project areas, magnetic resonance spectroscopy of biological molecules, development of clinical applications of stable isotopes, circadian cybernetics, and X-ray crystallography of immunoglobulins. An underlying theme in all four is the use of sophisticated state-of-the-art biophysical and computational techniques for defining molecular events in biological systems, both *in vivo* and *in vitro*. The thrust of each project and progress in the past year are outlined in the following.

Biological processes occur in fluid media, and ultimately our knowledge of their mechanisms requires detailed information for chemical and molecular structural properties in biological fluids. Magnetic resonance spectroscopy has unique advantages over other approaches in this area that are being exploited in studies currently underway in the group. The program continues to develop along three interrelated lines, measurement and analysis of high resolution spectra for biological molecules (especially nucleic acid constituents and drugs), synthesis of selectively labeled nucleic acid fragments essential for complete spectral assignments, and computation of conformational properties from NMR parameters. This coordinated approach enabled the first complete conformation analysis for a dinucleoside monophosphate, ApA, in aqueous solution. It was found that the conformation is actually a time-average of right helical, loop, and extended conformations, the interchange being extremely rapid on an NMR time scale. Spectral analyses were also completed for all possible ribonucleotide dimers, the assignments again relying heavily on synthesis of appropriate deuterated counterparts. Preliminary studies of conformational flexibility in nucleic acid fragments showed that changes in hydrogen ion concentration and temperature produce correlated conformational changes specific for each nucleotidyl unit. Collaborative studies were also initiated in three new projects dealing with the effect of hapten binding on antibody structure, counter ion influence on nucleic acid free radicals, and membrane differences between normal and sickled erythrocytes.

In broadest terms, the ultimate goal of the Argonne program for clinical applications of stable isotopes is the replacement of radiotracers as clinical

diagnostic aids by stable isotope counterparts. This has involved development of sensitive analytical instrumentation, synthesis of appropriate labeled compounds, and validation of the stable isotope approach in actual clinical procedures. A major advance was made in the past year in development of simple, inexpensive, yet accurate, $^{13}CO_2$ breath tests for routine clinical applications. ^{13}C-labeled substrates capable of releasing $^{13}CO_2$ on metabolism were synthesized, tested, and validated for use in testing regimens for hepatic microsomal function, bacterial overgrowth in the small intestine, and bile salt malabsorption. These breath tests are currently being exploited in diagnosis of disease in children and other individuals where use of $^{14}CO_2$ is contraindicated. Significant progress occurred in development of a ^{13}C-labeled chenodeoxycholic acid approach for measuring bile acid kinetics utilizing serum samples, a procedure with obvious advantages over conventional intubation methods, especially for routine tests. Methods were also developed for measuring methadone levels at very low concentrations in physiological fluids. The procedures were used to monitor pool size, turnover rate, and drug half-life in subjects on methadone maintenance programs. An important step in instrument development was made with successful testing of a stable isotope ratiometer-multiple ion detector expressly designed for routine measurement of stable isotope ratios in bioorganic molecules.

All biological organisms, from the simplest eucaryotes to man, follow a circadian periodicity ($\sim$ 24 hours) in their biological behavior. What form this periodicity takes at the molecular level, and the existence (or non-existence) of a molecular biological clock common throughout the phyla are two of the most important questions addressed by investigators in this field and by the current project in circadian cybernetics. In the past year, advances were made along several directions. Studies of light-entrained *Tetrahymena* cultures showed circadian regularities in respiratory CO_2, glycogen, ATP, cyclic AMP, and tyrosine aminotransferase that parallel rather closely the chronotypic properties for much higher organisms such as rats. This raises the intriguing possibility of common circadian regulatory pathways in these phylogenetically diverse systems. An equally exciting result, and one which has elicited much lay interest, was the discovery that circadian rhythms of light/food 'conditioned rats can be reset by administration of stimulants (advance) or depressants (delay) depending upon phase of the cycle.

One of the main goals of research workers in the field of immunology has been the interpretation of antibody function and specificity in terms of molecular structural properties. The work of the crystallography group focuses directly on this problem, and the X-ray diffraction studies of Mcg (Bence-Jones) and IgG1 myeloma proteins have yielded a wealth of information since inception of the project. The initial phase of the refinement of the Bence-Jones structure at 2.3 Å was carried out in the past year, following the construction of an atomic model from the 2.3-Å map. Such accurate 3-D structures are essential for intercomparison of structural features, e.g. immunoglobulin fold, with other immunoglobulin fragments. Furthermore, the availability of the complete Mcg amino acid sequence (J. F. Deutsch, University of Wisconsin) permitted a correlation of sequence with 3-D structure for the first time, and has led to perhaps the most exciting discovery to come out of the Bence-Jones work, namely, the existence of rotational allomerism between V and C domains of immunoglobulin light chains. This finding is of immense importance in defining the evolution of antibody diversity.

Solid progress was also made in the crystallographic study of the IgG1 molecule. A serious problem in preparing crystals of suitable size for diffraction measurements was solved by development of new purification techniques; crystals prepared by the new procedure diffract to 3.5 Å, sufficient to trace the polypeptide chains for the molecule. Attempts are currently underway to solve the IgG1 structure by multiple isomorphous replacement techniques supplemented with a relatively new "molecular replacement" procedure whereby the known Bence-Jones structure is used to simulate nearly identical regions of the IgG1 structure.

Among other activities of Biophysics group members that deserve mention are the extensive involvement in collaborative research activities with outside investigators (more than 20 projects currently underway), the aggresive pursuit of new sources of funding support (NIH, EPA), and the continued organization and participation in major national and international conferences and symposia. Of special note was the Second International Conference on Stable Isotopes in Chemistry, Biology, and Medicine held in Oak Brook, October 20-22. The conference was again organized by P. D. Klein and proved to be an unqualified success and a credit to Argonne and BIM.

Finally, the studies of Edmundson's group on immunoglobulin structure and their significance to antibody evolution and function were subjects of two lengthy articles, in Science (189, 1075, 1975) and Nature (257, 447, 1975). Mention of Hardman and Ainsworth's work on concanavalin A structure was also made in Nature (255, 278, 1975). Considerable excitement was generated in a number of science newsmagazines and the popular press by the work of Ehret's group on clock resetting in rats, cf., Science News 108, 17, 1975; Selecta 41, 3760, 1975; Harper's Bazaar, Sept. 1975.

BIOPHYSICS STAFF

REGULAR STAFF

Ainsworth, Clinton F. (Scientific Assistant)
Danyluk, Steven S. (Senior Chemist)
Edmundson, Allen B. (Senior Biochemist)
Ehret, Charles F. (Senior Biologist)
Ely, Kathryn R. (Scientific Associate)
Groh, Kenneth R. (Scientific Assistant)
Hachey, David L. (Assistant Biochemist)
Klein, Peter D. (Senior Biochemist)
Meinert, John C. (Scientific Assistant)
Schiffer, Marianne (Biophysicist)
Szczepanik, Patricia A. (Scientific Associate)
Westholm, Florence A. (Scientific Assistant)

TEMPORARY STAFF DURING 1975

Abola, Enrique (Postdoctoral Appointee)
Dobra, Kenneth W. (Postdoctoral Appointee)
Ezra, Fouad S. (Postdoctoral Appointee)
*Fiat, Daniel (Visiting Scientist)
Firca, Joseph R. (Postdoctoral Appointee)
Mede, Karin (Postdoctoral Appointee)
Panagiotopoulos, Nicolas C. (Postdoctoral Appointee)
Schoeller, Dale A. (Postdoctoral Appointee)
Tewari, Ravindra (Postdoctoral Appointee)
Tserng, Kou-Yi (Postdoctoral Appointee)
Wyrwicz, Alice M. (Postdoctoral Appointee)

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*Terminated during 1975.

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STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: CONFORMATIONAL PROPERTIES OF NUCLEIC ACIDS IN AQUEOUS SOLUTION; ADENYLYL-3'-5'-ADENOSINE

Steven S. Danyluk, Principal Investigator
Clinton F. Ainsworth, Fouad S. Ezra, Norman S. Kondo, John V. Nelson,† and Alice Wyrwicz, Participating Investigators*

OBJECTIVES

Our aims are threefold: (1) to obtain quantitative conformational data for nucleic acid segments of variable chain length, (2) to show the relative importance of various intramolecular forces (H-bonding, π electron interaction, etc.) in stabilizing these conformations, and finally (3) to relate conformational properties to interaction mechanisms in biological processes, e.g., transcription, replication, and protein synthesis. Much of the recent effort has concentrated on ribonucleic acid constituents with the study progressing in a systematic manner from the simplest constituents, containing all conformational bonds for a nucleotidyl unit, i.e., monoribonucleotides, to more complex dinucleoside monophosphates, i.e., adenylyl-3'-5'-adenosine, ApA, incorporating not only all nucleotidyl conformational bonds but also bonds along the phosphodiester backbone. Although this project deals primarily with time-averaged conformational properties, it is coupled closely to parallel investigations of conformational dynamics of nucleic acid segments, and synthesis of selectively labeled (^2H, ^{13}C) di- and trinucleotides (see following sections).

BACKGROUND AND PREVIOUS FINDINGS

Ribonucleoside monophosphates are the simplest repeating chemical and structural constituent units of polyribonucleotides, tRNA and mRNA. Accordingly, they are excellent models for evaluating conformational properties relevant to RNA tertiary structures, and for assessing the importance of short-range intramolecular forces as conformational determinants in nucleic acids. Recent X-ray crystallographic studies (Kim, S-H. et al., Acta Crystallogr. B29, 703, 1973) have reported 3-dimensional structures for several dimeric ribonucleosides, but no comparable comprehensive conformational data existed for such molecules in solution until the present. In previous preliminary approaches to the problem, NMR measurements of base and anomeric H1' signals for dimers were used to monitor base-base orientations qualitatively (Hruska, F. E., and S. S. Danyluk, Biochim. Biophys. Acta 157, 238, 1968) and show the existence of conformational flexibility in ribose rings (Hruska, F. E., and S. S. Danyluk, J. Amer. Chem. Soc. 90, 3266, 1968). However, a large amount of spectroscopic information essential for quantitative evaluation of ribose ring and phosphodiester backbone conformations was unavailable because direct unequivocal assignments of dimer spectra were impossible.

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*Federal City College, Washington, D. C.

†Summer 1975 participant in the Undergraduate Honors Research Participation Program, Carnegie-Mellon University.

EXPERIMENTAL METHODS

Selectively deuterated adenylate dimers, *ApA, Ap*A (asterisk denotes fully deuterated nucleotidyl unit) were synthesized by procedures described earlier (Kondo, N. S., and S. S. Danyluk, J. Am. Chem. Soc. 94, 5121, 1972); ApA was obtained commercially. Solutions (0.02 M) were prepared in 100% D_2O and spectra were measured with a Varian 220 MHz spectrometer operating in the Fourier Transform mode. Spectral analyses and NMR parameters were derived by computer simulation-iteration methods (Davies, D. B., and S. S. Danyluk, Biochemistry 13, 4417, 1974).

MAJOR NEW FINDINGS

Synthesis of selectively deuterated adenylate dimers, *ApA, Ap*A, and D-8ApA permitted the first direct assignment of all signals in the 220 MHz proton spectrum of ApA. This has been followed by determination of a complete set of NMR parameters for the entire molecule, and the δ and J values in turn have been used to compute conformational properties for all eight conformational bonds. Very briefly the results support the following important conformational conclusions:

1) The conformational properties for ApA are consistent with two compact, folded dynamically averaged structures, a base-stacked right helical structure, I, characterized as *anti*, C3'-*endo* (^{3}E), g^-, $\omega\omega'$ (320°,330°), $g'g'$, gg, C3'-*endo* (^{3}E), *anti*, and a more loosely stacked loop structure, II, with *anti*, ^{3}E, g^-, $\omega\omega'$ (80°,50°), $g'g'$, gg, ^{3}E, *anti* orientations.

2) Conformations I and II differ only in torsion angle values about P-O3' and P-O5' bonds ($\omega\omega'$); hence these bonds are pivotal for the overall dimer conformation.

3) Dimerization produces a shift in ribose equilibrium, $^2E \rightleftarrows {}^3E$, in favor of ^{3}E in both Ap- and -pA fragments (60:40 vs. 35:65 in monomers); a change in glycosidic torsion angle toward 0°, and a greater locking-in of rotamers along the phosphodiester backbone. Furthermore, there is clear evidence that transitions $^2E \rightarrow {}^3E$ and $\chi \rightarrow 0°$ are directly related to base stacking in ApA.

4) Conformations I and II undergo base destacking to form an extended conformation primarily by rotation of P-O3' and P-O5' bonds.

SIGNIFICANCE

Transfer RNA molecules are key links in protein synthesis, serving the dual role of message recognition and amino acid charging in the overall process. Both functions are critically dependent upon tRNA molecular geometry, and local conformational features of short-chain segments at anticodon and charging sites. The present investigation and further planned studies will provide information regarding favored conformational structures of such segments, their relative conformational stabilities, and the effect of base sequence thereon. All of this knowledge is essential for sorting out the complex structural mechanisms occurring at mRNA-tRNA and tRNA-polypeptide chain interfaces.

PROPOSED COURSE OF THE PROJECT

Following protocols established in the ApA work, the project will be extended to include all of the other 15 pairs of ribodinucleoside monophosphates (16 in total). Emphasis will be placed on measurement, assignment, and determination of accurate δ and J values. Signal assignments will be validated with selectively deuterated dimers, *XpX etc., prepared as part of the synthesis program. Conformational properties will be calculated for all dimers and detailed comparisons made with crystal structure data where available (UpA, ApU, GpC).

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ANL 61200

STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: SYNTHESIS OF SELECTIVELY LABELED OLIGORIBONUCLEOTIDES

Clinton F. Ainsworth, Principal Investigator
*Steven S. Danyluk, Fouad S. Ezra, and Norman S. Kondo,** *Participating Investigators*

OBJECTIVES

The objectives of this project are: (1) the synthesis of selectively labeled (^{2}H,^{13}C) di- and trinucleotides, and (2) utilization of these compounds for direct assignment of ^{1}H and ^{13}C spectra for the oligomers.

BACKGROUND AND PREVIOUS FINDINGS

Conformational analyses of nucleic acid fragments require as the first step an unambiguous assignment of signals for their NMR spectra. Such assignments are often impossible to make because of signal overlap, a situation particularly troublesome for ^{1}H spectra of homo-oligomers, XpXpXp. To circumvent this difficulty we have developed procedures whereby one or more of the nucleotidyl fragments is replaced by its fully labeled counterpart at a known position(s) (Kondo, N. S., and S. S. Danyluk, J. Am. Chem. Soc. 94, 5121, 1972). The approach was instrumental for achieving a complete conformational analysis for the diadenylate, ApA (Kondo, N. S., and S. S. Danyluk, Biochemistry, in press).

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*Federal City College, Washington, D. C.

EXPERIMENTAL METHODS

Both chemical (Lohrmann, R., and H. G. Khorana, J. Am. Chem. Soc. 86, 4188, 1964) and enzymatic methods (Mohr, S. C., and R. E. Thach, J. Biol. Chem. 244, 6566, 1969) are used to incorporate a fully labeled nucleotidyl unit(s) into a known oligomer position. Fully labeled ribomononucleotides were extracted from labeled algae, *Synechococcus lividus*, by procedures described earlier (Kondo, N. S., et al., J. Labelled Compd. 9, 497, 1973). All synthesized oligomers were purified by standard chromatographic techniques.

MAJOR NEW FINDINGS

The labeled dimer *CpC (asterisk denotes fully deuterated nucleotide) corresponding to a section of the -CpCpA charging end of tRNA molecules was synthesized from benzoylated 3'-CMP and 5,6,1',2',3',4',5',5"-octadeuterio isopropylidinecytidine. Preliminary analysis showed the unexpected presence of two components in the product in the ratio 2:1. NMR analysis showed the major component to be *CpC with a 3'→5' linkage while the second component has a 2'→5' linkage.

SIGNIFICANCE

Synthesis of labeled nucleic acid fragments makes possible the study of conformational properties and dynamics for these molecules and their likely conformational features in the naturally occurring state. Reincorporation of labeled segments into the parent molecule, i.e., *C$\overset{*}{p}$C$\overset{*}{p}$A in tRNA, also opens up the possibility of investigating a host of molecular interactions and motional processes for the biopolymer.

PROPOSED COURSE OF THE PROJECT

Future work will focus on the synthesis of doubly labeled (^{2}H) A$\overset{*}{p}$A$\overset{*}{p}$A, *ApApA*, and *A$\overset{*}{p}$ApA to complete the conformational study for the lysine codon ApApA (Kondo, N. S., F. Ezra, and S. S. Danyluk, FEBS Letters 53, 213, 1975). Efforts will also be directed at the synthesis of CpCpA trimers ^{2}H labeled at all possible nucleotidyl positions.

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STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: CONFORMATIONAL DYNAMICS OF PYRIMIDINE MONONUCLEOSIDES AND NUCLEOTIDES; pD EFFECTS

Fouad S. Ezra, Principal Investigator
Clinton F. Ainsworth, Steven S. Danyluk, and Ronald R. Piester, Participating Investigators*

OBJECTIVES

The aims of this work are: (1) to investigate the effect of pD upon the conformational properties of ribomononucleosides and nucleotides and, thereby, (2) to clarify the role of specific intramolecular forces as conformational determinants.

BACKGROUND AND PREVIOUS FINDINGS

Recent NMR studies (Davies, D. B., and S. S. Danyluk, Biochemistry 13, 4417, 1974) have yielded a wealth of information about time-averaged conformational properties of nucleic acid constituents in aqueous solution at neutral pH. These properties are describable in terms of three conformational parameters: (1) base-ribose ring orientation, *-anti* for the majority of nucleotides; (2) ribose conformational equilibrium, $^2E \rightleftarrows {}^3E$, with ^3E for pyrimidine and ^2E for purine nucleotides; and (3) exocyclic group orientation--preferred *gg* and *g'g'* rotamers about C4'-C5', C5'-05' bonds, respectively, and g^- about C3'-03'. For the most part the conformations were determined at pH $\sim$ 7; however, purine and pyrimidine nucleotides typically have groups that can undergo changes of ionization state within the fluctuating pH range encountered in reacting biological systems. It is of obvious importance to investigate what influence charged groups have on nucleotide conformation; the pH study is designed to provide this information.

EXPERIMENTAL METHODS

Commercially purchased pyrimidine nucleosides (uridine, cytidine) and nucleotides (3'-UMP, 5'-UMP, 3'-CMP, 5'-CMP) were made up to 0.01-0.02 M concentrations in 100% D_2O over a pD range from 1.0 to 9.0. pD values for the solutions were adjusted by addition of concentrated NaOD or DCl; no further salts or buffers were added. High resolution ^1H spectra were recorded in the Fourier Transform mode with a Varian 220 MHz spectrometer equipped with a Nicolet FT accessory (20K-20 bit). NMR parameters were estimated directly from the spectra and then refined further by computer simulation-iteration (Davies, D. B., and S. S. Danyluk, ibid.).

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*Spring 1975 participant in the Undergraduate Honors Research Participation Program, Sterling College, Sterling, Kansas.

MAJOR NEW FINDINGS

The results of this study demonstrate that the conformational properties of mononucleosides and nucleotides are closely coupled to solution pH; the perturbations are most clearly evident at pH values of 4-7 and < 2. Moreover, the extent of conformational change is primarily related to phosphate group location (3' or 5' position), and secondarily to the nature of the pyrimidine ring, i.e., to the presence of ionizable amino group. An analysis of chemical shift and coupling constant variations shows conclusively that a conformational change in one region of a nucleoside/tide correlates with and/or produces changes at other conformational bonds. Thus, a decrease of pH from 8 to 5 encompasses a change of phosphate charge from -2 to -1 and produces a sequential increase in magnetic nonequivalence of the two C5' protons that correlates linearly with increase in shielding of H1'. Both shift changes result from a perturbation of the base-ribose glycosyl torsion angle; the latter, in turn, parallels rotamer population changes at C4'-C5' (increase in *gg*) and C5'-O5' (increase in *g'g'*). A change of ionization state also affects the ratio $^3E/^2E$ of ribose conformer populations, the changes being more marked for 3'- than 5'-pyrimidine nucleotides.

SIGNIFICANCE

The findings of this study are important for clarifying the role of intramolecular interactions in stabilizing the overall conformations of nucleic acid constituents. Since most of the electrostatic interactions existing at the monomer level are also possible in naturally occurring polynucleotides, the monomer studies are of direct relevance to RNA and DNA structures.

PROPOSED COURSE OF THE PROJECT

Similar studies of pH effects on conformational dynamics are planned for a series of dinucleoside monophosphates. Since base-base interaction is a dominant feature of dimer conformation, the effect of base ionization on base stacking, glycosyl torsion angle, and ribose conformation will be of special interest. Results of the pH study will be coordinated with concurrent measurements of temperature effects on ^1H spin-lattice relaxation times of dimers.

ERDA RT-03-02
ANL 61200

STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: CONFORMATIONAL DYNAMICS OF URIDYLYL-(3'-5')-ADENOSINE IN SOLUTION; T_1 MEASUREMENTS

Alice M. Wyrwicz, Principal Investigator
Steven S. Danyluk, Participating Investigator

OBJECTIVES

A detailed spin-lattice relaxation (T_1) study is being undertaken of diribonucleoside monophosphates with the objectives of (1) sorting out intra- and intermolecular dipolar contributions to T_1, (2) determining reorientational dynamics for these molecules in solution, and (3) identifying regions of greater conformational flexibility in dimer molecules.

BACKGROUND AND PREVIOUS FINDINGS

In addition to molecular reorientational processes, a dinucleoside monophosphate such as UpA has intramolecular conformational flexibility about eight conformational bonds. The overall conformation in solution is thus actually a time-average of favored rotational possibilities about all eight molecular conformational bonds. NMR studies currently underway in a related project (see preceding report by Ezra et al.) are primarily concerned with determining time-averaged conformational properties for dimers (cf. Kondo, N. S., and S. S. Danyluk, Biochemistry, in press) from CW high-resolution ^1H spectra. Such measurements, however, do not yield molecular reorientational correlation times, nor are they suited for following intramolecular dynamics. An especially pertinent question to be resolved for UpA deals with the kinetics of interconversion between UpA 1 and UpA 2 conformations (Rubin, J., et al., Biochemistry 11, 3112, 1972) in solution.

EXPERIMENTAL METHODS

Carefully degassed solutions of uridylyl-(3'-5')-adenosine, UpA, were made up in 100% D_2O (0.02 M), and proton T_1 values were measured by the 180°-τ-90° inversion recovery method with a Varian 220 MHz spectrometer equipped with an FT accessory. A total of 250 repetitive scans was accumulated in the time domain to obtain a single FT spectrum.

MAJOR NEW FINDINGS

This is a new program, initiated in September 1975.

SIGNIFICANCE

Results of this investigation will provide currently unavailable information for the effects of base sequence and chain length on molecular dynamics

and conformational flexibilities of nucleic acid fragments. Such data have a direct bearing on the stabilities of key loop and terminal ends of tRNA molecules and will ultimately be useful in developing mechanistic models for sequential melting and/or unwinding of the tRNA cloverleaf structure.

PROPOSED COURSE OF THE PROJECT

The initial phase of the study will concentrate on measurement of accurate T_1 values for UpA as a function of temperature and pD, and the interpretation of these data in terms of a dipole-dipole relaxation model (Akasaka, K., Biopolymers 13, 2273, 1974). Two selectively deuterated analogues, *UpA and U*pA (asterisk denotes fully deuterated nucleotide), will be used to sort out intranucleotide contributions to dipolar relaxation processes. Microdynamic models for molecular and conformational reorientational processes will be developed from the relaxation data.

ERDA RT-03-02
ANL 61200

STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: EPR STUDIES OF FREE RADICALS IN γ-IRRADIATED NUCLEIC ACIDS; COUNTER ION EFFECTS

*Alonzo J. Fairbanks,** *Principal Investigator*
Clinton F. Ainsworth, Steven S. Danyluk, and Fouad S. Ezra, Participating Investigators

OBJECTIVES

The objectives of this project are: (1) to characterize free radicals produced in ribose-phosphate regions of nucleic acids by γ-irradiation, (2) to show the influence of cations on the nature of these free radicals, and (3) to explore the implication of such radicals to biological damage, specifically DNA breaks.

BACKGROUND AND PREVIOUS FINDINGS

Although much progress has been made in identifying free radicals produced in irradiated nucleotide components of RNA and DNA, a number of important questions still remain. These are particularly concerned with the types and abundance of radicals formed along the phosphodiester backbone and the influence of counter ions bound to negatively charged phosphate groups on such radical formation. In earlier studies (Bernhard, W. A., and S. S. Danyluk,

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*Trinity College, Deerfield, Illinois.

Radiat. Res. 53, 169, 1973) a weak doublet observed on the outermost fringes of the central, strong radical signal in electron paramagnetic resonance (EPR) spectra of γ-irradiated monomers and dimers was tentatively identified as a phosphorus based radical of form $RO(RO')PO_2$, but this interpretation has subsequently been questioned by other workers (Van Worst, A., et al., Int. J. Radiat. Biol. 24, 605, 1973), who suggest a radical of type $R\dot{C}H_2$ instead. Because either or both of these radicals may be involved in key initial steps leading to strand breakage, further investigation is clearly warranted.

EXPERIMENTAL METHODS

Commercial samples of uridine mononucleotides (further purified in some instances on cation exchange columns) were sealed in EPR tubes in a vacuum chamber and were γ-irradiated at liquid N_2 temperature, 77°K, and at room temperature. EPR spectra were recorded with a Varian Century line spectrometer operating at X-band.

MAJOR NEW FINDINGS

This project was initiated in July 1975. In preliminary work completed thus far, there is a strong indication that the nature of the outermost "doublet" EPR signal depends upon the type of cation associated with the nucleotide; i.e., uridine 5'-monophosphate prepared as the sodium salt yields a different spectrum from the lithium salt.

SIGNIFICANCE

There is strong evidence to suggest that the primary lesions produced in genetic material of biological systems by γ-rays are due to actions of chemically reactive free radicals. The conditions for formation of such radicals and their subsequent reactions are largely unknown. Results of this project will provide answers to these questions. The results will also be useful for devising strategies for control of adverse effects of radiation by control of conditions under which free radicals are formed.

PROPOSED COURSE OF THE PROJECT

Highly purified samples of mononucleotides, prepared carefully with selected alkali metal and alkaline earth cations, will be subjected to fractionated doses of γ-radiation at liquid N_2 and He temperatures, and their EPR spectra will be recorded. Comparable studies will also be made of corresponding fully deuterated monomers. Spectra will be analyzed by appropriate computer programs to derive g values and hyperfine splitting parameters. Irradiated samples will also be subjected to various thermal cycles to establish sequential free radical interconversions.

ERDA RT-03-02
ANL 61200

STRUCTURAL STUDIES OF BIOLOGICAL MOLECULES: A SPIN-LABEL STUDY OF SICKLE ERYTHROCYTES

Michael E. Johnson, Principal Investigator*
Steven S. Danyluk, Participating Investigator

OBJECTIVES

The immediate goal of these studies is to determine structural and functional differences between membranes of sickle cells and normal erythrocytes. Specifically, it is intended to determine (1) any intrinsic structural differences between the two membrane systems; (2) the source of observed abnormal sickle membrane interactions with ions (particularly Ca^{+2} and Zn^{+2}) and the heavy metals, lead and mercury; and (3) the source of observed strong membrane-hemoglobin interactions in the sickle erythrocyte. The ultimate goal of these studies is the rational design of a drug therapy that will alleviate the symptoms of the disease while inducing minimal side effects.

BACKGROUND AND PREVIOUS FINDINGS

Much experimental and theoretical work has been done in investigating the polymerization reaction in sickle cell hemoglobin (Proceedings of the 1st Natl. Symp. on Sickle Cell Disease, Eds. J. I. Hercules, et al. NIH, Bethesda, 1974). There is also evidence that hemoglobin and ionic interactions with the membrane play a significant role in determining the deformability of the erythrocyte (Fisher, S., et al., Biochim. Biophys. Acta 375, 422, 1975; LaCelle, P., et al., Semin. Hematol. 7, 3551, 1970). However, very little work has been done in examining the effects of sickling upon the structural properties of the erythrocyte membrane or in determining the role of the membrane in inducing sickling.

EXPERIMENTAL METHODS

The membranes of intact erythrocytes will be spin-labeled with nitroxide derivatives of stearic acid and analogs of cholesterol. The electron spin resonance spectra of these labels, measured with a Varian E-line Century Series EPR spectrometer, will then be used to monitor the membrane environment as a function of experimental conditions. Ultracentrifugation density gradient techniques will be used to separate cell samples into populations of equal cellular age.

MAJOR NEW FINDINGS

This is a new program, initiated in June 1975.

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*University of Illinois at the Medical Center, Chicago.

SIGNIFICANCE

Because this research is directed toward clarifying the type of drug therapy needed to alleviate symptoms of sickle cell disease, the medical significance is obvious. In addition, this research is also expected to clarify ion-membrane and protein-membrane interaction mechanisms, two areas in which understanding is currently quite limited.

PROPOSED COURSE OF THE PROJECT

The project is still in its beginning phases. Essentially it will follow a course outlined by the objectives above. Blood samples will be obtained from sickle patients and normal adults at the University of Illinois Research Hospital, and comparative studies will be made using appropriate spin labels. After the intrinsic differences are understood, comparative studies are planned with the focus on ionic interactions and hemoglobin-membrane interactions.

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NIH AM 15351-05
EPA-IAG-D6-E681

03085

STABLE ISOTOPE STUDIES: INTRODUCTION

Peter D. Klein, Principal Investigator

The following reports describe a series of projects directed to expanding the clinical use of stable isotopes. They reflect a continuation of the collaborative activities of the program which involve a number of clinical centers throughout the country. In addition, integral parts of our overall program are concerned with the synthesis of appropriate substrates labeled with stable isotopes, and with the development of instrumentation for their measurement.

In cooperation with associates at Mayo Clinic, we have synthesized a variety of ^{13}C-labeled bile acids for use in humans as tracers of bile acid kinetics. A comprehensive study on the gas chromatographic, electron impact and chemical ionization mass spectrometry of bile acids has provided a unique screening system for the identification of novel or unusual bile acids occurring in human bile in disease states, such as gallstone formation, cirrhosis, cholestasis, or chronic liver disease. The first measurement of bile acid kinetics in man, using peripheral blood samples instead of biliary intubation has been carried out and validated against conventional techniques and ^{14}C-labeled tracers.

Collaborative studies are underway on the metabolism of folic acid in association with Dr. I. H. Rosenberg, principal investigator, at the University of Chicago.

We have collaborated with Dr. Mary Jeanne Kreek at Rockefeller University in the first long-term (8-day) study of methadone metabolism, using d_5-labeled methadone prepared by Dr. Hachey. The existence of a metabolic pool with $T_{1/2}$ of 30-40 hours was shown for the first time.

The clinical testing and validation of ^{13}C "breath tests," modeled on successful ^{14}C tests already in use in clinical diagnosis, showed that these tests have identical reliability and value in diagnosis. We have applied them to children with cystic fibrosis, infants with bacterial overgrowth, and individuals with primary biliary cirrhosis.

A stable isotope ratiometer-multiple ion detector, designed to provide low cost instrumentation for the measurement of stable isotope ratios in organic molecules was designed, constructed, and evaluated in routine applications. Following the publication of its description, a commercial version, embodying further simplification, has appeared on the market. This unit has attracted great customer interest since it provides the capability of a computer system at one-third to one-sixth the cost.

Work of this group was presented in six papers at the Second International Conference on Stable Isotopes, Oak Brook, Illinois, October 20-23, 1975. These were part of more than 80 papers contributed to this Conference by the 230 participants from the U. S., Canada, Europe, and Asia.

