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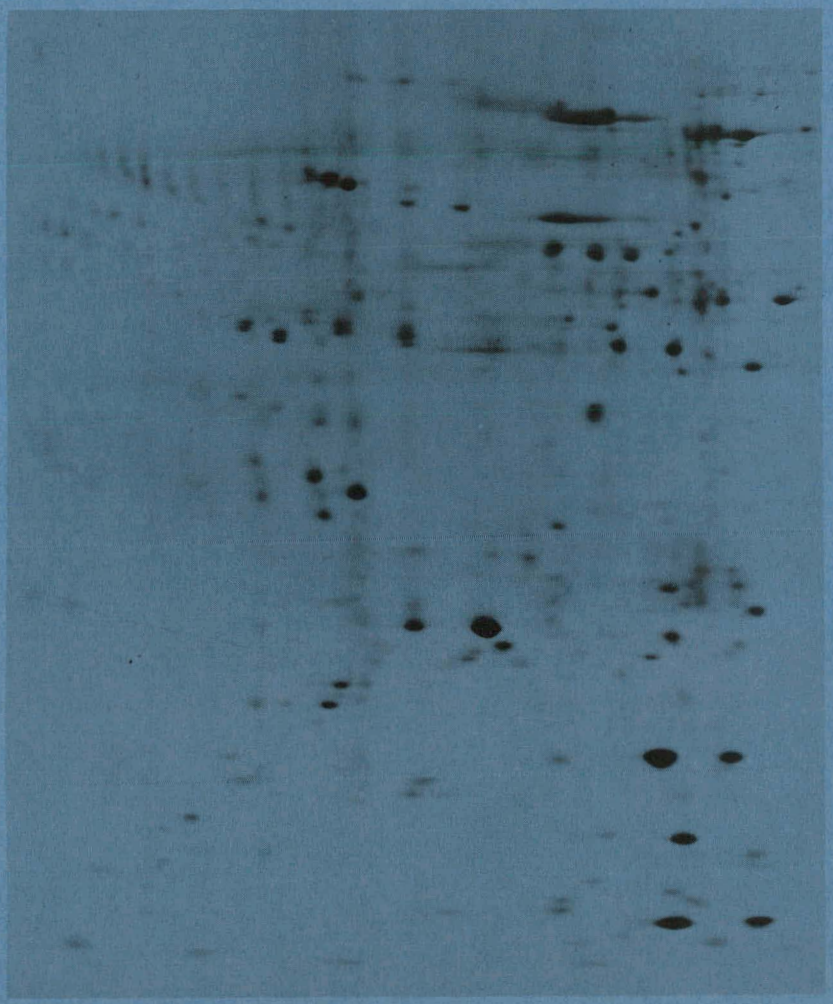
DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

Annual Report 1977



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DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

ANNUAL REPORT 1977

Douglas Grahn, Director
Steven S. Danyluk, Associate Director
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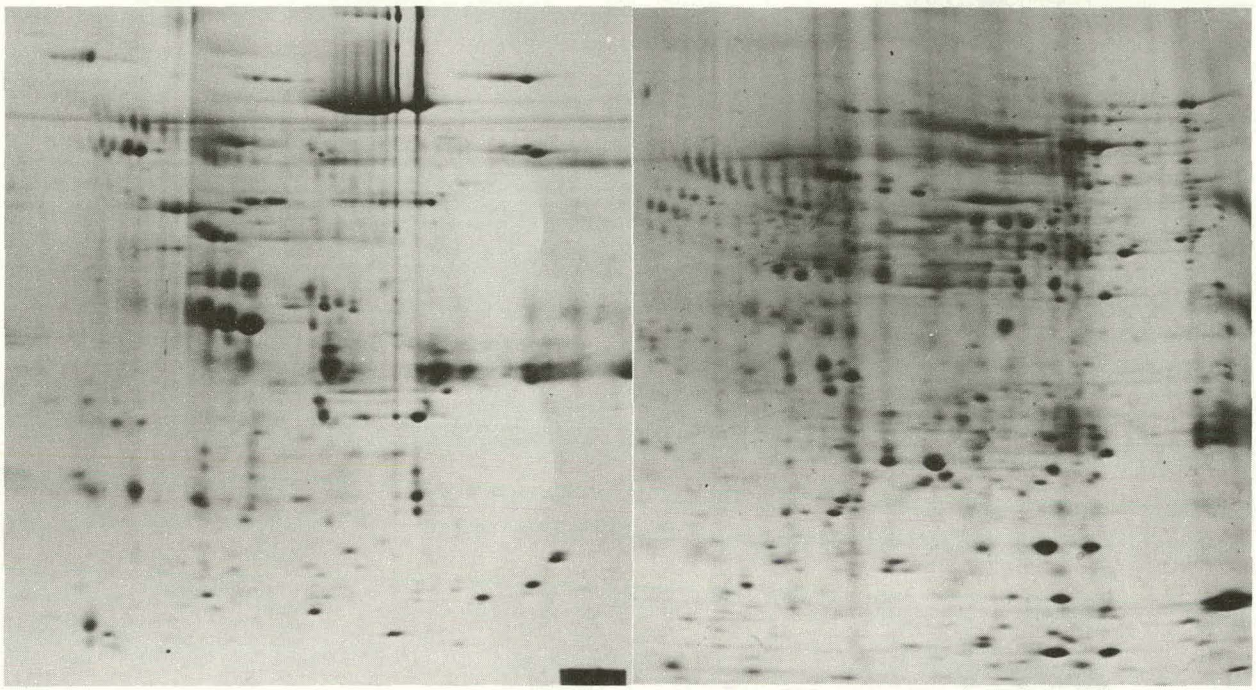
Marcia W. Rosenthal, Editor

Preceding Report
ANL-77-55, December 1976



COVER: Part of a two-dimensional electrophoresis pattern of the urinary proteins from a patient with cancer of the bladder, prepared by the Molecular Anatomy Program. For comparison, the complete patterns are shown from the patient (right) and from a normal individual (left). The normal pattern is reproducible, but small differences are found among individuals. Each spot represents a polypeptide chain which may either be a complete protein gene product or part of one. Position on the pattern is a function of isoelectric point and molecular weight.

To assign each spot an identifying number and to study its appearance or disappearance in cancer or after exposure to energy-related pollutants, it is necessary to have internal standards for position on the pattern. Quantitation must also be feasible. To intercompare large numbers of patterns from human populations, computerized image analysis and data reduction are also needed. The Molecular Anatomy Program is developing solutions to all of these problems.

Such two-dimensional mapping of urinary proteins, illustrated here, is a noninvasive technique useful for identification of mutagenesis, carcinogenesis, and the toxicology of energy related pollutants in man. (See Section 5.)

TABLE OF CONTENTS

1. INTRODUCTION

Douglas Grahn, Division Director

The Division continues to address a wide range of biomedical topics; the brief introductions to the separate chapters give the reader a sense of the objectives and accomplishments for the diverse subjects under investigation. Within this diversity, the Division is steadily devoting more of its energies into what can be termed modern environmental toxicology. The three-point foundation of this effort incorporates studies in mutagenesis, carcinogenesis, and cytotoxicity. The activities in environmental toxicology therefore arise from our basic research, primarily in the molecular and cellular aspects of biological systems.

Concurrently, the large-scale radiation toxicity programs have undergone some curtailment; the net effect has been to sharpen the focus on their principal objective, the collection of data that will have predictive value for the assessment of low-level radiation hazards to man.

The Molecular Anatomy Program and the Bioanalytical Center, which involve extensive collaborative relationships with other institutions and universities, bring the Division an awareness of the other end of biological problems: the manifestations and diagnosis of human disease.

Although this report is primarily concerned with the scientific progress of the Division in 1977, it is appropriate to discuss some of the new research areas that have developed in the first months of 1978. One of these, the program in Toxicity of Energy Storage Systems, is principally concerned with the health hazards associated with the increased use of novel battery systems for both electric vehicles and utility load leveling. This is a cooperative venture with the Division of Environmental Impact Studies. The environmental and health effects assessment phase of the project has reached the point were specific research programs are being formulated to answer questions pertaining to the biological effects of chemicals involved in manufacture, use, and disposal of these batteries.

Another new program, the Biomedical Effects of Energy Transmission, addresses the potential biological hazards of extremely low frequency energy transmission systems. Construction of exposure facilities is nearly complete, dosimetric measurements are

under way, and the initial biological studies, which employ a number of circadian behavioral and neurophysiological end points, have begun.

The Toxicity Program for Coal Combustion and Conversion Effluents, first identified in last year's annual report, continues to progress in the areas of instrumentation, chemical and physical characterization, and biological studies pertaining to effluents from fluidized bed combustors. In addition, the study of effluents associated with the conversion of coal to high-BTU gas has emerged as a significant commitment. We are fortunately able to obtain direct process stream and effluent samples for chemical and biological analysis for both technologies.

Some organizational changes within the Division may be noted. On May 1, 1978, Douglas Grahn was appointed Director, succeeding Warren K. Sinclair who had served as Acting Director since November 22, 1977. Donald D. Grube became Acting Executive Assistant on April 1, 1978.

In order to deal more efffectively with the need to develop and execute new programs relating to the health effects of different energy technologies, a reorganization of the Division has taken place (Figure 1.1).

Finally, two comments on the organization and logistics of this report are in order. First, the sections within the report represent areas of scientific interest, rather than a specific Divisional group structure. Thus, the personnel listed at the beginning of each section are the Divisional staff and temporary appointees whose work is represented in the section. Second, the publications listed at the end of the report (Section 18) are the articles, reports, and abstracts from the entire Division during 1977. The publication lists following the individual sections are designed for readers interested in specific topics. These lists cover only the pertinent subject matter and are expanded to include all publications appearing or accepted for publication from January 1977 through July 1978; abstracts are not included.

Douglas Grahn
Director
Division of Biological
and Medical Research

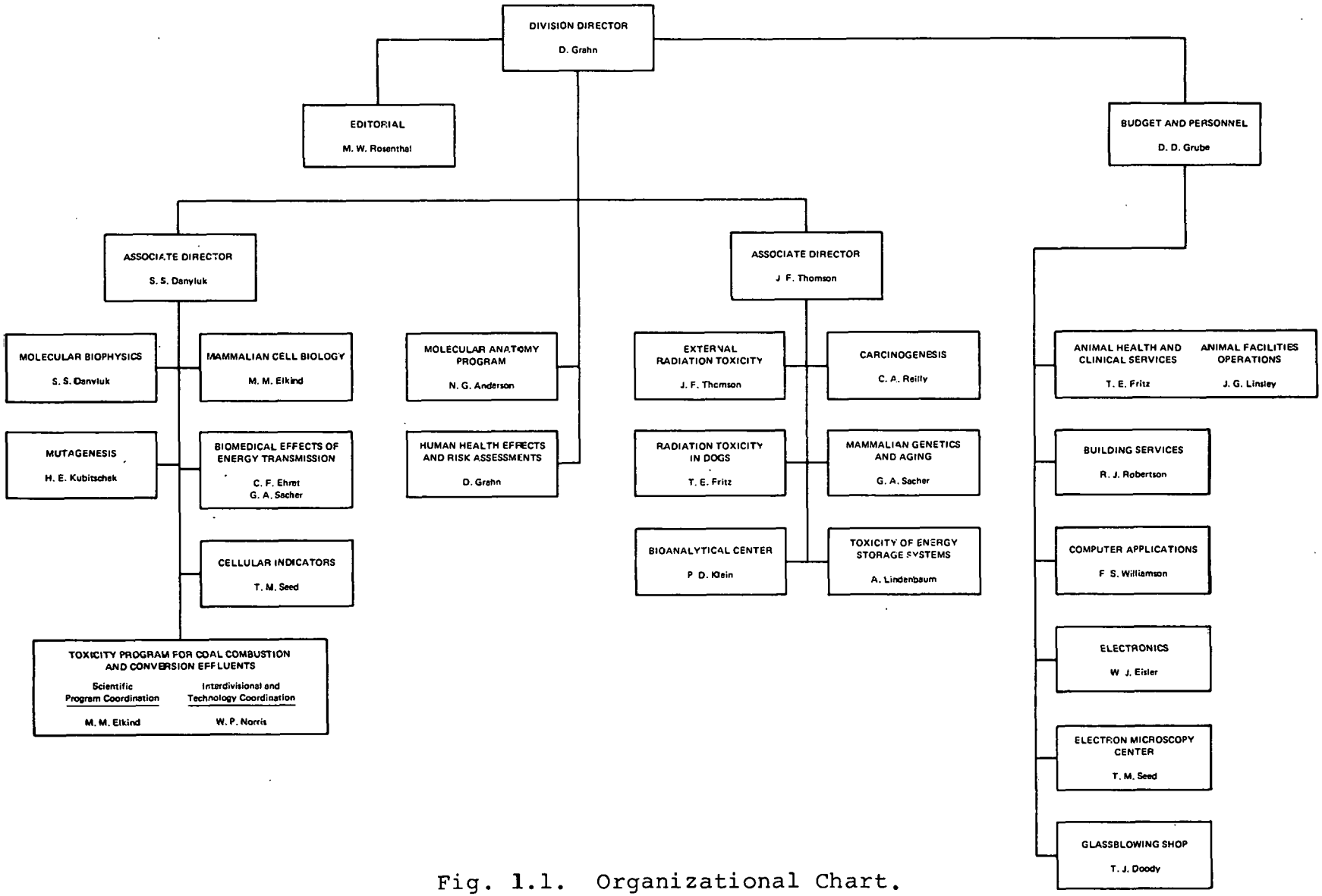


Fig. 1.1. Organizational Chart.

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2. ENERGY PRODUCTION AND HUMAN HEALTH

Jane R. Benson (Scientific Assistant)
Charles D. Brown (Scientific Assistant)
Diana K. Dixon-Davis (Scientific Assistant)
Douglas Grahn (Senior Biologist)
Robert T. Lundy (Assistant Biologist)

The prevention of undue risks to the public health and safety from expanding uses of existing energy technologies and from the development of new technologies requires an assessment of the potential health effects at all phases of the various fuel cycles. This project provides an analytical approach to the analysis of potential health effects, their production under different economic or resource utilization situations, and an appraisal of positive or negative health effects and population changes that are concomitant with change in the energy resource base.