ERDA RT-03-02
ANL 61200
NIH AM 17862-01

STABLE ISOTOPE STUDIES: MEASUREMENT OF BILE ACID KINETICS IN HUMAN SUBJECTS WITHOUT DUODENAL INTUBATION; KINETIC STUDIES OF SERUM BILE ACIDS FOLLOWING ADMINISTRATION OF ^{13}C-LABELED BILE ACIDS

Peter D. Klein, Principal Investigator
Patricia A. Szczepanik, Kou-Yi Tserng, Alan F. Hofmann, and Peter Y. Ng,* Participating Investigators*

OBJECTIVES

The objectives of this study are: (1) to validate the use of ^{13}C-labeled bile acids in the measurement of biliary bile acid kinetic measurements against

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*Mayo Clinic, Rochester, Minn.

conventional ^{14}C-labeled bile acids; (2) to validate the measurement of serum bile acid kinetics against biliary bile acid measurements, and (3) to establish procedures for the simultaneous measurement of the five major bile acids--lithocholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, and cholic acids--in serum samples from patients to whom these bile acids have been administered as the ^{13}C-labeled tracers. This will permit the first complete characterization of bile acid kinetics in man and, more significantly, will eliminate the present requirements of hospitalization and duodenal intubation to obtain the analytical samples. This will permit extension of these measurements to children and pregnant women for whom the invasive procedures of intubation are not advisable.

BACKGROUND AND PREVIOUS FINDINGS

The measurement of bile acid pool sizes and synthesis rates is a recurrent necessity in the study of gallstone formation, cystic fibrosis, cholestasis, biliary cirrhosis, and other gastroenterological disorders. To date, these measurements have required hospitalization of the patient, the inconvenience and discomfort of duodenal intubation, and often the fluoroscopic positioning of the duodenal tube, with the attendant radiation exposure. Four elements now permit the establishment of bile acid kinetic measurements in serum. These elements are: (1) the demonstration by Hofmann and co-workers (personal communication) that, following a meal, the serum concentrations of bile acids rise from a fasting level of $\sim$ 200 nanograms/ml to 10 µg/ml within 90 minutes; (2) the ability to administer sufficient ^{13}C-labeled bile acid ($\sim$ 25-50 mg) to produce adequate isotopic levels for measurement over 5-7 days; (3) the demonstration by Hachey and Szczepanik that chemical ionization of bile acids prior to mass spectrometric analysis produces no cleavage of the side chain where ^{13}C labeling is most feasible, hence permitting detection of the label with great sensitivity; and (4) the construction of the SIRMID unit which permits measurements of isotopic ratios in successive gas chromatographic peaks, thus permitting simultaneous measurements of all major bile acids in the same analytical sample.

EXPERIMENTAL METHODS

^{13}C-labeled bile acid (e.g., chenodeoxycholic acid-24-^{13}C) is administered together with the ^{14}C-labeled form to patients in the Gastroenterology Unit at Mayo Clinic. The next morning, duodenal intubation is used to obtain a sample of bile in the fasting state, and 90 minutes after the patient has eaten breakfast, a sample of serum is obtained. The bile acids are isolated from bile and serum by established procedures and the methyl ester acetate derivatives are prepared for gas chromatography-mass spectrometry. The isotope ratio of the chenodeoxycholic acid is measured using the Biospect gas chromatograph-chemical ionization mass spectrometer and the stable isotope ratiometer-multiple ion detector (SIRMID) unit developed by Klein et al. (Clin. Chem. 21, 1253, 1975). The disappearance of ^{13}C and of ^{14}C (determined by scintillation counting of isolated chenodeoxycholic acid) follows a semi-log plot versus time, and the pool size is obtained from the intercept of the regression line; the synthesis rate is calculated from the slope of the line.

MAJOR NEW FINDINGS

The first series of patients have been studied at the Mayo Clinic in which ^{13}C-labeled chenodeoxycholic acid was administered simultaneously with ^{14}C-labeled chenodeoxycholic acid. Isolation of the serum bile acids has shown that there is sufficient chenodeoxycholic acid in 2 ml of serum to permit accurate measurement of the isotopic content over a period of 5-7 days, and these measurements are in very close agreement with values obtained on biliary bile acids. There appears to be no interference from other serum constituents and the amounts of deoxycholic and cholic acid present in the samples from serum also should be adequate to permit isotopic ratio measurements.

SIGNIFICANCE

At present, bile acid kinetic measurements require a minimum of 5 days hospitalization, the use of fluoroscopy to position the duodenal tube, the discomfort and inconvenience of an indwelling tube, and the use of 2-5 μCi radioactive bile acid for each bile acid measured. The validation in progress can be expected to show that with five 4-ml samples of whole blood it will be possible to obtain the same information with no more discomfort than 5 venipunctures, on an outpatient basis. We expect that this will have a considerable impact on epidemiological and pediatric studies, as well as those of pregnant women, for whom intubation is contraindicated in the last trimester because of the fluoroscopic positioning required. We are, for example, presently barred from conducting epidemiological studies on American Indian children of prepubertal, pubertal, and postpubertal ages (a period during which lithogenic bile first makes its appearance) because of the refusal of the tribal council to permit intubation procedures to be used on these children. The high incidence of cholesterol gallstones in this population can now be related to bile acid kinetics during puberty, using these procedures. Similarly, the cholestasis and gallstone formation during pregnancy can now be studied with minimal discomfort and an absence of hazard.

PROPOSED COURSE OF THE PROJECT

Following the validation of individual bile acid kinetic measurements in serum vs. bile, we shall conduct a limited series of validations involving multiple kinetic studies within the same patient to demonstrate that independent measurements on each major bile acid can be conducted simultaneously. As indicated, there exist a number of clinical applications that have awaited this capability, and there appears to be no shortage of opportunities for its use. This work illustrates the unique contributions that can be made through collaborative support by ERDA and the National Institutes of Health (through the National Institute for Arthritis, Metabolic and Digestive Diseases, Grant AM-17862).

ERDA RT-03-02
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NIH AM 17862-01

STABLE ISOTOPE STUDIES: BILE ACID TRANSFORMATIONS OCCURRING IN GALLSTONE THERAPY; DEVELOPMENT OF AN ANALYTICAL PROCEDURE FOR BILE ACIDS BY INVERSE ISOTOPE DILUTION ASSAYS WITH DEUTERIUM-LABELED BILE ACIDS

Karin A. Mede, Principal Investigator
*Peter D. Klein, David L. Hachey, Alan F. Hofmann,**
G. P. van Berge Henegouwen, and Peter Y. Ng,* Participating Investigators*

OBJECTIVES

The main objectives of this study are (1) to develop quantitation techniques by inverse isotope dilution for the accurate measurement, at the nanogram level, of bile acids, especially lithocholic acid, in plasma and liver biopsy samples; and (2) to establish the form of deuterium-labeled standard best suited for use as a carrier and internal standard. The techniques developed will be applied to samples obtained at Mayo Clinic, and the values obtained for lithocholic acid in liver biopsies will be correlated with light and electron microscopic studies performed at Mayo. The use of these techniques will be extended from gallstone patients to patients with other forms of liver disease, including liver cirrhosis and chronic active liver disease.

BACKGROUND AND PREVIOUS FINDINGS

Gallstones, predominantly cholesterol stones, are present in about 15 million people in the United States. The condition may be caused by a decreased amount of total bile acids, abnormal proportions of bile acids, an increased amount of cholesterol, or a combination of factors that cause the cholesterol, normally kept solubilized in the bile by the detergent effect of the bile acids, to precipitate. Currently the efficacy of administering chenodeoxycholic acid to dissolve cholesterol gallstones is being investigated. This acid, generally a major constituent of bile, is present in decreased quantities in some persons with gallstones. The administered chenodeoxycholic acid is absorbed from the intestine and enters the enterohepatic circulation. In the gall bladder, its detergent effect helps to resolubilize the cholesterol in the gallstones. The enteric flora are able to convert chenodeoxycholic acid to lithocholic acid, which is hepatotoxic. Although only a small portion of the lithocholic acid is believed absorbed from the intestine, the actual amount absorbed is not known. Very little also is known of the lithocholic acid pool size in patients receiving therapeutic doses of chenodeoxycholic acid, in patients with other liver diseases, or in healthy man.

EXPERIMENTAL METHODS

Inverse isotope dilution curves are constructed with unlabeled bile acid

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*Mayo Clinic, Rochester, Minn.

in labeled bile acid solutions. These deuterium-protium mixtures are assayed as the methyl ester acetates on Poly S 179 columns using the Biospect mass spectrometer and the stable isotope ratiometer-multiple ion detector (SIRMID) unit developed at Argonne (Klein, P. D., et al., Clin. Chem. 21, 1253, 1975). The bile acids and added labeled bile acid spike are isolated from plasma by use of the nonionic polymeric absorbent, Amberlite XAD-7. The dried extract from this material is subjected to basic hydrolysis at 120°C in a Teflon-lined acid digestion bomb. Following work-up and derivatization, the isotope ratio of each sample is determined by GC/MS as described above, and the quantity of bile acid present in the original plasma sample is calculated from this ratio. The GC/MS procedure in this study is widely used to quantitate individual compounds in a mixture of similar substances. The use of a labeled spike added at the start of the procedure, to determine an unlabeled entity, ensures identical behavior with respect to losses and chemical and physical behavior throughout the procedure.

MAJOR NEW FINDINGS

The preliminary data obtained to date in this new project are mainly concerned with the feasibility of isolating bile acids from plasma and the quantitation of these acids. The initial studies focus on cholic acid, one of the major bile acid constituents, rather than on lithocholic acid which is normally present only in trace quantities in plasma. The use of varying amounts of plasma and labeled cholic acid spike show that recovery of both labeled and unlabeled cholic acid is facile and reproducible. Use of a threefold range of labeled spike in plasma shows good correlation of results at the three levels. It is concluded that the experimental techniques required to meet our objectives are quite feasible.

SIGNIFICANCE

Routine testing for circulating lithocholic acid in patients undergoing chenodeoxycholic acid therapy for gallstones will be facilitated, and the safety of the therapy can be evaluated with the development of routine techniques for measurement of lithocholic acid. Correlation of lithocholate levels in the liver with microscopic morphology of the liver will confirm or deny the possible hazard of lithocholate production from chenodeoxycholic acid in the intestine. The ability to measure bile acids in blood should also be of value as a liver function test, since the normal liver removes most of the bile acid from the blood and increased levels of plasma bile acids may indicate liver disease. This test of liver function has the advantage over currently used tests in that it does not require administration of another substance to the patient.

PROPOSED COURSE OF THE PROJECT

This work, which is part of the National Cooperative Gallstone Study, will continue from cholic acid to other bile acids with emphasis on lithocholic acid. Labeled conjugated and sulfated forms of the bile acids will also be studied to establish the form best suited for use.

ERDA RT-03-02
ANL 61200
NIH AM 17862-01

STABLE ISOTOPE STUDIES: BILE ACID TRANSFORMATIONS OCCURRING IN GALLSTONE THERAPY; CHARACTERIZATION OF BILIARY BILE ACIDS BY MASS SPECTROMETRY

Patricia A. Szczepanik, Principal Investigator
Peter D. Klein, David L. Hachey, John L. Thistle, and*
Alan F. Hofmann, Participating Investigators*

OBJECTIVES

The objectives of this work are (1) to develop a simple, sensitive, and selective technique for the routine screening of complex bile acid mixtures, (2) to use this methodology to detect and identify unusual bile acids that may be associated with cholelithiasis, and (3) to monitor the effects of bile acid therapy on biliary bile acid composition in gallstone patients.

BACKGROUND AND PREVIOUS FINDINGS

Chemical ionization mass spectrometry of bile acids yields extremely simple mass spectra with only two or three ions predominating. The mass ion data combined with gas chromatographic retention data form a unique coordinate system, with a specific index for each of the bile acids, and provide the basis for the utilization of selective ion monitoring techniques for the characterization of bile acid mixtures. Because of its simplicity and sensitivity, this technique is particularly useful for screening samples from patients with gallstones (cholelithiasis) and for monitoring changes in patients participating in chemotherapy trials for the dissolution of gallstones.

Although cholelithiasis is common, relatively little is known about the biliary bile acid composition of patients with the disease. Atypical bile acids are sometimes found in gas chromatographic analyses of samples from gallstone patients. In patients treated with large doses of a particular bile acid, it may comprise up to 80% of the total bile composition, and alterations in metabolism may result. Therefore, our main concerns were to confirm the identities of primary and secondary bile acids found in bile samples from treated patients and to detect and identify any abnormal bile acids.

EXPERIMENTAL METHODS

A Biospect chemical ionization mass spectrometer (Scientific Research Instruments, Baltimore, Md.) employing a stable isotope ratiometer-multiple ion detector (SIRMID) (Klein, P. D., et al., Clin. Chem. 21, 1253, 1975) was used to monitor up to six mass ions in a cyclic manner. The output from the ion monitoring process was recorded on a multipen recorder.

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*Mayo Clinic, Rochester, Minn.

MAJOR NEW FINDINGS

Using the methodology developed in our laboratory, we have examined the biliary bile acids from groups of five patients at Mayo Clinic on placebo, chenodeoxycholic acid, or cholic acid therapy, before and after treatment for 1 year. The identities of all primary and secondary bile acids found to be present by gas chromatography were confirmed. Specific searches were made for over 50 possible compounds, including keto hydroxy bile acids as well as all epimers of di- and trihydroxy bile acids. No abnormal bile acids were found in any of the patients studied, regardless of treatment. Several other interesting observations were made: Lithocholic and ursodeoxycholic acids, both considered to be transformation products of chenodeoxycholic acid, were present in samples from all patients. Moreover, placebo patients showed concentrations of lithocholic acid as high as those found in patients on chenodeoxycholic acid therapy. The 3β-epimer of chenodeoxycholic acid was also found to occur randomly in 7 of 30 samples examined. Several keto bile acids were detected in concentrations between 0.5 and 5.0% of the total bile acid sample, but the significance of their presence is unknown at this time.

PROPOSED COURSE OF THE PROJECT

This project is part of an ongoing collaborative program to define and document the constituents of biliary bile in cholelithiasis before and during trial chemotherapy. Studies are presently underway in healthy volunteers administered deoxycholic acid. Further investigations of gallstone patients after 1 year of chenodeoxycholic acid treatment, as well as studies on rhesus monkeys maintained on high levels of chenodeoxycholic acid, are scheduled.

ERDA RT-03-02
ANL 61200

STABLE ISOTOPE STUDIES: BILE SALT METABOLISM IN INFANCY AND CHILDHOOD; BILE SALT KINETICS IN CYSTIC FIBROSIS

Patricia A. Szczepanik, Principal Investigator
Peter D. Klein, John B. Watkins, and Ann M. Tercyak,**
Participating Investigators

OBJECTIVES

The objectives of this study were to examine the enterohepatic circulation of bile acids in children with cystic fibrosis and to determine the effect of pancreatic enzyme replacement therapy on the bile acid pool size, synthesis, and fractional turnover rate.

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*Children's Hospital Medical Center, Boston, Mass.

BACKGROUND AND PREVIOUS FINDINGS

Cystic fibrosis is a recessive genetic disease characterized by abnormal secretion of mucoproteins and malfunction of the exocrine glands, with insufficiency of the pancreas occurring in 80% of those affected. Since intraluminal digestion of fat depends on the lipolytic activity of pancreatic enzymes, a failure to absorb dietary fat and the elimination of large amounts of lipid in the feces are manifestations of this disease; bile acid excretion is also elevated. Children with cystic fibrosis excrete seven times the normal levels of bile acids. Very little is known about the kinetics of lipid hydrolysis and absorption in cystic fibrosis, and there is no information about bile salt synthesis or secretion, or amount of bile salt available for lipid solubilization. Moreover, studies indicate that even with pancreatic enzyme replacement, lipid malabsorption persists. The effect of pancreatic enzyme therapy on bile acid pool size is unknown.

EXPERIMENTAL METHODS

Bile salt kinetics were studied using ^{2}H-labeled chenodeoxycholic and cholic acids in five patients with newly diagnosed cystic fibrosis, ranging in age from 3 1/2 months to 4 1/2 years. Bile samples collected by duodenal intubation were purified and derivatized, and the isotope ratios were measured using the gas chromatography-mass spectrometry-accelerating voltage alternation method (Klein, P. D., et al., Anal. Chem. 44, 490, 1972). The pool sizes and synthesis rates for chenodeoxycholic and cholic acids were calculated using the standard Linstedt method.

MAJOR NEW FINDINGS

Our studies show that children with cystic fibrosis have abnormally high losses of bile acids and correspondingly high synthesis rates, as indicated by very high fractional turnover rates. However, the increase in synthesis rate is insufficient to maintain normal bile acid pool size as evidenced by the exceedingly low levels of chenodeoxycholic and cholic acids observed in these patients.

Within five days after initiation of pancreatic enzyme replacement, there are a doubling of the bile acid pool and a dramatic fall in the synthesis rate, indications that conservation of bile acid is occurring. These data suggest that bile acid reabsorption and regulation of bile acid synthesis are normal in patients with cystic fibrosis, and that the excessive fecal bile acid losses observed in this disease are due primarily to the presence of large amounts of unhydrolyzed fats and other undigested nutrients in the intestine which entrain the bile acids and prevent their reabsorption, causing subsequent elimination in the stool.

SIGNIFICANCE

Investigations into the metabolic processes of infants and children have been limited because of the lack of suitable procedures. Stable isotope

techniques, which eliminate the radiation hazards associated with tracer studies, and the development of instrumental methods for stable isotope analysis now permit these studies. The use of these techniques to study bile acid kinetics in normal developing infants has already provided much information about their pool size and synthesis rates and consequently about insufficiencies which lead to distress states. These methods, especially when combined with the $^{13}CO_2$-trioctanoin breath test, have enabled description of the entero-hepatic circulation of children with cystic fibrosis and demonstrated our ability to monitor fat absorption and bile acid kinetics in enzyme replacement treatment.

PROPOSED COURSE OF THE PROJECT

This study is part of an ongoing collaborative program to determine the developmental characteristics of bile acid metabolism in infancy and childhood. Abnormalities that lead to disease states are of particular interest, and studies are presently underway in children with type II hyperlipidemia, a condition characterized by high levels of plasma cholesterol and early death due to coronary artery disease or stroke.

ERDA RT-03-02
ANL 61200
NIH AM 17862-01

STABLE ISOTOPE STUDIES: SYNTHESIS OF LABELED BILE ACIDS

Kou-Yi Tserng, Principal Investigator
David L. Hachey and Peter D. Klein, Participating Investigators

OBJECTIVES

Studies of bile acid metabolism and kinetics are useful tools for the diagnosis of liver disease and other disorders. In preliminary experiments, it was found that the pool size and synthesis rate obtained in patients by using carbon-13 labeled cholic acid correlated very well with the results from conventional carbon-14 labeled cholic acid. A program was set up to develop the use in clinical investigations of these bile acids labeled with stable isotopes. It is our purpose to synthesize and provide the clinical investigators with the required labeled bile acids. Because large quantities are needed (gram level), substantial efforts are devoted to the development of more efficient synthetic routes, with higher yield, to reduce the cost of labeled compounds.

BACKGROUND AND PREVIOUS FINDINGS

Carbon-13 labeled bile acids have been synthesized in our laboratory on a small scale as described by Hachey, D. L., et al. (J. Labelled Compd. 9, 703, 1974). The acetylated bile acids were degraded to 23-*nor* chloride bile acids by using the Kochi reaction. These chlorides were then reacted with $Na^{13}CN$, and hydrolysis of the cyano compounds yielded the carbon-13 labeled bile acids. Although this reaction sequence was superior in several aspects to the method described by others using the classical Hunsdicker reaction, this method also required substantial effort and gave low overall conversion yield.

EXPERIMENTAL METHODS AND MAJOR NEW FINDINGS

The synthetic method of Hachey et al. was reviewed and repeated. Every step was monitored carefully by thin-layer and gas chromatography. It was concluded that the low overall yield and extensive purification required for the reaction products were caused by two steps, namely, the acetylation step and the Kochi reaction.

Acetylation of bile acids usually produced oily products. These were a mixture of several compounds, including incompletely acetylated bile acids, mixed anhydrides, and several other unidentified minor products. Upon subsequent lead tetraacetate degradation, these products resulted in even more complicated mixtures. In contrast, the formyl derivatives of bile acids were all well-defined products which could be easily prepared. By switching from acetyl derivatives to formyl derivatives, some of the troublesome by-products produced in subsequent reactions can be avoided. In some cases, the pure 23-*nor* chloride can be isolated very easily in high yield, even without a chromatographic separation step.

Two equivalents each of lead tetraacetate and lithium chloride were used in the previous procedure for the conversion of acetylated bile acids to the 23-*nor* chlorides. This combination not only resulted in incomplete conversion (typically below 50%), but also required a complicated isolation procedure, due to the unreacted lead tetraacetate. It was found that by using an excess of lithium chloride (2- to 3-fold excess), added in several portions over a 1 to 2 hour interval, the conversion was substantially increased (to an almost quantitative reaction), and the isolation was simplified because the reaction had used up all of the lead tetraacetate.

Using the modified procedure, we have synthesized gram quantities of cholic-24-^{13}C acid, chenodeoxycholic-24-^{13}C acid, deoxycholic-24-^{13}C acid, and lithocholic-24-^{13}C acid (previously prepared in small quantities by the method of Hachey et al.). Furthermore, gram quantities of ursodeoxycholic-24-^{13}C acid were also synthesized, for the first time, by the modified procedure.

SIGNIFICANCE

These procedures, when published and made available to commercial suppliers of ^{13}C-labeled compounds, will significantly reduce the cost of synthesis

and, thereby, the ultimate cost of labeled compounds to the clinician. The achievement of a uniformly applicable synthesis procedure for ^{13}C labeling of bile acids will stimulate study of those bile acids, such as ursocholic, which have been inaccessible to date.

PROPOSED COURSE OF THE PROJECT

A number of deuterated bile acids and their glycine, taurine, or sulfate conjugates will be synthesized for use as internal standards in inverse isotope dilution studies to check the recovery of each step.

ERDA RT-03-02
ANL 61200

STABLE ISOTOPE STUDIES: APPLICATION OF STABLE ISOTOPIC TRACERS TO THE STUDY OF THE CLINICAL PHARMACOLOGY OF METHADONE IN MAINTENANCE PATIENTS

David L. Hachey, Principal Investigator
Mary Jeanne Kreek, David Mattson,† and Peter D. Klein, Participating Investigators*

OBJECTIVES

The major objective of this work is to define the pharmacokinetic behavior of methadone, i.e., to determine the metabolic disposition of the drug, to measure body pool size and clearance rate, and to study the mode of transmittal of the drug in pregnant women to the developing fetus and from nursing mothers to their newborn infants. This involves a two-part study: (1) the development of methods for measuring methadone down to nanogram levels in physiological fluids, using inverse isotopic dilution techniques; and (2) the study of the pharmacokinetic behavior of the drug following oral administration of a single dose of deuterium-labeled methadone to patients on long-term methadone maintenance.

EXPERIMENTAL METHODS

Deuterium-labeled methadone prepared previously in our laboratory was used for these studies. Standard isotope dilution curves were prepared to relate the observed isotope ratio to the mole composition of methadone/

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*The Rockefeller University.

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

deuterated methadone standard mixtures. Isotope ratios were measured using the Biospect chemical ionization mass spectrometer and stable isotope ratiometer-multiple ion detection system (SIRMID) described earlier (Klein, P. D., et al., Clin. Chem. 21, 1253, 1975). This allows us to measure isotope ratios over a wide dynamic range (40,000:1) and to measure quantitatively amounts as small as 5 ng/ml in plasma. Pharmacokinetic variables (pool size, turnover rate, drug half-life) were calculated using computer programs available through the BIM divisional computer facility.

MAJOR NEW FINDINGS

Methadone levels in urine were measured in several newborn infants whose mothers were maintained on methadone during pregnancy. With only a single exception (one infant had an abnormally high urinary methadone level of 19.0 μg/ml), urine levels were a factor of five lower than in a typical adult subject (4-10 μg/ml). Plasma methadone levels measured on young adolescent maintenance patients were found to be 2-4 times lower than in adults (150-400 ng/ml). The lower levels reflect the small dose/kg body weight received by these subjects.

Pharmacokinetic studies in three adult subjects revealed that methadone has a very slow clearance rate from the body ($T_{1/2}$ = 30-50 hours). In two subjects an initial rapidly turning over pool was observed ($T_{1/2}$ = 5-8 hours).

A major new physiological phenomenon related to drug tolerance was observed in two individuals. In these patients we frequently observed an abrupt rise in the plasma isotope ratio of the methadone following bowel movements after a period of severe or moderate constipation. Methadone has a strong anesthetic effect on intestinal smooth muscle that inhibits intestinal motility. Patients given an enema or who experience a bowel movement break up the cluster of fecal material containing isotopically enriched methadone, which is then reabsorbed by the gastrointestinal tract and re-enters the enterohepatic circulation. This, in effect, results in a delayed enterohepatic circulation of the drug. These findings will have significant importance in evaluating drug therapy in patients experiencing constipation. We were able to observe this phenomenon only because the stable isotope tracer technique developed at Argonne permits plasma kinetics measurements for periods up to 8 days. Since the plasma methadone levels are low at all times, similar studies using radioactive tracers could not be done.

SIGNIFICANCE

Stable isotopic tracer techniques are now shown to be a valuable tool in the clinical pharmacology of drug use. Since they may be used in situations where radioisotopes are contraindicated, they can be used in women of childbearing age and in small children. Of more subtle environmental importance, stable isotopes present no potential threat of contaminating the biosphere with radioactive waste products.

PROPOSED COURSE OF THE PROJECT

Two new areas will be explored in the coming year. First, we will try to stabilize those subjects experiencing a delayed enterohepatic circulation of methadone, due to severe constipation, by treatment with laxatives. We will then repeat the kinetic studies. Secondly, we plan to study the microsomal N-demethylation of methadone using $^{13}CO_2$ breath tests analogous to the aminopyrine $^{13}CO_2$ breath test.

ERDA RT-03-02
ANL 61200

STABLE ISOTOPE STUDIES: THE $^{13}CO_2$ BREATH TEST

D. A. Schoeller, Principal Investigator
J. B. Watkins, J. F. Schneider,† N. W. Solomons,† I. Rosenberg,†*
A. F. Hofmann,‡ A. Newcomer,‡ and P. D. Klein, Participating Investigators

OBJECTIVES

The research on the $^{13}CO_2$ breath tests is a part of two ongoing programs: the clinical applications program and the instrument development program. The objectives of these two programs are the development of clinical applications of stable isotopes and the development of instrumentation that will facilitate the use of stable isotopes in a routine manner with a minimum of cost. Achievement of these goals will increase desirability and practicality of using stable isotopes. This will stimulate the demand for isotopically labeled compounds and detection devices, and will encourage investment by private enterprise.

EXPERIMENTAL METHODS

The $^{13}CO_2$ breath test is performed following the administration of a substrate labeled with ^{13}C. Cleavage of the target bond linking the label to the substrate results in the formation of $^{13}CO_2$ which is expired in the breath. The expired CO_2 is collected from the patient, cryogenically purified, and analyzed for excess $^{13}CO_2$ by isotope ratio mass spectroscopy (McKinney, C. R., et al., Rev. Sci. Instrum. 21, 724, 1950). The amount of excess $^{13}CO_2$ and the rate of its appearance in breath give information about the metabolism of the substrate.

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*Harvard Medical School.

†Department of Medicine, University of Chicago.

‡Mayo Clinic, Rochester, Minn.

BACKGROUND AND PREVIOUS FINDINGS

The $^{13}CO_2$ breath test is modeled after the similar $^{14}CO_2$ breath tests, which have received wide clinical acceptance. However, because of the radiation hazard, the latter test is proscribed for use in children and pregnant women, a restriction which will be removed by the use of the stable isotope ^{13}C.

At present, three substrates, already shown to be of diagnostic value with the $^{14}CO_2$ breath test, are being used for the $^{13}CO_2$ breath tests. These substrates are 4,4-dimethyl-aminopyrine, a drug whose rate of metabolism is related to hepatic microsomal function (Lauterburg, B., and J. Bircher, Gastroenterology 65, A32/556, 1973); sodium glycocholate, a bile salt which has been used in the diagnosis of bile salt malabsorption and bacterial overgrowth in the small intestine (Fromm, N., and A. F. Hofmann, Lancet 2, 621, 1971); and trioctanoin, a neutral fat whose rate of metabolism has been utilized in the diagnosis of fat malabsorption (Kaihara, S., and H. N. Wagner, Jr., J. Lab. Clin. Med. 71, 400, 1968).

Except for the use of different substrates, all of the $^{13}CO_2$ breath tests are run in the same manner. This uniformity makes the use of automated analysis both desirable and practical. However, the method of collecting the CO_2 as carbonate and the subsequent release has not lent itself to automation; moreover, this method has been shown to fractionate the carbon isotopes (Schoeller, D. A., et al., ANL-75-30, 1974, p. 179).

MAJOR NEW FINDINGS

During 1975, all three of the substrates have undergone clinical testing. In work done in collaboration with Dr. Hofmann, the trioctanoin $^{13}CO_2$ breath test was validated against the $^{14}CO_2$ test. The high correlation ($r = 0.97$) indicated that identical information can be obtained from the two tests. The $^{13}CO_2$ test was then used in a study of fat malabsorption in infants with cystic fibrosis in collaboration with Dr. Watkins. The utility of the test in diagnosing fat malabsorption and in the management of pancreatic enzyme replacement therapy of these children was demonstrated.

The glycocholate $^{13}CO_2$ breath test was validated against its ^{14}C analog in collaboration with Dr. Solomons. It was found to correlate well ($r = 0.94$), and was shown to be useful in diagnosing moderate disorders in bile salt absorption. The test is presently being used in a study of Guatemalan children who have had intestinal resections following severe intestinal complications of typhoid fever. The preliminary findings indicate that bile salt malabsorption occurs only in those children undergoing radical resection.

The aminopyrine $^{13}CO_2$ breath test is being utilized in collaboration with Dr. Schneider to monitor the response of patients with cirrhosis of the liver to treatment with phenobarbital, a known stimulant of microsomal induction. Preliminary results using the breath test have shown the treatment to be effective in increasing the microsomal function and that this increased function persists for over 1 month after discontinuation of the treatment.

A new method of collecting CO_2 has recently been developed in which 50 ml of the patient's breath is collected and stored in an evacuated flask. After transportation to the central facility, an aliquot of this sample is taken and the CO_2 is cryogenically removed and purified. This technique has been shown to eliminate the fractionation of the carbon isotopes and to be a safe means of storing the samples when immediate analysis is not possible.

SIGNIFICANCE

The $^{13}CO_2$ breath tests have been shown to be a safe alternative to the $^{14}CO_2$ breath test, which cannot be used in many patients because of the radiation hazard. The test has been shown to be useful in the diagnosis and management of gastrointestinal and hepatic disorders and is potentially applicable in the study of numerous other disorders of oxidative metabolism. Because the test is noninvasive and involves no radioactivity, it is ideally suited for use in pediatrics.

PROPOSED COURSE OF THE PROJECT

Further research will continue in two directions. The first will be the development of new clinical applications of the $^{13}CO_2$ breath test in order to extend further the utility of the test. The second will be the development of an automated system to purify and analyze the CO_2 to obtain increased convenience and decreased cost.

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ANL 61200

03086

CIRCADIAN REGULATION: CONTROL AND REGULATION OF THE BIOLOGICAL CLOCK IN HIGHER ORGANISMS AND PROTISTANS

Charles F. Ehret, Principal Investigator
Kenneth W. Dobra, Kenneth R. Groh, and John C. Meinert, Participating Investigators

OBJECTIVES

All of the higher plants and animals, and even the eucaryotic microorganisms, are endowed with a biological clock that programs their daily activities, to wake or to sleep, to flourish or to vegetate, to live or to die. Our aim is to find out, in molecular terms common to all circadian systems, what makes the clock "tick," how to manipulate its period, and how to reset its phase.

BACKGROUND AND PREVIOUS FINDINGS*

Evidence that the programmer for the clock, and the period-measuring basis of the clock itself, is in eucaryotic gene action comes from many sources (Ehret, C. F., Advances in Biological and Medical Physics, Vol. 15. Academic Press, New York, 1974, pp. 47-77), but the evidence is to date inconclusive. Thus, although the basic rules governing circadian mode, period, and phase are now well known and strongly predictive, the underlying mechanisms remain conjectural. Several of the earliest predictions of the Chronon Theory (a polycistronic replicon is read out like a tape, to tell time) have been realized: molecular hybridization has demonstrated circadian chronotypic RNA, and Mendelian analyses have shown a tendency for clock mutants to cluster at a common locus. A critical test of the Chronon Theory requires additional studies using messengers specific for short-lived regulatory enzymes in pathways strongly associated with circadian phase control. Because highly polymerized energy reserves unique to eucaryotes, such as glycogen, are regulated in strict circadian fashion and are omnipresent in infradian mode cells (Sutherland, A., et al., J. Cell. Biol. 58, 240, 1973), and because their regulatory pathways are similarly unique, centering on biogenic amine derivatives of tyrosine and tryptophan and vulnerable to suspected chronobiotics (psychotropic drugs, hallucinogens) and known chronobiotics (theophylline, pentobarbital; Ehret, C. F., et al., Science 188, 1212, 1975), an intensive investigation of the circadian properties of these pathways, and of the short-lived but chronotypically inducible regulatory enzyme tyrosine aminotransferase, TAT, (Ehret, C. F., and V. R. Potter, Int. J. Chronobiol. 2, 321, 1974), was begun.

EXPERIMENTAL METHODS

The methods of comparative molecular biology and biochemistry are employed in association with novel circadian protocols for entrainment that include not only light, but also food and temperature cycling as zeitgebers. The organisms used, the unicellular *Tetrahymena pyriformis* (Ciliata) and the rat (Mammalia), are sufficiently diverse phylogenetically to permit comparisons of the most general physiological and regulatory significance, as well as comparisons at cellular and organismic levels of organization. The cellular studies presently focus upon glycogenolysis and the catecholamine and indoleamine regulatory pathways, and appropriate analytical procedures (e.g., analyses for tyrosine aminotransferase, cyclic AMP, glycogen, ATP and Energy Charge, and respiration) have been developed for measurements of relevant chronotypic properties. The organismic studies focus upon rapidly resetting the mammalian clock by food and drug protocols suggested by the imputed molecular mechanisms. Temperature telemetry units are implanted intraperitoneally in rats, and their circadian rhythm of deep-body temperature is measured and compared during entrainment, and during free-run following drug administration or dietary changes.

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*See "A Concise Circadian Glossary" (Ehret, C. F., ANL-75-30, 1975, p. 189) for definition of terms.

MAJOR NEW FINDINGS

At the cellular level, in *Tetrahymena* cells entrained by light-dark cycles to circadian synchrony (Dobra, K. W., and C. F. Ehret, ANL-75-30, 1973, p. 191), a characteristic chronology of molecular activity is seen: TAT reaches its peak in the middle of the dark phase, whereas cyclic AMP (cAMP) 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 hours) "out of phase" with the peak for TAT. Comparison of these findings with earlier measures of the chronotype of the rat (Ehret, C. F., Advances in Biological and Medical Physics, Vol. 15. Academic Press, New York, 1974, pp. 47-77) show that the circadian chronology of glycogen storage and depletion is remarkably similar in these two species. The technical findings, taken together with earlier studies, generate several important new theoretical perspectives about eucaryotic regulation that appear to be universally applicable: when external environmental pressures cause a virtual cessation of cell division, eucaryotic cells enter the infradian (slow exponential) growth mode endowed not only with a store of energy-reserve polymers (such as glycogen), but also with a clock that parsimoniously programs the metering-out in discontinuous daily rations of these reserves. The simplest theory consistent with the data is that the same gene-action clock that programs the ultradian cell division cycle in well-nourished cells continues to program the circadian cycle of energy-reserve depletion in infradian cells (Ehret, C. F., and K. W. Dobra, Chronobiologia, in press).

SIGNIFICANCE

Our new understandings about mechanisms have already led to the control of phase in circadian biological clocks by pharmacological means. New programs have been designed to reset the clock of the mammal. When extrapolated to man, these programs should have measurable impact upon time, life, and energy saving in industries dependent upon shift work and on transmeridianal travel. More importantly, similar applications of circadian environmental controls have been shown to have dramatic impact not only upon photoperiodically regulated plant and animal systems in agriculture, but even at the cellular level in organisms that play a crucial role in sewage disposal and environmental pollution control (Meinert, J. C., et al., Microbial Ecology 2, 201, 1975). We have long enjoyed bioengineering research breakthroughs through genetic exploitation; equivalent gains are to be expected on a significantly larger scale by proper attention to chronobiological and circadian engineering methods.

PROPOSED COURSE OF THE PROJECT

At the cellular level, the action of drugs that inhibit the enzymes of catecholamine metabolism, including especially reserpine and chlorpromazine, will be investigated for their potential action as zeitgebers in circadian-infradian cells. At the same time the putative target enzymes (tyrosine-3-hydroxylase and L-dopa decarboxylase) will be assayed to determine their chronotypic activity patterns. Because enzymes in the tryptophan-indoleamine

pathway are also of interest, we will search for serotonin-n-acetyl transferase, which plays a key role in circadian regulation in mammals. An immediate outcome will be a better understanding of chronotypic drug action at the cellular level and *in vitro* under infradian growth mode conditions, which conditions we postulate to be a *sine qua non* for valid extrapolation to *in vivo* organismic expectations. An ultimate goal is to learn how enzymes of these pathways may feed back on the gene-action clock to reset it. The way to this goal will be expedited by comparative studies at the cellular level between protistan cells and mammalian cells grown in circadian synchrony in the infradian growth mode in tissue culture; cell lines will preferably be derived from subjects suffering from genetic deficiencies in the mobilization of energy reserves (Parkinson's, McArdle's and other glycogen storage disturbances).

At the organismic level, a new data acquisition system capable of interrogating by telemetry the deep body temperatures of 40 rats simultaneously will be used to test new protocols for rapidly resetting the circadian clock. A central strategy is to influence the glycogen storage and depletion mechanisms by means of programmed-feeding patterns supplemented by chronobiotic drugs such as the methylated xanthines (caffeine, theophylline, theobromine) and ethanol. Since each resetting of the circadian clock may enhance risk of damage to the resetting clockworks, and since the circadian clock itself probably involves gene action, several cocarcinogens will be tested for their influence upon circadian phase, period, and degree of synchronization.

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ANL 61200

03087

CIRCADIAN REGULATION OF RESPIRATION, GLYCOGEN, TYROSINE AMINOTRANSFERASE, AND BIOGENIC AMINES IN CULTURES OF TETRAHYMENA PYRIFORMIS

Kenneth W. Dobra, Principal Investigator
Charles F. Ehret, Kenneth R. Groh, and John C. Meinert, Participating Investigators

OBJECTIVES

The objective of these studies is to test the hypothesis that circadian regulation of glycolysis (in this sense meaning that portion of energy production derived from the phosphorylation of glycogen reserves) depends upon the metabolism of tyrosine and tryptophan, at the cellular level; and furthermore, that activation of adenyl cyclase by products of tyrosine metabolism, specifically epinephrine and norepinephrine, is sufficient to account for the observed circadian change in total cellular glycogen, respiratory CO_2, and cyclic AMP. Regulation of the levels of tyrosine and tryptophan by the short-lived chronotypic (i.e., time-specific) enzyme tyrosine aminotransferase would therefore provide a link between genetic transcription of a temporally unique enzyme, and the control of the total energy potential available to the cell.

BACKGROUND AND PREVIOUS FINDINGS

Work from this and other laboratories has shown that hepatic glycogen in the rodent oscillates with a daily rhythm under light-dark entrainment during starvation and inanition and persists with a circadian period during the "free-run" conditions of constant darkness and starvation. Some enzymes associated with glycogen metabolism in the rat also exhibit circadian periodicity. The hepatic enzyme tyrosine aminotransferase (TAT) oscillates with a period of about 24 hours and can be regulated by light-dark (LD) and feed-starve (FS) cycles, changes in dietary tryptophan and tyrosine, and the administration of such drugs as norepinephrine, theophylline, and quinolinic acid.

When cultures of *Tetrahymena* enter into the infradian mode of growth after having been grown on a medium rich in glucose, they contain large stores of glycogen which can occupy much of the cellular endoplasm (Sutherland, A., et al., J. Cell. Biol. 58, 240, 1973). This glycogen storage is then available for metabolism during the relatively anaerobic infradian mode, during which it is gradually depleted. The findings from other laboratories that *Tetrahymena* has epinephrine and serotonin (Janakidevi, K., et al., J. Biol. Chem. 241, 2576, 1966), and that the levels of glycogen might be altered by reserpine, dichloroisoproterenol, and aminophylline (Blum, J. J., Proc. Nat. Acad. Sci. U.S.A. 58, 81, 1967) have suggested that the circadian regulation of glycogen metabolism in the ciliated protozoan may be very similar to that found in the vertebrate hepatic system.

EXPERIMENTAL METHODS

Tetrahymena pyriformis (W) were cultured as continuous monolayers of cells on the surface of enriched proteose peptone agar. Plates were inoculated with an infradian donor culture and placed into an air-tight plastic respiratory chamber with controlled air flow and constant temperature. Air containing 21% O_2 was hydrated and the CO_2 was removed by bubbling through a fritted disc scrubber containing an NaOH solution. The output air from the chamber was passed through an infrared CO_2 monitor and through a mass flow meter for recording the flow rate. Light entrainment began after 2 days of growth when the cells were beginning the infradian mode. Cells were washed from plates taken every 3 hours, and samples were taken for cell counts, protein, glycogen, TAT, ATP, and cyclic AMP determinations.

MAJOR NEW FINDINGS

We had previously shown that the circadian rhythm of respiratory CO_2 was light entrainable (Dobra, K. W., and C. F. Ehret, ANL-75-30, 1973, p. 191). The present studies showed that there is a circadian rhythm of total cellular ATP which ranged from a low during the dark to a maximum during the light phase of the LD cycle. Both the maximum and minimum of ATP concentration are correlated with respiratory CO_2 levels. In addition there is a twofold change in the amount of cyclic AMP during each 24-hour period which is also in phase with both the CO_2 and ATP rhythms. Total cellular glycogen appears to undergo a stepwise decrease in light synchronized cultures of *Tetrahymena*. As respiration increases, glycogen content falls from 20 $\mu g/10^5$ cells to 15 $\mu g/10^5$ cells. During the dark phase when respiration is minimal, the decline in glycogen content is more gradual. When net glycogen synthesis occurs, as in free-run, the synthesis happens during the time corresponding to the late dark phase.

The enzyme TAT undergoes a very striking increase in activity during the middle of the dark phase which coincides with the minima in respiration and glycogen utilization. The peak in TAT activity for the rat liver also occurs during the dark phase (Ehret, C. F., Advances in Biological and Medical Physics, Vol. 15. Academic Press, New York, 1974, pp. 47-77), but unlike the case in *Tetrahymena* is nearly coincidental with the peak in respiration. TAT activity in *Tetrahymena* was found to be suppressed by the addition of 1 µg/ml of norepinephrine to the cultures. The time of greatest suppression was during the dark phase.

Experiments utilizing cultures of *Tetrahymena* which experienced 2 days of entrainment (LD 6:18) and which were sampled during 2 days of free-run gave parallel results. The greatest utilization of glycogen occurred during the subjective light phase, and there was even a net synthesis of glycogen during the times corresponding to the subjective dark phase. TAT reached a peak during the end of the dark phase and the peak in cyclic AMP occurred during the time of greatest glycogen utilization. Our findings for *Tetrahymena* that both glycogen and TAT oscillate with a circadian period, suggest the involvement of chronotypically homologous regulatory pathways for the circadian metabolism of glycogen in very diverse phyla. This is further strengthened by the observation that catecholamines can alter the activity of adenyl cyclase in *Tetrahymena pyriformis* (Rozenzweig, Z., and S. Kindler, FEBS Letters 25, 221, 1972).

SIGNIFICANCE

The influence of diet and hormones has added to the complexity of determining the circadian control of energy metabolism in the rodent liver. By using a homogenous culture of eucaryotic cells (*Tetrahymena*), it is possible to bypass the higher order feedback controls in the multisystem organism thus revealing cellular mechanisms involved in metabolic control.

PROPOSED COURSE OF THE PROJECT

Future experiments will involve prolongation of the infradian mode of growth to facilitate longer free-run studies. The emphasis will be on establishing definite phase relationships between several parameters of energy metabolism and the chronotypic enzymes which may influence the circadian control of the utilization of energy reserves.

ERDA RT-03-02
ANL 61200

03088

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: INTRODUCTION

Allen B. Edmundson, Principal Investigator

The efforts of the group responsible for the following papers are directed toward the elucidation of the three-dimensional structures of the serum IgG1 immunoglobulin and the urinary Bence-Jones protein from a patient (Mcg) with multiple myeloma and amyloidosis. IgG immunoglobulins in both myelomatous and normal sera consist of two light chains (MW 23,000) and two heavy chains (MW 50,000) linked by interchain disulfide bonds. Bence-Jones proteins are excreted light chains, and their presence in urine is pathognomonic of multiple myeloma. The isolation of crystalline IgG and Bence-Jones proteins from one patient is particularly significant because the amino acid sequence of the IgG light chain constituent is identical with that of the Bence-Jones protein only when the proteins are obtained from the same individual. This combination provides a unique system for the determination of the three-dimensional structure of an antibody molecule. The high resolution study possible with the Bence-Jones protein can complement and extend the low resolution study attainable with the myeloma protein.

Antibodies are among the most fascinating proteins because they are biologically selected for variability and yet have common modular structures. Each modular unit ("domain") of $\sim$ 110 amino acid residues is under control of a separate gene. Immunoglobulin light chains consist of two domains, while the heavy chains have four domains. We set out to determine the structure of the common immunoglobulin fold, and to explain how divergence of this common structure can account for such different functions as antibody specificity, complement fixation, and interactions with cell surfaces.

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ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: PURIFICATION AND CRYSTALLIZATION OF THE Mcg IMMUNOGLOBULINS

Kathryn R. Ely, Principal Investigator
Enrique E. Abola, Allen B. Edmundson, Joseph R. Firca, Nicolas C. Panagiotopoulos, Marianne Schiffer, and Florence A. Westholm, Participating Investigators

OBJECTIVES

Our principal objective is the production of large single crystals essential for X-ray diffraction studies.

BACKGROUND AND PREVIOUS FINDINGS

The Mcg immunoglobulins were obtained in 1970. The Bence-Jones protein crystallized in the trigonal space group $P3_121$, with a = 72.3 and c = 185.9 Å. An electron density map was calculated at a resolution of 2.3 Å, and an atomic model was constructed with the aid of the amino acid sequence determined by J. W. Fett and H. F. Deutsch at the University of Wisconsin. The atomic coordinates were measured and used in the refinement procedure of Schiffer et al. The IgG1 protein crystallized in the orthorhombic space group $C222_1$ with a = 87.8; b = 111.3, and c = 186.3 Å.

MAJOR NEW FINDINGS

For binding studies it has been necessary to prepare and maintain a large inventory of Bence-Jones crystals of the appropriate size. Procedures established for the native protein were modified to obtain trigonal crystals of chemically modified or reconstituted proteins described in the report of J. R. Firca. Crystallographic techniques are currently being used to determine the structural implications of these chemical studies.

Major emphasis has been placed on the crystallization of the IgG1 immunoglobulin. This protein tends to aggregate or to form degradation products during prolonged storage. Consequently, since 1973, the protein has only formed small irregular crystals. However, within the past year, gel filtration with Sephadex G-200 at 4°C has been used to remove the aggregates and degradation products from the IgG1 protein. The purified protein is concentrated and centrifuged to remove denatured protein, debris, etc. The pH, ionic strength, and temperature are regulated at all times, and care is exercised to minimize surface denaturation during transfers. Strict adherance to the purification procedure has been successful to the extent that large crystals ($\sim$ 0.4 x 1.0 mm) are now produced in 2-3 months by dialysis against de-ionized water. These crystals diffract to about 3.5-Å resolution, and data collection is currently in progress for the native protein.

In initial efforts to solve the structure of the IgG1 protein by multiple isomorphous replacement, derivatives have been prepared by diffusion of heavy atom salts into the crystals. Since they are grown in water, the crystals are readily dissolved in media of even low ionic strength. Successful derivatives are made only if the concentrations of heavy atom compounds are kept low (e.g., 5×10^{-4} M) and if the crystals are observed closely to ensure that X-ray data collection is initiated before physical damage becomes apparent.

SIGNIFICANCE

There are only 3 other IgG proteins that have been crystallized for X-ray diffraction and none of these is from a patient with a Bence-Jones protein of known crystal structure. The expected rewards for the diffraction study therefore outweigh the technical difficulties in obtaining suitable crystals and derivatives.

PROPOSED COURSE OF THE PROJECT

We shall continue to attempt to improve crystallization techniques for the IgG1 protein. Our goal is to produce a sufficient number of crystals to obtain a structure at a resolution of at least 3.5 Å. The crystal inventory will be expanded to permit an extensive search for successful heavy atom derivatives. We shall also continue to prepare crystals of chemically modified or reconstituted light chain dimers and IgG1 proteins.

ERDA RT-03-02
ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: INTERCONVERSION OF Mcg LIGHT CHAIN ISOMERS IN SOLUTION

Joseph R. Fırca, Principal Investigator
Enrique E. Abola, Allen B. Edmundson, Kathryn R. Ely, Nicolas C. Panagiotopoulos, Marianne Schiffer, and Florence A. Westholm, Participating Investigators

OBJECTIVES

The light chains in the Mcg Bence-Jones dimer and IgG1 protein are conformational isomers. The principal objective of our study is an elucidation of the interconversion of these isomers in solution. The binding properties of the Bence-Jones dimer have previously been studied in the crystals. Our second objective is to extend these results to the binding of hapten-like molecules in solution.

BACKGROUND AND PREVIOUS FINDINGS

The light chains in both the Bence-Jones dimer and the IgG1 are covalently connected by an interchain disulfide bond. However, the principal noncovalent interactions in the IgG1 protein occur between light and heavy chains, rather than between the two light chains. The presence of a crystallographic twofold axis of rotation between halves of the IgG1 molecule indicates that the light chains have identical conformations when associated with heavy chains (Edmundson, A. B., et al., J. Biol. Chem. 245, 2763, 1970). In contrast, the crystallographic analysis of the Bence-Jones dimer revealed a striking difference in the two light chains (Schiffer, M., et al., Biochemistry 12, 4620, 1973): the conformation of monomer 1 was similar to that of a heavy chain, while monomer 2 closely resembled the light chain component of the antigen-binding fragment (Fab) of an antibody molecule.

The Bence-Jones dimer was found to bind Dnp* compounds and other hapten-like molecules in sites similar to the binding regions in Fab fragments. The recognition of conformational isomerism, which results in the formation of such binding sites, and the demonstration of actual binding in these sites, led us to the proposal that the Bence-Jones dimer be considered a model for a primitive antibody.

EXPERIMENTAL METHODS

The Bence-Jones dimer was dissociated into monomers in 0.4 M propionic acid after reduction and reversible alkylation of the disulfide bond linking the two polypeptide chains. One fraction of the alkylated monomers was mixed with heavy chains isolated from the IgG1 protein, while the second fraction was reconstituted to the original dimeric form. In each sample, the dissociating agent (i.e., the acid) was removed by dialysis against pH 5.4, 0.004 M sodium acetate, which favors noncovalent reassembly of immunoglobulin complexes. After this step, sample 1 contained an IgG1-like complex of two heavy chains and two alkylated light chains, and sample 2 consisted of an alkylated dimer of light chains. The alkyl groups were removed and the interchain disulfide bond between light chains was restored ("covalent reassembly") by dialysis against 0.01 M reducing agent in pH 8.7 borate buffer. The reassembled complexes were compared with the native IgG1 and Bence-Jones proteins by disc electrophoresis.

Light chains in the IgG1 protein were reduced and alkylated, prior to dissociation from heavy chains in 0.2 M acetic acid. These alkylated light chains were reassembled into covalent dimers by the technique used for the Bence-Jones protein. Attempts were made to crystallize the reassembled light chain dimers, as well as the recombinant IgG1 and Bence-Jones proteins (see the preceding report of Ely et al.).

The binding constant for di-Dnp lysine to the Bence-Jones protein in solution was determined by the method of equilibrium dialysis.

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*Dnp: dinitrophenyl.

MAJOR NEW FINDINGS

Bence-Jones monomers could be substituted for light chains in the reassembly of the IgG1 protein. However, the yields of the recombinant IgG1 molecules were limited to about 10% because the heavy chain components formed inactive aggregates in the dissociating media. The Bence-Jones dimer could be dissociated and reassembled in yields > 80%. Light chains from the IgG1 protein could be converted into a dimer indistinguishable from the Bence-Jones protein isolated from urine. All three types of recombinant molecules were crystallized (see preceding report by Ely et al.). Crystallographic studies of the reassembled Bence-Jones and light chain dimers have been initiated.

Di-Dnp lysine was found to be tightly bound to the Bence-Jones dimer in crystals (Edmundson, A. B., et al., Biochemistry 13, 3816, 1974). In solution the Bence-Jones dimer binds di-Dnp lysine with a binding constant of $\sim 8 \times 10^4$ liters per mole. This value compares favorably with binding constants of Fab fragments from other myeloma immunoglobulins. Significantly, the Bence-Jones dimer binds two molecules of di-Dnp lysine in solution, but only one in the crystal. We previously found two sites capable of binding such molecules in the crystal, but access to one of these sites was blocked by another closely packed protein molecule in the crystal lattice.

SIGNIFICANCE

These studies indicate that the conformational isomers of light chains can be interconverted in solution. Since the biological activity of antibodies is associated with a complex of two or more interacting chains, the flexibility permitting conformational isomerism during the formation of these complexes is of fundamental importance in the physiology and evolution of the immunoglobulins.

PROPOSED COURSE OF THE PROJECT

We shall attempt to improve the conditions for the reassembly of IgG1 molecules. The molecular features of the recombinant Bence-Jones and light chain dimers will continue to be investigated by crystallographic methods. The binding of hapten-like molecules by the immunoglobulins in solution will be examined in greater detail.

ERDA RT-03-02
ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES: CRYSTALLOGRAPHIC REFINEMENT OF THE Mcg BENCE-JONES PROTEIN

Marianne Schiffer, Principal Investigator
Martin Kraimer, Enrique E. Abola, Allen B. Edmundson, Kathryn R. Ely, Joseph R. Firca, Nicolas C. Panagiotopoulos, and Florence A. Westholm, Participating Investigators*

OBJECTIVES

The principal objective is to obtain the most accurate coordinates of the nonhydrogen atoms permitted by the diffraction data for the Bence-Jones dimer. A refined and revised atomic structure is desirable for documentation of previous structural studies and for interpretation of future results like those presented in the preceding report of Firca et al.

BACKGROUND AND PREVIOUS FINDINGS

Electron density maps for the Bence-Jones dimer have been calculated to 6.0, 3.5, and 2.3 Å (Schiffer, M., Biochemistry 12, 4620, 1973; Edmundson, A. B., et al., Biochemistry 14, 3953, 1975). An atomic model has been constructed from the 2.3-Å map, and coordinates have been measured (report of Ely et al.). The refinement procedure is the last phase of the structural study. The associated investigations of the hapten-binding regions, the conformational isomerism, and the rotational allomerism can all be related to the refined structure.

EXPERIMENTAL METHODS

The procedure for "constrained crystallographic refinement" is similar to that described by Deisenhofer and Steigemann (Acta Crystallogr. B31, 238, 1975).

MAJOR NEW FINDINGS

The atomic coordinates were subjected to a "model building" program (Diamond, R., Acta Crystallogr. 21, 253, 1966) to obtain an accurate set of coordinates within the limits of standard bond distances and angles. These coordinates were then refined, new "phases" were calculated for the protein, and a new electron density map was calculated using these phases. The success of the initial refinement is shown by the appearance of electron density for 90 atoms for which there was no density in the map calculated with isomorphous phases. When identical alpha-carbon atoms in very regular segments of the two monomers were superimposed by a least squares procedure, the average deviation decreased from 0.95 Å to 0.65 Å during the refinement.

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*Computer Scientist, Administrative Staff.

SIGNIFICANCE

The Bence-Jones dimer is the largest molecule to which the refinement procedure has been applied. Accurate knowledge of the structure will facilitate comparisons between the Mcg protein and other immunoglobulins. It will also aid us in interpreting the hapten-binding results in crystals and in correlating these results with the solution studies.

PROPOSED COURSE OF THE PROJECT

The mathematical refinement procedure will be continued to convergence. New diffraction data will be collected to obtain a better scale for data measured on different crystals. Model building procedures using skeletal models and interactive computer controlled graphics will be performed on poorly defined sections of the molecule.

ERDA RT-03-02
ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: ROTATIONAL ALLOMERISM AND DIVERGENT EVOLUTION OF DOMAINS IN IMMUNOGLOBULIN LIGHT CHAINS

Allen B. Edmundson, Principal Investigator
Enrique E. Abola, Kathryn R. Ely, Joseph R. Firca, Nicolas C. Panagiotopoulos, Marianne Schiffer, and Florence A. Westholm, Participating Investigators

OBJECTIVES

The three-dimensional structure of the Bence-Jones protein indicated that the V and C domains were probably derived from a common primordial gene. The principal objective of the present study was to use the atomic model and the amino acid sequence of the protein to determine what changes led to what C. C. F. Blake (Nature 257, 447, 1975) calls "functional differentiation" of the V and C domains.

BACKGROUND AND PREVIOUS FINDINGS

The structural analysis of the Bence-Jones protein indicated a basic "immunoglobulin fold" in both the V and C domains (Schiffer, M., et al., Biochemistry 12, 4620, 1973). This fold consists of two layers of beta-pleated sheets, one with three antiparallel segments and the second with four antiparallel strands. The two layers are connected near the middle of each domain by an intrachain disulfide bond. The interior of each domain is composed mainly of hydrophobic side chains. For comparative purposes, the domains were considered as cylinders of beta-pleated sheets enclosing hydrophobic interiors.

In the Bence-Jones dimer, the three-chain layers of the V domains face each other across the solvent channel in which hapten-like molecules are bound (see report by Firca et al.). The corresponding layers provide most of the external surfaces of the dimer of C domains. In contrast, the important interactions stabilizing the compact arrangement of the C domains are across the solvent-free zone between the four-chain layers. These layers form the external surfaces in the V dimer. We attempted to define the structural basis for these functional changes during evolution, and at the same time provide an explanation for retention of a common fold.

EXPERIMENTAL METHODS

The atomic model was constructed by Kathryn Ely and the amino acid sequence was determined by Fett and Deutsch at the University of Wisconsin (Biochemistry 13, 4102, 1974). Pairs of 38 homologous amino acid residues from the V and C domains were chosen from the most highly ordered regions (the beta-pleated sheets). The coordinates of the alpha-carbon atoms of these residues were compared with a least-squares procedure adapted by Marianne Schiffer from a program written by Steigemann in Huber's laboratory in Munich.

MAJOR NEW FINDINGS

Divergence of the V and C domains during evolution can be explained in terms of rotational allomerism (allomers have similar three-dimensional structures, but different sequences). The cylinders of beta-pleated sheets have rotated in such a way that the homologous three- and four-chain layers in the two domains perform different functions in the association of pairs of light chains. The comparison of 38 homologous pairs of residues indicates that the two domains are related by an angle of rotation of about 165°. The patterns of amino acid sequences in the pleated sheets are consistent both with the changes resulting from rotational allomerism and with the structural similarities of the V and C domains.

In pleated sheets the side chains emerge on alternating sides of the polypeptide backbone. With only two layers of pleated sheets in a domain, one set of side chains in each layer is directed toward the interior and the other set is directed toward the surface of the cylinder. To make functional changes in a cylinder by rotation, it is necessary only to change the set of surface residues. The internal set can be left relatively undisturbed.

The pleated sheets providing the external surfaces of the dimer generally have an alternating pattern of external polar and internal hydrophobic residues. This alternating pattern is broken in the three-chain layers of the V domains by the substitution of aromatic for aliphatic polar residues in positions important for maintenance of the general architecture of the hapten-binding sites. Similarly, the alternating pattern in the four-chain layers of the C domains is interrupted by substitution of hydrophobic for hydrophilic residues at the sites of interaction in the C_1-C_2 interface.

The preservation of the hydrophobic character of residues in the key internal sites largely explains the marked similarities in the overall structures of the V and C domains.

Because of rotational allomerism, the surface properties of the V dimer are different from those of the C dimer. We have proposed that these differences are important in the formation of amyloid fibrils and in the characteristic thermal behavior of Bence-Jones proteins, since both phenomena are associated only with the V domains.

SIGNIFICANCE

Rotational allomerism appears to be a fundamental feature of modern immunoglobulin structures. The genetic changes leading to this allomerism are critical in the evolutionary pathway from a primordial domain to the present Bence-Jones dimer.

PROPOSED COURSE OF THE PROJECT

We shall use the conclusions from this study in the interpretation of all future results obtained with immunoglobulins.

ERDA RT-03-02
ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: STRUCTURAL INVESTIGATION OF THE IgG1 PROTEIN BY MULTIPLE ISOMORPHOUS REPLACEMENT METHODS

Enrique E. Abola, Principal Investigator
Alan Winiecki, Allen B. Edmundson, Kathryn R. Ely, Joseph R. Firca, Nicolas C. Panagiotopoulos, Marianne Schiffer, Florence A. Westholm, and Kevin Williams,† Participating Investigators*

OBJECTIVES

The principal objective of this study is the determination of the structure of the IgG1 protein by the method of multiple isomorphous replacement. Crystals and derivatives of this protein are difficult to prepare (see report of Ely et al.), and we have attempted to optimize their use by improving methods for data collection and processing.

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*Electronics Division.

†Summer 1975 participant in the Undergraduate Honors Research Participation Program, Harvard University.

BACKGROUND AND PREVIOUS FINDINGS

Salts of heavy atoms have damaged the crystals of IgG1 protein in many cases (see report of Ely et al.). As a result of the low concentrations used to minimize this damage, the heavy atoms are usually found in low occupancies. Multiple site substitution also complicates interpretation of the results, and we have had to examine many data sets in hopes of finding suitable derivatives.

Since special modifications of the software for data collection were commercially unavailable at a reasonable cost, we devised a new system in collaboration with Alan Winiecki of the Electronics Division. A new system for data management was also desirable to store the large quantities of data from previous crystallographic studies of the Bence-Jones dimer, as well as to handle current studies of the Bence Jones and IgG1 protein (see report of Firca et al.). Plotting programs were useful in examining the packing of molecules in the crystal lattice and in evaluating the possible effects of chemical modifications on the protein structures.

EXPERIMENTAL METHODS

The multiple isomorphous replacement method is used as described by Green, D. W., et al. (Proc. R. Soc. London A225, 287, 1954). The "DOS" software system for the Picker FACS-I diffractometer was modified to utilize special data collection methods. The "System 2000®" data base management system was also introduced. In collaboration with Kevin Williams, a summer student, we developed programs to plot protein structures in two dimensions or in stereo pairs.

MAJOR NEW FINDINGS

The locations of major sites occupied by some heavy atoms have been identified by crystallographic techniques (difference Patterson syntheses). However, the vector maps for multiple site derivatives are difficult to interpret, and are continuing to be evaluated to locate minor sites in three dimensions.