The program has three objectives, which are being pursued concurrently:

1) The baseline mortality structure of populations is being defined in terms of the relationships between observed mortality and the social, economic, demographic, and cultural factors.

2) Demographically and actuarially correct models of the relationships between mortality and various risk factors are being developed to provide reliable measures of health-effects variables and an analytical framework into which studies under objectives (1) and (3) will fit.

3) Assessment of the health risks associated with all phases of the coal-fuel cycle, from extraction to combustion, are under development.

During the past year, the group has also responded to short-term requirements for health assessments, for program inventory and development planning, and for new program proposals. A portion of the work under objective (1) is funded by the U. S. Nuclear Regulatory Commission and is therefore dedicated to establishing a broadly utilitarian computerized approach to the assessment of potential human health risks in populations in the vicinity of nuclear power stations.

Highlights of recent progress toward these objectives include the following findings. Initial analyses of the effect of socioeconomic factors on life expectancy and age-adjusted total death rate indicate that between 35% and 50% of their total variance can be attributed to economic and cultural factors routinely annotated by the U. S. Bureau of the Census. There are also significant regional differences in the U. S. that must be considered in the analysis of variation in death rate.

A new analytical model has been developed to analyze the expected influence on human health from an increased use of coal as a primary energy source. This model employs the demonstrated quantitative effect of cigarette smoking on human health (total mortality) as a proxy for air pollution exposure history and relates air quality measures to cigarette equivalents. The resulting dose-response model, when applied with DEMPAK (the BIM/ANL mortality projection model), gives projections of excess mortality that are both age- and sex-specific and also include latency. These methods have been applied to several health effects assessments and are a demonstrable improvement over other "single factor" approaches.

DEVELOPMENT AND EVALUATION OF SOCIOECONOMIC AND DEMOGRAPHIC FACTORS

D. K. Dixon-Davis, J. R. Benson, and P. M. Fuja[1]

Fifteen sites, each centered on an energy production facility and with a total of 191 counties, are being studied for their applicability to the problem of relating socioeconomic variables with human health and energy production. The selected sites sample the full spectrum of geographic, economic, and social variables in the U. S. and represent the present full complement of electric power generation modes (gas, oil, coal, nuclear, and hydro).

An initial multivariate regression analysis of the relationships between socioeconomic and cultural characteristics and mortality among the white population reveals that socioeconomic factors could account for up to half of the variance in the mortality measures selected. These measures were expectation of life at birth, expectation of life at age 10, age-standardized death rate, and infant mortality rate. Male mortality was more strongly and significatly related to income, education, occupation, ethnicity, and other social variables than female mortality. Additionally, those variables that related best to adult mortality did not necessarily relate to infant mortality. Forward selection multivariate regression analyses were applied to 39 selected socioeconomic variables. The amount of explained variance for all four mortality measures, using the best 10 variables, ranged from 26.7% to 55.0% (mean = 36.6%), and using the best 20 variables, from 35.6% to 73.2% (mean = 46.0%). Using dummy variables to substitute for unmeasured site (regional) variations in socioeconomic characteristics increased explained variance by an average of only 4.8%. The use of population-weighted data in the regression increased explained variance in every instance, by an average of 16.4%. An examination of the residual variances around the regressions showed the smallest residuals in the east coast counties that have the largest populations. This level of explained variance in widely varying mortality data indicates that mortality can be standardized for variation in the common socioeconomic factors tabulated by the U. S. Census Bureau.

An information summary of the factors influencing human mortality has been prepared from the available literature. Over two dozen cultural, social, personal, and environmental factors were reviewed. In addition to a qualitative summary, an initial quantitative summary was derived for those factors known to influence either infant (0-1 year old) or adult (ages 25-64) mortality.

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[1]Energy and Environmental Systems Division.

The three most important factors affecting infant mortality are birth weight, maternal age, and parents' socioeconomic status. Infant mortality has a parabolic relation with both birth weight and maternal age. The optimum childbearing interval is 25-29 years of age, and the infant death rate is lowest for birth weights of 3.5-4.0 kg. Parents' socioeconomic status is negatively associated with infant mortality.

As a general rule, factors that improve an adult's financial ability to cope with illness usually have an inverse relationship with mortality. For example, as the number of years of educational attainment increases, there is a significant reduction in the total mortality ratio (observed number of deaths in each education group/age-adjusted expected number of deaths in each education group). Significant regressions have also been seen for a number of cancer sites and for two forms of heart disease. In addition, strongly significant relationships occur with occupational status; each class interval increase in status (laborers low, professionals high) decreases the mortality ratio. Perhaps indirectly related to the financial ability to cope are the financial and emotional benefits of marital status. With few exceptions, within each age cohort married persons have the lowest death rates of all marital groups--married, single, widowed, and divorced.

Most of the remaining factors influencing mortality relate to the probability of exposure to the etiologic agent(s) of the disease process(es) leading to death, either as a result of the workplace environment, life-style, or place of residence. Many occupations are known to have very high death rates for specific causes, e.g., the mortality rate for respiratory diseases among coal miners is fivefold higher than the U. S. average.

Personal habits and life-style strongly influence mortality rates and causes of death. Lung, kidney, and bladder cancer death rates are highly correlated with cigarette smoking ($r = 0.43$, 0.49, and 0.76, respectively). The positive regressions of gastrointestinal tract cancer death rates on alcohol consumption are significant. Differences in personal habits are also reflected in the low cancer and heart disease death rates for certain religious groups (Mormon and Seventh-Day Adventist).

Regarding place of residence, there is a significant rise in the mortality rate as the degree of urbanization increases (total mortality rate/10^5 increases 0.14 ± 0.03 for each percentage increase in urbanization). Skin cancer is the only cause of death with a true geographic influence; rates are higher in the southern states where the ultraviolet irradiation is greatest. In most other comparisons, region seems to be a surrogate for other factors, such as educational attainment, industrial mix, income levels, etc.

Finally, there are many recognized toxic agents that are locally or regionally distributed via diverse environmental pathways. Categorically, both airborne and waterborne pollutants have been statistically and, on occasion, clinically associated with increased levels of morbidity and mortality. Death rates from all causes have been increased as have rates for many specified causes, e.g., lung cancer, bladder cancer, chronic lung disease, and cardiovascular disease. The results of some of these studies have been applied to the development of the air pollution-health effects model discussed below.

QUANTITATIVE ASPECTS OF THE IMPACTS OF ENERGY-RELATED EFFLUENTS ON HUMAN HEALTH

R. T. Lundy, C. D. Brown, and D. Grahn

Because all energy systems release effluents in concentrations well below the level of acute toxicity for most people, the effects may not be apparent for several years. Thus it is particularly important to consider the factors of age, sex, and duration of exposure in determining the observable effects, and it is necessary to incorporate these factors into any calculation of health effects. Early approaches to prediction of health effects of energy production by others used only a single dose-response coefficient and applied it to the total population without regard for latency periods or temporal variations in the numbers of persons at risk.

We have developed a system of general demographic models (DEMPAK) for projecting number of deaths and population size by sex, age, and cause of death forward through time for any defined initial population and set of vital rates. The expected change in mortality is obtained by projecting the death rates through time, based on the exposure history of the population at risk to the effluent of interest in conjunction with appropriate dose-response coefficients. These projected changes in death rates are then used to update the projection model in each time interval. Our models generate estimates of average life shortening as well as estimates of premature deaths and increased death rates.

At present, two dose-response models have been developed. In the first, the effects of a given exposure are assumed to be linear with respect to cumulative dose, subject to the reservation that a given increment of dose can have an effect for only a specified period of time following an initial latent period. This model is most useful for analyzing the effects of radiation exposure. Coefficients for this model are derived from appendix VI of the Reactor Safety Study [U.S. Nuclear Regulatory Commis-

sion Report WASH-1400 (NUREG-75-014), 1975] and the BEIR report (Report of the Advisory Committee on the Biological Effects of Ionizing Radiations, U. S. National Academy of Sciences-National Research Council, 1972).

The second model has been developed for the analysis of the effects of airborne combustion products, the principal source of concern for the increased utilization of coal. It uses cigarette smoke as an archetype for all forms of air pollution. Exposure to a given dose rate (in cigarette equivalents per day) produces in the long term an increase in total age-specific mortality rates. This increase is linear with respect to dose at any age, but exponential with respect to age. Latency effects are included by having the long-term effects approached asymptotically as a function of duration of exposure. Exposure to mixed urban and industrial air pollutants is commonly identified by indicators of polluted air, such as SO_2, total suspended particulates (TSP), suspended sulfates (SO_4), and benzo(a)pyrenes. Our model converts indicator values from $\mu g/m^3$ concentrations in ambient air to cigarette per day equivalents, based on the effects demonstrated by other studies (Finch, S. J., and S. C. Morris, Brookhaven National Laboratory Report BNL 218081, 1976; Carnow, B. W., and P. Meier, Arch. Environ. Health 27, 207, 1973).

The importance of latency and age composition factors in quantitative estimates of health effects was shown in the course of our work for the National Coal Utilization Assessment (Lundy, R. T., and C. D. Brown, ANL Report AA-11, 1977, 9-1--9-21). This study included an analysis of an assumed 9 $\mu g/m^3$ average increase in suspended sulfate concentration over a 50-year period in a 30-state region as a result of increased coal use in the Midwest. A comparison of the results from the single coefficient approach and those from our second dose-response model revealed that the "excess mortality" generated by the single factor approach overstated excess deaths by 36,000, or 300%, in 1985, agreed with the present model in 2000, and understated the number of deaths by 17,000, or 23%, in 2020 (Lundy, R. T., and D. Grahn, Proceedings of the American Statistical Association, Social Statistics Section, Part II, pp. 672-677, 1977).

We also applied our demographic models in an Argonne study for the Nuclear Regulatory Commission, "The Environmental Effects of Using Coal for Generating Electricity" (NUREG-0252). In this study, the effects to be expected from a standard 1000-MWe coal-fired power plant on the population within an 80-km radius were analyzed for six generalized combinations of coal and topographic environments. Because the average concentration of airborne effluents in each scenario varied irregularly as a result of topographic and meteorological factors, the effects of population distribution on the net impact were checked using two assumptions: first, the population was evenly distributed within the area of impact; and second, the population was concentrated

in the area of the highest decile air pollution concentration. The latter assumption yielded the maximum impact. The study showed that even if "New Source Performance Standards" (NSPS) are adhered to, the increase in death rates in the affected population could range from 5 to 29 deaths per million exposed person-years.

Early versions of our model were also used to assess human health effects of the use of fluidized-bed combustion of coal compared to conventional combustion for steam generation. It was determined that there would probably be no significant difference between the two systems in their impact on human health, as both can easily meet NSPS for controlled substances.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

Dvorak, A. J., C. D. Brown, E. H. Dettman, R. A. Hinchman, J. D. Jastrow, F. C. Kornegay, C. R. LaFrance, B. G. Lewis, R. T. Lundy, R. D. Olsen, J. I. Parker, E. D. Pentecost, J. L. Saguinsin, and W. S. Vinikour. The Environmental Effects of Using Coal for Generating Electricity. U. S. Nuclear Regulatory Commission, NUREG-0252, 1977.

Lundy, R. T., and C. D. Brown. Health risks. National Coal Utilization Assessment, An Integrated Assessment of Increased Coal Use in the Midwest: Impacts and Constraints. ANL/AA-11 Draft, 1977, Vol. II, Chap. 9, pp. 9-1--9-21.

Lundy, R. T., and D. Grahn. Predictions of the effects of energy production on human health. Proceedings of the Social Statistics Section, 1977, Part II, Chicago, August 1977. American Statistical Association, Washington, DC, 1978, pp. 672-677.

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3. THERAPY OF POISONING BY RADIOACTIVE AND NONRADIOACTIVE METALS

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

Raymond A. Guilmette (Postdoctoral Appointee)[1]
Elsie M. Sorensen (Postdoctoral Appointee)

The overall objective of this program is the development of methods for decorporation of toxic metals whose compounds deposit primarily in the skeleton and liver. In the prosecution of this work it is recognized as a fundamental principle that the development of effective therapeutic procedures is dependent upon results from parallel physiological experiments and morphological observations designed to elucidate the interactions between mammalian tissues and the toxic metal compounds under study. Accordingly, three secondary research objectives are presently being pursued: (1) Biochemical and physicochemical studies, *in vitro*, of the interactions of toxic metals and/or therapeutic substances with tissue components. (2) Morphological studies, including autoradiography and histology, designed to demonstrate (a) gross and microscopic localization of toxic metals in living tissues, (b) the pathological sequelae of toxic metal retention, and (c) the possible reversal of such sequelae by treatment. (3) Generation of metabolic and therapeutic data regarding the interactions of toxic metals with experimental animals, with special attention toward applicability to man. Because the highly dangerous radiation from plutonium, americium, and other alpha emitters is of great concern, a considerable portion of this program is directed toward the actinide metals. Recently, however, using concepts, procedures, and therapies developed in the course of our "nuclear" studies, we have obtained useful new metabolic and therapeutic information regarding nonradioactive toxic metals such as lead.

Significant advances toward the above research goals over the past year are described in the following sections. To summarize some of the highlights from recently obtained results:

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[1]Terminated during 1977.

1) From carefully designed experiments with beagles, based on earlier therapeutic results with rodents, we have further defined the variables promoting an optimal treatment regimen with diethylenetriaminepentaacetic acid (DTPA) for decorporation of plutonium (and probably americium) in man.

2) In young, rapidly growing rats, a short (2 week) regimen of treatments with DTPA achieved a striking 5-fold reduction in the skeletal burden of plutonium.

3) In collaborative studies with D. Grahn (reported in Section 10) of the genetic consequences of high LET irradiation of the gonads, new information was developed to allow more precise calculation of the radiation dose received specifically by spermatogonial stem cells from plutonium retained in the mouse testis.