A satisfactory prototype software system for data collection has been put into operation in the FACS-I diffractometer. The modifications to this system are nearing completion. Damage to crystals by both X-ray exposure and waiting time (crystals are markedly sensitive to fluctuations in air temperature) is already being decreased by introduction of the more efficient data collection system. The "System 2000®" is presently in use to arrange and catalogue the large amounts of diffraction data and to store data sets in a format permitting convenient access. Packing diagrams of the Bence-Jones dimers in trigonal crystals have been obtained with the plotting programs. These programs have also been used to produce different views of regions of interest in the chemical modification studies (see report of Firca et al.).

SIGNIFICANCE

If we succeed in solving the "heavy atom problem," we can determine the structure of the IgG1 molecule by the methods used in the Bence-Jones study. Efficient data collection and management are essential for the success of our large crystallographic program.

PROPOSED COURSE OF THE PROJECT

We shall continue to search for heavy atom compounds which bind in fewer sites with higher occupancies. Existing computer programs will be further modified to improve data collection and management.

ERDA RT-03-02
ANL 61200

X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS: STRUCTURAL INVESTIGATIONS OF IgG1 AND BENCE-JONES PROTEINS BY MOLECULAR REPLACEMENT METHODS

Nicolas C. Panagiotopoulos, Principal Investigator
Enrique E. Abola, Allen B. Edmundson, Kathryn R. Ely, Joseph R. Firca, Marianne Schiffer, and Florence A. Westholm, Participating Investigators

OBJECTIVES

The principal objective of this study is the determination of the three-dimensional structure of the IgG1 protein. The Bence-Jones dimer can be crystallized in an orthorhombic form by dialysis against water. We hope to determine whether the conformational isomerism noted in trigonal crystals (grown in ammonium sulfate) also is manifest in water.

BACKGROUND AND PREVIOUS FINDINGS

Molecular replacement methods have been used in other laboratories in investigations of noncrystallographic symmetries of molecules in crystals (Rossman, M. G., The Molecular Replacement Method; A Collection of Papers. Gordon and Breach, New York, 1972, 267 pp.). Of more immediate interest to us is the fact that these methods have also been applied in determinations of the crystal structures of proteins nearly identical with molecules with known structures. For example, Schiffer participated in this type of study on the Au V domain dimer (a fragment of a Bence-Jones protein; see Fehlhammer, H., et al., Biophys. Struct. Mechanism 1, 139, 1975). The Au protein fragment closely resembled a V dimer (Rei), whose structure had been previously determined by conventional isomorphous replacement methods (Epp, O., et al.,

Eur. J. Biochem. 45, 513, 1974). When applicable, the molecular replacement procedures appreciably reduce the effort and time required for a structural determination, mainly because isomorphous derivatives do not have to be found. As indicated in the preceding report of Abola et al., it is difficult to obtain isomorphous derivatives of the IgG1 protein. Therefore, we are using the known structure of the Bence-Jones dimer (trigonal form) in attempts to solve the structures of both the IgG1 protein and the Bence-Jones dimer in the orthorhombic form.

EXPERIMENTAL METHODS

Attempts are being made to determine the structures by "molecular replacement methods" (Rossman, M. G., ibid.). This approach obviates the need for isomorphous derivatives.

Molecular replacement methods utilize the known structure of a subunit of the parent molecule to determine the relative orientation and position of the subunit in the unit cell of crystals of the parent protein. The Patterson functions of the known and unknown structures are rotated and translated relative to each other. The position of maximum overlap between the functions determines the orientation and position of the known subunit in the unknown structure.

MAJOR NEW FINDINGS

The molecular replacement methods require an extensive computational effort, and we have developed or adapted a series of computer programs for this purpose. Initial results for both the IgG1 protein and orthorhombic form of the Bence-Jones dimer are promising, though incomplete. The general mode of packing of the IgG1 molecules in the crystal has been defined, and the overall shape of each molecule has been determined. The low-resolution comparison of the Bence-Jones dimers in the orthorhombic and trigonal crystals suggests that the molecules are probably similar in the two forms.

SIGNIFICANCE

The application of molecular replacement methods may lead to the first structure of an intact antibody-like molecule. Tentatively, the results for the orthorhombic form of the Bence-Jones dimer indicate that the biologically important conformational isomers are present in physiological solutions as well as in crystals grown in ammonium sulfate.

PROPOSED COURSE OF THE PROJECT

These investigations will be extended to higher resolution. Attempts will be made to produce more highly ordered and stable crystals of both IgG1 and orthorhombic Bence-Jones proteins to obtain diffraction data to or beyond 3.5-Å resolution (see report by Ely et al.).

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10. MAMMALIAN CELL BIOLOGY

ERDA RT-03-02
ANL 61300
NIH CA 18081-01
NIH CA 18434-01
EPA-IAG-D6-E681

GROUP LEADER'S OVERVIEW

Mortimer M. Elkind, Group Leader

The research of this Group has progressed in several separate but related areas based upon the use of mammalian cells cultivated *in vitro*. The strength of the *in vitro* system reflects the degree of quantitation and precision inherently possible compared to animal studies. This is true particularly when molecular mechanisms are of interest in connection with, for example, end effects such as cell killing, mutagenesis, and neoplastic transformation. While cells in culture cannot simulate in all regards the interactions that occur in animals and humans, they permit a more rapid and incisive convergence upon hypotheses which can then be tested in the whole organism.

In respect to mechanisms of damage and repair associated with nuclear radiations, new results and insights have emerged. The sulfhydryl inhibitor N-ethylmaleimide sensitizes the cell to radiation only in phases of the cell cycle where cells have a significant capacity for sublethal damage. The sensitization results from an inhibition of the repair of such damage, and cells ordinarily made less responsive by being X-rayed in the absence of oxygen are sensitized in the same way and to the same degree. These results indicate that response variations through the cell cycle mainly reflect a cyclic ability to accumulate sublethal damage; the repair of this damage probably involves an enzyme containing sufhydryl.

Past work on the nature of the lethal lesion(s) due to ionizing radiation has led to the hypothesis that distortion of the DNA double helix is involved. In part further to test this hypothesis, and in part to initiate studies relative to epidemiological questions, damage interaction experiments were started involving ionizing (X-rays) and nonionizing (ultraviolet and near ultraviolet "sunlight-like" light) radiations. The principal lesions in DNA produced by nonionizing radiations were confirmed to be quite different from those due to X-rays; still, the damage produced by the former interacts with the latter in support of a role for helix distortion in lethal damage registration and/or expression.

A comparison of the molecular and biological effects of ultraviolet light and "sunlight" showed that there are both qualitative and quantitative differences. Pyrimidine dimers--major photoproducts resulting from ultraviolet light--are thought to be responsible for killing, mutation, and skin cancer (e.g., in sufferers from the autosomal recessive disease xeroderma pigmentosum). However, the qualitative differences observed between these radiations place in question projections customarily made from the effects of ultraviolet light to those to be expected from sunlight.

Neutron radiobiology was pursued in a more intensive way than heretofore. The interest derives from two sources: (1) a need to understand the biological effects (e.g., cell killing, mutation, and neoplastic transformation) of radiations associated with nuclear power production; and (2) the advent of neutron radiation as an advanced mode of cancer therapy. Accordingly, with support from a one-year grant from the National Cancer Institute plus a request for consulting services from the Fermi National Accelerator Laboratory, Batavia, Illinois, preclinical radiobiological studies have been initiated of the properties of a neutron beam developed at the Fermi National Laboratory with the intent of developing a regional center for the treatment of cancer with neutrons.

Finally, to initiate studies of hazards that may result from low levels of pollutants due to nuclear and nonnuclear energy-production technologies, measurements of neoplastic transformation *in vitro* have been started. Advantage is to be taken of the availability of a variety of nuclear radiation sources: X- and γ-rays at the Argonne National Laboratory; neutrons from the Fermi Laboratory proton accelerator, from the Argonne JANUS Reactor, and from the 30" cyclotron at the University of Chicago, as well as nonnuclear radiations such as ultraviolet and simulated sunlight sources. Measurements of the reversal of transformation induction--i.e., repair processes--also are underway.

Among the foregoing are several projects further to be pursued in the future work of this Group. These, and other initiatives to which they lead, may be summarized as follows: (1) The biology of the cyclic response of cells to various radiations (ionizing and nonionizing), chemicals, and drugs with emphasis on the functional and molecular aspects of damage-repair processes; (2) the application of cell-based studies to the improvement of cancer treatment by radiation and drugs, administered separately and together; and (3) the development of additional cell systems suitable for a broad assessment of cell changes (e.g., transformation and mutation) resulting from exposure to nuclear and nonnuclear pollutants.

MAMMALIAN CELL BIOLOGY STAFF

REGULAR STAFF

Elkind, Mortimer M. (Senior Biophysicist)
Geroch, Mary E. (Scientific Assistant)
Ley, Ronald D. (Assistant Biophysicist)
Liu, Chin-Mei (Scientific Assistant)
Long, Melvin D. (Scientific Assistant)
Sedita, Beverly A. (Scientific Assistant)

TEMPORARY STAFF DURING 1975

Han, Antun (Visiting Scientist)
Jacobson, Gunnard K. (Research Associate)
Kautzky, Eva E. (Research Associate)
*Kimler, Bruce F. (Postdoctoral Appointee)
Ngo, Frank Q. (Research Associate)
Utsumi, Hiroshi (Postdoctoral Appointee)

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THE EFFECTS OF N-ETHYLMALEIMIDE AND HYDROXYUREA ON CHINESE HAMSTER CELLS (V79) IN CULTURE

Warren K. Sinclair, Principal Investigator*
Antun Han, Bruce F. Kimler, and Melvin D. Long, Participating Investigators

OBJECTIVES

The purpose of this study is to elucidate the main factors that control the lethal response to X-irradiation of Chinese hamster cells as their molecular and biochemical composition alters during the cell cycle.

BACKGROUND AND PREVIOUS FINDINGS

Previous studies have shown (1) that DNA synthesis is one primary factor controlling the lethal response; (2) that another factor (probably a small fraction of the protein sulfhydryl in the cell) which varies during the cell cycle also controls response. DNA synthesis can be blocked by hydroxyurea (HU) and the second factor can be modified by N-ethylmaleimide (NEM), a sulfhydryl binding agent. The fact that NEM does not have to be present during irradiation indicates that NEM interferes with repair mechanisms rather than modifying primary damage.

EXPERIMENTAL METHODS

Cultures of Chinese hamster V79 cells synchronized by mitotic selection are exposed to inhibitors, such as hydroxyurea and N-ethylmaleimide, and to X-irradiation at various points during the cell cycle. Cultures are subsequently incubated for 8 days and survival is determined by the number of colonies formed. Degree of synchrony and other experimental variables are determined for each experiment.

MAJOR NEW FINDINGS

NEM sensitizes cells both in G_1 (a phenomenon better demonstrated in HeLa cells (see the following report by Han et al.) and in late S. Suitable combinations of HU and NEM can reduce the resistance of the Chinese hamster cell at all stages of the cell cycle to a level at or below that observed for mitotic cells; i.e., at a given dose, the response can be rendered almost "flat" throughout the cycle. Mitosis is the most sensitive stage of the cell cycle for the lethal response to X-irradiation, and mitotic cells are insensitive to NEM and to HU. Consequently, it appears that most, if not all, of the

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*Associate Laboratory Director for Biomedical and Environmental Research.

variable response of these cells during the cell cycle may be due to a changing capacity for repair throughout the cycle. This hypothesis is strengthened by additional recent experiments using both HU and NEM, especially when these compounds are added after irradiation. Cells exposed to HU before and after irradiation cannot be further sensitized to X-irradiation by the addition of NEM--i.e., cells exposed in this way are similar to mitotic cells in their response and lack the capacity to repair sublethal (and potentially lethal) damage.

SIGNIFICANCE

These studies are at the heart of our understanding of radiation damage and repair processes in mammalian cells, and their dependence upon changing molecular and biochemical processes. Consequently, they are of considerable theoretical importance. They may also lead to practical means of modifying radiation damage to cells either to increase it or to ameliorate it. Furthermore, radiation is not the only noxious agent that causes sublethal damage and thus invokes repair mechanisms in cells. Many chemical pollutants act similarly. Identification of similar, perhaps the same, repair mechanisms following the administration of other energy-related pollutants could be very important to a better understanding of the nature of specific insults to cells.

PROPOSED COURSE OF THE PROJECT

The principal new experiments that need to be undertaken are to elucidate better the variability of primary damage through the cell cycle, in order to provide a necessary baseline for assessing the effects of a varying repair capacity. An important question here is the extent to which the primary response of the cell depends upon the amount of DNA in it. Experiments must be designed to cast new light on this. In addition, the role of DNA synthesis with respect to repair mechanisms now requires some new scrutiny.

ERDA RT-03-02
ANL 61300

SENSITIZATION OF SYNCHRONIZED HUMAN CELLS TO X-RAYS BY N-ETHYLMALEIMIDE

Antun Han, Principal Investigator
Warren K. Sinclair, Bruce F. Kimler, and Melvin D. Long, Participating Investigators*

OBJECTIVES

The objective is to develop a more complete understanding of the factors governing cell cycle dependent response variations after exposure to ionizing radiation.

BACKGROUND AND PREVIOUS FINDINGS

The lethal response of mammalian cells to radiation fluctuates through the cycle. Studies with synchronized Chinese hamster cells showed that these fluctuations are controlled by two factors: semiconservative DNA synthesis; and a material containing sulfhydryl (Sinclair, W. K., Radiat. Res. 55, 41, 1973). Further, the pattern of sensitization of Chinese hamster cells by the sulfhydryl binding agent N-ethylmaleimide (NEM) indicated that NEM interferes with the repair of sublethal damage when registered during the period of DNA synthesis.

EXPERIMENTAL METHODS

Conventional methods for cell survival determination and the initiation of synchronous growth with cultured human cells (i.e., HeLa cells) were used.

MAJOR NEW FINDINGS

In contrast to Chinese hamster cells, HeLa cells have a peak of radiation resistance before, as well as during, DNA synthesis. Thus sensitization by NEM during both periods of resistance can be examined in the same cell line. While quantitative differences were observed between these two periods, it was found (1) that NEM sensitizes only in periods of the cell cycle where cells ordinarily have a significant ability to accumulate sublethal damage; and (2) that NEM sensitization results presumably from the inhibition of repair of sublethal damage. Combined treatment with NEM and a DNA inhibitor produced the same effect in minimizing cyclic variations in survival response as in Chinese hamster cells (see Sinclair et al., preceding report).

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*Associate Laboratory Director for Biomedical and Environmental Research.

SIGNIFICANCE

(1) The action of the sulfhydryl poison (at low, nontoxic concentrations) appears to be independent of the length of the cell cycle. (2) Repair of sublethal damage in the X-ray resistant phases of the growth cycle involves a molecule containing sulfhydryl, probably a sulfhydryl enzyme.

PROPOSED COURSE OF THE PROJECT

Treatments have been discovered that reduce the ability of mammalian cells to accumulate sublethal X-ray damage, e.g., the antibiotic actinomycin D (Elkind, M. M., et al., Nature 214, 1088, 1967) and ultraviolet or near ultraviolet light (see report by Han et al., p. 202). Operationally, these act by introducing damage additive to ionization damage. In contrast NEM is effective at essentially nontoxic concentrations; it appears to inhibit the repair of sublethal damage. We shall inquire: (1) Are molecules containing sulfhydryl involved in the repair of damage that is additive to the damage due to ionizing radiation? (2) Can a distinction be made between "damage addition" and "repair inhibition"? (3) Radiations of high linear energy transfer--e.g., fast neutrons--have killing modes involving a much reduced accumulation of sublethal damage compared to X-rays. Is NEM correspondingly less effective under such circumstances?

ERDA RT-03-02
ANL 61300

SENSITIZATION OF HYPOXIC MAMMALIAN CELLS WITH A SULFHYDRYL INHIBITOR

Bruce F. Kimler, Principal Investigator
Warren K. Sinclair, Mortimer M. Elkind, and Melvin D. Long, Participating Investigators*

OBJECTIVES

The sulfhydryl binding reagent N-ethylmalemide (NEM) has been shown to inhibit the repair of sublethal X-ray damage in aerobic cells (see the preceding two reports by Sinclair et al. and Han et al.). It is possible that damage-repair processes in hypoxic cells differ from those in aerobic cells; hence the potentiation of cell killing by NEM under hypoxia was examined.

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*Associate Laboratory Director for Biomedical and Environmental Research.

BACKGROUND AND PREVIOUS FINDINGS

As described further in this report, NEM in low concentration sensitizes aerobic cells to X-ray damage by inhibiting the repair of sublethal damage (see the preceding two reports by Sinclair et al. and Han et al.). This inhibition occurs primarily in the X-ray resistant phases of the cell cycle and, accordingly, the shape of the survival curve of asynchronous, aerobic cells reflects the sensitization of these resistant cells.

EXPERIMENTAL METHODS

Conventional cell culture techniques were used for assessing by colony formation the survival of Chinese hamster cells. Hypoxia was established by gassing glass culture vessels with an appropriate mixture of N_2 and CO_2 (Elkind, M. M., et al., Cellular Radiation Biology, Williams & Wilkins Co., Baltimore, 1965, pp. 442-461) in the presence or absence of NEM. Cells were synchronized by the mitotic-cell selection technique.

MAJOR NEW FINDINGS

Chinese hamster cells have a short cell cycle. Consequently, as found with aerobic cells, the influence of NEM present at the time of irradiation of hypoxic cells is mainly during the DNA synthetic phase, being both qualitatively and quantitatively similar to that for aerobic conditions. A comparison of the survival curves of asynchronous aerobic and hypoxic cells showed that: (1) in both cases, the effect of NEM was to reduce the ability to accumulate sublethal damage; and (2) the magnitude of the reduction was independent of state of oxygenation.

SIGNIFICANCE

In these experiments, survival was assayed under aerobic conditions and therefore oxygen was available to the cells promptly after the radiation-NEM treatment. Consequently, the results support the following: (1) NEM sensitization of hypoxic cells does not reflect, to any significant extent, electron affinic, oxygen-like action by NEM. (2) The molecular lesions produced under hypoxia are qualitatively the same as those produced under aerobic conditions. (3) The sensitivity variation through the cell cycle under hypoxic conditions reflects the same ability for damage accumulation and repair as under aerobic conditions.

PROPOSED COURSE OF THE PROJECT

This study is completed.

ERDA RT-03-02
ANL 61300

DAMAGE INTERACTION DUE TO IONIZING AND NONIONIZING RADIATION IN MAMMALIAN CELLS

Antun Han, Principal Investigator
Mortimer M. Elkind and Chin-Mei Liu, Participating Investigators

OBJECTIVES

Our objectives are (1) to test further the hypothesis that agents that distort the DNA duplex produce interactive damage, and (2) to develop the background cytotoxicity data needed to understand possible interactive effects produced by ionizing and nonionizing radiations in connection with end points like mutation and neoplastic transformation.

BACKGROUND AND PREVIOUS FINDINGS

The shapes of the survival curves of mammalian cells when exposed to X-rays, ultraviolet light (UV), or near-ultraviolet light (NUV) indicate that each of these radiations kills cells by a process of damage accumulation. Furthermore, the principal lesion for all three radiations is thought to be in, or assocated with, DNA. Experiments were performed, therefore, to test for the interaction of damage caused by these radiations.

EXPERIMENTAL METHODS

Standard techniques for quantitative experimentation with cultured mammalian cells were used. Populations of synchronized cells were obtained by mitotic selection.

MAJOR NEW FINDINGS

Exposure of Chinese hamster or mouse cells to a conditioning dose of UV or NUV reduces the sublethal X-ray damage they can accumulate and *vice versa*. Experiments with synchronized cells revealed that the foregoing is a consequence of damage interaction and not population selection. Cells treated in their DNA synthetic phase can repair the interactive damage although the rate of repair depends upon the order in which the radiations are applied; i.e., rapid when X-rays are given first and slow when UV or NUV precedes X-rays.

SIGNIFICANCE

These results indicate that damage interaction can result even when, quantitatively at least, the primary molecular lesions might be quite different (i.e., primarily bond breakage due to ionizing radiation and primarily production of pyrimidine dimers plus other DNA photoproducts due

to UV and NUV). It is a possibility, however, that this interaction reflects in part common pathways for damage expression rather than the interaction of initial lesions in target molecules. Relative to possible effects in man, these data indicate that ionizing and nonionizing radiation together (e.g., X- or γ-rays plus sunlight) may produce enhanced effects.

PROPOSED COURSE OF THE PROJECT

Additional measurements are needed relative to the degree of interaction at various phases of the growth cycle and the possible repair of interactive damage at these phases. Since UV and NUV damage may interact with X-ray damage because of distortion of the DNA duplex, other strand distorting treatments will be used to see if their effects interact with UV or NUV damage. In addition, to pursue mechanisms, as well as for epidemiological considerations, tests for possible damage interaction between high linear energy transfer radiations (e.g., fast neutrons) and UV and NUV light will also be performed.

ERDA RT-03-02
ANL 61300

DNA DAMAGE RELATIVE TO CELL KILLING: COMBINATIONS OF IONIZING AND NONIONIZING RADIATIONS

Mortimer M. Elkind, Principal Investigator
Antun Han and Mary E. Geroch, Participating Investigators

OBJECTIVES

Our objective is to develop an understanding, at a molecular level, of how nuclear and nonnuclear radiations kill mammalian cells.

BACKGROUND AND PREVIOUS FINDINGS

Earlier studies of repair of sublethal damage in mammalian cells (Elkind, M. M., et al., Nature 214, 1088, 1967) led to evidence that: (1) nuclear DNA, or nuclear DNA in association with its molecular and structural environment (e.g., the nuclear envelope), is the principal site of ionizing radiation damage and repair in a mammalian cell; and (2) distortion of the DNA double helix could give rise to damage functionally equivalent to that damage produced by ionizing radiation. These conclusions came from X-ray experiments involving a DNA intercalating agent, actinomycin D. Subsequently, it was shown that actinomycin D alone produces strand breaks in comparable numbers, at equivalent levels of cell killing, to X-rays (Elkind, M. M., Biophys. J. 11, 502, 1971). Thus, it remained a possibility that distortion of the double helix was not a necessary condition for the reduced ability to accumulate sublethal X-ray damage produced by actinomycin D.

Ultraviolet (UV) and near ultraviolet (NUV) light are thought to kill mammalian cells primarily by the production of intrastrand cross-links between adjacent pyrimidine bases in DNA. Since the resulting cyclobutane ring causes a distortion in the strand in question and since we found in a separate study (see preceding report by Han et al.) that X-ray and UV or NUV damage potentiate X-ray killing and *vice versa*, it became necessary to compare with X-rays the production of single-strand breaks in DNA due to UV and NUV.

EXPERIMENTAL METHODS

Conventional techniques for measuring cell survival after irradiation were used. DNA breakage was assessed using velocity sedimentation in alkaline sucrose gradients (Elkind, M. M., and C. Kamper, Biophys. J. 10, 237, 1970).

MAJOR NEW FINDINGS

For the same level of survival, about 10 times as many single-strand breaks are present immediately after irradiation by X-rays, at about 0°C, than after UV or NUV light. Breaks were also measured up to 5 hours after exposure, to see if significant numbers of additional breaks appear with incubation after nonnuclear irradiation. These breaks might result from the initiation of repair processes which excise pyrimidine dimers in surviving cells. For UV and NUV doses in the biologically relevant range (e.g., about 10 percent survival), a small number of additional breaks appeared, but they were still far too few to cause a significant increase in the total number.

SIGNIFICANCE

These results support the hypothesis that damage interaction with ionizing radiation occurs when strand distortion, as opposed to strand breakage, occurs. Strand distortion over long stretches of DNA could be cumulative. Consequently, impairment of structural relationships, as opposed to the kinds of short-range changes associated with mutation, may be relevant to cell killing.

PROPOSED COURSE OF THE PROJECT

Additional measurements of DNA damage and its connection with cell killing will be made. The relationship of DNA intercalators in general to ionizing and nonionizing damage potentiation will be tested further. Also, strand breakage in cells where pyrimidine dimer excision is easily detected--which is not the case with rodent cells--will be examined to see if differences exist relative to the production and/or development of DNA damage.

ERDA RT-03-02
ANL 61300

SPURIOUS PHOTOLABILITY OF DNA LABELED WITH (METHYL-^{14}C)-THYMIDINE

Mortimer M. Elkind, Principal Investigator
Ronald D. Ley and Mary E. Geroch, Participating Investigators

OBJECTIVES

As part of a study of the DNA breakage induced by nonionizing radiation, ultraviolet (UV) and near ultraviolet (NUV) light, the DNA from Chinese hamster cells grown in culture appeared quite photosensitive. Because this sensitivity seemed abnormally high, an investigation of the source of this photolability was undertaken.

BACKGROUND AND PREVIOUS FINDINGS

In contrast to ionizing radiation, nonionizing radiation is generally known to be inefficient in the production of strand breaks in the DNA of mammalian cells when compared to equivalent lethal doses of ionizing radiation. When the DNA from Chinese hamster cells was examined following UV exposure, it appeared damaged to an abnormal degree even though the survival response of these cells, in parallel experiments, was normal.

EXPERIMENTAL METHODS

Conventional cell culture techniques were used to assess mammalian cell survival. The assessment by the use of velocity sedimentation of single-strand breaks in radioactively labeled DNA was as previously described both for mammalian cell DNA (Elkind, M. M., and C. Kamper, Biophys. J. 10, 237, 1970) and for the DNA from bacteriophage T4 (Tyrrell, R. M., R. D. Ley, and R. B. Webb, Photochem. Photobiol. 20, 395, 1974).

MAJOR NEW FINDINGS

A careful examination of cell culture variables (e.g., medium, serum, cell subline, etc.) did not reveal the cause of the apparent photolability. However, marked photolability was observed only when the DNA was labeled with a particular lot of ^{14}C-thymidine (New England Nuclear Corporation, Compound NEC-568, Lot No. 824-137).

Since the thymidine analog 5-bromodeoxyuridine (BUdR), upon incorporation into the cell, renders the DNA photolabile (Ben-Hur, E., and M. M. Elkind, Biophys. J. 12, 636, 1972), a determination was made of the proportion of BUdR relative to thymidine in the growth medium required to produce the same rate of strand breakage, both with Chinese hamster cells and with bacteriophage T4. In both instances, and for both UV and NUV irradiations, a 1-2% addition of BUdR to thymidine produced a photolability equivalent to that observed with New England Nuclear's Lot No. 824-137 of ^{14}C-thymidine.

A sample of the latter material placed in the CP-5 Research Reactor at the Argonne National Laboratory for neutron activation--along with suitable Br standards--indicated that Lot No. 824-137 contained a molecular ratio of 1-2% Br relative to thymidine.

The data sheets accompanying shipments of labeled thymidine purchased from the New England Nuclear Corporation indicate that deoxyribose is added enzymatically to (methyl-^{14}C)-thymine from a "pyrimidine deoxyriboside" followed by purification by partition column chromatography. The foregoing results suggest that the pyrimidine deoxyriboside used is 5-bromodeoxyuridine and that the purification procedure can result in residual levels of BUdR of 1-2% relative to thymidine.

SIGNIFICANCE

Manufacturers, in addition to New England Nuclear Corporation, may use methods of producing labeled thymidine that result in significant levels of contamination with a halogenated pyrimidine. In addition to the DNA instability produced by BUdR or IUdR (e.g., the induction of virus release), spurious results may be obtained in photochemistry and photobiology. Indeed, photochemically it was found that BUdR contamination levels of 0.01% could be readily detected.

PROPOSED COURSE OF THE PROJECT

No further work specifically addressed to this question is planned.

ERDA RT-03-02
ANL 61300

RADIATION-INDUCED MALIGNANT TRANSFORMATION OF CULTURED MOUSE CELLS

Antun Han, Principal Investigator
Mortimer M. Elkind, Frank Q. Ngo, Eva E. Kautzky, and Chin-Mei Liu, Participating Investigators

OBJECTIVES

Our aim is to investigate radiation-induced oncogenesis at the cellular level, particularly in respect to molecular mechanisms, repair processes, and radiation quality.

BACKGROUND AND PREVIOUS FINDINGS

Thus far two systems have been mainly used for successful quantitative studies of radiation-induced cellular oncogenesis: The Syrian hamster embryo cell system (Borek, C., and E. J. Hall, Nature (London) 243, 450, 1973) and C3H mouse embryo cells, introduced by C. Heidelberger and associates (Reznikoff, C. A., et al., Cancer Res. 33, 3239, 1973). The data obtained with these systems show a strong dependence on radiation dose, although the shapes of the induction curves display significant quantitative and qualitative differences.

EXPERIMENTAL METHODS

Conventional techniques for quantitative measurements with cultured cells were used plus the method of assessing neoplastic transformation *in vitro* as developed for C3H, 10T-1/2 mouse cells (Reznikoff, C. A., et al., ibid.).

MAJOR NEW FINDINGS

Survival curves of 10T-1/2 cells have been measured after X-rays, fission spectrum neutrons, ultraviolet light, and near ultraviolet light. X-ray dose fractionation resulted in a typical increase in survival--indicative of repair of sublethal injury during the fractionation interval--accompanied by a reduction in the proportion of transformants per surviving cells. As is the case for Chinese hamster cells (see report by Han et al., p. 202), X-ray and ultraviolet damage interact.

SIGNIFICANCE

These initial results with fractionated X-ray doses suggest that cells can repair damage related to transformation as well as that related to cell killing. From this the important implication follows that the transformation frequency should decrease with decreasing dose rate.

PROPOSED COURSE OF THE PROJECT

1) Cells transformed *in vitro* will be tested for their tumorigenicity by inoculations into appropriate hosts.

2) Repair studies using ionizing and nonionizing radiations, singly and together, will be extended relative to cell killing and transformation.

3) Neutrons of various qualities will be assessed to see if the properties of high linear energy transfer radiation associated with cell killing also apply to transformation.

4) Transformation at low dose rates of γ-rays will be measured to see if predictions from high dose rate fractionation studies are borne out.

5) The development of new cell lines for transformation studies will be undertaken to assess the degree to which the results with 10T-1/2 mouse cells are general.

EPA-IAG-D6-E681

ULTRAVIOLET AND NEAR ULTRAVIOLET LIGHT: A COMPARISON OF PROPERTIES RELATIVE TO CELL FUNCTION AND DNA DAMAGE

Mortimer M. Elkind, Principal Investigator
Antun Han, Chin-Mei Liu, and Eva E. Kautzky, Participating Investigators

OBJECTIVES

Sunlight on the surface of the earth is filtered by the ozone layer and consequently wavelengths shorter than about 290 nm are severely discriminated against. However, most of the photobiology of ultraviolet radiation has been performed with 254 nm, the principal emission of a low-pressure mercury germicidal fluorescent lamp. The purpose of this study is to compare the properties of the latter radiation with those of a near ultraviolet light source that simulates sunlight. This is a necessary step prior to the use of the simulated-sunlight source for studies of cell killing, mutation, and neoplastic transformation.

BACKGROUND AND PREVIOUS FINDINGS

As reviewed by R. B. Setlow (Proc. Nat. Acad. Sci. 71, 3363, 1974), a number of effects produced by ultraviolet light in bacteria and bacterial viruses appear to have the same action spectrum (i.e., dependence of effect on wavelength). Since these effects include the production of photoproducts in DNA, since pyrimidine dimers are dominant among the latter, and since cells from sufferers of xeroderma pigmentosum are deficient in their ability to manage dimers, it may be inferred that Setlow's action spectrum applies to a number of end effects such as cell killing, mutation, and neoplastic transformation. A knowledge of the action spectrum offers a way, therefore, of quantitatively comparing the effects of a "sunlight" source (NUV) with those of the 254-nm mercury discharge emmission (UV) from a germicidal lamp.

EXPERIMENTAL METHODS

Conventional techniques for measuring cell survival after irradiation were used. DNA breakage was assessed using velocity sedimentation in alkaline sucrose gradients (Elkind, M. M., and C. Kamper, Biophys. J. 10, 237, 1970).