4) Early and continuing DTPA therapy was found to reduce sharply (by at least 10-fold) the testicular burden of monomeric plutonium in both the mouse and dog, thus reducing the genetic risk from alpha irradiation of stem cells.

5) In studies of simulated lead poisoning in mice we have found that therapy with two alkyl esters of DTPA--compounds developed in our actinide research to promote increased membrane penetration--resulted in reduced lead retention in brain (a critical tissue), as well as in liver and kidney.

6) In DTPA-treated mice, monomeric plutonium definitely has been shown to be excreted as the Pu-DTPA complex.

INTERACTION OF MONOMERIC PLUTONIUM WITH THE LIVER AND SKELETON, AND WITH DTPA, AS RELATED TO DECORPORATION

M. H. Bhattacharyya and D. P. Peterson

A study was completed on the metabolism of ^{14}C-labeled DTPA in rats whose bile ducts and urinary bladders had been cannulated. By 24 hours after injection of the labeled chelating agent, 84% was found in the urine. In addition, a second, much smaller excretory pathway for DTPA *via* the liver into bile was identified; 0.28% of the injected ^{14}C activity was measured in bile collected for 24 hours after injection. Analysis of the bile by anion-exchange column chromatography revealed that about 50% of the ^{14}C was present as unchanged DTPA. In another study, the form of plutonium appearing in the bile following DTPA administration to rats previously injected with monomeric plutonium was analyzed by anion-exchange column chromatography. Nearly all the plutonium appearing in bile was in the form of the plutonium-DTPA complex. Thus, although the bile represents only a minor excretory pathway for DTPA, these two studies indicate that the amount of biliary DTPA is sufficient to induce, by direct chelate formation, biliary excretion of nearly all the monomeric plutonium removed from the liver.

Other experiments were conducted to determine whether a great decrease in bone density, brought about by exposure to weanling rats to a low-phosphate diet, might result in a corresponding release of plutonium from the skeletons of plutonium-treated animals. In plutonium-injected weanling rats subjected to a low-phosphate diet for 2 weeks, a 21-25% decrease was found in the ratios of ash to dry weight in femurs and vertebrae. However, these changes in bone mineral density could be accounted for entirely by increases in the dry weights of the bones, with no changes in ash weights. Furthermore, there was no additional release of plutonium from the skeleton over plutonium-injected controls receiving a normal diet. In the same experiment, a 5-fold reduction in the skeletal plutonium levels of weanling rats was achieved following 2 weeks of intraperitoneal $Na_3[ZnDTPA]$ (0.25 mmoles/kg, 5 days/wk). Thus, DTPA was surprisingly effective in reducing the skeletal plutonium burden of the young, rapidly growing rat. In contrast, similar treatment to adult mice resulted in only a 2-fold reduction in skeletal plutonium content.

DEPOSITION AND RETENTION OF PLUTONIUM IN THE TESTIS OF MOUSE AND DOG: EFFECTS OF THERAPY WITH DTPA

J. J. Russell and A. Lindenbaum

Results of a 1-year autoradiographic and radiochemical study in B6CF$_1$/Anl male mice followng intravenous injection of 10 μCi/kg of plutonium citrate include: (1) a 30% testicular weight loss; (2) a nearly constant testicular plutonium burden; (3) an average radiation dose accumulated by the testes of 70 rad by 348 days, at the average rate of 0.8 rad/day over the first 6 days and 0.2 rad/day thereafter; (4) an increasing association of alpha activity with glycolipid-positive droplets in the interstitial tissue; (5) a reduction in the number of mature spermatozoa and mitotic figures (with D. Grahn); and (6) calculation that a factor close to 4 is required to convert the overall testicular "smear" dose to the rad dose actually delivered to the spermatogonial stem cells (see Section 10).

An interspecies study of the testicular distribution and concentration of ^{239}Pu in the mouse and dog was made to compare the effects of DTPA, given twice a week for 3 months, when therapy was initiated early (6 hours after plutonium injection) and when delayed (6 days after plutonium). In all cases, the testicular plutonium burden was sharply reduced by DTPA. In the dog, it was reduced to one tenth and one fifth of the control level by early and delayed treatment, respectively. In the mouse, the reductions were to one eighth and one fourth for early and delayed treatment. Autoradiographic track counts indicated approximately equal loss of alpha activity from interstitial tissue and seminiferous tubules in both species.

DETERMINATION OF AN OPTIMAL THERAPEUTIC REGIMEN WITH DTPA FOR ACTINIDE DECORPORATION

A. Lindenbaum, R. A. Guilmette, J. J. Russell, and E. S. Moretti

Further experiments with dogs, designed to determine the optimum treatment frequency and dose levels, and to compare two chemical forms of DTPA (the calcium and zinc chelates) for removal of plutonium in case of human contamination, have been completed. Beagles received daily intravenous injections of either 70 mg/kg or 14.2 mg/kg Na_3[Ca-DTPA] for 5 days, beginning at 6 hours following intravenous administration of ^{239}Pu citrate. From this point on, intravenous injections of 14.2 mg/kg Na_3[Zn-DTPA] were given for 3 months at six daily treatments per

week to some dogs, and twice per week to the others. Radiochemical analysis and quantitative autoradiography are now being carried out on a standard array of tissues to obtain retention and tissue distribution data for therapeutic evaluation.

THORIUM-227 DISTRIBUTION IN MOUSE SARCOMA TISSUES

A. Lindenbaum and J. J. Russell

A cooperative project with A. Friedman of the Chemistry Division aims at tumor therapy based on preferential uptake of radioactive substances within and around tumors. In recent work, we have made autoradiographic measurements at the light and electron microscopic levels to determine any preferential microdistribution of thorium-227 citrate in mouse sarcoma #180 tumor tissues. Preliminary autoradiographic evidence shows a diffuse distribution of thorium throughout the tumor, with lesser amounts in necrotic centers and in fatty areas of the tumor. The hoped-for autoradiographic evidence of cascading daughters of ^{227}Th (i.e., ^{223}Ra, ^{219}Ra, ^{215}Po, etc.) was not found.

LEAD METABOLISM AND EFFECTS OF THERAPY WITH DTPA ESTERS

E. M. Sorensen, E. S. Moretti, and A. Lindenbaum

The objectives of this research are to (1) compare the lead distribution in the mouse with and without chelation therapy, with emphasis on critical target organs in lead poisoning, (2) assess dysfunction of the blood-brain barrier and resultant compartmentalization of lead in the neonatal rat brain (with and without chelation therapy), and (3) evaluate lead-induced changes in diel (daily) cycles of both the mouse and rat.

Recent studies are aimed at the development of methodology to assess the pathological effects of lead when the levels in tissues approach those likely to be encountered in the general population. In one effort, transmission electron microscopy was used to examine mouse liver and kidney. A single intravenous injection of lead acetate (80 mg Pb/kg) was found to produce intracellular inclusions in sufficient numbers to permit easy recognition and scoring. As the administered dose was decreased, however, both the size and number of inclusions declined markedly. Other studies attempt to measure lead-induced changes

in diel cycles of oxygen metabolism, deep body temperatures, and activity in the $B6CF_1$/Anl mouse (work in collaboration with G. A. Sacher).

In an effort to compare the therapeutic advantage of several chelators (including those in current clinical use) on lead decorporation in the mouse, an experimentally elevated body burden of lead was challenged with several chelators recently developed in this laboratory. Six treatments over 3 days, initiated on the third day after injection of 30 mg Pb/kg in the form of lead citrate, with either the dibutyl or dihexyl DTPA esters resulted in significant reductions in lead burdens in the brain, kidney, femur, and whole body compared to the levels in the tissues of untreated controls at 21 days. Rapid decreases in fecal lead excretion, and a leveling off of tissue lead burdens within a few days following the sixth treatment with the dibutyl DTPA ester indicate that prolonged therapy with this chelator (work now in progress) might reduce lead levels even further.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

Baxter, D. W., N. G. Doan, and A. Lindenbaum. Differences in early retention of lead acetate and lead citrate in mouse tissues. Biological Implications of Metals in the Environment. Proceedings of the Fifteenth Annual Hanford Life Sciences Symposium, Richland, WA, September 29-October 1, 1975. Eds. H. Drucker and R. E. Wildung. USERDA CONF-750929, 1977, pp. 560-569.

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4. DEVELOPMENT OF NEW TECHNOLOGY FOR THE USE OF STABLE ISOTOPIC TRACERS IN THE STUDY OF HUMAN HEALTH AND DISEASE

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Peter D. Klein (Senior Biochemist)
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This program has five major aspects: first, the development of analytical instrumentation of requisite sensitivity, stability, and simplicity to conduct stable isotope measurements in a routine manner; second, the development of appropriately labeled compounds for metabolic investigations, initially through custom syntheses but eventually through commercial sources; third, development of analytical methodology to isolate, purify, and determine the isotopic content of specific organic compounds reflecting metabolic processes or disease states; fourth, collaborative development of clinical applications and testing on a routine basis, through a network of clinical centers around the country; and finally, the collection and dissemination of stable isotope information on an international scale through survey publications and conferences.

During the past year, we have expanded our ability to trace ^{13}C by the construction of a combustion system for solid samples that permits recovery of label from lyophilized stool or urinary samples, and by the construction of a highly efficient CO_2 trapping system that is designed for use in incubators for premature infants. These developments permit extension of the use of ^{13}C-labeled substrates to new areas of pediatric research.

Synthesis of a variety of stable isotopically labeled compounds has been accomplished in response to specific clinical protocols. These compounds include d_3-, d_5-, and d_8-labeled forms of methadone, ^{13}C-triolein (from methyl oleate supplied by Los Alamos ICONS program), and, in departure from stable isotopes, ^{11}C-labeled bile acids, in conjunction with Paul Harper, Franklin McLean Memorial Research Institute, The University of Chicago.

The clinical application of the dimethylaminopyrine breath test has shown exceptional ability to predict patient response in

liver disease treatment protocols, as measured by clinical evaluation and liver biopsy. A series of children with Zellweger's syndrome has been found to have an atypical bile acid, trihydroxycoprostanic acid, in their bile. This bile acid, normally found in alligator bile, appears to reflect a primary defect of bile acid synthesis in this syndrome. Finally, we have begun development of isotope ratio methodology applicable to clinical studies of calcium absorption, using ^{42}Ca and ^{44}Ca.

The activities of this program contribute to the medical applications of stable isotopes as nonradioactive, noninvasive probes of specific metabolic processes that may be affected by environmental contaminants. This program provides a means of studying the metabolism and fate of pollutants in the examination of disease states and lays the basis for estimating levels of human exposure through clinical studies of selected populations. These studies can be extended into epidemiological examinations of occupational and general populations as they prove to have significant diagnostic values.

Finally, this program represents the confluence of a number of research programs within DOE and also in various institutes of the National Institutes of Health, and it serves to link the network of stable isotope users within a national and an international framework.

INSTRUMENTATION

D. A. Schoeller,[1] F. Stellaard, and H. C. Niu

Two new instruments have become operative during 1977, a Nuclide 3-60 Sector mass spectrometer and a solvent programmer for the Waters high pressure liquid chromatograph (HPLC). The Nuclide 3-60 is specifically designed for precise carbon and oxygen isotope ratio analysis. This instrument has demonstrated a precision of 0.5°/oo which is 10 times more precise than the PE-270 which was first used for isotopic measurements of CO_2 samples. The increased precision permits measurements of natural abundance ratios and is needed for most $^{13}CO_2$ breath test applications in order to keep the ^{13}C substrate dosage and cost to a minimum.

The solvent programmer has vastly improved the performance and versatility of the HPLC system. The use of programming to increase the solvent strength has shortened analysis time, while improving chromatographic separations relative to the single solvent systems that had been used previously. Also, mixtures of greater solute diversity can be analyzed without the inherent loss of sensitivity for those components that elute late. The HPLC is routinely used in checking the purity of newly synthesized ^{13}C substrates or labeled internal standards.

We have developed a number of new ancillary apparatuses. Of major importance to the clinical breath test program has been the development of an automated CO_2 purification system and isotope ratio analyzer. This system accepts up to 50 breath samples for sequential analysis. An aliquot is withdrawn from each sample and the CO_2 is cryogenically purified over dry ice and liquid nitrogen. The CO_2 is introduced into the sample side of the dual inlet of the Nuclide 3-60, and the CO_2 sample pressure is adjusted to equal that of the standard gas which is in the other half of the dual inlet. The isotope ratios are measured by peak stepping and the isotopic enrichment of the sample relative to the standard is calculated. The pressure matching of the sample and standard gases is not fully automated and requires operator intervention. The analysis time is 15 min/sample, which is a 4-fold improvement over the manual technique. The automated isotope analysis has been shown to be accurate and to have a precision within 1°/oo.

A combustion system has been built to augment the CO_2 analyzer by converting solid biological samples to CO_2 for isotopic

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analysis. Samples are burned over a platinum catalyst at 1000°C in the presence of 150 torr of oxygen. Replicate combustions of isotopic standards have shown the system to be accurate to within 1‰. The combustion line will be used for measuring the natural abundances of intravenous nutritional fluids and for measuring ^{13}C incorporation in solids.

Finally, a new CO_2 collection apparatus has been developed and tested for use in sampling expired CO_2 from infants in incubators. The apparatus consists of an enclosed glass spiral into which 5 ml of 1 N NaOH solution is added. The breath is drawn from the incubator, and through the NaOH where the CO_2 is absorbed and concentrated. The spiral provides the long absorption path that is necessary to absorb the CO_2 quantitatively. Without this apparatus it is not possible to perform $^{13}CO_2$ breath tests on premature and newborn infants.