MAJOR NEW FINDINGS

Three percent of the emission of the "Sun Lamps" manufactured by the Westinghouse Corporation is "dimer producing." A detailed comparison of the survival curves of two lines of mammalian cells--V79 Chinese hamster cells and 10T-1/2, C3H mouse cells--showed that a constant dose factor--i.e., 0.03--would not translate the NUV curve into the UV curve because the curves are different in shape. As for strand breaks in DNA, the efficiency of their production by NUV in Chinese hamster cells was larger than that attributable to the proportion of dimer-producing dose; and with incubation after UV or NUV exposure, the dependence on time of the inventory of breaks differed qualitatively for the two radiations.

SIGNIFICANCE

These results indicate that dimer production alone is not the principal lesion after NUV exposure as it appears to be after UV. Consequently, inferences of effects of sunlight based upon measurements made with a 254-nm emmission may not be accurate. Further, nonnuclear agents in general (e.g., chemical carcinogens) whose action is thought to be "UV-like" may not produce interactive effects with sunlight predictable from UV data alone.

PROPOSED COURSE OF THE PROJECT

In addition to cell killing and strand breaks in DNA, a comparison of the properties of UV and NUV emissions will be extended to neoplastic transformation and mutation. By the use of an intense, variable-wavelength source of monochromatic light in the UV-NUV region, the hypothesis will be tested that pyrimidine dimers are primarily responsible for end points such as cell killing, mutation, and transformation in mammalian cells. Finally, the interaction of nonnuclear pollutants (e.g., mutagens and carcinogens) with UV or NUV will be compared to determine if UV may be used with confidence for epidemiological questions involving sunlight.

EPA-IAG-D6-E681

MONTE CARLO SIMULATION OF DNA DAMAGE AND REPAIR MECHANISMS

Thomas B. Borak, Principal Investigator*
Mortimer M. Elkind, Participating Investigator

OBJECTIVES

Our objectives are to develop a computer code that will calculate the breakage and repair of DNA molecules as predicted by different models, and to derive the parameters involved in breakage and repair processes by simulating best fits to experimental data.

BACKGROUND AND PREVIOUS FINDINGS

Lethality in elementary biological systems subjected to nuclear and nonnuclear pollutants has been associated with lesions produced in DNA. Normally the spatial separation of radiation-induced lesions is assumed to

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*Neutron and Gamma-Ray Toxicity Group.

be random. However, the repair and reorganization of the DNA fragments may depend on their separation and their chemical environment. For example, actinomycin D, added to mammalian cells just prior to or just after a dose of radiation, adds damage similar to X-ray damage (Elkind, M. M., et al., Nature 214, 1088, 1967). In addition to producing frank breaks, the experimental technique of lysing cells in alkaline solutions produces alkali-labile lesions that appear as single-strand breaks produced by the radiation. These factors must be accounted for when interpreting experimental results.

EXPERIMENTAL METHODS

Computer codes are generated which simulate and integrate various damage and repair processes in DNA. The computational results are used to formulate molecular weight distributions and velocity sedimentation profiles that are compared with experimental observations.

MAJOR NEW FINDINGS

The combined effects of the initial distribution of single-stranded DNA and various distributions after breakage have demonstrated significant changes in the shapes of the resulting sedimentation profiles. These generally conform to expectations, indicating that the computer code is working properly.

SIGNIFICANCE

This technique is valuable for rapid assay and interpretation of DNA damage, and will provide insight into the molecular biology of changes in cell functions effected by environmental pollutants.

PROPOSED COURSE OF THE PROJECT

The code is being extended to incorporate the complementary strand of the DNA double helix. This will enable the examination of the interdependence of single- and double-stranded lesions, as well as offer an opportunity to introduce normal and chemically induced cross-links. In addition to the qualitative features, quantitative parameters associated with damage and repair processes will be computed.

NIH CA 18081-01
NIH CA 18434-01

RADIOBIOLOGY OF FAST NEUTRONS

Frank Q. H. Ngo, Principal Investigator
Antun Han and M. M. Elkind, Participating Investigators

OBJECTIVES

This research is to determine the radiobiological properties of the fast neutron beam recently made available at the Fermi National Accelerator Laboratory (Fermilab) for purposes of guiding the use of this beam for the treatment of cancer. The objectives of these studies are twofold: (1) to study the biological effects of fast neutrons currently generated at Fermilab; and (2) to provide basic radiobiological information necessary for the clinical application of this neutron source.

BACKGROUND AND PREVIOUS FINDINGS

A major problem in radiotherapy is that hypoxic tumor cells are relatively resistant to radiation of low linear energy transfer (e.g., X-rays). High linear energy transfer radiations (e.g., fast neutrons) appear to be more effective for two reasons: (1) relative to X-rays, hypoxic cells are more sensitive; and (2) the repair of sublethal damage is reduced after neutron irradiation compared to X-rays. These two properties could increase the margin between tumor damage versus normal tissue damage.

EXPERIMENTAL METHODS

Standard cell culture techniques for measuring survival after irradiation are performed at ANL with V79 Chinese hamster cells irradiated at the Fermilab facility.

MAJOR NEW FINDINGS

Experimental difficulties arising from the separation of ANL and the Fermilab have been overcome.

Initial survival determinations of aerated cells indicate an enhanced killing response of Fermilab neutrons vs. X-rays because (1) the cells have a reduced capacity to accumulate sublethal damage, and (2) their survival curve drops off more rapidly with dose after the neutrons than the X-rays.

Experience has taught that no therapeutic trial should be undertaken with a new type of radiation without adequate radiobiological information beforehand. The present studies, when evaluated along with data from other neutron studies, will provide the radiotherapists with the information needed to initiate clinical studies. The clinical use of this neutron source, under Dr. Lionel Cohen of the Michael Reese Medical Center, is scheduled to commence during the next calendar year.

PROPOSED COURSE OF THE PROJECT

The following major items will be pursued: (1) cells will be irradiated under hypoxic as well as aerated conditions to measure the degree to which hypoxia protects against cell killing by Fermilab neutrons, and (2) sublethal neutron damage and its repair will be studied and compared to X-rays. The foregoing results will then be compared to similar measurements made with neutrons available from the JANUS reactor at the Argonne National Laboratory and fast neutrons from the 30-inch cyclotron at the University of Chicago, since these neutron spectra are distinctly different from that available at Fermilab.

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11. GENETICS

ERDA RT-03-03
ANL 62100
ANL 62200
ANL 62201

GROUP LEADER'S OVERVIEW

Herbert E. Kubitschek, Group Leader

OBJECTIVES AND ORGANIZATION

The primary objectives of the Genetics Group are to determine the mechanisms of production, action, and repair of lethal and mutagenic DNA lesions produced by environmental and other chemical and physical agents, and to appraise their potential genetic hazards. We are therefore concerned with lethal and genetic lesions of all kinds, including those produced by transuranic elements and other internally incorporated radioisotopes, by nonionizing and ionizing radiation, and chemical agents. Present programs include one in Mammalian Genetics, which emphasizes the potential genetic hazards of transuranic elements, primarily plutonium, and two in Molecular Genetics, which emphasize the molecular action of lethal and mutagenic lesions and the processes by which these can be repaired.

MAMMALIAN GENETICS

The new direction of this program was established only relatively recently. Present results are necessarily preliminary, but they agree with expectations that higher LET radiations carry higher associated genetic risks. Future experimental series are expected to define genetic dose-rate and total-dose factors influencing neutron/gamma RBE, and to determine whether neutrons can be used as a basis of comparison with continuous exposures to plutonium alpha particles.

MOLECULAR GENETICS

The major deleterious effects of chronic exposure to pollutants 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, while 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, other cellular effects generally diminish far more rapidly because of the physicochemical redundancy in those structures.

Because DNA has a relatively simple structure, only a limited number of different kinds of lesions can be produced. For this reason, we are concerned with mechanisms of lethality and mutagenesis produced not only by environmental pollutants, but by other agents as well, since these can provide vital information on shared mechanisms. Again, because certain lesions specifically inhibit DNA replication, or alter initiation or termination of replication, we also carry out associated research in these areas. The same is true for those lesions affecting cell growth or division. 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 microorganisms as in man. Other advantages of using bacteria are the availability of a tremendous number of different genetically marked strains, as well as the fact that many experiments can be done more cheaply and far more rapidly with these than is possible in higher organisms. Despite these advantages, many experiments require the use of mammalian cells, such as the ongoing study of mutagenesis in mouse myeloma cells to investigate possible mutational mechanisms of antibody diversity.

In addition to the research detailed in the individual project reports that follow, collaborative research was also carried out with two resident Faculty Research Participants on sabbatical leave to the Molecular Genetics Group, Drs. Ronald J. Doyle and Arthur L. Koch. Dr. Doyle is Associate Professor in the Department of Microbiology and Immunology, University of Louisville Schools of Medicine and Dentistry. Dr. Koch, Professor in the Department of Microbiology, University of Indiana, was a recipient of the fifth annual Argonne Universities Association Distinguished Appointment Awards for 1974. The results of their collaborative studies on near-UV inactivation of cell transport have already led to the submission of several papers for publication. With their departure, however, research in this area has been put in abeyance.

Major findings during the year include:

1) Unrepaired double-strand breaks produced by the incorporated radionuclide ^{125}I are lethal (R. E. Krisch). Single-strand breaks also occur but are not lethal.

2) Single low doses of UV induced conjoint bacterial mutations to bacteriophage resistance and to resistance to azide (H. E. Kubitschek). These results support earlier findings of clustered, multiple mutations in another bacterial system, and imply the presence of a previously undiscovered strong mutational process that is not explained by classical point mutations or frameshift mutations.

3) Results of comparative action spectra for near-UV induced lethality in bacteria in the presence and absence of 8-methoxypsoralen void some proposed generalizations on the mechanism of photodynamic action (B. S. Hass and R. B. Webb). These results also indicate that therapeutic dermatological doses of UV could be reduced, with decreased risk of mutagenesis or carcinogenesis, by using shorter wavelengths than are now employed.

4) The terminus of the bacterial chromosome is attached to the cell membrane during replication, and its release at the end of chromosome replication requires protein synthesis (T. Matsushita). These results indicate the possibility of a hitherto unknown regulatory mechanism in DNA replication.

SIGNIFICANCE AND PROPOSED COURSE

The results of our in-depth studies on the mutagenic and lethal lesions produced by particular radionuclides and environmental agents, such as ^{125}I, near-UV, and far-UV, provide important information on their biological and molecular mechanisms of action. In addition, our studies have already provided fundamental, and in some cases the first, information allowing assessment of potential hazards to man. For example, Webb's collaboration on investigations of the potential hazards of a commercial UV dental lamp, used primarily to polymerize adhesive resins after their application to the teeth of children, led to an FDA warning and design improvement. Again, on the basis of their most recent work, Hass and Webb suggest a method for improving dermatological treatments. Again, based partly on Krisch's findings with microorganisms, a clinical group at Harvard recently initiated animal experiments to test ^{125}I as an antineoplastic agent.

In addition, there is a broader level of significance arising from our concerted studies of variety of genetic lesions. These lesions appear to produce only a limited number of kinds of genetic effects, such as dimers, adjuncts, single-strand and double-strand breaks, and cross-links, each of which leads to different degrees of genetic damage. Thus, if potential damage from each of these basic genetic lesions can be assessed, even qualitatively, we should then be able to estimate the relative genetic hazards of different environmental pollutants. Furthermore, to the extent that carcinogenesis involves one or more mutagenic prelesions, it may ultimately be possible to associate risk of carcinogenesis with type of genetic lesion.

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 (Assistant Geneticist)
Prioleau, John C. (Scientific Assistant)

*Sauri, Catherine J. (Scientific Assistant)
Shotola, M. Anita (Scientific Assistant)
Venters, Dace (Scientific Assistant)
Webb, Robert B. (Bacteriologist)

TEMPORARY STAFF DURING 1975

Hass, Bruce S. (Research Associate)
†Koch, Arthur L. (Visiting Scientist)
Lee, Chung H. (Research Associate)
Newman, Chester N. (Postdoctoral Appointee)

PUBLICATIONS

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*Terminated during 1975.
†Argonne Universities Association Distinguished Appointment for 1974-1975.

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ERDA RT-03-03
ANL 62100

MAMMALIAN GENETICS: GENETIC EFFECTS OF HIGH LET RADIATIONS

Douglas Grahn, Principal Investigator
Barbara H. Frystak, Chung Hee Lee, Arthur Lindenbaum, and John J. Russell,* Participating Investigators*

OBJECTIVES

The potential genetic hazards from exposure to the high LET radiations need further evaluation for two reasons. One, there is increasing use of neutrons and other high LET radiations in therapy and, two, there is an increasing potential for environmental contamination by the alpha-emitting element plutonium and other transuranic elements from the expanding use of the light water reactor (LWR) and its associated fuel cycle. Small quantities of these elements are expected to be released to the environment during normal fuel reprocessing and to become available to man by inhalation or ingestion.

BACKGROUND AND PREVIOUS FINDINGS

Concern has been developing about the genetic hazards of high LET radiations as evidenced by an ICRP report that emphasized the uncertainties of RBE values for high LET radiation-induced mutation (ICRP-18, 1972). Some preliminary studies and analyses on plutonium-induced genetic damage in mice have also been reported from the British Medical Research Council (The Toxicity of Plutonium, Med. Res. Council, London, 1975; Beechey, C. V., et al., Nature 256, 577, 1975) and by the Institute for National Defense in Sweden (Lüning, K. G., et al., Proceedings of the IAEA Symposium on Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975, in press). The British efforts have identified a low level of ^{239}Pu-induced cytogenetic injury, while the Swedish study has shown significant dominant lethal effects at a rate greater than the MRC data would have predicted. Preliminary dosimetry determinations show a very heterogeneous exposure of the male germinal elements (Green, D., et al., Nature 255, 77, 1975).

EXPERIMENTAL METHODS

The JANUS reactor offers the opportunity to examine the genetic hazards of neutrons under differing exposure parameters and to provide a basis for comparison with a low LET radiation (^{60}Co γ-rays) and the ^{239}Pu alpha emissions. The standard method of detecting dominant lethal mutations in the mouse ($B6CF_1/Anl$) constitutes the central effort, which requires evaluation of uterine contents 14 days after conception. Supplementary information is

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*Therapy of Metal Poisoning Group.

obtained on testis weight, epididymal sperm count, frequency of sperm abnormalities (Searle, A. G., and C. Beechey, Mut. Res. 22, 63, 1974; Bruce, R., et al Mutat. Res. 23, 381, 1974), and on the induction of chromosome translocations in the spermatogonial stem cell population, utilizing an air-drying method of preparing samples of suspensions of testicular contents (Evans, E. P., et al., Cytogenetics 3, 289, 1964). For the internal radiation exposures, monomeric plutonium in citrate solution is injected intravenously at an initial body burden of 10 μCi/kg, and exposures to ^{60}Co γ-rays and fast fission neutrons are executed in accordance with procedures established for the JANUS program (see Section 4 of this report). Dosimetry for the plutonium burden in the testes will be done by autoradiographic procedures.

MAJOR NEW FINDINGS

Ideally, one wishes to compare continuous γ-radiation and continuous neutron irradiation with the plutonium alpha emissions, as the latter can be studied only as a continuous irradiation emitted from the retained burden. Since it is technically impossible to use the JANUS neutron source on a continuous basis, we approach the problem indirectly by comparing plutonium with continuous γ-irradiation, fractionated γ-irradiation with fractionated neutron exposures, and single exposures of γ-rays with single dose neutron irradiation, in order to form a matrix of comparative data that should permit a comparison of plutonium effects against one or more standard exposure situations. A summary of initial findings is given below.

1) Single dose gamma versus neutron irradiation. Dominant lethal mutation rates, frequency of induced sperm abnormalities in postmeiotic germ cell stages, and testis weight changes during both loss and recovery periods show a consistent neutron/gamma RBE of about 5.5 to 6.0.

2) Weekly fractions of gamma or neutron irradiation. Complete sterility, as measured by sperm count, is induced within 8 weeks by neutron exposures above 5 rads/week and gamma-ray exposures above 100 rads/week, so these levels are of minimal interest. There is strong evidence for the equilibration of germinal tissue injury after 10-12 weeks at neutron doses below 5 rads/week and γ-ray exposures below 50 rads/week. Testis weight change is a sensitive measure of injury, and it is possible to observe the RBE increase from 6 to 10 between 4 and 12 weeks of exposure. Preliminary evaluation for dominant lethals after 9 weekly exposures revealed consistent evidence of their presence, though a dose-response relation did not emerge. Comparison of neutron dose accumulations of 7.5, 15, and 22.5 rads with 77, 156, and 360 rads of γ-rays suggests an RBE of about 10 for dominant lethal mutations, comparable to the testicular measures.

3) Continuous ^{60}Co gamma irradiation. Daily exposures of 3.6 and 6.4 rads/day (22.5 hours) are available in the Intermediate Level Gamma Room, and initial samples taken after 7 weeks exposure (176 and 314 rads accumulated) show changes in testis weight and sperm count comparable to those seen following weekly exposure to about the same total dose.

4) Internal plutonium burdens. Male mice have been injected with ^{239}Pu citrate solution at 10 μCi/kg, approximately 0.25 μCi per mouse, and gonad retention after 6, 30, and 75 days was steady at about 5 x 10^{-4} of the initial

amount injected. The data on the testicular measures were equivocal, but dominant lethal testing at about 150 days indicated a 2% increase in both preimplantation and postimplantation losses.

5) Cytogenetic analyses. Five to 10 weeks after single dose irradiation, preparations of meiotic spermatocytes were made and up to 400 metaphase figures were analyzed. Reciprocal translocation frequencies increased from 1.4% after 90 rads to 10.0% after 569 rads of gamma-ray exposure. For the neutron-irradiated group, translocations increased in the dose range from 20 rads (0.4%) to 60 rads (2.9%), but decreased at higher doses.

SIGNIFICANCE

The emerging observations on the occurrence of low levels of genetic injury from ^{239}Pu, which concur with early findings at other laboratories, are important to engineering design considerations of the breeder reactor and its associated fuel cycle, the LWR fuel cycle, and proposals concerning the use of mixed oxide fuels. The risk cannot yet be evaluated on a relative scale, but the planned comparisons with ^{60}Co γ-rays and the JANUS neutrons should place the risk into proper perspective. At the same time, the ability to measure genetic damage at neutron dose levels below 10 rads may provide an opportunity to make significant evaluations of risk and attendant RBE at neutron dose levels near those of the working environment.

PROPOSED COURSE OF THE PROJECT

During the next year, more attention will be given to the estimation of dominant lethal mutation rates, testicular weight changes, and frequency of abnormal sperm and of reciprocal translocations in the gonia; less attention will be given to the sperm count because of high intrinsic variance. Additional replications of the single dose series will be carried out, and at least one additional fractionation series will be started in early 1976 (this series runs concurrently with JANUS program exposures). These two series will define the basic dose-rate, total-dose factors that influence neutron/gamma RBE for the genetic measures and will identify whether weekly fractions of neutrons can be used as a basis of comparison with continuous exposure to the plutonium alpha particles. The continuous exposure series, gamma ray and plutonium, will be especially emphasized in order to define the actual level of injury associated with diverse plutonium burdens and to develop a basis for comparison with the external radiations, which will permit an evaluation of the risk in comparative exposure terms more commonly dealt with in health physics. An initial effort may be made with the more potent mutagen that characterizes the actual effluent released to the environment--reactor-grade plutonium.

ERDA RT-03-03
ANL 62200

MOLECULAR AND RADIATION GENETICS: GENETIC REGULATION, ALTERATION, AND REPAIR

Herbert E. Kubitschek, Principal Investigator
Dace Venters, Participating Investigator

OBJECTIVES

We are examining 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.

BACKGROUND AND PREVIOUS FINDINGS

Early calculations indicated that the high rates of UV-induced mutation observed in a repair-deficient bacterial strain of *Escherichia coli* might be due to genetic recombination involving several contiguous genes. To test this possibility, UV-induced mutations to antibiotic resistance were examined in a strain of *Bacillus subtilis* chosen because the genes for these antibiotics are known to be close enough to one another to permit genetic recombination. Studies with this system carried out in The Netherlands (Kubitschek, H. E., and G. Venema, ANL-75-30, 1974, p. 236) confirmed that UV does indeed induce multiple, clustered mutations in this system. Of 136 streptomycin-resistant mutants examined, all but one were additionally mutated to resistance to rifampicin, neomycin, kanomycin, or erythromycin. Moreover, about 6% of these mutants were resistant to every one of these five different antibiotics, although in all cases the cells were exposed only to a single, low dose of UV.

EXPERIMENTAL METHODS

Studies of mutational clustering require the use of organisms with well-defined genetic maps. We employ the two strains of bacteria (*B. subtilis* and *E. coli*) with the most complete genetic maps, and use standard techniques of microbial genetics to detect bacterial mutations, and well-characterized repair-deficient strains to accentuate or inhibit certain mutational processes.

MAJOR NEW FINDINGS

Because the multiply resistant mutants might have occurred as some kind of genetic "hotspot" phenomenon peculiar to one region of the genome of *B. subtilis*, we have begun a search for clustered mutations in a different genetic region of the unrelated microorganism *E. coli*. Results have been obtained so far only with two genes: these are conjointly mutated by single low doses of UV. These genes, for resistance to the bacterial virus T5 and resistance to the metabolic poison azide, are known to be separated by approximately 1% of the genome. Nevertheless, conjoint mutation frequencies were 1000-fold higher than are accounted for by chance mutations of the

individual genes. These findings clearly support the earlier results for clustered mutations.

SIGNIFICANCE

Any mutational process that produces clustered mutations is of special concern in studies of mutagenesis and carcinogenesis. First, such processes produce multiple gene mutations rather than affecting genes one at a time. Second, many of the mutations are induced at some distance from the original genetic lesion, increasing the effective range of potency of such lesions and increasing the efficacy of the mutagen out of all proportion to the original rate of induction of lesions. Third, if, as has been suggested, carcinomas are produced as a result of accumulation of several mutations, then mutagens capable of producing multiple mutations may be especially dangerous.

PROPOSED COURSE OF THE PROJECT

The degree of multiplicity of mutation in this second system (*E. coli*) needs further examination to characterize the mechanism of multiple mutation. Other important questions are: Is this multiple mutational process characteristic of other mutagens in addition to UV, and if so, which? To what extent is this process dependent upon recombinational repair mechanisms? And how do other repair processes affect multiple mutation? Answers to these questions can be obtained with the present bacterial systems, which should permit efficient design of future experiments to determine the role of this process in higher organisms.

ERDA RT-03-03
ANL 62200

MOLECULAR AND RADIATION GENETICS: CHROMOSOME REPLICATION AND THE DIVISION CYCLE OF ESCHERICHIA COLI

Chester M. Newman, Principal Investigator
Herbert E. Kubitschek, Participating Investigator

OBJECTIVES

The primary objective is to determine whether chromosome (DNA) synthesis during the growth cycle in slowly growing bacteria is parallel to that found in higher (eucaryotic) organisms. In addition, we plan to examine the role of DNA synthesis as a regulating element of cell division in slowly growing cells.

BACKGROUND AND PREVIOUS FINDINGS

There is a difference of opinion concerning DNA synthesis in slowly growing bacterial cultures. C. E. Helmstetter (J. Mol. Biol. 24, 417, 1967) and others maintain that DNA synthesis occurs over the first two-thirds of the cell cycle. H. E. Kubitschek and M. L. Freedman (J. Bacteriol. 107, 95, 1971) measured the average amount of DNA per cell in unperturbed slowly growing cultures. Their results support (1) a constant period of replication of DNA at slow growth rates, and (2) DNA replication near the end of the division cycle, as occurs in eucaryotic organisms. The present study should put the controversy to rest.

EXPERIMENTAL METHODS

Measurements of the mean time to complete a round of replication (C) and of the variation in C are obtained from radiation "suicide" experiments. Exponential-phase cultures are exposed briefly to iodine-125, incorporated in iododeoxyuridine. Cells not synthesizing DNA at the time of exposure will not incorporate this radioisotope, and therefore only these will survive "suicide" by radioactive decay.

MAJOR NEW FINDINGS

Preliminary estimates from three experiments indicate that C is about 80 minutes at all slow growth rates, supporting the findings of Kubitschek and Freedman.

SIGNIFICANCE

Because *Escherichia coli* has been the organism used for numerous cell and molecular biological studies, we need to know how similar these bacteria are to eucaryotic cells so that we may assess the degree to which results from bacterial experiments are valid for higher cells and to what extent bacteria may provide model systems for development of tests and procedures with eucaryotic cells. Also, some experiments that may reveal the role of DNA synthesis in regulating cell division are presently only possible in bacteria, in which widely different growth rates can be maintained.

PROPOSED COURSE OF THE PROJECT

Our immediate proposed course is to settle once and for all the difference about the timing of DNA replication in slowly growing bacteria by use of this new method. An alternative approach involving cell killing by photolysis of incorporated bromodeoxyuridine will also be examined.

ERDA RT-03-03
ANL 62200

MOLECULAR AND RADIATION GENETICS: EFFECTS OF RADIOISOTOPE DECAY IN THE DNA OF MICROOGANISMS

Robert E. Krisch, Principal Investigator
Donna M. Darby, Participating Investigator

OBJECTIVES

Radioactive isotopes incorporated into the genetic material of living cells cause death or genetic damage. The goal of our experiments is to relate the observed biological effects to specific physical and chemical changes accompanying radioactive decay, as well as to physicochemical damage to the genetic material. Iodine-125 decays by electron capture and is known to cause severe molecular damage ("molecular explosions") when its decay occurs in small organic molecules. This isotope therefore is utilized as a model source for highly localized and very severe damage to DNA molecules in living systems. On the other hand, carbon-14 decays by electron emission and causes relatively little molecular damage. However, its biological effects are of widespread interest because this isotope is present in the environment in very large quantitites, both from natural processes and as a result of nuclear explosions.

BACKGROUND AND PREVIOUS FINDINGS

We demonstrated previously that double-strand breaks (DSBs) in DNA from ^{125}I decay in bacteriophage result primarily from local molecular damage, presumably via vacancy cascades, while single-strand breaks (SSBs) result primarily from self-absorption of emitted ionizing radiation (Krisch, R. E., et al., ANL-75-30, 1974, p. 224). We also demonstrated that ^{125}I decay in the DNA of bacteriophage T4 or of wild-type (*rec*$^+$) or repair-deficient (*rec*A) *Escherichia coli* cells causes DSBs with approximately unit efficiency. Every such break appears to be lethal for phage or for *rec*A bacterial cells, but only one in three is lethal for wild-type cells. We know of no previous work that has attempted to correlate the biological effects of ^{14}C decay with DSBs in DNA.

EXPERIMENTAL METHODS

^{125}I is incorporated into the DNA of bacterial cells (*E. coli*) or bacteriophage (T1 or T4) in the form of 5-iododeoxyuridine (IUdR), an analogue of thymidine. Carbon-14 is incorporated as ^{14}C-2-thymidine. Labeled microorganisms are stored in a metabolically inert state (in liquid nitrogen at -196°C) and samples are periodically thawed after varying amounts of radioactive decay, and assayed for loss of viability, for damage to DNA, and for biological repair of the damage, using standard techniques. Accuracy is increased by use of very large molecular weight bacterial DNA, obtained by gentle lysis of cells directly on the surface of the sucrose gradient (Bonura, T., et al., Radiat. Res. 63, 567, 1975).

MAJOR NEW FINDINGS

Our data indicate that for small numbers of ^{125}I-induced DSBs, up to about 3.5 DSBs per bacterial genome, *rec*$^+$ cells can repair about 70% of such breaks while *rec*A cells show no evidence for any repair of DSBs. Additional DSBs beyond this number are not repaired at all. Furthermore, it appears that all lethal events in both strains can be accounted for by unrepaired DSBs, that is, every unrepaired DSB is lethal. The greater resistance of the *rec*$^+$ strain to the lethal effects of ^{125}I decay is accounted for by its ability to repair DSBs.

In experiments with ^{14}C decay no DSBs were observed. However, about 2-4% of all decays in *E. coli* B/r are lethal, as a result of some undetermined mechanism. The data suggest extensive cellular repair of potentially lethal lesions.

SIGNIFICANCE

On the basis of our results we believe that DSBs probably play a crucial role in biological damage from all forms of ionizing radiation and possibly from other environmental agents. We have established ^{125}I decay in DNA as a practical tool to study the induction, repair, and biological significance of DSBs in the DNA of simple test organisms. In the case of ^{14}C decay, we have carried out pioneering experiments on the biological effects of DNA damage by this widely distributed isotope.

PROPOSED COURSE OF THE PROJECT

To uncover the biochemical mechanisms involved in the repair of DSBs in DNA, we plan to study the effects of ^{125}I decay in bacterial cells that are mutant in various genes governing repair of DNA damage. We also plan to do parallel studies on the induction and repair of single-strand breaks. Because the current emphasis of our research is to clarify the important role of DSBs in biological damage from environmental insults, we plan to put in abeyance our studies of the biological effects of ^{14}C decay, which do not appear to involve DSBs. It may be possible, through collaboration with others in this Division, to initiate analogous experiments on the role of DSBs in radiation damage to the cells of higher organisms.

ERDA RT-03-03
ANL 62201

MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS: INITIATION AND TERMINATION OF DNA REPLICATION IN BACILLUS SUBTILIS

Tatsuo Matsushita, Principal Investigator
Anita Shotola and Scott Winston, Participating Investigators*

OBJECTIVES

Our basic purpose is to study mechanisms of bacterial DNA replication and control using both genetic and biochemical techniques for the analysis of replication enzymes and proteins, and to relate replication mechanisms to other processes such as recombination, DNA repair, mutagenesis, and control of cell division. An understanding of DNA replication mechanisms should aid studies of related processes at all phylogenetic levels where DNA synthesis or DNA modification is involved.

BACKGROUND AND PREVIOUS FINDINGS

Both DNA replication and DNA repair occur in toluene-treated cells of *Bacillus subtilis* (Matsushita, T., and N. Sueoka, J. Bacteriol. 118, 974, 1974), but new replication forks are not formed. Our specific objectives were to study the mechanisms for this loss of initiation *in vitro*, and also to investigate the termination of replication.

EXPERIMENTAL METHODS

We study DNA replication in *B.subtilis* because, in addition to having a well-mapped chromosome and to the availability of required mutants, genetic transformations can be performed with this bacterium. Thus specific genes can be synthesized and the positions of newly synthesized genes can be located on the chromosome by standard density transfer techniques and transformation analyses. (O'Sullivan, A., and N. Sueoka, J. Mol. Biol. 27, 349, 1967). We also use the method of *in vitro* synthesis of genes in toluene-treated cells (Matsushita, T. et al., Nature [London], New Biol. 232, 111, 1971), and the method of isolating membrane-attached DNA of N. Sueoka and J. Hammers(Proc. Nat. Acad. Sci. U.S.A. 71, 4787, 1974) to examine the role of these attachments.

MAJOR NEW FINDINGS

First we have established the mechanism of loss of initiation of DNA

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*Participant in the Summer Graduate Student Program in Biology, University of Rhode Island.

replication in toluene-treated cells. We found that toluene acts to inhibit initiation by stopping protein synthesis.

Also there is a concomitant increase in DNA terminus attachment to cell membranes in these cells. *In vivo* studies show that the terminus DNA-membrane attachment can be released, and that this terminus release depends on protein synthesis.

SIGNIFICANCE

Previously it was thought that the terminus was permanently attached to the cell membrane. Our new evidence suggests a dynamic model of terminus attachment and release, and a new regulatory role for the terminus-attachment site. These findings suggest that similar intermittent attachment of eucaryotic DNA to nuclear membrane may also occur, and would explain some of the conflicting published results on DNA-nuclear membrane attachments.

PROPOSED COURSE OF THE PROJECT

The role of the cell membrane in DNA replication will be examined as follows: (1) The origin-membrane attachment site will be studied for a possible regulatory role during initiation. (2) Studies with 6-(p-hydroxyphenylazo)-uracil (HPUra), a specific replication inhibitor, will test whether the release of the DNA terminus from the membrane is part of the replication cycle or independent of the replication process and involved in control of cell division. (3) Studies of nuclear membrane-DNA attachment sites will be initiated in eucaryotic cells (the mouse myeloma system).

ERDA RT-03-03
ANL 62201

MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS: A STUDY OF MUTAGENESIS IN MOUSE MYELOMA CELLS

Tatsuo Matsushita, Principal Investigator
Bernard Jaroslow and Pattle Pun,† Participating Investigators*

OBJECTIVES

The overall purpose of this project is to examine the role of DNA replication and mutagenesis in several specific eucaryotic processes: the generation of antibody diversity, cell transformation, and differentiation. Our approach involves generating immunological and biochemical genetic mutants in the mouse myeloma system. Immunological mutations are located in the immunoglobulin variable ("V") gene region. Experiments first require isolation of V-gene mutants. We then plan to measure mutation rates with different mutagens to determine whether the V-region of a myeloma cell is hypermutable. This will test the merit of the somatic mutation theory for the generation of antibody diversity. We also hope to generate biochemical genetic mutations, like those in microorganisms, affecting eucaryotic repair, replication, and recombination. Used in combination with the immunological mutants, the biochemical genetic mutants would be useful for relating the repair, replication, and recombination processes to mutagenesis in the myeloma cell.

EXPERIMENTAL METHODS

The mouse myeloma cell is a plasma cell that has been transformed into a tumor cell (plasmacytoma). Since it is an end cell tumor (as opposed to a stem cell tumor), the antibody-producing tumor cell is monoclonal, and each clone produces one specific myeloma protein as the major cell product. As a result, these mutants are easily detectable by use of antimyeloma antisera. A second potential advantage of this system is that high mutation rates are expected because rates for generating heavy chain and Fc fragment mutants are known to be quite high (Preud'homme, J., et al., Proc. Nat. Acad. Sci. U.S.A. 72, 1427, 1975). Another very practical advantage is that the cloning and mutant detection system already has been worked out. (Coffino, P., and M. D. Scharff, Proc. Nat. Acad. Sci. U.S.A. 68, 219, 1971). This system consists of a rat embryo feeder layer, and three sequential overlays of agarose-medium, agarose-myeloma cells, and agarose-antisera.

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*Aging Research Group.

†Visiting Resident Associate, Wheaton College.

BACKGROUND AND PREVIOUS FINDINGS

Preliminary accomplishments are: (1) The myeloma light chain secreting cell MOPC-11, 662 variant (kindly supplied by M. D. Scharff), was successfully grown *in vivo* and in *in vitro* suspension cultures. (2) Myeloma protein was purified, and anti-662 antisera were prepared. (3) The cloning system was perfected with around 50% plating efficiency.

MAJOR NEW FINDINGS

None thus far.

SIGNIFICANCE

The study of mutation rates in the hypervariable region is expected to test the validity of the somatic mutation theory for generating antibody diversity. In addition, if the expected high rates of mutagenesis prevail in these cells, a study of the molecular mechanisms of mutagenesis in the immunoglobulin genes may reveal a novel mutagenic process. Lastly, the isolation of immunological myeloma mutants and the preparation of V-gene antisera for two differing myelomas will allow a quick and convenient mutagen screening system in a eucaryotic system.

PROPOSED COURSE OF THE PROJECT

The preparation of a high titer, anti-V region antiserum will continue in preparation for mutational studies. Biochemical studies will be initiated to characterize myeloma repair and replication enzymes. Also UV-irradiation studies will be initiated to characterize myeloma excision repair and postreplication repair. The biochemical and UV work will also facilitate the future isolation of repair and replication mutants.

ERDA RT-03-03
ANL 62201

MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS: LETHAL AND MUTAGENIC EFFECTS OF NEAR-ULTRAVIOLET RADIATION

Robert B. Webb, Principal Investigator
Mickey S. Brown and Bruce S. Hass, Participating Investigators

OBJECTIVES

Our aim is identification of the lethal and mutagenic lesions induced by near ultraviolet (near-UV) radiation, and their biological consequences.

BACKGROUND AND PREVIOUS FINDINGS

Pyrimidine dimers in DNA are induced at the rate of 5.5×10^{-7} dimers per genome per J/m^2 and single-strand breaks (or alkali-labile bonds) in DNA are induced at the rate of 3.6×10^{-7} breaks per genome per J/m^2 in *Escherichia coli* by 365-nm radiation (Tyrrell, R. M., Photochem. Photobiol. 17, 69, 1973; Tyrrell, R. M., R. D. Ley, and R. B. Webb, Photochem. Photobiol. 20, 249, 1974). These dimers were demonstrated to be the major component of lethal damage in certain repair-deficient mutants of *E. coli* (Brown, M. S., and R. B. Webb, Mutat. Res. 15, 348, 1972).

Damage to repair systems by near-UV radiation was demonstrated. Photoreactivating enzyme activity is destroyed by 365-nm radiation within the biologically effective dose range (Tyrrell, R. M., R. B. Webb, and M. S. Brown, Photochem. Photobiol. 18, 249, 1973) and dimer excision is impaired, as shown biochemically.

Also, inactivation of bacteria at wavelengths longer than 313 nm is strongly oxygen dependent in contrast to the absence of oxygen dependence at 313 nm and below (Webb, R. B., and J. R. Lorenz, Photochem. Photobiol. 12, 283, 1970).

EXPERIMENTAL METHODS

Monochromatic near-UV radiation is obtained from a 2.5-kW mercury xenon arc lamp in combination with a monochromator. Broad-spectrum near-UV is obtained from a fluorescent BLB source of special design. High intensity visible radiation is obtained from a Marc 300 arc lamp in a housing of special design with heat resistant interference filters. Illumination for photoreactivation is provided by a 2 x 2 inch projector with a liquid filter to isolate a band between 370 and 440 nm, or by a mercury xenon monochromator adjusted to 405 nm.

Single-strand breaks are analyzed by the alkaline sucrose gradient technique. Pyrimidine dimers are assayed by established procedures with paper chromatography.

Various bacterial strains differing in known repair capability and other characteristics are used. Mutant assays utilize both continuous cultures (chemostat and turbidostat) and batch cultures. Various mutational systems are used as they are deemed appropriate.

MAJOR NEW FINDINGS

1) Near-UV inactivation of excision repair and recombination repair systems is strongly oxygen dependent, consistent with earlier observations of the oxygen-dependent lethal effects of near-UV.

2) The absence of enzymatic photoreversibility in excision repair deficient and proficient strains could not be accounted for by the destruction of the photoreactivating enzyme, suggesting that pyrimidine dimers (although present) are not the significant lethal lesions in these strains.

3) The major lethal lesions are strongly oxygen dependent. This finding rules out pyrimidine dimers as the lethal lesion since they are not induced by an oxygen-dependent mechanism. The finding is consistent with single-strand breaks (or alkali-labile bonds) in the DNA as the major lethal lesion at 365 nm.

4) The absence of a strong lethal effect associated with the pyrimidine dimers induced at 365 nm implies that dimers induced at 365 nm are repaired more efficiently than dimers induced at 254 nm. The molecular basis of this difference has not been determined.

5) Significant shoulders in the action spectrum for lethality in two strains of *E. coli* in the regions of 340, 410, and 500 nm are consistent with absorption by endogenous chromophores such as quinones, porphyrins, and flavins and, along with the oxygen effect, implicate photodynamic mechanisms.

6) The photoreversibility of mutational lesions, together with the absence of a strong oxygen dependence for mutation induction by near-UV at high fluence rates, indicates that pyrimidine dimers are the major mutational lesions induced at 365 nm. Single-strand breaks, which are strongly oxygen dependent and should not be subject to enzymatic photoreactivation, are at most weakly mutagenic.

SIGNIFICANCE

Because of the known carcinogenicity of sunlight, together with the increasingly widespread use of intense near-UV sources in dentistry and medicine (for example the use of BLB sources in the treatment of psoriasis), this new information about near-UV lesions and their biological consequences in bacterial systems strongly suggests that similar investigations be made for mammalian cells.

The damage to DNA repair systems in bacteria suggests the corresponding damage in humans from the strong synergism between certain near-UV wavelengths

(320-370 nm) and shorter ultraviolet wavelengths. These results also affect calculations of biological damage resulting from depletion of the earth's ozone layer.

The induction of the various DNA lesions by wavelengths longer than 320 nm is more efficient by several orders of magnitude than predicted from the estimated absorbance of DNA, indicating that DNA is not the chromophore for these effects. Identification of the true chromophores and photochemical reactions might permit development of preventive measures.

PROPOSED COURSE OF THE PROJECT

Emphasis will be placed on the synergistic interactions between different near-UV wavelengths and kinds of cellular damage. In addition, repair of near-UV lesions and protection against near-UV damage will be examined in greater detail, with the collaboration of R. D. Ley. The study of apparent photodynamic nature of lethal and mutagenic effects of near-UV radiation will be extended to include photodynamic processes involving added sensitizers.

ERDA RT-03-03
ANL 62201

MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS: EFFECT OF 8-METHOXYPSORALEN ON PHOTODYNAMIC LETHALITY AND MUTAGENICITY IN ESCHERICHIA COLI

Bruce S. Hass, Principal Investigator
Robert B. Webb, Participating Investigator

OBJECTIVE

Our objective is to determine the biophysical mechanisms involved in the action of 8-methoxypsoralen (8-MOP) as it affects lethality and mutagenicity induced by near- and far-UV radiation.

BACKGROUND AND PREVIOUS FINDINGS

Because of their ability to promote melanin formation in the presence of sunlight, the psoralens, and particularly the furocoumarin 8-MOP, are used in conjunction with photoirradiation in the clinical treatment of some dermatological conditions, such as psoriasis and vitiligo. However, 8-MOP has been reported to be carcinogenic, and to be mutagenic in conjunction with near-UV radiation, antimutagenic with far-UV, and an inhibitor of postirradiation excision repair as well. (Igali, S., et al., Mutat. Res. 9, 21, 1970; Bridges, B. A., Photochem. Photobiol. 14, 659, 1971).

EXPERIMENTAL METHODS

Experiments dealing with lethality follow the procedures and techniques described by R. M. Tyrrell et al. (Photochem. Photobiol. 18, 249, 1974). Resting state cells of the strain *Escherichia coli* WP2 Hcr, deficient in excision of DNA photoproducts, are used to minimize complications due to excision repair processes.

MAJOR NEW FINDINGS

We obtained an action spectrum showing the effect of 8-MOP on cell survival as a function of wavelength of the lethal radiation. This was compared with a similar spectrum in the absence of 8-MOP. 8-MOP increased photosensitization by about 100-fold at 390 nm and by even more at the maximum sensitivity peak at 340 nm. In contrast, however, 8-MOP acted as a photoprotective agent at wavelengths shorter than 300 nm, decreasing lethality by more than 10-fold at 254 nm. The crossover point was found to lie at 304 nm, coincident with the absorption peak of 8-MOP in ethyl alcohol.

SIGNIFICANCE

Our results void certain classical generalizations on mechanisms of photodynamic action; the biological action spectrum is not necessarily coincident with the chemical absorption spectrum of the photodynamic agent. In addition, our studies provide a rationale for the time-honored method of performing experiments at the two convenient wavelengths of 365 and 254 nm. These two wavelengths can therefore be used with greater assurance.

These results will have significant clinical applications. They already suggest that present UV doses in dermatological irradiations could be reduced at shorter wavelengths, and that potentially mutagenic and carcinogenic wavelengths in the near- and mid-UV should be eliminated or reduced.

PROPOSED COURSE OF THE PROJECT

Projected goals are twofold: (1) to continue our study of the mechanisms of sensitization and protection occurring in the photodynamic action of 8-MOP in the bacterial cell, and (2) to determine common physical and chemical elements in the photodynamic mechanisms of 8-MOP and acridine orange as an approach to better understanding of photodynamic processes.

ERDA RT-03-03
ANL 62201

MOLECULAR GENETICS AND ENVIRONMENTAL MUTAGENS: DNA REPAIR OF THE LETHAL EFFECTS OF FAR-ULTRAVIOLET AND NEAR-ULTRAVIOLET IRRADIATION ON BACTERIAL CELLS

Mickey S. Brown, Principal Investigator
Robert B. Webb, Participating Investigator

OBJECTIVES

Our objectives are (1) to determine the near-UV repair systems and their efficiencies at various ultraviolet wavelengths; and (2) to examine the protection by carotenoids against the lethal effects of UV radiations.

BACKGROUND AND PREVIOUS FINDINGS

Photorepair of near-UV lethal lesions was discovered earlier in our laboratory. Near-UV lesions, like far-UV lesions, were observed to be photoreactivable in strains unable to carry out postreplication recombination repair.

EXPERIMENTAL METHODS

These experiments employ isogenic wild-type and repair-deficient mutants of *Escherichia coli* and *Sarcina lutea* wild type and mutant (without pigment). Experimental methods used have been described earlier (Brown, M. S., and R. B. Webb, Mutat. Res. 15, 348, 1972).

MAJOR NEW FINDINGS

While pyrimidine dimers are the primary lethal lesions for far-UV (below 320 nm), wavelengths above 320 nm effectively kill cells by another mechanism, as yet unknown.

Carotenoid pigments in *S. lutea* shield these cells at radiations between 300 and 500 nm, although not at shorter wavelengths.

SIGNIFICANCE

Ultraviolet irradiations are known to produce several kinds of biological damage, including skin cancer. These effects can be greatly increased through synergistic reactions from exposures to more than one wavelength or, alternatively, reduced by the photorepair system. Separation of these effects requires an analytical investigation throughout the entire ultraviolet spectrum.

PROPOSED COURSE OF THE PROJECT

The research on carotenoid protection is nearing completion. Further biological and molecular experiments are required to identify the newly found lethal mechanism at wavelengths above 320 nm.

03091

12. ANALYSIS AND ASSESSMENT

ERDA RT-04-04
ANL 68100

GROUP LEADER'S OVERVIEW

Douglas Grahn, Group Leader

This program was initiated in the past year and most of the effort has been directed toward recruitment, data-base resource identification, and definition of the scope of the study. This is a collaborative study with the Energy and Environmental Systems Division (EES), though its present activity is largely centered in the Division of Biological and Medical Research, where the effort is presently concentrated on the basic demographic aspects of disease incidence and death rate. As the program expands, studies on cost-benefit analysis will become a more important aspect, and that effort should center in EES. The results of these latter studies should find their most effective application in the environmental impact analysis for the extensive synthetic fuels industry presently in the planning stage. In addition, however, research and analytical results should continue to serve the expanding nuclear industry. For example, during the year, Dr. Grahn acted as a consultant to the Nuclear Regulatory Commission on the final judgments for the rulemaking known as Appendix I of Title 10 CFR Part 50, "Numerical guides... to meet the criterion 'as low as practicable'...in...nuclear power reactor effluents." He was also called upon to assist ERDA in responding to criticism regarding the potential health hazards of plutonium,* and he continues his activity with NCRP regarding the reexamination of risks to man from low dose rate, low dose, low LET irradiation. Another member of the staff, Mr. Lundy, assisted in the organization of a two-week meeting held in Palo Alto on the "Application of Monte Carlo Techniques to the Analysis of Social and Demographic Problems."

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*Grahn, D. Comments on "The cancer hazard from inhaled plutonium" and "Estimated production of human lung cancer by plutonium from worldwide fallout." J. W. Gofman, Congressional Record, July 31, 1975; submitted via ERDA/DBER to the Joint Committee for Atomic Energy, U. S. Congress.

ANALYSIS AND ASSESSMENT STAFF

REGULAR STAFF

Benson, Jane R. (Scientific Assistant)
Grahn, Douglas (Senior Biologist)
Lundy, Robert T. (Assistant Biologist)

TEMPORARY STAFF DURING 1975

Dixon-Davis, Diana (Research Associate)

PUBLICATIONS

Grahn, D. Analysis of population, birth, and death statistics in the counties surrounding the Big Rock Point Nuclear Power Station, Charlevoix County, Michigan. ANL-8149 (1975).

Grahn, D. Book Review: Review of the current state of radiation protection philosophy NCRP Report No. 43. Int. J. Appl. Radiat. Isot., in press.

ERDA RT-04-04
ANL 68100

SOCIOECONOMIC AND DEMOGRAPHIC ASPECTS OF SELECTED MORTALITY PATTERNS IN THE UNITED STATES, 1950-1970

Douglas Grahn, Principal Investigator
Robert Lundy, Diana Dixon-Davis, Jane Benson, and Phyllis Walker, Participating Investigators*

OBJECTIVES

The ultimate objective is to predict potential health costs to man accruing from the effluents or by-products of any energy system or mix of systems, but the establishment of reliable prediction equations first requires a baseline analysis of those preexisting and essentially uncontrolled factors known to have significant influence on patterns of mortality. These factors are the cultural, social, economic, and demographic traits of a defined local or regional population. Thus, the immediate objective is the rigorous statistical definition of consistent relationships that may exist among the above traits and between them and selected causes of death, especially those causes that may have interpretive value for the detection of environmental pollutants.

BACKGROUND AND PREVIOUS FINDINGS

The health status of most individuals is strongly influenced by their personal habits, occupational history, educational level, economic status, and cultural background [Kitigawa, E., et al., Differential Mortality in the U.S., Harvard Press (1973); Daric, J., Mortality, occupation and socioeconomic status, USDHEW (1951)]. Recently, evidence has also begun to accumulate on the acute and chronic health effects of environmental pollutants (Lave, L., et al., Science 169, 723, 1970; Sagan, L., Nature 250, 107, 1974), although it is usually not possible to correlate directly a toxic agent with an end effect, or to attribute the action of any agent independent of associated sociocconomic traits. Thus, the cost-benefit analysis of a control technology, for example, remains moot, until the diverse effects of both population traits and pollutants on an individual's present and future health are better delineated.

EXPERIMENTAL METHODS

The data will come primarily from official sources of demographic and health statistics. The analysis will use standard demographic, actuarial, and multivariate procedures, as well as some special adaptations of multivariate techniques. These procedures will consist primarily of life table manipulations, creation of synthetic variables via factor analysis, and stepwise multiple regression.

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*Energy and Environmental Systems Division, Argonne National Laboratory.

MAJOR NEW FINDINGS

The initial course of the study has been divided into five activities:

1) A literature review leading to the preparation of a technical memorandum on the socioeconomic and demographic variables that influence human health.

2) Assembly of a library of tested statistical programs.

3) Identification of data-base sources, their acquisition, and the development of data management programs.

4) Selection of proposed or active energy facility sites for the initial analyses.

5) Identification of the potentially significant health-related socioeconomic and demographic traits and cause-of-death categories, including those that index the impact of occupational and environmental pollutants, social stability forces, and economic status.

Fifteen energy facility sites have been chosen for their divergent socioeconomic, geographic, and vital statistical characteristics. Among the selected demographic and socioeconomic variables, in addition to age, race, and sex, are educational attainment, occupation, income, urbanization, migration, housing, marital status, and medical services. Several of these are exceedingly complex and not uniformly comparable among different regions of the U.S. Standardizing indices are being developed as needed. For example, a standardized index of income is being constructed for comparisons of real variations in income and mortality levels. The conventional definition of urban vs. rural is not meaningful to this study, so "urban" is being redefined to provide an index that is also a proxy for numerous social, cultural, and economic differences between urban and rural life. The quality of medical services cannot yet be quantified because of the social and cultural attitudes toward medicine that influence the use of medical care.

The natality and mortality data for 1970 have been purchased but other years will be accessed at Brookhaven National Laboratory. The data volume and random order make them difficult to use directly, so they have been entered into the data-base management SYSTEM 2000, where the entries are indexed for retrieval, either interactively or by the batch computer. Direct acquisition of the census data is also impractical because of cost and quantity. Access methods have therefore been set up at the University of Chicago. Computer input/output facilities have also been made available at Argonne to both Brookhaven and Lawrence Berkeley Laboratory for direct use of data.

The study will concentrate on causes of death that are either a major proportion of total deaths (heart and circulatory diseases, infant mortality, cancer), sensitive to carcinogens (bladder cancer, leukemia, lung cancer, etc.), sensitive to social and cultural patterns (cervical and intestinal cancer, homicides, lung cancer, infant mortality, etc.), or relatively insensitive to sociocultural variations (diabetes). All the demographic variables were chosen for their potential predictive value in a final model, and how they would correlate with specific causes of death and general levels of mortality.

We will study the 20-year period from 1950-1970 because of the comparability of the data bases, and because this period avoids the immediate mortality stresses introduced by the depression and World War II. During this 20-year period, there were rapid changes in the U.S. industrial structure, and extensive internal migrations from rural to urban and urban to suburban living. Though complex, these changes should enhance correlation analyses. In addition, the chosen time span will make it possible to study cancer mortality and other chronic diseases with long development periods.

SIGNIFICANCE

The National Environmental Policy Act of 1969 (NEPA) requires that ERDA and other agencies act responsibly to prevent undue risks to the public health and safety that may be associated with expanding uses of existing energy technologies and developing new ones. Health impacts will be or are part of the cost-benefit analysis of effluent control technologies, of energy facility site selection, and of the choice of technology mix. The output of this project will provide a "handbook" approach to analysis of potential health effects, their prediction under different economic or resource utilization scenarios, and a realistic appraisal of the health effects, positive or negative, or population changes concomitant with stimulated economic change. Thus, spurious changes in vital statistics parameters can be more easily identified, and equally important, unexpected effects may be detected early enough to permit activation of effective controls.

PROPOSED COURSE OF THE PROJECT

Consequent to the final selection of energy sites, demographic traits, and vital statistical parameters, the course of action will be to assemble the data, to prepare a computer file, and to initiate the statistical processing, The data will first be subjected to path analysis and factor analysis to detect the most sensitive relations between the independent (demographic) and dependent (mortality) variables. This will be followed by detailed intrasite regression analysis and subsequently by intersite comparison for repeatability of relationship. Sensitivity testing may later be done with a completely different array of regional or local populations.

Concurrent to the statistical studies, interpretive studies will be carried out on the cultural and social anthropology of disease and death for those sites characterized by a more divergent array of population traits, such as social and/or ethnic mix, migratory labor patterns, or extreme economic factors.

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13. SUPPORT FACILITIES

COMPUTER SUPPORT ACTIVITIES

Frank S. Williamson

FACILITIES

During the year we acquired a 1,000 line per minute printer (Mohawk Data Sciences) for our remote data station (RADS). Although we consume only 2.1 percent of the batch computing resources, we rank high in terms of print traffic at RADS, and our Potter printer was breaking down with increasing frequency. We have converted obsolete 026 card punches to 029-equivalent so that we now have two keypunches and two verifying keypunches. Indeed we seem to have introduced this cost-effective upgrade to the laboratory. We encourage the use of microfiche computer output and provide a viewer in the Rads User Area.

CONSULTING SUPPORT

The number of computer users in the Division fluctuates, and is currently 48. All of these individuals rely on rapidly available, usually immediate, consultation to resolve abortive computer runs, and most users depend on help to set up programs and run them.

The heaviest users of the facilities are the crystallographic studies, then the molecular studies. Both the facilities and the programming services are used extensively by the Neutron and Gamma-Ray Toxicity and the Radiation Toxicity in Dogs Groups for management and analysis of data; and they are also used by many experimenters in the Division to process and analyze data obtained with data acquisition systems.

PROGRAMMING

The accompanying three individual project reports illustrate particular aspects of our activities.

The BIMFILE System is a filing system that is particularly well suited to biological or medical needs, and has great flexibility. We recently evaluated System 2000 as an alternative and concluded that it lacked features that we already enjoyed. We believe that the BIMFILE System, which is already in use by the JANUS and Dog Projects, will be critically important to future nonnuclear toxicological studies in the Division. The other two reports give

examples of programs that attack the problem of data entry errors. We feel very strongly that assurances of quality cannot be overemphasized, but that rejection of errors is not enough.

Many other programs have been written for users, and many existing programs have been modified on request.

We also run data analysis jobs for various scientific staff, though this service is provided at the expense of programming services.

ERROR DETECTION AND RECOVERY IN PROCESSING EXPERIMENTAL DATA

Jeanne A. Blomquist, Principal Investigator
Frank S. Williamson, Participating Investigator

OBJECTIVES

Our objective is to process the data output from experimental equipment with speed and convenience, while detecting data transmission errors and offering a plausible substitution for each erroneous value.

METHODS

Commercially available laboratory instrumentation in the Division is equipped with Teletype Printers in numerous cases. Computer-compatible output is obtained at low cost by buying the paper-tape punch option. This paper tape uses even parity, allowing detection of punching or reading errors. The tape is read into the ANL Central Computing Facility via the Division's remote batch station. Computed results, and low-resolution graphs, are returned from the same station on a high speed line printer, usually within 30 minutes.

BACKGROUND

In the case of interest, a Beckman scintillation counter measures radioactivity of tritium, carbon-14, and phosphorus-32 on fractions of sedimented, irradiated DNA released from Chinese hamster cells lysed on top of an alkaline sucrose gradient. These data are punched on paper tape.

Errors in punching or reading the tape, due to dirt accumulations in the holes, must be detected and, if possible, corrected.

Spillover of counts from each radioisotope into detector channels set for the other two isotopes must be compensated, then the weight-average and number-average molecular weights are calculated for each gradient (a set of fraction samples).

MAJOR NEW DEVELOPMENTS

The computer program recognizes parity errors from the paper tape input and flags the erroneous values. Since the data values from successive samples form a related set, polynomial interpolation is used to substitute for the erroneous value and the substitution is flagged. Later, if the user decides that the substitution is not acceptable, the data can be rerun with manual corrections.

Spillover corrections are rigorously performed by solving the three simultaneous equations for the three channel sensitivities to all three radioisotopes.

SIGNIFICANCE

In processing automatically collated data, assurance of freedom from errors is of paramount importance. However, the rejection of data by the computer program can lead to processing delays, and manual correction procedures severely burden the research worker. Sophisticated attempts at error recovery through the use of redundant data almost completely eliminate the need for manual correction and reprocessing.

PROPOSED COURSE OF THE PROJECT

This technique will be applied wherever possible in future cases.

BIMFILE--THE DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH FILE MANAGEMENT SYSTEM

Frank S. Williamson, Principal Investigator
Martin R. Kraimer and Jeanne A. Blomquist, Participating Investigators

OBJECTIVES

The objectives are to provide means for the scientist having modest proficiency in the PL/I programming language to save, correct, retrieve, and print reports of large quantities of data.

BACKGROUND

The first indexed-sequential files to use BIMFILE principles were used in the JANUS project in 1970. A more elaborate version of BIMFILE was defined in 1972. Specifications for a report generator were outlined, and this software implemented, in 1974. During this period, Dog Project files were converted to the system. It was decided to limit the immediate objective to access of BIMFILE from PL/I programs, with the future possibility of developing a higher level language to specify file operations.

METHODS

PL/I was chosen as our programming language for data management because it combines sophisticated file-access techniques, character string handling, and full floating-point mathematical capability. Sequential files (e.g., magnetic tape) and indexed-sequential files (on disk for random, or sequential, access) are supported. The operational features of BIMFILE are dependent upon the existence of a characteristic group of fields (the Header group) in each record. A large number of records may be filed under a single, unique identifi... associated with a sequence number.

MAJOR NEW DEVELOPMENTS

In the past year, BIMFILE-2 has been developed and implemented. This requires a change of file format and Dog Project files are being converted (without impacting Project operations). New features include: (1) Variable-length comments and data field arrays, spanned over multiple records. (2) PL/I-callable routines to add records, delete records, modify records, and retrieve records. Retrieval is either random or by sequences (either forward or backward). Use of these interface routines creates a separate audit trail of record additions and changes in a "journal" file which is used in conjunction with backup tapes for rapid recovery from file errors or loss. (3) Routines to print reports and tabulations from retrieved, or computed, data. (4) A utility program to allow correction of fields in file records, such fields being specified by name. (5) Built-in checks to eliminate accidental duplication of records added to files. (6) Access to several files at the same time.

SIGNIFICANCE

BIMFILE-2 minimizes the programming needed to retrieve, select, and print a report from stored data. It maximizes the security of stored data and reduces errors. It has a flexibility, and a variety of stored data types, not found in other systems available to us.

PROPOSED COURSE OF THE PROJECT

We plan (1) to implement access protection so that users may only perform file operations for which they are authorized, and (2) to support direct-access files (PL/I Regional-2 Type). After experience with BIMFILE-2, the possibility of higher-level language development will be studied.

ENTRY AND VERIFICATION OF HEMATOLOGY DATA

Martin R. Kraimer, Principal Investigator
Donald E. Doyle, Participating Investigator*

OBJECTIVES

Our objectives are to screen hematology data as they are entered into the computer and to identify unusual values so that samples can be remeasured, if necessary, while they are viable.

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*Radiation Toxicity in Dogs Group.

BACKGROUND AND PREVIOUS FINDINGS

The ultimate goal is to have a local minicomputer control the taking of data from blood samples and perform checks and screening. The current development was planned in 1974 as an interim stage which would afford the desired facilities, though needing manual input of data.

EXPERIMENTAL METHODS

A program was written in the PL/I language, to be run on the ANL Time Sharing Computer so that the operator can interact with the program. Other programs, also in PL/I, perform calculations and update master files.

MAJOR NEW DEVELOPMENTS

As now operating, the operator enters hematology data at a time-sharing computer terminal and those that lie within predetermined limits are accepted. Outlying values are flagged, whereupon the operator compares them with the last five readings for that dog. A truly aberrant value can then be remeasured, while the sample is still available.

Additional programs produce tabulations of values and identify dogs that merit examination, based on blood parameters.

SIGNIFICANCE

This system has worked smoothly and engenders confidence in the transcription of data values. Few data errors have been observed, and they are extremely easy to correct.

PROPOSED COURSE OF THE PROJECT

This system will serve until further progress is made in automating the data collection.

ELECTRON MICROSCOPY CENTER

R. J. Michael Fry and Thomas M. Seed

During calendar 1975, eight of the eleven research groups within the Division made active use of the Electron Microscopy Facilities in support of various ongoing programs. Four of these groups, namely Carcinogenesis, Aging, JANUS, and the Dog Program, had one or more full-time microscopists employed in evaluating ultrastructural features of varied biological problems. The number of individual EM users currently numbers eighteen, although this number tends to fluctuate.

In addition, the EM Center provides support to other ANL divisions, when requested. At present we are assisting five separate research groups within four divisions (Chemistry, Physics, Solid State Science, and Radiological and Environmental Research) in projects ranging from "Ecological studies of Lake Michigan plankton and the relationship to pollutant transport" (J. G. Ferrante, RER) to "Metal analysis for fusion reactors" (S. D. Das, PHY).

FACILITIES

During the year, we acquired an Ortec X-ray Microanalysis System for use in conjunction with the already housed Cambridge Mark II Steroscan electron microscope. This addition will give us new analytical capabilities in the area of high resolution detection of trace elements and heavy metals within tissues and cells. Although the system is not yet fully developed, it has already been employed, primarily in experiments designed to localize plutonium within liver tissue.

Each investigator has complete access, upon request, to scope time, dark-room time (for photographic processing), and preparative EM equipment and supplies. In addition, technical help with micrograph interpretation, tissue preparation techniques, and photographic and microscope assistance is also immediately and readily available to the divisional researchers. Mr. G. T. Chubb continues to provide this technical assistance to the Division staff.