SYNTHESES

K. Y. Tserng and D. L. Hachey

In continuation of the program of synthesizing bile acid sulfates, the syntheses of all monosulfates of four major bile acids were finished this year. The syntheses of 3-monosulfates were reported in the previous year. The preparation of 7- and 12-monosulfates was achieved by using similar procedures with several modifications. The methyl esters of 3-carbethoxy derivatives of chenodeoxycholic acid and deoxycholic acid or diacetates of cholic acid were used as starting materials. The reaction solvent was changed to pyridine from dimethylformamide-ether mixture to facilitate the isolation of products. The glycine conjugates (except for those from cholic acid) were obtained by starting with 3-carbethoxy derivatives of ethyl esters of bile acid glycine conjugates. For the syntheses of glycine conjugates of cholic acid monosulfates, the sodium salts of unconjugated sulfates were converted to triethylammonium salts using an ion-exchange column. The triethylammonium salts were then dissolved in chloroform and conjugated with ethyl glycinate in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. The taurine conjugates of all bile acids were prepared by conjugating sodium salts of unconjugated sulfates with taurine in dimethylformamide. Besides the unlabeled sulfates, which are to be used as standards in validation of analytical procedures, the 3β-d_1 labeled sulfates of lithocholic acid and its conjugates were also synthesized. They were used by Dr. John Watkins of Harvard Medical School in validation of the isolation, separation, and solvolysis procedures used in the study of lithocholic acid metabolism in infants. The disulfates of conjugated chenodeoxycholic acids

were prepared. They have provided Dr. Alan F. Hofmann of the University of California at San Diego with the authentic samples required to study sulfation of chenodeoxycholic acid in man.

For the clinical ^{13}C breath tests program, a number of new substrates were synthesized. The ^{13}C-triolein was prepared by esterification of 1-^{13}C-oleic acid and glycerol in the presence of sulfuric acid. The preparation of methyl-^{13}C- and ^{14}C-labeled methacetin and phenacetin by etherization of p-acetaminophenol with labeled methyl iodide or ethyl iodide provided possible alternative compounds to aminopyrine for determining the N- or O-dealkylation capacity of the hepatic P-450 mixed function oxidase system. These new compounds involve a lower risk to the patient.

The synthesis of ^{11}C-labeled bile acids as possible liver scanning agents in nuclear medicine was explored in this laboratory. The short half-life of ^{11}C ($t_{1/2}$ = 20.4 min) required that the synthetic procedure be completed in less than 2-3 half-lives in order to obtain compounds of sufficient activity. This problem was overcome by carbonation of the Grignard reagent derived from 23-chloronorcholanes to form the labeled compounds. The procedure took only 15-20 minutes after the introduction of $^{11}CO_2$ from the cyclotron, and a product with a total activity of 2 mCi was obtained. The preliminary study on human volunteers has indicated that intravenously administered chenodeoxycholic acid is rapidly concentrated in liver and a good image of liver can be obtained.

In order to study the metabolism and pharmacokinetics of methadone and its enantiomer, a variety of labeled methadones, e.g. methadone-d_3, methadone-d_5, methadone-d_8, and their enantiomers, were synthesized. The combined use of these labeled methadones will enable the simultaneous measurement of plasma levels of individual methadones.

ANALYTICAL DEVELOPMENTS

D. L. Hachey, H. Niu, D. A. Schoeller,[1] F. Stellaard, and K. Y. Tserng

Keto bile acids found in human bile are believed to result from bacterial oxidation of the hydroxy bile acids as they traverse the gastrointestinal tract. During an investigation of the hydrolysis procedures used to isolate bile acids, we observed partial destruction of keto bile acids. Products obtained from the hydrolysis were identified as hydroxy bile acids, which probably result from a coupled oxidation:reduction process in the hydrolysis solvent system. Reduction of keto bile acids was influenced by high temperature, high concentration of alkali, and the presence of alcohol in the solvents. Similarly, oxidation of hydroxy bile acids was observed when acetone was present in the hydrolysis solvent. The amount of keto bile acids naturally present in bile could be estimated first by reduction with sodium borodeuteride, and then using an alcohol-free hydrolysis mixture for deconjugation. The products were examined by isotope ratio mass spectrometry, and the increase above the natural abundance of the M+1 ion was used to determine the amount of keto bile acids originally present in bile. The results indicated that many widely used isolation procedures may substantially underestimate the content of keto bile acids in biological samples.

Most biological samples require some form of derivatization prior to mass spectrometric analysis in order to enhance their volatility. Derivatization procedures should be rapid, quantitative, and free of undesirable artifacts. Most common procedures involve methylation, acetylation, or trimethylsilylation; all produce enol ethers or enol esters of 3-keto bile acids. We have developed methylation procedures using dimethoxypropane and acetylation procedures using 4-dimethylaminopyridine/acetic anhydride that eliminate the problems of formation of enol ethers or enol esters.

In order to obtain high precision isotope ratio analyses by chemical ionization mass spectrometry on ions that are separated by only one or two mass units, it is necessary to minimize, or at least stabilize, the hydride abstraction phenomenon. We have observed that hydride abstraction ion intensity was consistently lower when we used the direct insertion probe than it was when we used the gas chromatograph inlet. We also observed that the temperature of the gas chromatograph transfer lines influences the amount of hydride abstraction; greater amounts of hydride

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abstraction were obtained at temperatures greater than 300°C, but the problem was minimal at temperatures below 250°C.

Measurement of intestinal malabsorption in infants is important in assessing nutritional status. We have developed procedures for determination of ^{13}C content of fecal samples. Data were obtained from two infants who were on a standard formula diet of defined composition. The results indicate that precision of the combustion process and isotope ratio analysis was within 0.3‰ (n = 4). The variation within a single subject for successive 96-hour collection samples was 1‰ (n = 7), and the variation was only slightly larger between individual subjects. This low sample variation makes it possible to detect excretion of approximately 0.8 μmoles of singly labeled substrate per kg of body weight.

Methadone pharmacokinetic studies have heretofore been limited to data obtained from urine because no suitable procedure existed for selectively extracting methadone from plasma. We have developed a procedure for recovering a very pure methadone from plasma. The technique involves a complex extraction:back extraction procedure to remove undesirable lipid components of blood that interfere with the mass spectrometric assay. The procedures permit about 50-60% recovery efficiency and are now routinely used to study plasma methadone pharmacokinetics (see Clinical Applications, below).

Three deuterium isotope labeled forms of methadone have been prepared which contain 3, 5, and 8 deuterium atoms. Techniques have been developed to synthesize isotopically pure compounds so that overlap with adjacent ions does not interfere with quantitation of the individual isotopic forms. Two of these compounds, S-(+)-d_3 and R-(-)-d_5, are being used as *in vivo* tracers; while the third, (±)-d_8, is being used as an internal standard to quantitate plasma drug levels by inverse isotope dilution.

High pressure liquid chromatographic techniques have been developed to determine purity of methacetin and phenacetin in various syntheses of the isotopically labeled forms. The assay is sensitive enough to detect nanogram quantities of these drugs in biological fluids.

We have begun preliminary work on a study of the absorption and kinetics of metals in biological systems, using a nonconventional approach to measuring metal isotopes that involves formation of a volatile metal chelate. We have started working with calcium because of its clinical importance in vitamin D metabolism. Calcium is an especially challenging element because its chelates have a number of troublesome properties, including a strong tendency to hydrolyze, formation of heteropolymers in the gas phase, decomposition under chemical ionization mass spectrometry conditions, and very low sample volatility. We have prepared a number of β-diketone calcium chelates and related

β-ketoamine Schiff base chelates and evaluated their suitability with respect to stability, volatility, and solubility. The most promising chelating agents seem to be fluorinated β-diketones that contain a tertiary butyl group. Such a chelate of calcium possesses most of the desired qualities, although it is extensively polymerized and the major ions in the electron ionization mass spectrum occur near m/e 965. From isotope ratios measured on a related β-diketone calcium chelate we found that it is possible to measure isotope ratios to 1 part in 2,000 for both the ^{42}Ca and ^{44}Ca isotopes. Mass spectral studies indicate that electron ionization must be used in order to obtain a monomeric species containing a single metal and a single chelate ligand. We have successfully derivatized 10 μg of calcium, and we expect that 0.5 to 1.0 μg calcium is sufficient to obtain accurate isotope ratio measurements.

CLINICAL APPLICATIONS

P. D. Klein, P. A. Szczepanik, D. A. Schoeller,[1] D. L. Hachey, K. Y. Tserng, and F. Stellaard

The clinical applications of the dimethylaminopyrine (DAP) demethylation $^{13}CO_2$ breath tests have been pursued in collaboration with Dr. John Schneider, The University of Chicago. In a study of 30 subjects suffering from alcoholic hepatitis, the DAP breath test gave a very good measure of liver function over the course of patient recovery. The DAP test was shown to be more reproducible and less ambiguous than the standard liver function tests--SGOT, serum albumin, serum bilirubin, and prothrombin time--and was in good agreement with clinical evaluation of the patient. We have performed preliminary trials of new liver function breath tests using the newly synthesized ^{13}C-methacetin and ^{13}C-phenacetin. Both of these drugs undergo hepatic O-dealkylation. Preliminary results indicate that the methacetin test may provide a better liver function breath test than aminopyrine because methacetin undergoes almost complete dealkylation in the normal liver, so that any reduction indicates impairment of the first pass hepatic clearance capacity.

The DAP $^{14}CO_2$ breath test was used in collaboration with Dr. Carl Peraino to ascertain its ability to detect the formation of hepatic tumors in carcinogen-treated rats. The large variations

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observed in the breath test response obscured the decreased response to the breath test expected in those animals in which tumors had formed and essentially ruled out the breath test for diagnosis of hepatic carcinoma.

Five $^{13}CO_2$ breath tests using ^{13}C-labeled fats have now been used in the investigation of fat malabsorption in infants. The tests use the free fats, sodium octanoate and palmitic acid, and the triglycerides, trioctanoin, tripalmitin, and triolein. From these, a battery of three substrates--palmitic acid, trioctanoin, and triolein--have been chosen for use in identifying whether fat malabsorption is due to a pancreatic, intraluminal, or mucosal disorder. The battery of tests has been performed in three subjects. In two patients with celiac disease, the two long-chain fats were poorly absorbed, as expected in a mucosal disorder, whereas absorption of the medium chain fat, trioctanoin, was normal. In a child with Schwachman's syndrome, the two triglycerides were poorly absorbed while the free fat absorption was normal, as would be expected for pancreatic insufficiency.

Preliminary studies of lipid metabolism in premature infants using $^{13}CO_2$ breath tests have begun in collaboration with Dr. Tibor Heim, Hospital for Sick Children, Toronto, Canada. Using substrates that were either naturally enriched or were depleted in ^{13}C, we have shown that we can distinguish between lipid and carbohydrate oxidation as the metabolism switches in response to changes in the composition of intravenous infusions.

Because the use of radioactive isotopes is contraindicated in children and women of childbearing age, studies that deal with the determination of bile acid kinetics using ^{2}H- or ^{13}C-labeled bile acids continue to generate a number of collaborative programs. The measurement of bile acid kinetics in approximately 85 Pima Indian children to determine the age of onset of lithogenic bile among southwest American Indians (collaborator: Dr. L. Bennion, Phoenix Clinical Research Section, NIAMDD) is nearly completed. Using a single sampling of duodenal bile after administration of 11,12-$^{2}H_2$-chenodeoxycholic acid, the pool size and turnover rate of this compound have been determined in both male and female children, before, during, and after onset of puberty. Chenodeoxycholic acid has been shown to be important in maintaining cholesterol solubility in the gall bladder. Because American Indians are known to have a high incidence of cholesterol cholelithiasis, the evaluation of the chenodeoxycholic acid pool size data in these children may elucidate the onset and extent of this disease in the Pima Indian children.

Another study designed to focus on the higher incidence of cholesterol gallstones in women, in collaboration with Dr. A. Hofmann and Dr. P. Ng, previously of the Mayo Clinic, Rochester, is nearly completed. Administration of stable labeled cholic acid and chenodeoxycholic acid during pregnancy and during the use of oral contraceptives will enable, for the first time, the

definition of bile acid kinetics in normal ovulating women and in pregnant women, as well as the evaluation of the biliary lipid composition and bile acid kinetics due to changes in specific sex hormones. These studies should also lead to an understanding of how contraceptive steroids cause the bile to become lithogenic.

The availability of our gas chromatographic/mass spectrometric/computer techniques and analytical expertise used to quantitate bile acids continues to attract collaborative studies which deal with the detection and identification of the bile acid components of biological samples. Two of these studies have been outlined in the Bile Acid Transformations in Gallstone Therapy and the National Cooperative Gallstone Study grants. For the latter study, bile samples obtained from volunteer cholelithiasis patients before and during treatment with chenodeoxycholic acid continue to be examined for the presence of any atypical, and possibly toxic, bile acids formed from the chenotherapy. The study is expected to be completed by the end of the next calendar year.

Because chenodeoxycholic acid can be converted by 7-alpha-dehydroxylase to the toxic lithocholic acid, analyses of the sulfate and nonsulfate bile acid fractions of human meconium have been carried out in collaboration with Dr. John Watkins, Harvard Children's Hospital, Boston, to establish that the sulfation of lithocholic acid is a well-developed detoxification mechanism in the human fetus. Validation of this mechanism in the fetus is essential if chenotherapy is to be approved for women of childbearing age.

The identification of atypical bile acids in infants and children presenting with various liver diseases has become increasingly important. The following collaborators have requested assistance with specific problems: Dr. Barbara Kirschner, The University of Chicago, with the analysis of serum bile acids from infants having various combinations of liver diseases such as neonatal hepatitis, extrahepatic biliary atresia, and neonatal cholestasis; Dr. R. Wilson, Harbor View Medical Center, Seattle, with serum samples from two brothers suffering from a condition characterized by cholestasis and cirrhosis since the first year of life; Dr. Norman Javitt, Cornell University Medical Center, New York, with the identification of hyocholic acid in a child with cholestatis; Dr. Russell Hanson, University of Minnesota, with the identification of trihydroxycoprostanic acid and other C-27 bile acids in patients with Zellweger's syndrome; Dr. John Watkins, with the identification of hyocholic acid in children with Byler's disease; and Dr. T. R. Holzbach, Cleveland Clinic, with the analysis of bile acids from the portal blood of patients undergoing colectomy for inflammatory bowel disease.