RESEARCH SUPPORT

Specifically, intradivisional technical assistance is being given to the following programs: (1) The Carcinogenesis Group, under the direction of R. J. M. Fry, by R. L. Devine and T. N. Tahmisian, is studying the ultra-structural characteristics of cells in pituitary isografts and tumors in host animals. (2) B. J. Wright is assisting Y. E. Rahman and the Biochemistry Group in determining the cellular fate of liposome-encapsulated drugs which are potentially useful therapeutic compounds. (3) V. V. Yang and R. L. Devine, in conjunction with S. P. Stearner, are evaluating the ultrastructural consequences on the microvasculature of the JANUS mouse of protracted gamma and neutron irradiation. (4) T. M. Seed is currently analyzing the path-ological consequences of low doses of continuous gamma irradiation in the beagle dog, with particular reference to hemopoietic and reproductive tissue

injury. (5) I. Greco and C. K. Lee have been assisting M. P. Finkel and C. A. Reilly in the Experimental Radiation Pathology Group in identifying oncogenic viruses within bone tumors from man, dog, mouse, and hamsters.

In addition, two Resident Associates, Eugene W. McArdle of Northeastern Illinois University and D. G. Oldfield of DePaul University, have carried out collaborative research (with C. F. Ehret and R. J. M. Fry, respectively) in the Center. Dr. McArdle undertook an ultrastructural analysis of ultradian and infradian *Tetrahymena* cells from agar plate cultures, while Dr. Oldfield has attempted to develop a method for luminescence analysis of biological materials using the scanning microscope.

FUTURE OUTLOOK AND DIRECTIONS

The current heavy use of the facilities is expected to continue, and we anticipate that the Center will be extensively used as new toxicological studies are initiated on nonnuclear pollutants. We have already begun developing new analytical microscopic systems, as well as updating many of the older preparative procedures; these activities should increase the flexibility of our support capabilities.

LABORATORY ANIMAL FACILITIES

Robert J. Flynn

Approximately 60% of the studies funded within the Division of Biological and Medical Research involve animal resources, and the housing, use, care, and management of these resources consume a significant fraction ($\sim$ 17%) of the Division's budget. Further studies, particularly on nonnuclear pollutants, could well require further animal resources and additional construction. Thus, the efficient, economic, and appropriate management of these resources is critical to the success of the Division's research programs.

The management staff of the facilities includes a veterinarian certified in laboratory animal medicine, a clinical veterinarian, a veterinary microbiologist, a veterinary pathologist, appropriate assisting staff members, technicians, animal care supervisors, and animal care personnel. Each individual has specific, needed capabilities and it is the task of the Assistant Director for Animal Facilities to see that each is utilized appropriately.

The major problem of the facilities continues to be one of overcommitment. The research staff, in its enthusiasm to perform more and more research, has continually demanded more and more services from the facilities, without a commensurate increase in animal care staff or physical plant. At the same time, the staff has demanded better and better animals. The facilities' management staff has the knowledge and professional capabilities to produce

and maintain the highest quality research animals, but the present physical plant was not designed to maintain such animals. Steps are being taken to correct this latter problem with the renovation of E wing, while the problem of overcommitment is being approached through scheduling and priority management of animal experiments.

Some added space, in the form of two new dog gamma rooms, plus a small increase in animal care manpower will become available during 1976; a modest rodent breeding wing planned for 1977 will probably be ready for occupancy about 1978; and an environmental pollutants animal wing tentatively planned for about 1978-1979 will probably become operational about 1980. These added resources should mitigate the problem of overcommitment.

The success of the facilities' professional staff is evident in many ways. The quality of animals produced by the staff, with some minor exceptions to be corrected when the rodent colonies are re-cesarean derived and when the E wing renovations are completed, is superior to that of any animals commercially available. The quality of care, while not meeting the ideal as set by the professional staff itself, is superior to most comparable institutions and exceeds that required for full accreditation by the American Association for Accreditation of Laboratory Animal Care.

LABORATORY ANIMAL FACILITIES STAFF

REGULAR STAFF

*Brennan, Patricia C. (Biologist)
†Fritz, Thomas E. (Veterinary Pathologist)
Flynn, Robert J. (Senior Veterinarian)
Keenan, William G. (Scientific Associate)
Poole, Calvin M. (Veterinarian)
Simkins, Richard C. (Scientific Assistant)
†Tolle, David V. (Scientific Associate)

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*Neutron and Gamma-Ray Toxicity Group.

†Radiation Toxicity in Dogs Group.

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14. EDUCATIONAL ACTIVITIES

POSTGRADUATE TRAINING

During 1975, a total of 36 postdoctoral appointees, visiting scientists, and research associates contributed to the research programs of the Division. Eighteen of these were new appointments in 1975, 12 more 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
David W. Baxter*	Chicago College of Osteopathic Medicine	A. Lindenbaum
Jerome C. Cater*	University of Glasgow	B. N. Jaroslow
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
Daniel Fiat†	Weizmann Institute of Science	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
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
Bruce F. Kimler	University of Texas	M. M. Elkind
Arthur L. Koch†	Indiana University, Bloomington	H. E. Kubitschek
Chung Hee Ryu Lee*	University of Illinois, Urbana	D. Grahn
Chung K. Lee	University of Illinois, Urbana	M. P. Finkel
Ronald G. Lindahl	Wayne State University	R. N. Feinstein
Rand E. McNitt	University of North Carolina	J. Shen-Miller

*Research Associate

†Visiting Scientist

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
John E. Parks	University of Wisconsin, Madison	A. Lindenbaum
Dale A. Schoeller	Indiana University, Bloomington	P. D. Klein
Bobby R. Scott	University of Illinois, Urbana	E. J. Ainsworth
Surendra T. Shenoy	University of California, Davis	C. Peraino
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 13 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 1975, their schools, and their staff sponsors were as follows:

Marion A. Bakula	St. Louis University	Y. E. Rahman
Mabel Conley	Southern University, Louisiana	P. C. Brennan
Richard A. Cutler	University of Texas, Dallas	G. A. Sacher
Ronald J. Doyle	University of Louisville	H. E. Kubitschek
Alonzo J. Fairbanks	Trinity College, Illinois	S. S. Danyluk
Louis Glatzer	University of Toledo	R. B. Webb
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
Maria E. Ortiz	California State Polytechnic	P. C. Brennan
Basavaraju P. C. Rao	Bennett College	M. P. Finkel
John F. Schneider	University of Chicago	P. D. Klein
Thomas A. Victor	Northwestern University	S. S. Danyluk

SUMMER GRADUATE STUDENT PROGRAM IN BIOLOGY

Twenty students from 17 different universities were enrolled in the 1975 program. Dr. Edgar Reilly of the University of Iowa served as coordinator of the course, in cooperation with Dr. Walter Kisieleski. The program, which ran for 8 weeks, consisted of 18 "core" lectures in radiation biology by Dr. Reilly, followed by a series of 11 specialized lectures given by

*Research Associate

Dr. Walter Kisieleski, Dr. Arthur Lindenbaum, Dr. Ronald Ley, Dr. John Ainsworth, Dr. Bernard Jaroslow, Dr. Michael Fry, Dr. Douglas Grahn, Dr. Steven Spigarelli (Division of Radiological and Environmental Research), Dr. Ray Hinchman (Environmental Statement Project), and Dr. Norman Frigerio (Environmental Statement Project).

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 Mr. Connie Smyros (Reactor Research Operations Division), Mr. Emil Johnson, Mr. George Chubb, Ms. Rosemarie Devine, and Ms. Betty Jean Wright. Mr. Donald Moore gave a demonstration of liquid scintillation counting methods at Packard Instrument Company, and Mr. Michael Sloan showed radioimmunoassay methods 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:

Cecelia J. Babcock	University of Illinois, Urbana	C. Peraino
Patrick K. Bender	Kent State University	A. B. Edmundson
William F. Blakely	University of Illinois, Urbana	G. A. Sacher
John Christopher	Oregon State University	C. Peraino
George Conder	University of New Mexico	S. A. Spigarelli (RER)
David E. Denekas	Stanford University	S. P. Stearner
Cynthia Durbahn	Gustavus Adolphus College	M. Walgren (RER)
Stephen A. Goldstein	University of Tampa	S. S. Danyluk
Adele M. Hathaway	Northern Illinois University	J. Shen-Miller
Carol A. Hoenich	West Chester State College	Y. E. Rahman
James B. Kobler	Vassar College	R. D. Ley
Charles M. Lombard	University of Chicago	W. E. Kisieleski
Lauri R. Robertson	Vassar College	R. D. Ley
Daniel P. Rosenberg	University of Illinois, Urbana	R. J. Flynn
Robert S. Schoefield	San Diego State University	T. B. Borak
David W. Uzzell	Williams College	P. D. Klein
Gail S. Vogelzang	University of Illinois, Circle Campus	G. T. Chubb
Richard A. Wielgos	Western Illinois University	P. C. Brennan
Scott E. Winston	University of Rhode Island	T. Matsushita
Roger W. Zygmunt	Purdue University	E. J. Ainsworth

OTHER GRADUATE PROGRAMS

Three 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 and their staff sponsors were as follows:

John P. Christopher*	Oregon State University	C. Peraino
Alfred G. Garcia	Northern Illinois University	R. J. M. Fry
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 1975, five students held such appointments in the Division:

Howard W. Brahm	Ohio State University	G. A. Sacher
James I. Fast	Northern Illinois University	M. P. Finkel
Patricia M. Irving	University of Wisconsin, Milwaukee	J. Shen-Miller
Bradford B. Smith	San Diego State University	J. F. Thomson
Charles J. Zeller	Northern Illinois University	R. J. M. Fry

In addition, two students held Guest Graduate Student appointments. They were Avrom M. Brendzel, University of Illinois, Circle Campus, under the supervision of Y. E. Rahman, and John H. Wulf, University of Texas, under the supervision of G. A. Sacher. G. Steven Kalesperis, Chicago College of Osteopathic Medicine, was a Resident Student Associate under T. Matsushita.

UNDERGRADUATE TRAINING

During 1975, a total of 29 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

Marlane J. Angle	Eastern Montana College	W. P. Norris
Paul C. Chalmers	University of Bridgeport	J. Shen-Miller
Michael R. Haley	Willamette University	B. N. Jaroslow
Linda M. Klemka	Augustana College, Illinois	T. Matsushita
James B. Kobler	Vassar College	R. J. M. Fry
Richard La Fountain	Mount Union College	A. B. Edmundson
Ronald R. Piester	Sterling College	S. S. Danyluk
Donna M. Williams	Northern Illinois University	H. E. Kubitschek

SUMMER PROGRAM

Janice L. Arnold	Illinois Institute of Technology	R. B. Webb
Jason Chao	Northwestern University	P. C. Brennan
Cynthia L. Cilyo	University of Illinois, Urbana	C. F. Ehret
Iola Earley	University of Illinois, Circle Campus	M. M. Elkind

*Also participant in the Summer Graduate Student Program in Biology.

Karl I-Ming Li	Massachusetts Institute of Technology	P. C. Brennan
Clifron M. Martin	Prairie View A & M University	Y. E. Rahman
Robert C. Melville	University of Delaware	F. S. Williamson
John V. Nelson	Carnegie-Mellon University	S. S. Danyluk
Marilyn J. Schneider	State University College of New York at Brockport	P. D. Klein
Timothy M. Sigmon	Lenoir Rhyne College	F. S. Williamson
James A. Slavin	Case Western Reserve University	T. Borak
Kevin Williams	Harvard University	A. B. Edmundson
Martin Wojciechowski	Northern Michigan University	J. Shen-Miller
Robert Yeager	California State College, San Bernardino	R. N. Feinstein

FALL PROGRAM

Roger Edvenson	Coe College	H. E. Kubitschek
Mason B. Hunter	Carroll College	J. Shen-Miller
David H. Mattson	Carleton College	P. D. Klein
Mary C. Oskowski	Washington and Jefferson College	R. B. Webb
Kevin M. Strathy	St. Olaf College	Y. E. Rahman
Jill G. Warner	St. Olaf College	R. J. M. Fry
William D. Wickart	Knox College	S. A. Tyler

OTHER TRAINING

The following high school students worked as High School Trainees:

Michael Gholston	Lockport Central High School	J. Cater
Cynthia Heeg	Lemont High School	P. Polk
Beverly Woods	Joliet Central High School	M. Brown

In addition, James Spotts of Illinois State University worked under the sponsorship of W. J. Eisler as a Neighborhood Youth Corps trainee.

JOINT ARGONNE-UNIVERSITY APPOINTMENTS

During 1975, 20 staff members held a total of 30 faculty appointments at universities in the Chicago area. These appointments comprise limited teaching activities, generally of a specialized nature, at the graduate level, which involve regular contact with students. They have led to co-sponsorship of graduate students and to collaborative research efforts with faculty members, some of which are described in this report.

In addition, George A. Sacher gave a course on Mathematical Models in Social and Biological Gerontology, September through December, 1975, at the University of Waterloo, Waterloo, Ontario, Canada. Mortimer M. Elkind holds an appointment in the Department of Radiotherapy, Tufts University Medical School, Medford, Massachusetts.

The affiliations with Chicago area universities were as follows:

University of Chicago

Mortimer M. Elkind
Robert N. Feinstein
R. J. Michael Fry
Peter D. Klein
Timothy E. O'Connor
George A. Sacher
Fritz Schlenk

University of Illinois at Chicago Circle

Douglas Grahn
Bernard N. Jaroslow
Herbert E. Kubitschek
Carl Peraino
Fritz Schlenk
Jane Shen-Miller
John F. Thomson

Loyola University

Thomas E. Fritz
Bernard N. Jaroslow
Walter E. Kisieleski
Arthur Lindenbaum

Northern Illinois University

R. J. Michael Fry
Douglas Grahn
Bernard Jaroslow
Herbert E. Kubitschek
Carl Peraino
Y. E. Rahman
Christopher A. Reilly, Jr.
Fritz Schlenk
John F. Thomson
Robert B. Webb

Northwestern University

Peter D. Klein

SECOND INTERNATIONAL CONFERENCE ON STABLE ISOTOPES

October 20-23, 1975, the Second International Conference on Stable Isotopes was held at the Oak Brook Hyatt House under the sponsorship of the Division of Biological and Medical Research and under the auspices of the U. S. Energy Research and Development Administration. Additional support was received from Merck, Sharp and Dohme, Ltd., G. D. Searle and Co., Inc., and the University of Chicago. Dr. Peter D. Klein was the Conference chairman, heading a Committee of 11 members from laboratories and universities in Belgium, Sweden, Japan, and the United States.

The Conference, whose theme was "Meeting Human Needs," stressed the application of stable isotopes to those pharmacological, clinical, and environmental problems that require the safety of non-radioactive tracers for their solution. The program was composed of 78 scientific papers plus five read by title. The papers were divided among sessions on clinical, pharmacological, nuclear magnetic resonance, and environmental applications of stable isotopes, on isotope effects, and on the use of ^{13}C breath tests as diagnostic tools. Another session and a workshop-demonstration dealt with techniques and methodological developments.

A total of 240 graduate students and academic and clinical research scientists from 12 foreign countries, plus the United States and Canada, attended and participated in the discussions and activities.

The Conference Proceedings, edited by Roseland Klein, will include full texts of the papers presented. They will be published in 1976 as ERDA CONF 751027 document, available from the National Technical Information Service, U. S. Department of Commerce, Springfield, Virginia.

No AUA-ANL Biology Symposium was held during 1975.

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15. OUTSIDE LECTURES BY DIVISIONAL STAFF DURING 1975

Ainsworth, E. J. "Experimental Radiobiology" (two lectures)
Loyola University School of Dentistry, Maywood, Ill., January, 1975.

Ainsworth, E. J. "Late Effects of Neutron or Gamma Radiation"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Ainsworth, E. J. "Mammalian Radiobiology: Radiation Syndromes"
Barat College, Lake Forest, Ill., October, 1975.

Ainsworth, E. J. "Life Shortening, Neoplasia, and Systemic Injuries in Mice after Single or Fractionated Doses of Neutron or Gamma Radiation"
IAEA International Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975.

Ainsworth, E. J. "Late Effects of Radiation"
Barat College, Lake Forest, Ill., November, 1975.

Baxter, D. W. "Differences in Early Retention of Lead Acetate and Lead Citrate in Mouse Tissues"
Fifteenth Hanford Life Sciences Symposium, on the Biological Implication of Metals in the Environment, Richland, Wash., September 29-October 1, 1975.

Bhattacharyya, M. "The Association of Monomeric Plutonium with Mouse Liver Parenchymal Cells"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Bhattacharyya, M. "Monomeric Plutonium and Mouse Liver Parenchymal Cells: Deposition and DTPA-Induced Removal"
Workshop on the Biological Effects and Toxicity of Pu-239 and Ra-226, Sun Valley, Idaho, October 6-9, 1975.

Brennan, P. C. "Cell-Mediated Immunity in Beagle Dogs: Effect of Age, Radiation, and Genetic Background"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Brennan, P. C. "Biological Safety"
American Chemical Society, Chicago, Ill., October 11, 1975.

Brennan, P. C. "Effect of Single or Fractionated Doses of Neutron or Gamma Radiation on Cell-Mediated Immune Function"
Centro di Studi Nucleari della Casaccia, Rome, Italy, November 26, 1975.

Brown, M. S. "Microbial Enzymology"
College of DuPage, Glen Ellyn, Ill., February 12, 1975.

Crouse, D. A. "Effects of *In Vitro* X-Irradiation on the Homing and Function of Memory Cells in LBN Rats"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Crouse, D. A. "Radiobiological Studies of a Murine Leukemia"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Danyluk, S. S. "NMR Spectroscopy of Nucleic Acids"
Dept. of Chemistry, University of Guelph, Ontario, Canada, June, 1975.

Danyluk, S. S. "NMR Spectroscopy of Nucleic Acids"
Dept. of Chemistry, University of Manitoba, Winnipeg, Canada, June, 1975.

Dobra, K. W. "Growth of High Density Monolayers of *Tetrahymena pyriformis* W. on Solid Agar"
Midwest Protozoology Conference, University of Western Michigan, Kalamazoo, April, 1975.

Dobra, K. W. "Circadian Regulation at the Cellular Level of Glycogen, Tryptophan, Tyrosine, and Catecholamine Metabolism"
XII International Conference of the International Society for Chronobiology, Washington, D.C., August 10-13, 1975.

Dobra, K. W. "Light Entrainment of Circadian Respiration, Glycolysis, and Catecholamine Metabolism in Free Living Cells"
International Congress on Rhythmic Functions in Biological Systems, Vienna, Austria, September 10, 1975.

Dobra, K. W. "Circadian Control of Glycogen Metabolism and Respiration in High Density Monolayers of *Tetrahymena pyriformis*"
Ciliate Genetics Conference, Münster, West Germany, September 22, 1975.

Dobra, K. W. "Circadian Rhythm of Respiratory CO_2 in High Density Monolayers of *Tetrahymena pyriformis*"
Midwest Protozoology Conference, Miami University, Oxford, Ohio, October 25, 1975.

Doyle, R. J. "Inactivation of Membrane Transport in *Escherichia coli* by Black Light"
75th Annual Meeting of the American Society for Microbiology, New York, N.Y., April 27-May 2, 1975.

Doyle, R. J. "Induction of Growth Delay by Inactivation of Membrane Transport after Exposure to Near-UV"
3rd Annual Meeting of the American Society for Photobiology, Louisville, Ky., June 23-27, 1975.

Doyle, R. J. "Near-Ultraviolet Light Inactivation of Membrane Carriers in *Saccharomyces cerevisiae*"
American Society of Microbiology Branch Meeting, Louisville, Ky., November 8, 1975.

Edmundson, A. B. "The Three-Dimensional Structure of Immunoglobulins"
Symposium on the Evolution of Proteins, Federation Meetings, Atlantic City, N.J., April 13, 1975.

Edmundson, A. B. "The Structures and Evolution of Immunoglobulins" (two lectures)
University of Utah, Salt Lake City, Utah, June 21-25, 1975.

Edmundson, A. B. "The 3D Structure of Immunoglobulins" (three lectures)
Woods Hole Oceanographic Institute, Woods Hole, Mass., July 8-12, 1975.

Edmundson, A. B. Chairman, Immunochemistry Session at Gordon Conference on Immunobiology and Immunochemistry, Andover, N.H., August 11-15, 1975.

Edmundson, A. B. "The 3D Structure of Immunoglobulins"
Royal Society of Medicine Symposium at the Rockefeller University, October 20-23, 1975.

Edmundson, A. B. "The 3D Structure of Immunoglobulins"
Kansas State University, Manhattan, Kans., November 24, 1975.

Ehret, C. F. "Circadian Cybernetics: An Integrating Discipline in the Biology of Higher Organisms"
Dept. of Biological Sciences, University of Illinois at Chicago Circle Campus, February 4, 1975.

Ehret, C. F. "Circadian Regulation at Cellular and Organismic Levels"
Dept. of Pathology, University of Chicago, Chicago, Ill., April 21, 1975.

Ehret, C. F. "The Infradian Eukaryotic Cell: A Circadian Energy-Reserve Escapement"
XII International Conference of the International Society for Chronobiology, Washington, D. C., August 10-13, 1975.

Ehret, C. F. "Circadian Cybernetics: An Integrating Discipline in the Biology of Higher Organisms"
Symposium on Biophysical Bases for Rhythmic Activity, International Congress on Rhythmic Functions in Biological Systems, Vienna, Austria, September 8, 1975.

Ehret, C. F. "Circadian Chronotypic Efficiency of Theophylline as an Enzyme Inducer and as a Zeitgeber in the Rat"
Anatomisches Institut der Medizinischen Hochschule, Hanover, Germany, September 17, 1975.

Ehret, C. F. "Circadian Rhythm of the Pasteur Effect and of Hypoxic Cytotoxia in Protistans"
Max-Planck-Institut für Zellbiologie, Wilhelmshaven, Germany, September 19, 1975.

Ehret, C. F. "Cell Cycle and Circadian Regulation: One Clock, One Clock"
Conference on Ciliate Genetics, Zoologisches Institut der Universität, Münster, Germany, September 22, 1975.

Ehret, C. F. "The Role of Genes and Their Expression"
Dahlem Konferenzen Workshop on Molecular Basis of Circadian Rhythms, Berlin, Germany, November 5, 1975.

Elkind, M. M. "Relevance of the Initial Part of the Survival Curve in Fractionated Radiation Therapy"
Victoria Hospital, University of Western Ontario, London, Canada, January 21, 1975.

Elkind, M. M. "Cytotoxic Action of Radiation and Some DNA Interactive Drugs"
Health Sciences Centre, University of Western Ontario, London, Canada, January 22, 1975.

Elkind, M. M. "DNA Repair Studies Relative to Radiation and Drug Damage"
Northern Illinois University, De Kalb, Ill., February 20, 1975.

Elkind, M. M. "Fractionation Radiation Therapy and its Dependence on the Initial Part of the Survival Curve"
Shields Warren Radiation Laboratory, Harvard University Medical School, Boston, Mass., February 27, 1975.

Elkind, M. M. "Studies of DNA Damage and Repair and the Influence of Hyperthermia"
Massachusetts General Hospital, Boston, Mass., February 28, 1975.

Elkind, M. M. "Radiation and Drugs as Tools in the Study of DNA Damage and Repair"
Illinois Institute of Technology, Chicago, Ill., March 10, 1975.

Elkind, M. M. "Damage-Repair Studies of DNA from Irradiated and Drug-Treated Chinese Hamster Cells"
Mallinckrodt Institute of Radiology, Washington University Medical School, St. Louis, Mo., March 18, 1975.

Elkind, M. M. "The Relevance of Survival Curve Shape in Dose Fractionation: A Review of the 6th L. H. Gray Conference"
Mallinckrodt Institute of Radiology, Washington University Medical School, St. Louis, Mo., March 19, 1975.

Elkind, M. M. "Radiation Cell Transformation: What is it?"
The Biology of Radiation Carcinogenesis, Gatlinburg, Tenn., April 7-10, 1975.

Elkind, M. M. "Radiobiological Principles of Importance to Radiotherapy"
University of Chicago, Chicago, Ill., June 2, 1975.

Elkind, M. M. "How Cellular Radiobiology Folds into Radiotherapy"
University of Chicago, Chicago, Ill., June 9, 1975.

Elkind, M. M. "Misrepair, a Proposed Lethal Process in Mammalian Cells"
Atomic Energy Commission of Canada, Ltd., Whiteshell Nuclear Research Establishment, Manitoba, Canada, June 18, 1975.

Elkind, M. M. "Fundamentals of Cellular Radiobiology"
Barat College, Lake Forest, Ill., September 24, 1975.

Elkind, M. M. "Drug Lethality and Sublethal Damage"
Conference on Cell Kinetics and Cancer Chemotherapy, Annapolis, Md., November 4-6, 1975.

Elkind, M. M. "Mechanisms of Action and Interaction of Ionizing Radiation and Chemotherapeutic Drugs"
Rush Presbyterian St. Luke's Medical Center, Chicago, Ill., December 19, 1975.

Ezra, F. "Proton Magnetic Resonance Studies of Uridylyl-(3'-5')-Adenosine. Conformational Analysis"
19th Annual Meeting of Biophysical Society, Philadelphia, Pa., February 19-21, 1975.

Ezra, F. "Proton Magnetic Resonance of Nucleic Acid Constituents"
Midwest Nuclear Magnetic Resonance Discussion Group Meeting, University of Notre Dame, South Bend, Ind., November 15, 1975.

Feinstein, R. N. "Role of the Carcinogen in Production of New Forms of Aldehyde Dehydrogenase in Rat Hepatomas"
10th Annual Isozyme Conference, St. Croix, U. S. Virgin Islands, December 1-3, 1975.

Finkel, M. P. "Plutonium Incorporation Through Ingestion by Young Animals"
Workshop on the Biological Effects and Toxicity of Pu-239 and Ra-226, Sun Valley, Idaho, October 6-9, 1975.

Fritz, T. E. "Effect of Continuous Irradiation on Beagles Irradiated During Fetal Life"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Fritz, T. E. "Chronic Low-Level Irradiation of Dogs: Immunologic, Hematopoietic, and Neoplastic Effects"
Dept. of Microbiology, Ohio State University, Columbus, Ohio, October 2, 1975.

Fritz, T. E. "Endocrine Diseases in Beagles"
Workshop on Beagles in Health Effects Research, ASILOMAR Conference Grounds, Pacific Grove, Calif., December 1, 1975.

Fry, R. J. M. "The Design of Cell Renewal Systems"
De Paul University Seminar, Chicago, Ill., January 17, 1975.

Fry, R. J. M. "Cell Proliferation Studies"
University of Michigan Seminar, Ann Arbor, Mich., March 21, 1975.

Fry, R. J. M. "Cell Renewal Systems"
University of Chicago, Chicago, Ill., March 31, 1975.

Fry, R. J. M. "Experimental Mammary Tumorigenesis"
Bureau of Radiological Health, Rockville, Md., April 5, 1975.

Fry, R. J. M. "Cell Proliferation: Normal and Abnormal"
University of Chicago, Chicago, Ill., April 11, 1975.

Fry, R. J. M. "Design Factors in Proliferative Systems: Is There Any Relation to Control?"
Tenth Paterson Symposium, Manchester, England, September 22, 1975.

Fry, R. J. M. "Radiation Oncogenesis *In Vivo*"
International Workshop on Particle Radiation, Key Biscayne, Fla., October 1-3, 1975.

Fry, R. J. M. "Acute Effects of Radiation in Mammals"
Northern Illinois University, De Kalb, Ill., October 13, 1975.

Fry, R. J. M. "Late Effects of Radiation in Mammals"
Northern Illinois University, De Kalb, Ill., October 27, 1975.

Fry, R. J. M. "The Effect of Pituitary Isografts on Radiation Carcinogenesis in the Mammary and Harderian Glands of Mice"
IAEA International Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975.

Fry, R. J. M. "The Selection of Mouse Strains and Tissues for Studies of Oncogenesis"
Interagency Collaborative Group on Environmental Carcinogenesis, National Library of Medicine, Bethesda, Md., December 17, 1975.

Grahn, D. "Health Effects Estimates"
Chicago Chapter, American Nuclear Society, Tutorial Seminar on the Reactor Safety Study, Draft Report (The Rasmussen Report), February 8, 1975.

Grahn, D. "Cost-Benefit as Weighed on Genetic Scales"
Georgia Institute of Technology, School of Nuclear Engineering, Atlanta, Ga., June 26, 1975.

Groh, K. R. "Polymorphism in *Tetrahymena*"
Northeastern Illinois University, Chicago, Ill., November 10, 1975.

Grube, D. D. "The Tumorigenic Effects of Psoralen and Ultraviolet Light"
American Association of Cancer Research Meeting, San Diego, Calif., May 8-10, 1975.

Guilmette, R. "Progress in the Therapeutic Removal of Plutonium from Experimental Animals"
NYU Institute of Environmental Medicine, Tuxedo, N. Y., May 5, 1975.

Guilmette, R. "Effectiveness of Interferon-Inducing Agents for Removal of Polymeric Plutonium from the Mouse"
20th Annual Meeting of Health Physics Society, Buffalo, N.Y., July 13-17, 1975.

Guilmette, R. "Progress in the Use of Pyran Copolymers for Decorporation of Polymeric Plutonium"
Workshop on the Biological Effects and Toxicity of Pu-239 and Ra-226, Sun Valley, Idaho, October 6-9, 1975.

Hachey, D. L. "Biomedical Applications of Stable Isotopes"
North Jersey Section of the American Chemical Society, January 27, 1975.

Hachey, D. L. "High Precision Isotope Ratio Measurements by CI/MS"
23rd Annual Conference on Mass Spectrometry and Allied Topics, Houston, Texas, May 23-30, 1975.

Hachey, D. L. "Quantative Analysis of Methadone in Biological Fluids Using Deuterium Labeled Internal Standards"
Second International Conference on Stable Isotopes, Oakbrook, Ill., October 20-23, 1975.

Hachey, D. L. "Synthesis of Pteroylglutamic Acid-3',5'-$^2H_2$ by Trifluoroacetic Acid Catalyzed Exchange with Deuterium Oxide"
Second International Conference on Stable Isotopes, Oakbrook, Ill., October 20-23, 1975.

Han, A. "The Effect of N-ethylmaleimide on the Response to X-rays of Synchronized HeLa Cells"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Han, A. "Interaction of Damage Due to Ionizing and Nonionizing Radiation in Chinese Hamster Cells"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Jaroslow, B. N. "Cell Proliferation and Aging"
Biology Dept., St. Louis University, St. Louis, Mo., March 10, 1975.

Jaroslow, B. N. "Immunity and Aging"
Biology Dept., St. Louis University, St. Louis, Mo., March 10, 1975.

Jaroslow, B. N. "Radiation and Immunity"
Biology Dept., Wheaton College, Wheaton, Ill., May 28, 1975.

Jaroslow, B. N. "Restoration of Antibody-Forming Capacity in Old Mice"
10th International Congress of Gerontology, Jerusalem, Israel, June 22-27, 1975.

Jaroslow, B. N. "Immunity and Aging"
Gerontology Research Center, Baltimore, Md., October 6, 1975.

Jaroslow, B. N. "Radiation and Immunity"
Argonne Community College Association, Argonne, Ill., October 14, 1975.

Jaroslow, B. N. "Immunity and Carcinogenesis"
Microbiology Dept., Ohio State University, Columbus, Ohio, October 16, 1975.

Jaroslow, B. N. "Immunity and Aging"
Microbiology Dept., Ohio State University, Columbus, Ohio, October 16, 1975.

Kimler, B. F. "Sensitization of Aerobic and Hypoxic Chinese Hamster Cells to X-Irradiation by N-Ethylmaleimide"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Kisieleski, W. E. "Species Susceptibility to Chemical Carcinogens"
Morraine Valley Community College, Palos Hills, Ill., August 14, 1975.

Kisieleski, W. E. "Environmental Carcinogens"
Chicago Section, American Association for Dental Research, Chicago, Ill., October 23, 1975.

Klein, P. D. "A Stable Isotope Ratiometer-Multiple Ion Detector (SIRMID) Unit for Quantitative and Qualitative Stable Isotope Studies by Gas Chromatograph-Mass Spectrometry"
Seventh Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Oak Ridge, Tenn., March 13-14, 1975.