Clinical studies using deuterium-labeled methadone are continuing with Dr. Mary Jeanne Kreek at Rockefeller University. The main thrust of this work is to study the kinetic behavior of

the individual methadone enantiomers. To facilitate this work, two scientists from Dr. Kreek's laboratory came to Argonne and spent a total of 5 weeks at the laboratory learning stable isotope tracer techniques. Urinary excretion studies have confirmed our earlier observations that inactive S-(+)-methadone is eliminated from the body almost twice as fast as the active R-(-)-methadone. Preliminary studies using noninvasive saliva drug levels were not encouraging. Saliva methadone levels are much higher than plasma levels, show considerable hourly variation, and are not sufficiently reliable to obtain pharmacokinetic data. More encouraging results were obtained from plasma pharmacokinetic data. These data support the elimination rate difference between R-(-)- and S-(+)-methadone previously observed in urine. In addition, they provide evidence for either a differential gastrointestinal absorption of the two forms or a differential hepatic clearance of methadone. In these plasma studies, we observed a consistently higher isotope ratio for the inactive S-(+)-methadone than for the active R-(-)-methadone. Studies designed to uncover the mechanism behind different plasma levels are in progress. These studies will involve simultaneous use of S-(+)-d_3 and R-(-)-d_5 methadone in a single study. Methadone-d_8 will be used as an internal standard to quantitate plasma methadone levels for d_0, d_3, and d_5 species.

Folic acid metabolism studies are continuing with Dr. I. H. Rosenberg at The University of Chicago. Two new patients have been studied in this series. One patient received a 5-day oral folic acid loading dose regimen, which permits kinetic studies for 6 days following the last day of isotope administration. The second patient received only a single dose of deuterated folic acid, which permits studies for 5 days. These experiments enable a much simpler treatment of the kinetic data than previously possible.

A new collaborative effort was begun this year with Drs. J. Robb and I. H. Rosenberg at The University of Chicago. This is a program to study metal metabolism using stable isotopes. These studies are designed to investigate the gastrointestinal absorption of metals, primarily calcium, but also magnesium, copper, iron, and zinc. In addition, we plan to study the pharmacokinetics of trace metals. Most of the preliminary work has been concerned with developing analytical methods to recover metals from plasma, developing chelation and extraction techniques, and developing mass spectrometric procedures.

INFORMATION DISSEMINATION

R. Klein[1] and P. D. Klein

Selected bibliographies of biomedical and environmental applications of ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{18}O, and ^{34}S for the years 1971-1976 have been compiled and are in press in Biomedical Mass Spectrometry. These bibliographies total nearly 1400 references and are indexed by subject and author to increase their utility. Assembly of this information has made it possible to see new trends in isotope usage and to identify active areas of research more clearly. We have received information on the formation of stable isotope research associations in Germany, in Japan, and in Eastern Europe, and are maintaining liaison with these groups. The Third International Conference on Stable Isotopes has been announced and will be held in Oak Brook, Illinois, during the current fiscal year (May 23-26, 1978).

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5. MOLECULAR ANATOMY PROGRAM

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A central issue in the choice of energy sources is their direct or indirect effects on human health. The expectation that large-scale animal studies on the effects of radiation and other energy-related pollutants would yield results that could be directly extrapolated to man has not always been realized. It has become of critical importance therefore to fill a gap in human health science studies by developing methods for detecting mutagenic, toxic, and carcinogenic effects in human populations.

The central aim of the Argonne Molecular Anatomy Program is to develop methods to determine such effects. The common denominator of this effort is two-dimensional electrophoretic mapping of human proteins and protein subunits. Two-dimensional mapping, based largely on the work of O'Farrell, is ordinarily done manually, allowing only a few analyses per day, and spot detection is by autoradiography, which is a time-consuming process. To do the work outlined here, large numbers of samples must be analyzed. A semiautomated system for analyzing up to 120 samples per day, called the ISO-DALT system, has therefore been developed at Argonne (Anderson, N. L., and N. G. Anderson, Proc. Nat. Acad. Sci. 74, 5421, 1977; Anderson, N. G., and N. L. Anderson, Anal. Biochem. 85, 331, 1978; Anderson, N. L., and N. G. Anderson, Anal. Biochem. 85, 341, 1978).

The first dimensional separation, termed ISO, employs isoelectric focusing, which gives the highest resolution separation of any technique currently available. The second dimension separation, termed DALT, utilizes electrophoresis in the presence of sodium dodecyl sulfate (SDS). SDS coats the peptide chains and gives them a charge very nearly proportional to their length. In free electrophoresis, all molecules would have nearly identical mobilities. However, in a microporous acrylamide gel, small molecules in SDS migrate faster than large ones simply due to the retarding effects of the small holes in the gel. The net effect is that the second dimension separation is almost entirely based on differences in molecular weight.

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Thus, the two-dimensional separation is based on two completely independent variables, isoelectric point (a reflection of amino acid composition) and molecular weight. The gels are fixed, stained, and photographed, and the photograph is scanned with an electrooptical scanner to give 2.5 x 10^6 or more measurements. Computer systems for image analysis are being developed to normalize patterns and to give spot lists by position plus integrated density.

It is estimated that the human genome contains 30-50,000 genes that code for proteins or their subunits. Many mutations result in a substitution of one amino acid for another, and about one third of such substitutions result in a change in the electrical charge of the protein involved. Single charge changes are visible as positional shifts in two-dimensional electrophoretic maps; the affected protein appears shifted to the left if a charge is added, and to the right if one is lost. Thus mapping is directly applicable to the problem of finding mutant proteins in man and ultimately to the problem of measuring the rate at which human mutations occur. If the mutation rate is constant, then no serious damage to the human genome is being done.

In any one human cell type, 10% or less of all structural genes are turned on. This means that there are compositional differences among different cell types and suggests that there may be many proteins unique to each cell type in man. Toxic agents generally make cells leaky, regardless of the precise molecular mechanism by which their effects are produced. Many of the leaked proteins are small enough to pass through the kidney and appear in urine. Maps of human urinary proteins therefore will provide a wealth of toxicological information as an increasing number are described, and their origin found.

In cancer, the pattern of gene expression is altered, and genes normally silent in the affected tissue are switched on, while some genes normally active are turned off. Mapping of tissues and, in some instances, of urinary proteins allows such alterations to be detected.

Experimentally, the problems are first, to develop techniques for two-dimensional mapping to the point where large numbers of samples may be mapped reproducibly and with high resolution, and second, to extract and use the enormous amount of data such maps provide.

We have begun by analyzing human plasma proteins and identifying the major ones in the two-dimensional ISO-DALT patterns (Anderson, N. L., and N. G. Anderson, Proc. Nat. Acad. Sci. 74, 5421, 1977). The exploration of human molecular anatomy is continuing, as described in the following reports, with mapping of

proteins of muscle, red cells, white cells, hair follicles, and urine. An essential aspect of this work is the development of methods for identifying activities associated with spots.

URINARY PROTEINS IN THE RAT: SEX DIFFERENCES AND USE AS INDICES OF TISSUE DAMAGE

F. A. Giere,[1] J. J. Edwards, S. L. Tollaksen, and N. G. Anderson

Sex Dependent Proteins of the Rat

This laboratory has shown that there are two major proteins present in the soluble phase of liver extracts from male rats that are lacking in the extracts from female rat livers (Anderson, N. G., and N. L. Anderson, Proc. of the Behring Institute Symposium, in press). Additionally, there is apparently a protein in the female liver that is lacking in the male. It was deemed desirable to examine additional tissues of the normal rat for the presence of sex-dependent proteins. Because numerous examinations of serum/plasma by others in this laboratory had not revealed obvious sex differences in rat blood, we directed our first investigations to the urine.

Adult male and female Sprague-Dawley rats were housed in individual metabolism cages. The urine was collected twice daily in a graduated cylinder in ice, filtered or centrifuged, and frozen (-70°C) if there was to be a delay before processing. After dialysis against 0.1% NaCl, biurets were run on the centrifugal fast analyzer to determine the protein concentration. The samples were freeze-dried and then reconstituted to an approximate concentration of 50 mg/ml. Five to 10 µl of this solution was applied to each isoelectric focusing gel, as described earlier (Anderson, N. L., and N. G. Anderson, Proc. Nat. Acad. Sci. 74, 5421, 1977).

Examination of the ISO-DALT two-dimensional slab gels of the urinary proteins revealed sex dependent differences in adult rats, but not in weanling rats. Male-dependent protein(s) appear at about 6 weeks and female protein at about 10 weeks of age. The male protein appears to consist of about four isoelectric species. These sex-dependent proteins are small (< 20,000 daltons). They are thus considerably smaller than any of the sex-dependent liver proteins described in the literature.

There is no sex difference in urinary proteins if the rats are castrated as weanlings. Furthermore, the sex differences are lost upon castration of the adult male and female.

That these sex differences are dependent upon the sex steroids was demonstrated by an experiment employing "reciprocal therapy." Once weekly for 4 weeks castrate males were given an

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intramuscular injection of 0.25 mg of estradiol cypionate in sesame oil, and castrate females were given an intramuscular injection of 5 mg depo-testosterone cypionate in sesame oil. In this experiment, the urinary protein map of the testosterone-treated castrate females did not appear to differ from the normal male urine map when the two were examined on the visual comparator.

Experiments are in progress to isolate and purify the sex-dependent male urinary protein and to make antibodies against it in rabbits. Antibodies to unfractionated male rat urine and unfractionated female rat urine will also be made. It is expected that experiments using these antibodies will resolve the tissue origins of sex-dependent urinary proteins and confirm the presence of a female urinary sex protein.

A clear understanding of normal male and female urinary protein maps is crucial to the utilization of information from urine examination as an index to tissue/organ damage.

Urinary Proteins as Indices of Organ/Tissue Damage

It is generally agreed that injured or diseased cells may leak protein to the extracellular fluid compartments of the body and that these proteins may be lost from the body *via* urine in the absence of a specific transfer mechanism to reabsorb them from the glomerular filtrate. The presence of protein in urine following muscle damage was perhaps the first example of this condition to be described. The presence of heart muscle enzymes in blood serum is used by the clinical laboratory to assess heart damage.

We have initiated a series of experiments with rats in which selected organs are insulted by various agents, and the urine from the treated animals is applied to the ISO-DALT system for protein mapping. The liver is being selectively damaged by carbon tetrachloride (by gavage) and by phenobarbital (by diet), the kidneys by boric acid (by gavage) and lead (by intravenous injection), and the pancreatic beta cells by alloxan (intraperitoneal injection). Additional animals will be subjected to an $LD_{50/30}$ dose of whole-body irradiation in the ^{60}Co High Level Gamma Facility. The two-dimensional maps of urinary proteins will be compared with the protein maps of the tissue homogenates from the injured tissue.

METHODOLOGY FOR ANALYSIS OF PROTEINS IN ERYTHROCYTE LYSATES

J. J. Edwards, S. L. Nance, and N. G. Anderson

Analysis of Human Erythrocyte Lysate Proteins by Two-Dimensional Electrophoresis

The human erythrocyte has one of the simplest biochemical organizations of any human cell type and offers many advantages for genetic, enzymatic, and clinical investigations. It is presently known that there are deficiencies of seven enzymes of the Embden-Meyerhoff pathway, five deficiencies of the hexose monophosphate shunt pathway, and four nonglycolytic deficiencies, all of which lead to the development of hemolytic anemias. It is also known that genetic variation occurs in many of the enzymes of the red cell. For example, there are over 80 known variants of glucose-6-phosphate dehydrogenase (G-6-P DH). Despite this, only limited information is available concerning the characterization of nonhemoglobin soluble proteins. Progress in the analysis of these proteins has been limited by the lack of suitable analytical systems capable of resolving the proteins in the presence of massive amounts of hemoglobin.

During the past year, studies have focused primarily on the isolation of highly purified soluble proteins from the human erythrocyte and the subsequent identification of these proteins in a two-dimensional electrophoretic map. Techniques of analytical chemistry involving affinity chromatography, gel filtration, selective precipitation, and selective elution with substrate analogs have been utilized to purify five enzymes from red cell lysates: carbonic anhydrase, catalase, G-6-P DH, pyruvate kinase, and hypoxanthine phosphoribosyltransferase. Enzymatic activities throughout all purification procedures were monitored with a GeMSAEC centrifugal fast analyzer. Electrophoresis of the purified enzymes followed by co-electrophoresis with whole lysate utilizing the two-dimensional ISO-DALT system has resulted in defining the map locations of these enzymes.

Figure 5.1 is a map of lysate protein subunits separated with the ISO-DALT system. Over 150 proteins or protein subunits are visible in this map. It is evident from the high resolution obtainable by this method that the system is useful for detecting mutant proteins in the study of genetic disease or to aid in the determination of the mutation rate in man.

Efforts are currently under way to identify the map locations of more enzymes, especially those of the glycolytic pathway, and to determine the effects of *in vivo* aging as it relates to posttranslational modification of the enzymes.

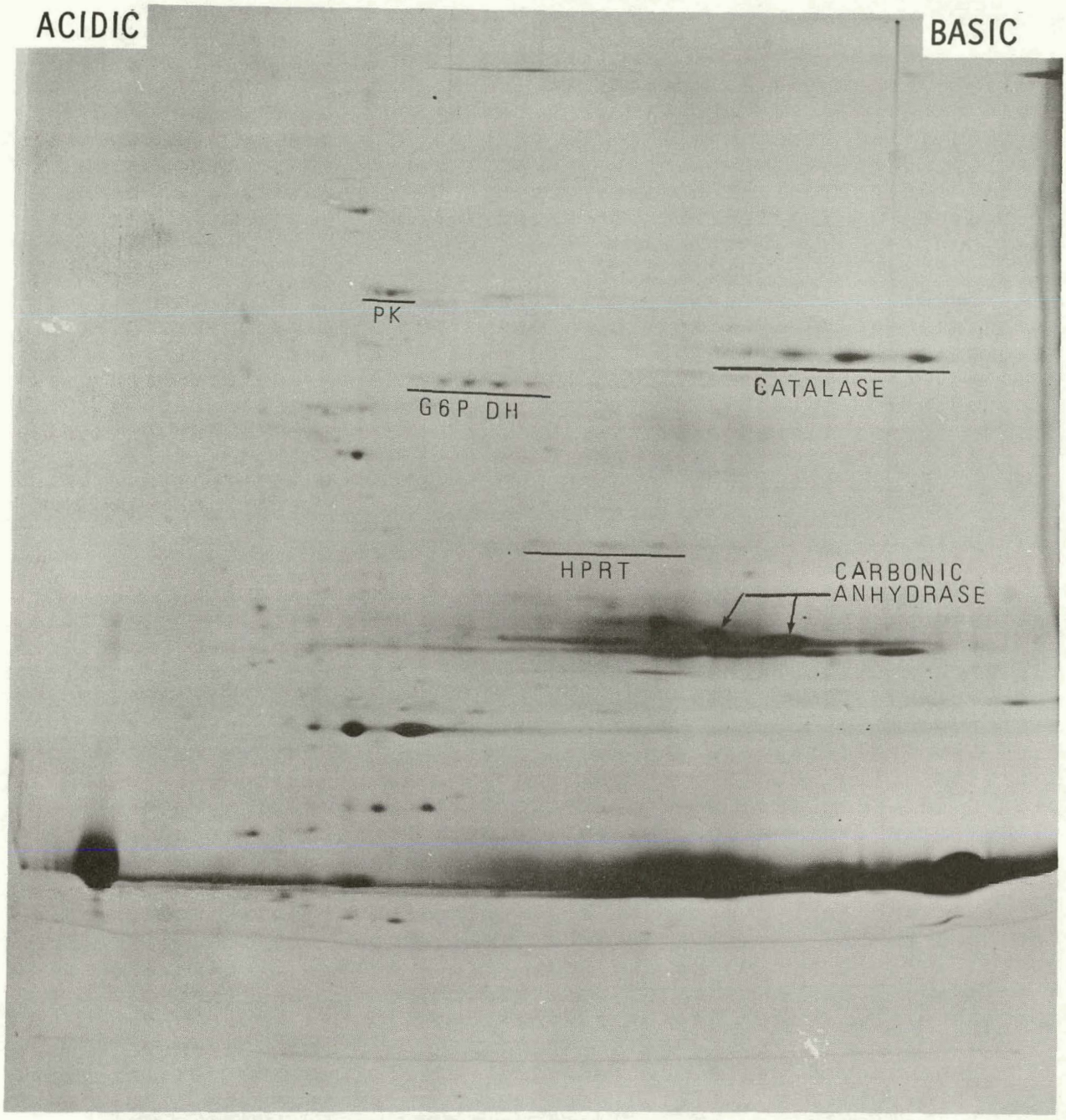

Fig. 5.1. Diagram of a two-dimensional electrophoretic (ISODALT) separation of human erythrocyte lysate proteins. The horizontal dimension is isoelectric focusing while the vertical dimension is sodium dodecyl sulfate electrophoresis. PK = pyruvate kinase; HPRT = hypoxanthine phosphoribosyltransferase.

Use of Schlieren Patterns for Position Location in Isoelectric Focusing Gels

The two-dimensional electrophoretic methods described above allow the majority of the protein gene products of mammalian cells to be mapped and described. In order to obtain more complete data from the protein maps, many of the map spots need to be identified functionally. To this end we have begun efforts to develop parallel preparative techniques of high resolution to isolate functionally active enzymes by essentially a one-step procedure of isoelectric focusing followed by rapid enzymatic analyses.

Isoelectric focusing gives the highest resolution separations currently available for one-dimensional systems, with a theoretical resolution of 0.01 pH units. We have recently developed a method for observing Schlieren patterns in isoelectrically focused slab gels by photographing diagonal lines through the gel. The Schlieren patterns observed appear to be due primarily to differences in gel thickness resulting from localized hydration. These differences have been shown to be related to discontinuities in the ampholyte spectrum but the mechanism(s) involved remain to be determined.

The experiment shown in Figure 5.2 illustrates the Schlieren pattern obtained with an ampholine range of 2-11 after electrofocusing at 30 watts for 3 hours. The pattern is highly reproducible with a given batch of ampholines. Following electrofocusing, photographs are rapidly prepared at a 1:1 magnification and are positioned immediately below the glass plate supporting the gel. With the Schlieren pattern as an outline, the gel is sliced longitudinally to conform to the major peaks. Each gel slice is placed into an appropriate buffer after a small piece of the slice is removed for pH determination. Protein is extracted from the gel and analyzed for enzymatic activity with the aid of a GeMSAEC centrifugal fast analyzer. Typically, 34 fractions are obtained and analyzed when a 2-11 ampholine range is used. Figure 5.3 is an example of the resolution obtained with this method. The graph shows the pH gradient with a linear range from 4 to 9 and the location of an erythrocyte enzyme, phosphohexose isomerase, as determined by enzymatic activity.

The use of this method will enable us to obtain small amounts of highly purified enzymes for analysis by two-dimensional electrophoresis.

Fig. 5.2. Schlieren pattern of isoelectrically focused slab gel containing ampholines in the pH range 2-11.

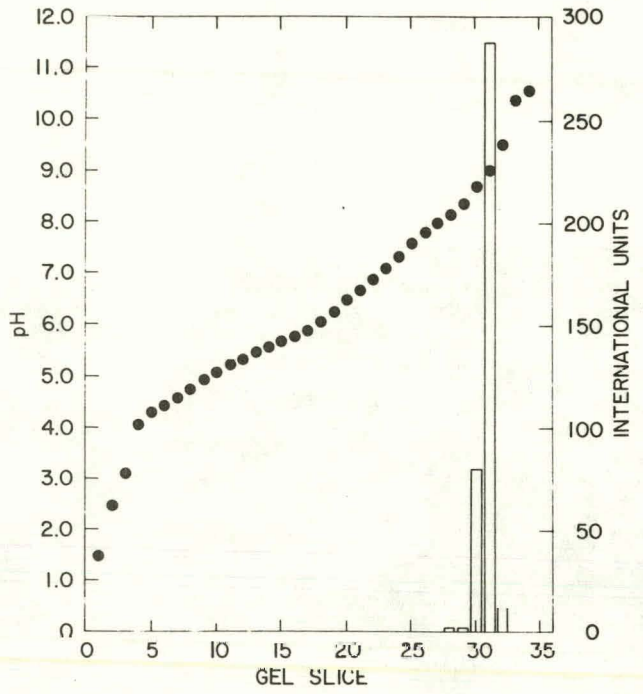

Fig. 5.3. Localization of human erythrocyte phosphohexose isomerase (bars) following isoelectric focusing. Enzyme activity is given in International Units.

TWO-DIMENSIONAL ELECTROPHORETIC MAPPING OF PROTEINS OF PLASMA, LYMPHOCYTES, AND HAIR FOLLICLES USING THE ISO-DALT SYSTEM

N. L. Anderson, B. J. Hickman, B. Bonner,[1] C. B. Shelman,[2] and R. H. Wehman[2]

Over thirty protein gene products (PGP's) have so far been identified in the two-dimensional electrophoretic (ISO-DALT) pattern of the human plasma proteins. About fifty visible PGP's remain to be identified, and it is likely that many if not most of these have not previously been described. The mixture of pro-

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[1]Fall 1977 participant in the Undergraduate Honors Research Participation Program, Lawrence University.

[2]Applied Mathematics Division.

Fig. 5.4. Portion of an ISO-DALT two-dimensional electrophoretic map of the proteins synthesized by a single human hair follicle during 24 hours *in vitro* incubation with ^{35}S-methionine.

teins in human serum has served as an excellent test system for establishing the usefulness and reliability of ISO-DALT protein mapping primarily because these proteins are more thoroughly characterized than any other collection of proteins. The major attributes of most of the plasma proteins (molecular weight, isoelectric point, degree of glycosylation, cysteine content, and genetic variability) can be determined directly from the two-dimensional gels. In particular, due to the enormous resolving power of the technique, the levels and types of microheterogeneity due to incomplete glycosylation and *in vivo* posttranslational modification are readily visible.

The reproducibility of the ISO-DALT system has also been exploited in a pilot study in human genetics. Serum samples from over two hundred matched trios (mother, father, newborn) obtained from J. V. Neel's laboratory at the University of Michigan were analyzed for about fifteen known and a number of unidentified proteins. Gene frequencies calculated for known polymorphisms, such as Gc-globulin, α-1 antitrypsin, and haptoglobin, agreed well with values from conventional electrophoretic analysis. In addition polymorphisms were observed in α-2 HS glycoprotein, as as yet unidentified carbohydrate-free protein, and the arginine-rich glycoprotein, and new variants were found in both the fibrinogen beta chain and in α-2 HS glycoprotein.

More recently, we have explored the attractive possibilities associated with ISO-DALT analysis of human hair follicles and lymphocytes radiolabeled *in vitro*. Following a brief period of culture in the presence of ^{35}S-methionine, several hundred thousand cpm can be incorporated by the contents of a single microtiter well (or by a single hair follicle). When subjected to ISO-DALT analysis such samples produce over 500 distinct protein spots (Figure 5.4). In a preliminary screening experiment, lymphocyte samples from 100 individuals were analyzed following radiolabeling. The results indicate that the average heterozygosity among cellular proteins is almost an order of magnitude less than among the plasma proteins. Since it is our plan to develop a system capable of measuring small increases in the human mutation rate, the use of such "low noise" cellular protein systems is obviously desirable.

In order to make feasible large-scale projects using the sensitive ISO-DALT technique, it has proved necessary to devote considerable attention to the development of hardware to run and to analyze the data from large numbers of gels. At present we have the capability to perform over 100 ISO-DALT analyses per day, and a computerized data-reduction system is under construction. When completed, the latter system will automatically detect and integrate the spots in ISO-DALT patterns and allow them to be merged into large, self-consistent files for detailed comparison.

CENTRIFUGAL ANALYSIS

S. L. Nance

Centrifugal analyzers are widely used by clinical chemists, but, due partly to expense, are almost unknown in the research community. We have found that many time-consuming projects formerly considered impracticable, or even impossible with some unstable enzymes, are now feasible with the use of a GeMSAEC centrifugal analyzer on loan from Electro-Nucleonics, Inc. Since we are primarily concerned with the separation and identification of individual proteins from a large number of sources, this analyzer, with its capacity for rapid and precise parallel measurements on 15 samples at a time, with computerized data acquisition and manipulation, is proving most convenient.

To date, more than 40 assays have been adapted for use in the GeMSAEC by this laboratory. These have been applied to (1) the identification of enzymatic proteins extracted from acrylamide gels after electrophoresis; (2) the location of enzymes in column chromatography fractions, $(NH_4)_2SO_4$ precipitates, and samples from other purification methods; (3) the characterization of proteins in cells of different ages; (4) the denaturation of enzymes at different temperatures; and (5) tissue surveys for the presence, or absence, of enzyme activities.

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6. CARCINOGENESIS

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One of the prime functions of a biological and medical research division within the Department of Energy's national laboratory structure is to provide the scientific data base for proper evaluation of health risks from the existing and emerging energy technologies. To fulfill this function Argonne's Division of Biological and Medical Research conducts a wide variety of carcinogenesis studies. In addition to the many groups, which, as parts of their general programs, address certain aspects of carcinogenesis, the Division has traditionally had a program specifically dedicated to carcinogenesis. That program was formerly under the direction of Dr. R. J. M. Fry. Since Dr. Fry's departure, the existing carcinogenesis program has been restructured and refocused, and two new studies have been initiated.

This report is divided into five subsections. Three of the sections describe long-standing, ongoing studies. The first of

these deals with the assessment of carcinogens and cocarcinogens and the underlying mechanisms of their actions. The second concerns cancer induction by bone-seeking radionuclides and seeks to provide a firm foundation for estimating cancer risks to human populations in the event of accidental incorporation of radionuclides. The third is aimed at defining the role of oncornavirus activation in tumor induction by radiation and other environmental pollutants. The other two sections describe the new studies, one dealing with the development of an *in vitro* cell system (murine teratocarcinoma cells) to screen chemicals rapidly for carcinogenic and mutagenic capacity, and the other investigating the potential use of plasma isozymes as indicators of mutagenesis in mammals. Accomplishments and projections for each of these studies follow.

CARCINOGENESIS AND COCARCINOGENESIS: INITIATION, PROMOTION, AND SYNERGISTIC ACTIVITY AMONG ENVIRONMENTAL POLLUTANTS IN THE PRODUCTION OF TUMORS

C. Peraino, R. N. Feinstein, Y. E. Rahman, D. A. Haugen, S. T. Shenoy, A. M. Prapuolenis, E. A. Cerny, E. F. Staffeldt, K. A. Rettman, and V. A. Ludeman

The human population is exposed to a multitude of environmental contaminants generated in part as by-products from a wide variety of industries, including those related to energy production. Many of these agents are known carcinogens or cocarcinogens. Cocarcinogens are defined here as agents that either increase the efficiency with which a carcinogen is activated, when given simultaneously with a carcinogen, or increase the probability of tumor formation when given after the carcinogen. The latter agents are termed promoters. The estimation of tumorigenic risk from promoters, however, is complicated by their presence in the environment as complex mixtures. Components of these mixtures may interact metabolically to produce synergistic increases in tumor formation. Depending upon the exposure modality, such interactions could consist of simultaneous and/or sequential exposure to both carcinogens and cocarcinogens.

The objective of this program is to study increases in oncogenesis produced by synergistic interactions among environmental contaminants emanating from energy-related technologies and other sources. We will carry out short-term and longer-term investigations, using interrelated *in vitro*/*in vivo* systems. The information obtained from our studies will be useful for the development of initial assessments of human tumorigenic risk from environmental factors. The research in this program centers around two systems, lung and liver.