Klein, P. D. "The Argonne Program for Clinical Applications of Stable Isotopes; A Model for Collaborative Interaction of a GC-MS Facility"
Conference on Clinical Applications of Mass Spectrometry, National Institutes of Health, Bethesda, Md., May 12-13, 1975.

Klein, P. D. "Instrumentation and Biomedical Applications of Stable Isotope Ratio and Selected Ion Measurements Using Chemical Ionization Mass Spectrometry"
U.S./Japan Seminar on Analytical and Biomedical Mass Spectrometry, Hakone, Japan, November 17-20, 1975.

Klein, P. D. "Instrumentation and Biomedical Applications of Stable Isotope Ratio and Selected Ion Measurements Using Chemical Ionization Mass Spectrometry"
Mass Spectrometry Symposium, Tokyo, Japan, November 21, 1975.

Klein, P. D. "$^{13}CO_2$ Breath Tests: Stable Isotopes Come of Age in Clinical Research and Diagnosis"
Symposium on the Uses of Mass Spectrometry and Stable Isotopes in Pharmacology and Medicine, Tokyo, Japan, November 25, 1975.

Klein, P. D. "Principles of Quadrupole Mass Spectrometry and the Use of Chemical Ionization Quadrupole Mass Spectrometry in the Measurement of Isotope Ratios in Organic Molecules"
Symposium on the Uses of Mass Spectrometry and Stable Isotopes in Pharmacology and Medicine, Tokyo, Japan, November 25, 1975.

Koch, A. L. "How Bacteria Face Depression, Recession, and Derepression"
1975 AUA-Argonne Distinguished Award Lecture, given at Argonne National Laboratory, Argonne, Ill., May 20, 1975.

Koch, A. L. "Does the Initiation of Chromosome Replication Regulate Cell Division?"
Regulatory Biology, Second Annual Colloquium, College of Biological Sciences, Ohio State University, Columbus, Ohio, September 4-6, 1975.

Kondo, N. S. "Proton Magnetic Resonance Studies on ApApA. The Unambiguous Assignment of Base and Anomeric Proton Signals"
19th National Meeting of the Biophysical Society, Philadelphia, Pa., February 19-21, 1975.

Krisch, R. E. "DNA Damage and Lethality from ^{125}I Decay in Wild Type and *rec*A Strains of *E. coli*"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Krisch, R. E. "Damage to the DNA of Microorganisms from Decay of Incorporated ^{125}I and the Relationship of DNA Damage to Lethal Effects"
International Conference on Molecular- and Microdistribution of Radioisotopes and Biological Consequences, Jülich, Federal Republic of Germany, October 2-4, 1975.

Kubitschek, H. E. "DNA Replication in Slowly Growing Bacteria"
Department of Microbiology and Immunology, Temple University, Pa., February 18, 1975.

Kubitschek, H. E. "D Periods In Slowly Growing Bacteria as Determined by Residual Division"
Biophysical Society Annual Meeting, Philadelphia, Pa., February 19-21, 1975.

Kubitschek, H. E. "DNA Replication during the Bacterial Cell Cycle"
Northwest Center, Indiana University School of Medicine, Gary, Ind., March 12, 1975.

Kubitschek, H. E. "UV-Induced Clustered Mutations in Bacteria"
Dept. of Biology, St. Louis University, St. Louis, Mo., November 14, 1975.

Kubitschek, H. E. "Clustered Mutations"
Dept. of Biological Sciences, University of Illinois at Chicago Circle Campus, November 27, 1975.

Ley, R. D. "Photolytic Sensitivity of DNA Labelled with (Methyl-^{14}C)-Thymidine"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Lindenbaum, A. "Bone Autoradiography with a Non-Light-Sensitive Film (Work in Progress Report)"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Lindenbaum, A. "Removal of Polymeric Plutonium from Mouse Liver by Interferon-Inducing Compounds"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-15, 1975.

Lindenbaum, A. "Retention of Polymeric Plutonium in Mouse Tissues as Affected by Anti-Viral Compounds and Their Analogs"
IAEA Seminar on Diagnosis and Treatment of Incorporated Radionuclides, Vienna, Austria, December 8-12, 1975.

Lombard, L. "Neoplasia in Laboratory Animals"
The Davis Foundation Lecture Series, University of Chicago, Chicago, Ill., March, 1975.

Lombard, L. "Hepatic Neoplasms in Mice"
Meeting of the Society of Pharmacologists and Experimental Pathologists, Bethesda, Md., May 23, 1975.

Lombard, L. "Histopathology of Lymphoreticular Tumors in Mice"
National Cancer Institute, Study Group, Bethesda, Md., June 23-27, 1975.

Lombard, L. "Lesions in Untreated B6CF$_1$ Mice"
Interagency Collaborative Group on Environmental Carcinogenesis, National Library of Medicine, Bethesda, Md., December 17, 1975.

Matsushita, T. "The Permanent Loss of Chromosome Initiation in Toluenized *Bacillus subtilis*"
Dept. of Biochemistry, University of Chicago, Chicago, Ill., January 24, 1975.

Matsushita, T. "Studies on the Initiation of Chromosome Replication in *Bacillus subtilis*"
Dept. of Biology, St. Louis University, St. Louis, Mo., February 14, 1975.

Matsushita, T. "On the Initiation of Chromosome Replication in *Bacillus subtilis*"
Dept. of Microbiology, University of Illinois Medical School, Chicago, Ill., May 5, 1975.

Matsushita, T. "Bacilli: Biochemical Genetics, Physiology and Industrial Applications"
American Society for Microbiology Conference, Cornell University, Ithaca, N.Y., August 7, 1975.

Matsushita, T. "Protein Synthesis and the Release of the Replication Terminus from the Cell Membrane in *Bacillus subtilis*"
Dept. of Biochemistry, University of Chicago, Chicago, Ill., September 19, 1975.

Matsushita, T. "Bacterial DNA Replication"
Dept. of Biology, Wheaton College, Wheaton, Ill., October 22, 1975.

McNitt, R. E. "Organelle Distributions in Geotropism of Corn Roots"
Meeting of the American Society of Plant Physiologists, Corvallis, Oreg., August 18-22, 1975.

Meinert, J. C. "Circadian Rhythms of Cyclic AMP and Glycogen in *Tetrahymena pyriformis*"
Midwest Protozoology Conference, Miami University, Oxford, Ohio, October 25, 1975.

Norris, W. P. "Interspecies Comparison of the Response of Mice and Dogs to Continuous ^{60}Co Gamma Irradiation"
IAEA International Symposium on the Biological Effects of Low-Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., November 3-7, 1975.

Norris, W. P. "Effects of Continuous ^{60}Co γ-Irradiation in Beagle Dogs"
Lawrence Livermore Laboratory, Livermore, Calif., December 3, 1975.

Panagiotopoulos, N. C. "Structures of Bence-Jones and IgG1 Immunoglobulins from One Patient"
Tenth International Congress of Crystallography, Amsterdam, The Netherlands, August 14, 1975.

Panagiotopoulos, N. C. "The Structure of Antibodies"
Nuclear Research Center "Demotritos," Athens, Greece, September 11, 1975.

Peraino, C. "Circadian Rhythm in the Regulation of Ornithine Aminotransferase (OAT) and Serine Dehydratase (SDH) Synthesis in Rat Liver *In Vivo*"
XII International Conference of the International Society for Chronobiology, Washington, D.C., August 10-13, 1975.

Peraino, C. "Immunochemical Analysis of Serine Dehydratase and Ornithine Aminotransferase Regulation in Rat Liver *In Vivo*"
Conference on Vitamin B_6 and its Functions in Enzymes, Iowa State University, Ames, Iowa, October 10-11, 1975.

Polk, P. "Application of a Biology Major in Radiation Research"
St. Edward's University, Austin, Texas, April 1, 1975.

Rahman, Y. E. "Functions of Lysosomes"
St. Louis University, St. Louis, Mo., April 7, 1975.

Rahman, Y. E. "Lysosomes, Membranes, and Aging"
St. Louis University, St. Louis, Mo., April 7, 1975.

Rahman, Y. E. "Drug Delivery by Liposome Encapsulation"
Lawrence Livermore Laboratory, Livermore, Calif., July 15, 1975.

Rahman, Y. E. "Liposome-Encapsulated Actinomycin D"
Biomedical Inventions Seminar at NIH, Bethesda, Md., November 7, 1975.

Reilly, C. A., Jr. "*In Vivo* Interference of Virus-Induced Osteosarcomas by a Benign Bone Tumor Virus"
VIIth International Symposium on Comparative Leukemia and Related Diseases, Copenhagen, Denmark, October 13-17, 1975.

Reilly, C. A., Jr. "Viral Interference with Bone Tumor Induction"
Mayo Clinic, St. Paul, Minn., November 25, 1975.

Russell, J. J. "A One-Year Autoradiographic Study of Polymeric ^{239}Pu in Dog Liver"
20th Annual Meeting of the Health Physics Society, Buffalo, N.Y., July 13-17, 1975.

Sacher, G. A. "Evaluation of Long-Term Environmental Impacts on Populations: An Unsolved Biological Problem"
American Association for the Advancement of Science Symposium, "Are There Thresholds in the Effects of Pollutants on Health?," New York, N.Y., January 30, 1975.

Sacher, G. A. "Age-Dependence of Number and Size of Optic and Vagus Nerve Fibers in Two Rodent Species"
10th International Congress of Gerontology, Jerusalem, Israel, June 22-27, 1975.

Sacher, G. A. "The Metabolic Constraint on Mammalian Longevity: Can It Be Lifted?"
Kansas City V. A. Hospital, Kansas City, Kans., April 15, 1975.

Sacher, G. A. "Dose, Dose Rate, Quality and Host Factors for Radiation Life Shortening: Implications for the Molecular Lesion"
Conference on Protein Adducts in DNA, Williamsburg, Va., May 2-6, 1975.

Sacher, G. A. "Body Temperature, Metabolic Rate, and Longevity of Vertebrates"
Symposium in Honor of Dr. C. L. Prosser, University of Illinois, Urbana, Ill., May 16-17, 1975.

Sacher, G. A. "Age-Dependence of Resting, Average, and Maximum Oxygen Consumption in *Mus* and *Peromyscus*"
Twenty-eighth Annual Meeting of the Gerontological Society, Louisville, Ky., October 26-30, 1975.

Sacher, G. A. "Life Tables of Small Rodent Species: Evolutionary Factors"
Queen's University, Kingston, Ontario, Canada, November 23, 1975.

Schiffer, M. "Comparison of κ and λ Bence-Jones Protein Structures"
Homogeneous Immunoglobulin Workshop VI, National Institutes of Health, Bethesda, Md., March 10-12, 1975.

Schiffer, M. "Crystallography of a Bence-Jones Protein"
Gordon Research Conference on Proteins, New Hampton, N.H., June 15-20, 1975.

Schiffer, M. "Conformational Differences in Light Chains"
Gordon Research Conference on Immunochemistry and Immunobiology, Andover, N.H., August 11-15, 1975.

Schlenk, F. "Some Aspects of Yeast Enzymology"
Dept. of Biochemistry, University of Chicago, Chicago, Ill., November 5, 1975.

Schoeller, D. A. "$^{13}CO_2$ Breath Tests in Clinical Chemistry"
National Bureau of Standards, Washington, D.C., August 29, 1975.

Schoeller, D. A. "Clinical $^{13}CO_2$ Breath Tests: Methodology and Limitations"
Second International Conference on Stable Isotopes, Oakbrook, Ill., October 21, 1975.

Schoeller, D. A. "Statistical Considerations Involved in the Treatment of Isotope Dilution Data"
Second International Conference on Stable Isotopes, Oakbrook, Ill., October 21, 1975.

Seed, T. M. "Mechanisms of Red Cell Destruction"
Dept. of Microbiology, Ohio State University, Columbus, Ohio, November 6, 1975.

Seed, T. M. "Membrane Structure of Pathogenic Protozoa"
Dept. of Microbiology, Ohio State University, Columbus, Ohio, November 6, 1975.

Shen-Miller, J. "Geotropism and Hormonal Effects on Plant Organelles"
Dept. of Biological Sciences, University of Illinois at Chicago Circle Campus, January 7, 1975.

Shen-Miller, J. "Geotropism and Hormonal Effects on Plant Organelles"
Symposium on Gravity and the Cell, NASA Ames Research Center, Moffett Field, Calif., March 20, 1975.

Simkins, R. C. "Gram-Staining Microorganisms"
Mayslake Seminary, Mayslake, Ill., November 1, 1975.

Sinclair, W. K. "The Effect of N-Ethylmaleimide on the Response to X-Rays of Synchronized HeLa Cells"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Stearner, S. P. "Late Injury to the Microvasculature after Neutron or Gamma Irradiation"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Vigneulle, R. M. "Repopulation Kinetics for CFU Derived from Aged, Irradiated Survivors of 780 R ^{60}Co Gamma Radiation"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

Webb, R. B. "Mutagenesis"
Dept. of Pharmacology, University of Illinois at the Medical Center, Chicago, Ill., March 4, 1975.

Webb, R. B. "Role of Pyrimidine Dimers and Damage to Repair Systems in Near-UV Lethality"
3rd Annual Meeting of the American Society for Photobiology, Louisville, Ky., June 23-27, 1975.

Winston, S. "The Permanent Loss of Chromosome Initiation in Toluene-Treated *Bacillus subtilis* Cells"
Student A. I. B. S. National Conference, Current Frontiers in Biology and Medicine, Philadelphia, Pa., March 22, 1975.

Yang, V. V. "The Fine Structure of the Irradiated Heart. Comparison of Fission Neutrons and ^{60}Co γ-Ray Effects in the Mouse"
23rd Annual Meeting of the Radiation Research Society, Miami Beach, Fla., May 11-18, 1975.

16. SEMINARS DURING 1975

During the first half of 1975, the Division of Biological and Medical Research Seminar Committee consisted of Drs. S. S. Danyluk, R. N. Feinstein, T. Matsushita, C. A. Reilly, and E. J. Ainsworth, Chairman. For the 1975-1976 seminars, the Committee consisted of Drs. M. H. Bhattacharyya, P. C. Brennan, R. D. Ley, C. A. Reilly, and T. Matsushita, Chairman.

The Seminar Program for 1975, selected by the Committee on the basis of recommendations from the staff, was as follows:

Dr. Douglas J. Pritchard, Mayo Clinic
"Clinical and Laboratory Investigations in Osteosarcoma"
January 16, 1975

Dr. Hugh H. Fudenberg, University of South Carolina Medical School
"Use of Tumor Specific Transfer Factor in Human Osteosarcoma"
January 29, 1975

Dr. Robert D. Phemister, Colorado State University
"Prenatal and Neonatal Radiation Injury in Dogs"
January 30, 1975

Dr. Alison P. Casarett, Cornell University
"Radiation Effects on Preimplantation Mouse Embryos *In Vitro*"
February 6, 1975

Dr. Lolita Daneo-Moore, Temple University School of Medicine
"Regulation of Cell Surface Growth and Division in a Streptococcus"
February 13, 1975

Dr. E. F. Riley, University of Iowa
"Recovery in Lens Epithelium During First 4 Weeks After Fission Neutron or X-Irradiation"
February 28, 1975

Dr. Arthur T. Winfree, Purdue University
"Unclocklike Behavior of a Biological Clock"
March 6, 1975

Dr. Maria de Sousa, University of Glasgow, Scotland
"Ecotaxis: Conception, Growth and Delivery of a Concept"
March 13, 1975

Dr. John D. Fernstrom, Massachusetts Institute of Technology
"The Influence of Food Ingestion on Brain Monoamine Synthesis"
March 20, 1975

Dr. Rainer Storb, University of Washington
"Experimental and Clinical Marrow Transplantation: The Seattle Experience"
March 27, 1975

Dr. William Regelson, Medical College of Virginia
"Host Resistance to Tumor Growth: Synthetic Polyanions and Other Compounds"
April 3, 1975

Dr. Nechama Haran-Ghera, The Weizmann Institute of Science, Israel
"Tumor Cell Host Relationship in Murine Experimental Leukemogenesis"
April 11, 1975

Dr. William H. Knospe, Presbyterian-St. Luke's Hospital
"Interrelationships Between Bone Marrow Stroma and Hematopoietic Cellular Proliferation"
April 17, 1975

Dr. Timothy E. O'Connor, National Cancer Institute
"Studies on Anti-Viral and Anti-Tumor Antibiotics"
April 22, 1975

Dr. Lionel Cohen, Michael Reese Medical Center
"Derivation of Cell Population Kinetic Parameters from Radiobiological Data"
April 24, 1975

Dr. A. C. Leopold, Purdue University
"Effects of Inorganic Solutes on the Actions of Plant Hormones"
April 29, 1975

Dr. R. C. Adelman, Temple University
"Impaired Hormonal Regulation of Enzyme Activity During Aging"
May 1, 1975

Dr. L. S. Lerman, Vanderbilt University
"DNA in the Compact State: Psi and Crystals"
May 6, 1975

Dr. H. S. van den Brenk, St. Thomas' Hospital, London, England
"Stimulation of Tumor Growth in States of Topical and Systemic Stress: Possible Common Mechanisms of Action"
May 22, 1975

Dr. Charles R. Shaw, M. D. Anderson Hospital and Tumor Institute, Houston, Texas
"Variation in Carcinogen Metabolism"
May 29, 1975

Dr. Andrew F. Stehney, Radiological and Environmental Research Division, Argonne National Laboratory
"Present Status of the Radium Studies of the Center for Human Radiobiology"
June 5, 1975

Dr. J. N. Stannard, University of Rochester School of Medicine
"Some Dilemmas, Mostly Scientific, in the Setting of Radiation Protection Standards"
June 12, 1975

Dr. Rex M. Tyrrell, Instituto de Biofisica, Brazil
"The Interaction Between Near-UV and Ionizing Radiation - Basis of a Tentative Model for Near-UV Lethality in Bacteria"
June 19, 1975

Dr. Edward Balish, University of Wisconsin
"The Germfree Animal: Some Examples of Its Research Applications"
July 1, 1975

Dr. Peter D. Klein, Division of Biological and Medical Research, Argonne National Laboratory
"$^{13}CO_2$ Breath Tests: Stable Isotopes Come of Age in Clinical Diagnosis"
September 18, 1975

Dr. William G. Quinn, Princeton University
"Genetic Studies of Learning in *Drosophila*"
October 16, 1975

Prof. H. F. DeLuca, University of Wisconsin
"The Vitamin D Endocrine System"
October 23, 1975

Dr. Phillip Y. Paterson, Northwestern University
"Viruses and Autoimmunity"
October 30, 1975

Prof. Alan Solomon, University of Tennessee
"Bence Jones Proteins and Light Chains of Immunoglobulins: Biochemical and Clinical Significance"
November 6, 1975

Dr. Albrecht M. Kellerer, Inst. für Med. Strahlenkunde, Wurzburg, Germany
"Problems in Radiation Carcinogenesis"
November 14, 1975

Dr. Norman G. Anderson, South Carolina Cancer Institute, Medical University of South Carolina
"Tumor Auto-Antigens"
November 20, 1975

Dr. T. Makinodan, Gerontological Research Center, Baltimore, Maryland
"Immunology of Aging"
November 26, 1975

Dr. R. E. Krisch, Division of Biological and Medical Research, Argonne National Laboratory
"DNA Breakage, Repair and Lethality Accompanying ^{125}I Decay in Microorganisms"
December 4, 1975

Dr. Robert Haselkorn, University of Chicago
"A Universal Procaryotic DNA Binding Protein"
December 11, 1975

Dr. Emerson W. Chan, Institute of Cancer Research, College of Physicians and Surgeons of Columbia University, Francis Delafield Hospital, New York
"Characterization of a Candidate Human Virus from Cultured Acute Myelogenous Leukemic Cells"
December 15, 1975

Dr. Otto G. Raabe, Inhalation Toxicology Research Institute, Albuquerque, New Mexico
"Respiratory Tract Deposition Models"
December 16, 1975

In addition, several informal seminars in specialized subjects were held during the year:

Dr. C. Cohen, University of Illinois
"A Model for the Origin of Antigen Change in Tumor Cells"
January 7, 1975

Dr. Arthur Koch, Indiana University Bloomington
"Ponderings on Past Procaryote Progress in Polypeticle Performance"
January 15, 1975

Dr. Ronald Doyle, Division of Biological and Medical Research, Argonne National Laboratory
"Chemical and Biological Properties of Concanavalin A"
January 22, 1975

Dr. Mortimer M. Elkind, Division of Biological and Medical Research, Argonne National Laboratory
"Spurious Photolytic Sensitivity of DNA"
January 29, 1975

Dr. Douglas Grahn, Division of Biological and Medical Research, Argonne National Laboratory
"Hot Particles, Facts and Fancies"
February 4, 1975

Dr. Tatsuo Matsushita, Division of Biological and Medical Research, Argonne National Laboratory
"The Initiation of Chromosome Replication in *Bacillus subtilis*"
February 5, 1975

Dr. L. Daneo-Moore, Temple University Medical School
"The Regulation of Macromolecular Composition in Cells Limited in Growth Rate by Nutritional and Osmotic Factors"
February 12, 1975

Dr. Warren Porter, University of Wisconsin
"Environmental Constraints on Predator-Prey Interactions"
February 13, 1975

Dr. Herbert E. Kubitschek, Division of Biological and Medical Research, Argonne National Laboratory
"DNA Unwinding Repair and Replication: Report on the Biophysical Society Meeting"
February 26, 1975

Dr. Bobby Scott, University of Illinois, Urbana
"A Mechanistic State Vector Model for the Interaction of Ionizing Radiation with Cells"
March 4, 1975

Dr. Robert E. Krisch, Division of Biological and Medical Research, Argonne National Laboratory
"Does Ultrafast (Type I) Repair of Single-Strand Breaks in DNA Really Occur?"
March 5, 1975

Dr. R. H. Pritchard, University of Leicester, England
"Control of DNA Synthesis in *Escherichia coli*"
March 11, 1975

Dr. Paul T. Cunningham, Chemical Engineering Division, Argonne National Laboratory
"Chemistry of Airborne Particulates"
March 17, 1975

Dr. Arthur Koch, Indiana University, Bloomington
"Does a Chromosome Always Initiate at the Appointed Time?"
March 19, 1975

Dr. James C. Copeland, Ohio State University
"Transformation Experiments with *Bacillus subtilis*"
March 24, 1975

Dr. Michael Chandler, University of Geneva, Switzerland
"Control of F Factor Replication"
March 26, 1975

Dr. Tatsuo Matsushita, Division of Biological and Medical Research, Argonne National Laboratory
"ICN-UCLA Winter Conference on the Regulation of DNA Synthesis"
April 1, 1975

Dr. M. Horisberger, Nestle Products, Switzerland
"Surface Structure of *Streptococcus faecalis*"
April 9, 1975

Dr. John Marshall, Radiological and Environmental Research Division, Argonne National Laboratory
"The Latent Period in Tumorigenesis"
April 15, 1975

Dr. M. G. Sargent, National Institute for Medical Research, England
"Cell Growth and Division in *Bacillus subtilis*"
April 16, 1975

Dr. Paul H. M. Lohman, Medical-Biological TNO, The Netherlands
"DNA Repair in Eukaryotic Cells"
April 17, 1975

Dr. Bruce G. Adams, University of Hawaii
"Role of GA1 3 Gene Locus Product in Carbohydrate Catabolism in Yeast?"
April 25, 1975

Dr. Wolfie Traub, Weizmann Institute of Science, Israel
"X-Ray Studies of Protein De- and Renaturation"
April 30, 1975

Dr. Robert Webb, Division of Biological and Medical Research, Argonne National Laboratory
"Dimers, Repair and Near-UV Lethality"
May 7, 1975

Dr. Richard Bockrath, Indiana University School of Medicine
"Specificity of Mutagenesis by UV Radiation"
May 14, 1975

Dr. Elizabeth Hamilton, Imperial Cancer Research Fund Laboratory, England
"Studies on Stem Cell Ageing *In Vivo* and *In Vitro*"
May 27, 1975

Dr. Richard Cutler, University of Texas
"Fixed Bacterial Cultures"
June 11, 1975

Dr. J. Hurley Myers, Southern Illinois University
"Microvascular Studies in Skeletal Muscle - Techniques for Study of Vascular Reactivity"
June 26, 1975

Mr. Scott Winston, University of Colorado
"The Regulation of Chromosomal Origin and Terminus-Membrane Attachment in *Bacillus subtilis*"
July 31, 1975

Dr. Hans Schrieber, University of Chicago
"Immunological Manipulation of *In Vitro* Growth of Plasmacytoma Cells"
August 20, 1975

Dr. J. R. Hippensteele, Illinois Wesleyan University
"Investigations of the Microvasculature of Rat Skeletal Muscles"
September 3, 1975

Dr. Robert E. Krisch, Division of Biological and Medical Research, Argonne National Laboratory
"DNA Breakage, Repair, and Lethality Following ^{125}I Decay in Microorganisms"
September 24, 1975

Dr. Tatsuo Matsushita, Division of Biological and Medical Research, Argonne National Laboratory
"Tryptophan Photoproducts and DNA Polymerase I Activity"
October 1, 1975

Dr. Gerald Cohn, Illinois Institute of Technology
"Photodynamic Effects in Yeast:
October 28, 1975

Dr. Arne Luz, Institute for Biology, Munich, W. Germany
"Experimental Studies with Short-Lived Bone-Seeking Radionuclides"
October 31, 1975

Dr. Herbert E. Kubitschek, Division of Biological and Medical Research, Argonne National Laboratory
"UV-Induced Clustered Mutations"
November 5, 1975

Dr. S. G. Sligar, University of Illinois
"Electron Transport and Mixed Function Oxidation by a *Pseudomonas* Mono-Oxygenase System"
November 19, 1975

Dr. Deepak Bastia, Yale University
"Isolation and Nucleotide Sequence Analysis of the Origin of DNA Replication of Col E1"
November 24, 1975

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17. PUBLICATIONS APPEARING IN CALENDAR YEAR 1975

JOURNAL ARTICLES

Balistreri, W. F., A. E. Cowen, A. F. Hofmann, P. A. Szczepanik, and P. D. Klein. Validation of use of 11,12-^{2}H-labeled chenodeoxycholic acid in isotope dilution measurements of bile acid kinetics in man. Pediat. Res. 9, 757-760 (1975).

Ben-Hur, E., and M. M. Elkind. Thermal sensitization of Chinese hamster cells to methyl methanesulfonate: Relation of DNA damage and repair to survival response. Cancer Biochem. Biophys. 1, 23-32 (1974).

Borak, T. B. A simple approach to calculating gamma ray SKYSHINE for reduced shielding applications. Health Phys. 29, 423-425 (1975).

Brennan, P. C., and B. N. Jaroslow. Age-associated decline in theta antigen on spleen thymus-derived lymphocytes of B6CF$_1$ mice. Cell. Immunol. 15, 51-56 (1975).

Bryant, W. F., and P. D. Klein. *N*-methylation of purines and pyrimidines. Anal. Biochem. 65, 73-78 (1975).

Cairnie, A. B., D. Grahn, H. B. Rayburn, F. S. Williamson, and R. J. Brown. Teratogenic and embryolethal effects in mice of fission-spectrum neutrons and gamma-rays. Teratology 10, 133-140 (1974).

Davies, D. B., and S. S. Danyluk. Nuclear magnetic resonance studies of 2'-and 3'-ribonucleotide structures in solution. Biochemistry 14, 543-553 (1975).

Edmundson, A. B., K. R. Ely, E. E. Abola, M. Schiffer, and N. Panagiotopoulos. Rotational allomerism and divergent evolution of domains in immunoglobulin light chains. Biochemistry 14, 3953-3961 (1975).

Ehret, C. F., and V. R. Potter. Circadian chronotypic induction of tyrosine aminotransferase and depletion of glycogen by theophylline in the rat. Int. J. Chronobiol. 2, 321-326 (1974).

Ehret, C. F., V. R. Potter, and K. W. Dobra. Chronotypic action of theophylline and of pentobarbital as circadian zeitgebers in the rat. Science 188, 1212-1215 (1975).

Epp, O., E. E. Lattman, M. Schiffer, R. Huber, and W. Palm. The molecular structure of a dimer composed of the variable portions of the Bence-Jones protein REI refined at 2.0-Å resolution. Biochemistry 14, 4943-4952 (1975).

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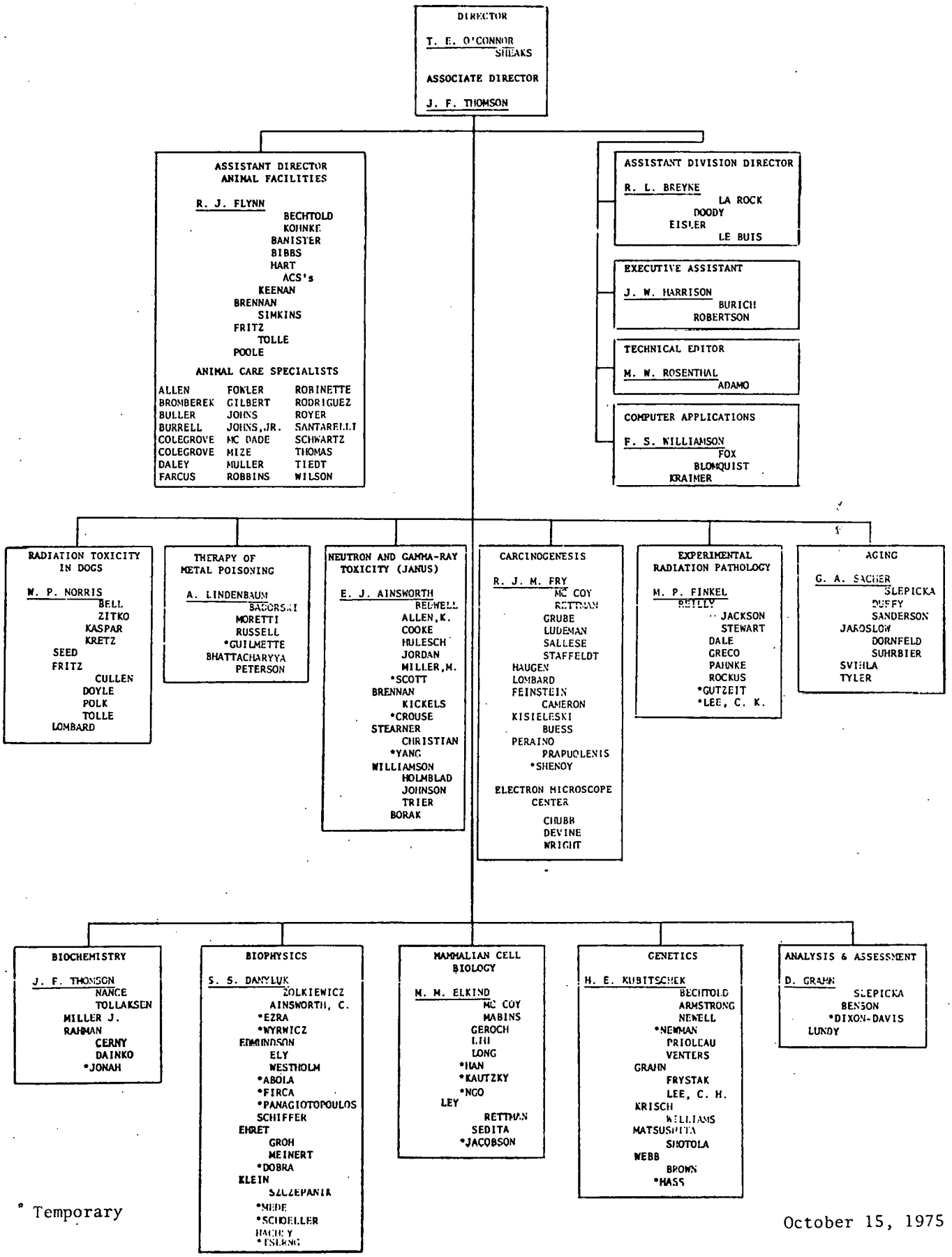

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