As outlined in the following report, this laboratory is in the process of establishing a multifaceted approach to the development of systems for detecting environmental carcinogens and tumor promoters. In part, this involves devising methods for identifying preneoplastic changes in lung and liver, using morphological and histochemical markers. Additional studies focus on the comparative effects of such agents on biochemical processes concerned with the control of gene expression and cell division. Correlation of the results from the two types of studies should facilitate the development of rapid tests for different types of environmental agents that increase tumorigenic risk and should also increase our understanding of the essential molecular events involved in the neoplastic transformation.

In Vitro Effects of Energy-Related Pollutants on Cultured Trachea

The lung is used to examine synergistic interactions of various energy-related pollutants in respiratory tumor production, with the expectation that (1) insight will be gained into the mechanisms of such interactions, and (2) direct, rapid tests for estimating the tumorigenic potential of these interactions can be developed. In this system, we will study the effects of the same test compounds in (1) a rapid cellular transformation assay utilizing tracheal organ cultures, and (2) tumorigenesis assays with tracheal implants or intratracheal instillations *in situ*. Correlations between changes in biochemical and morphological characteristics in the *in vitro* system and tumor production in the *in vivo* systems will then be undertaken.

Conditions for establishing long-term tracheal organ cultures are being evaluated. Rat tracheas are used in these studies because the rat serves as the general experimental model for several other aspects of the program, and because rats have been used widely in lung carcinogenesis studies. Assays for the following enzymes have been adapted to tracheal tissue: lactic dehydrogenase, ATPase, benzo(a)pyrene hydroxylase, acid phosphatase, and β-glucuronidase. Cultures have been prepared from rat macrophages obtained by lung lavage and assays for the enzymes indicated above have also been performed on these cells. Changes in enzyme levels as a function of culture duration are being examined.

Liver Tumor Incidence Studies

Studies of liver tumorigenesis in our laboratory showed that the short-term exposure of rats to 2-acetylaminofluorene (AAF), a liver carcinogen (tumor initiator), produced a low incidence of tumors with varying degrees of differentiation, from poorly differentiated hepatocellular carcinomas to highly differentiated benign adenomas. The subsequent feeding of phenobarbital or DDT, tumor promoters, not only increased overall tumor incidence markedly, but also shifted the distribution of tumor types toward highly differentiated adenomas. In connection with these observations, studies of skin and liver tumorigenesis in other laboratories have shown that increasing the duration of exposure to carcinogen increased the relative incidence of poorly differentiated tumors. The occurrence of such shifts in the spectrum of tumor types in response to initiator or promoter treatment raises the possibility that a screening system could be devised whereby an environmental pollutant would be identified as an initiator or a promoter on the basis of its effect on the tumor spectrum resulting from administration of the test agent under a standardized set of conditions. The use of tumor incidence as the test end point, however, suffers from the disadvantages of a necessarily long experimental duration, plus the requirement for a large number of experimental animals.

A rapid method for testing environmental contaminants for liver tumor initiating or promoting activity will therefore be developed. The procedure involves the exposure of rats to carcinogen or carcinogen-promoter treatments. Subsequently, the distribution of marker enzymes in preneoplastic hepatocytes will be examined histochemically and compared with the distribution of tumor types (relative degree of differentiation) that ultimately emerge. If consistent treatment-related shifts in tumor types occur, and if these shifts can be correlated with changes in enzyme marker distribution patterns, then the marker patterns can be used to differentiate between initiators and promoters.

The observations that phenobarbital and DDT act as liver tumor promoters suggest that promoters, like carcinogens, can differ substantially in molecular structure, but apparently possess a common molecular characteristic that enables them to modify tumorigenically important processes within the liver cell. A comparison of the relative tumor-promoting abilities of various agents has been undertaken, in conjunction with a comparison of their effects on molecular processes concerned with the replication and expression of genetic information. These processes include thymidine incorporation into hepatocyte DNA and the activities of enzymes involved in DNA synthesis, amino acid metabolism, and drug metabolism. Such studies should reveal the spectrum of molecular changes associated with promotion. These molecular changes, in turn, should provide insight into the sequence of molecular events involved in neoplasia. In addition, knowledge of the molecular processes associated with enhancement would be useful in the development of test systems for more rapid identifications of promoters in the environment.

An experiment designed to compare the tumorigenic enhancing activities of different levels of dietary phenobarbital has been under way for one year and is still in progress. The tumor incidence in all groups has been unexpectedly low to date. In part, a change in protocol may be responsible. The duration of exposure of the rats to dietary AAF had been reduced in this experiment from 18 days to 14 days in the hope of increasing the differential in tumor incidence between the control and the enhancer-treated groups. An additional contributory factor may be the nonuniformity of AAF activation-detoxification pathways in different lots of rats, leading to changes in susceptibility to AAF tumorigenesis. Such effects would be magnified in the marginal AAF treatments used in our experiments. In any event, the current tumor data are insufficient to permit definitive conclusions regarding phenobarbital dose effects, although there are indications that 0.001% and 0.002% dietary phenobarbital do not increase tumor incidence above control values whereas 0.05% phenobarbital (the level used in previous studies) has an enhancing effect which is as great or greater than that produced by 0.25% dietary phenobarbital. The results to date suggest that this experiment may provide important information on threshold dosage levels for liver tumor promotion as well as liver tumor initiation.

Studies were undertaken in which the treatment protocol (brief feeding of known hepatocarcinogen followed by prolonged feeding of test agent) that had successfully demonstrated liver tumor promoting activity for phenobarbital and DDT was used to test the polycyclic aromatic hydrocarbon, benz(a)anthracene, for such activity. In an initial experiment this hydrocarbon had no promoting activity when fed at a dietary concentration of 0.025% following the exposure of the rats to the hepatocarcinogen AAF. In a subsequent study benz(a)anthracene also failed to show any liver tumor promoter activity although the dietary level of the hydrocarbon was raised to 0.075%, a concentration 50% greater than the promoting dosage of phenobarbital used in our experiments. Since the cost of further experiments, involving still higher benz(a)anthracene dosage levels, would be prohibitive, this aspect of the work will be terminated with the conclusion that, under the experimental conditions used, benz(a)anthracene is not a liver tumor promoter. These findings, obtained over a 2-year interval, illustrate the need for the development of a more rapid and less expensive indicator of liver tumorigenic potential than is provided by the use of actual tumor incidence as the end point.

Tumorigenic Enhancement in Molecular Terms

In previous experiments, we observed that dietary phenobarbital and butylated hydroxytoluene (BHT) enhanced the production of hepatic tumors in rats previously fed the hepatocarcinogen AAF for a brief interval. However, phenobarbital proved to be a much more effective tumor promoter than BHT. A study was undertaken, therefore, to gain initial information on the comparative effects of these agents on a variety of biochemical responses in the liver. It is anticipated that such comparisons will lead to correlations of tumor-promoting activity and biochemical effects that will ultimately permit the identification of critical molecular events involved in liver tumor promotion.

Groups of parameters representing three different aspects of liver metabolism were compared in an effort to monitor the effects of phenobarbital and BHT on patterns of gene expression that might be controlled by different regulatory mechanisms. Biochemical effects of changing from a crude to a semipurified diet were also examined. The three aspects of liver metabolism compared were: (1) Liver growth; relative liver size, thymidine incorporation into DNA, thymidine kinase activity, and ornithine decarboxylase activity. (2) Amino acid metabolizing enzymes; serine dehydratase and ornithine aminotransferase adaptation. (3) Microsomal proteins; components of the mixed function oxidase system including cytochrome P-450, cytochrome b_5, and NADPH cytochrome P-450 reductase.

The data obtained (summarized in Table 6.1) do not yet provide a molecular basis for the difference in the efficiencies of

Table 6.1 Summary of Responses of Biochemical Parameters to Drug Treatment and Dietary Change[a]

Qualitative Response	Agent		
	Phenobarbital	BHT	Diet
Sustained increase	P-450,[1] b_5,[1] red.[1]	P-450,[1] b_5,[1] red.[1]	SDH,[3] OAT[3]
Transient increase	TK,[2] T-inc.,[2] ODC	TK,[2] T-inc.[2]	TK,[2] T-inc.[2]
Sustained decrease	OAT[3]	SDH,[3] OAT[3]	ODC
Transient decrease	SDH[3]	--	--

[a]The qualitative response of each parameter was placed in one of the four categories indicated in column 1. Parameters followed by the same superscript are related by having a common set of qualitative responses to the administration of xenobiotics and to the change from crude laboratory chow to the semipurified diet. (Both types of treatment caused an increase in liver size.) These sets are as follows: [1]sustained increase for xenobiotic treatment but no response to the dietary change, [2]transient increase for both xenobiotic treatment and dietary change, and [3]sustained or transient decrease for xenobiotic treatment, but sustained increase for dietary change.

The abbreviations are P-450, cytochrome P-450; b_5, cytochrome b_5; red., NADPH-cytochrome P-450 reductase; TK, thymidine kinase; T-inc., incorporation of [^{3}H]-thymidine; ODC, ornithine decarboxylase; SDH, serine dehydratase; and OAT, ornithine aminotransferase.

BHT and phenobarbital as tumor promoters. However, the study did reveal that the induction of hepatomegaly by these xenobiotics is a complex adaptive process involving both positive and negative biochemical responses in the liver. Moreover, the kinetics of these hepatic responses were also complex, with transitory as well as sustained changes occurring after the rats were shifted to, and maintained on, diets containing BHT or phenobarbital.

Structural Studies on Rabbit Liver Microsomal Cytochrome P-450

The mammalian microsomal monooxygenase system is found in most tissues, and functions in the oxidation of a wide variety of physiological, therapeutic, and environmental chemicals, including many carcinogens and mutagens. Some of these oxidations result in the formation of reactive metabolites believed to be responsible for the ultimate carcinogenic and mutagenic effect of the parent compounds. Various forms of cytochrome P-450 participate in the monooxygenase system, each form having a unique substrate specificity and other distinctive biochemical properties. The relative concentrations of the various forms determine to a significant degree the balance between oxidative detoxification of carcinogenic chemicals and their activation to reactive metabolites.

The structural analysis described here was done in collaboration with M. J. Coon (University of Michigan) and K. T. Yasunobu (University of Hawaii) in order to initiate studies designed to define relationships between the structure of different forms of cytochrome P-450 and their enzymatic specificity and their biological control. The amino-terminal amino acid sequence of the phenobarbital-inducible form of the cytochrome P-450 of rabbit liver microsomes was determined by automated Edman degradation. Methionine is the amino terminus, and 17 of the first 20 residues are hydrophobic, including two clusters of five consecutive leucines. The unusual composition and sequence of this region are similar to those of the short-lived hydrophobic amino-terminal regions present in certain preproteins, but not in the corresponding "mature" proteins. The preproteins are believed to be the initial product of transcription, and their occurrence has been described in the literature for several excretory proteins including the immunoglobulins, insulin, pancreatic zymogens, and parathyroid hormone.

This type of sequence has not, however, been previously described for any "mature" proteins. The presence of this unusual hydrophobic sequence may well be important in the interaction of the protein with the lipophilic components of the endoplasmic reticulum or with hydrophobic sites on other proteins also present in the membranes that participate in the complete electron transport system responsible for the monooxygenase activity.

Characterization and Concentration of Preneoplastic Foci in Liver

The histochemical visualization of gamma glutamyl transpeptidase (GGTP) in preneoplastic foci has been accomplished, and the technique is available for larger scale use. Histochemical assays for glucose-6-phosphatase and ATPase (either or both of which may be absent in preneoplastic foci) have also been worked out. The purification of GGTP from liver tumors has been initiated. Since GGTP is apparently localized in part on cell membranes in most preneoplastic foci, immunochemical methods can be devised whereby antibody to this enzyme can be used to isolate preneoplastic cells from a suspension of hepatocytes prepared from the livers of carcinogen-treated rats. The preneoplastic hepatocytes can then be subjected to a battery of biochemical tests designed to disclose the essential molecular differences between these cells and normal hepatocytes.

Metabolic Control in Normal and Neoplastic Liver

A study was completed which showed that phenobarbital, fed at the dietary concentration used in the tumor enhancement studies (0.05%) blocked the synthesis of rat liver ornithine aminotransferase as measured immunochemically. This response is similar to that produced by the injection of the glucocorticoid triamcinolone. Further study is required to determine whether

these inhibitory effects occur via similar or different mechanisms. Such studies should provide insight into the mechanisms by which phenobarbital controls gene expression and thereby tumor development in the liver.

In conclusion, the main contribution to date of the program on carcinogens and cocarcinogens has been the demonstration of the universality of the multistage concept of carcinogenesis. This concept suggests that increased tumor formation can result from interactions of different agents at different stages of the tumorigenic process. Some of these agents may represent a significant tumorigenic risk to humans, but they cannot be detected by currently available rapid screening methods that use end points based solely on the production of DNA damage. Therefore, increasing attention in this laboratory is being directed toward developing methods for detecting both complete carcinogens and agents that are not carcinogens themselves but instead facilitate tumor formation.

RADIONUCLIDE ONCOGENESIS: BONE CANCER INDUCTION BY INTERNAL AND EXTERNAL IRRADIATION

M. P. Finkel and L. S. Lombard

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

The original approaches of the radionuclide experiments were to determine the relative toxicities of different radionuclides, the toxicity of selected radionuclides in different species, and the dependence of oncogenicity upon exposure situation. Attention then focused on special problems related to the induction of bone cancer, objectives being: (1) to define the true latent period, or time to irreversible neoplastic change, and determine whether it varies with sex, age, dose, nuclide, radiation quality, or animal species; and (2) to locate the microscopic site of neoplastic change, estimate the actual amount of energy delivered to that site, and assess the influence of dose rate and exposure pattern on the oncogenic response.

During the past year little effort was devoted to this program because of the needs of the viral oncology program. Progress was made, however, on two long-term toxicity experiments. The first of these is concerned with the consequences of the exposure of beagles to ^{90}Sr as a function of age and exposure pattern. Five controls died during the past 2 years, when they were 13.4 to 17.5 years old. Their deaths were associated with heart and kidney disease, osteoarthritis, and neoplasms of mammary glands and clitoris. Of the 143 dogs in the total experiment, only three controls remain alive.

The second experiment that progressed during the year was designed to compare the consequences of the exposure of CF#1 mice to fission neutrons and gamma rays from CP-5, one of the first nuclear reactors, with the consequences of exposure to bone-seeking radionuclides. Histologic examination of the tissues was completed.

The white-footed mouse, Peromyscus leucopus, had been reported to have a high natural incidence of osteogenic sarcoma. In view of our interest in this disease and the availability of this species in the Division, we established a routine with P. H. Duffy and E. F. Staffeldt to make a roentgenographic examination of all animals coming to autopsy. Histologic examination of any interesting skeletal lesions followed. One hundred eighty-nine roentgenograms were studied of mice dying from 251 to 2011 days (5.5 years) of age. No bone cancers were detected, but there were 21 mice (11%) with odontomas, benign tumors of the jaw, associated with any one of the four incisor teeth. Six of these 21 animals had four odontomas each. A higher proportion of males (15.3%) than females (6.6%) was affected, and, contrary to expectation, this condition was more prevalent in animals coming to autopsy during the first 2 years of life than during the last 2 years.

TUMOR VIRUS ACTIVATION: ACTIVATION OF ENDOGENOUS ONCORNAVIRUSES BY RADIATION AND ENERGY-RELATED ENVIRONMENTAL POLLUTANTS

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A prevalent hypothesis suggests that radiation tumorigenesis may involve a viral mechanism. However, an unequivocal experimental demonstration is still lacking. We have previously shown that ^{90}Sr, a bone-seeking radionuclide, is an effective inducer of osteosarcomas in mice. In addition, we have isolated viruses, designated FBJ and FBR, from malignant bone tumors in two strains of mice, CF#1 and X/Gf, respectively. In the CF#1 strain, the tumor was spontaneous, whereas in X/Gf, it was induced by ^{90}Sr. In their respective hosts, these two viruses are potent, specific inducers of osteosarcomas. Since ^{90}Sr and our murine bone tumor viruses apparently act on the same target tissue, we have a unique system with which to determine whether radiation tumorigenesis involves the activation of these latent tumor viruses. With current techniques of molecular biology, we are searching for such evidence.

We aim to characterize these viruses and develop specific ^{3}H-cDNA probes and radioimmunoassays to monitor their expression in vivo. The demonstration of activation of latent cancer-causing viruses in our mouse systems would support the idea that viruses are fundamentally involved in cancer induction, and it would be a step toward understanding how radiation causes cancer. Following such a demonstration, there would be reason to suspect that chemotherapeutic and immunotherapeutic antiviral measures could be found for the prevention and cure of radiation-induced cancers.

We have partially characterized these malignant bone tumor viruses and developed appropriate cell culture systems for their large-scale production. Viral antigens and antisera as well as specific ^{3}H-cDNA probes are being prepared. Attempts are also being continued to recover tumor viruses from radionuclide-induced bone tumors.

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[1]Fall 1977 participant in the Undergraduate Honors Research Participation Program, Lawrence University.

[2]Participant in the 1977 Summer Research Institute in Cell Biology, University of Maine, Orono.

Biochemical Studies

Information basic to the assessment of the role of FBJ and FBR oncogenic viruses in ^{90}Sr-induced osteosarcoma was obtained. These two sarcoma viruses, probably the most relevant to sarcoma virus activation studies, were shown to possess 60-70S RNA, a reverse transcriptase, and a density in sucrose of 1.16 g/ml, all characteristic of type C RNA tumor viruses.

Of interest is the extent of relatedness of FBJ and FBR viruses to other murine oncornaviruses. Our preliminary molecular hybridizations indicated that there are substantial differences in sequence homology between RLV (murine Rauscher leukemia virus) and both FBJ and FBR viruses. A ^{3}H-cDNA probe made from RLV hybridized 86% to RLV DNA but only 69% to FBJ virus RNA and 68% to FBR virus RNA, as assayed by hydroxyapatite chromatography. There was also a significant drop in dissociation temperature--from 84°C to 70°C and 79°C, respectively. The same reaction samples, when assayed by S-1 nuclease, gave the following corresponding values: 50.0% to RLV, 40.2% to FBJ virus and 27.4% to FBR virus RNA's. These differences in sequence homology were also evident when the experiment was done in reverse. Our initial ^{3}H-cDNA probes, made from FBJ virus, hybridized 63% to FBJ virus RNA, but only 29.5% to RLV RNA. Similarly, a probe made from FBR virus, hybridized 84% to FBR virus RNA but only 62% to RLV RNA.

In a collaborative study with Arthur Frankel and Peter Fischinger (National Cancer Institute, Bethesda), we found that FBJ virus is also genomically different from Moloney sarcoma virus. Absent from FBJ virus are the Moloney sarcoma specific, or "sarc," sequences. Furthermore, the remaining shared, or "com," sequences are poorly related.

A step that often limits DNA probing studies is the synthesis of sufficient quantities of ^{3}H-cDNA by reverse transcriptase reactions. We recently discovered that reverse transcriptase reactions can be greatly stimulated by carrying out the reaction in the presence of polyethylene glycol--a neutral, oily, water-soluble polymer. Our preliminary experiments indicate that reaction kinetics are greatly enhanced and yields of faithful ^{3}H-cDNA probes are improved. These improved reaction conditions are potentially valuable in our proposed cDNA probing studies.

Tissue Culture Studies

We have found that FBJ and FBR viruses are capable of inducing morphological transformation of normal 3T3 fibroblasts derived from NIH Swiss or BALB/c mice. On dilution, they induce discrete foci of transformed cells, which can be quantitated for

a focus titration assay for these transforming viruses. The best indicator cell line for such focus titration assays is the D55 clone of BALB/c cells.

In association with the transforming agents, both virus preparations have nontransforming components 2-3 logs in excess of the transforming titers. These nontransforming components are readily detectable by reverse transcriptase assays and are routinely quantitated by a standard infectious center assay, the XC plaque assay.

Our proposed molecular studies require milligram quantities of these viruses. Therefore we have tested a number of cell lines for their large-scale production. The results indicated their host range of infectivity to be limited to murine cells. Whereas they infect and replicate efficiently in cell lines derived from NIH Swiss, BALB/c, C3H, and Sc-1 mice, they are noninfectious for cell lines of hamster, dog, mink, bat, sheep, and human origin. Among the murine cell lines, the best ones for producing FBJ and FBR viruses are the BALB/c and Sc-1 fibroblast, respectively. Firstly, these infected cell lines stably produce high titers of transforming virus (10^5 focus-forming units per ml). Secondly, the associated nontransforming components are present in excess by only one log, unlike the parent viral extracts and most sarcoma virus stocks. Such sarcoma-rich virus harvests make feasible the attempt to synthesize sarcoma-specific ^{3}H-cDNA probes. Thirdly, FBJ and FBR viruses derived from cell cultures retain their oncogenicity. They induce osteosarcomas in newborn mice of their respective host strains with potencies comparable to those obtained from tumor extracts. Fourthly, these infected cells adapt well to growth in roller bottles so that a constant supply source can be provided.

In collaboration with Jay Levy (Cancer Research Institute, University of California, San Francisco), a nonvirus producing culture was derived from an osteosarcoma induced in a Syrian hamster by FBJ virus. The cells, while not spontaneously releasing virus, do contain the FBJ sarcoma virus genome, which can be rescued by addition of helper oncornaviruses. This rescuable virus-cell system should be especially useful for viral activation studies.

Immunological Studies

In preparation for immunological studies, we have analyzed proteins of FBJ and FBR virus by SDS gel electrophoresis. Apart from the usual P30 and GP69-71 of murine C-type viruses, FBJ and FBR viruses have low molecular weight proteins of unique size classes. Instead of P10, P12, and P15, they have P9 and P11. This finding further indicates that they are distinct from other murine C-type viruses. Viral antisera and ^{125}I-labeled antigens are being prepared for the development of competitive radioimmunoassays for these viruses.

Virus Isolations from ^{90}Sr Tumors

Central to our study is the question of whether ^{90}Sr-induced osteosarcomas contain viruses capable of causing the same malignancy. The following observations were made with ^{90}Sr-induced osteosarcomas:

1) Most extracts made from ^{90}Sr-induced osteosarcomas contain viruses detectable by infectivity assays monitored by reverse transcriptase and XC plaque tests.

2) The majority of tumors placed in tissue culture or cocultured with susceptible host cells produce infectious virus detectable by the same tests. The transforming and tumorigenic potentials of these isolates are being evaluated.

3) Preliminary studies indicated that viruses, as detected by reverse transcriptase assays, are sometimes present in control cell cultures derived from kidneys, liver, spleen, and lung of normal CF#1 and X/Gf mice. The virus status in normal tissues will be confirmed and isolates will be characterized.

Biological Studies

Since FBJ and FBR viruses have such contrasting host ranges, we initiated studies designed to examine host genetic control of susceptibility to these viruses. FBJ virus is highly oncogenic in CF#1 mice and almost nononcogenic in the X/Gf strain, whereas FBR virus is highly oncogenic in X/Gf mice and almost nononcogenic in the CF#1 strain. We studied the cancer-inducing properties of each virus in the hybrid offspring of a CF#1-X/Gf cross. These hybrid offspring were susceptible to tumor induction by either sarcoma virus, but this susceptibility was significantly less than that of parental strains. This is true in all end points measured: the number of mice that developed sarcomas, the time at which death occurred from the sarcoma, and the mean number of sarcomas per animal.

In addition to the malignant FBJ virus we have previously isolated a second virus from CF#1 mice, the benign bone tumor virus, RFB, which induces osteomas. These two CF#1 viruses have similar host range specificities in tissue culture. They infect and replicate to high titers in whole embryonic cell cultures derived from CF#1, CBA, and NIH Swiss mice, but not from the X/Gf strain. Unlike the malignant virus FBJ, however, the benign virus RFB failed to induce morphologic transformation in cultures from susceptible hosts. It will be interesting to see, from our proposed genomic studies, whether there is a set of sequences present in FBJ, but absent in RFB. If so, such sequences could provide a molecular basis for the observed biological differences between FBR and RFB viruses with regard to transformation and malignancy.

Viruses from Fibrosarcomas Induced by Silastic Discs

We have also looked for viruses in fibrosarcomas induced by 15-mm silastic discs implanted under the skin of SKH hairless-1 mice which were supplied by R. J. M. Fry and D. D. Grube. Cultures derived from these tumors contained virus(es) detectable by XC plaque and reverse transcriptase assays. Their transforming and oncogenic potential are being evaluated.

In conclusion, the two osteosarcoma viruses central to our radiation/viral cocarcinogenesis studies, FBJ and FBR, have been partially characterized. With our previous morphologic studies, the present biochemical and biophysical data help establish that they belong to the Type C retrovirus group. Genomically, FBJ and FBR are different from at least two other murine leukemia and sarcoma viruses. This finding was not unexpected because our viruses have biological properties that are distinct from those of other murine viruses.

Several significant logistic and technical advances have been made that are important to the progress of our program. Firstly, we have established roller bottle cultures that stably yield large quantities of high-titered, transforming, and tumorigenic viruses without a vast excess of the nontransforming components. Secondly, the observed stimulatory effects of polyethylene glycol on reverse transcriptases should be valuable in our efforts to synthesize ^{3}H-cDNA probes. Thirdly, the unique P11 viral protein component may provide us a handle for developing specific radioimmunoassays for these viruses. These advances should facilitate the development of specific molecular probes and radioimmunoassays that we shall use together with other assays to monitor the expression of these viruses during ^{90}Sr induction of osteosarcomas.

CARCINOGEN DETECTOR SYSTEMS: STUDIES ON IN VITRO DETECTION SYSTEMS FOR CARCINOGENS AND BIOPHYSICAL AND IMMUNOLOGICAL MARKERS FOR TUMOR INITIATION AND PROGRESSION

T. E. O'Connor, B. A. Sedita, and D. D. Grube

Existing or new modes of energy production can potentially release carcinogenic agents into the workplace or general environment. An effective program for prevention of cancer by such energy-associated pollutants should include procedures for the detection of carcinogen effects in a simple biological system,

which can be routinely employed in monitoring exposure to carcinogenic pollutants, and, in addition, procedures for detecting early, preneoplastic changes in exposed populations. Such procedures for carcinogen monitoring and carcinogenesis detection in exposed populations should be based on molecular processes that are demonstrably relevant to the carcinogenetic process. As such, they should also be potentially useful in exploration of phenomena of tumor initiation and promotion in test animals and man.

The role of mutation in carcinogenesis remains controversial. Studies on the exposure of bacterial cell systems to mutagens have indicated an empirical correlation between mutagenicity in such systems and carcinogeniccity in animals. Nevertheless, the Boveri-Makino somatic mutation theory of cancer has not received definitive demonstration. Furthermore, the literature provides growing documentation of reversions of several tumor types to nonmalignant phenotypes under conditions where back-mutation appears excluded. Thus carcinogenesis appears to arise from a misdifferentiation in which somatic mutation may be incidental.

A number of observations in several laboratories suggests that chromatin preparations of animal tissues contain antigenic determinants and nonhistone acidic proteins that are tissue specific. Tumor induction involves generation of neoantigens and an altered set of chromatin nonhistone proteins that are tissue specific.

In a new program initiated in 1977, we are exploring the validity of these findings as a basis for the development of new carcinogen detector systems and as biophysical and immunological markers in tumor initiation and promotion.

The in vitro cell detector systems for carcinogens involve establishment of cell culture systems, derived from strain 129 SvSl mouse teratocarcinoma, that can undergo differentiation under controlled culture conditions. The cell types involved in these differentiations will be extensively characterized, with particular emphasis on their antigenicity and the composition of chromatin constituents, and on their patterns of protein synthesis as established by two-dimensional gel electrophoresis. The characterized cell types will then be exposed to a variety of mutagens and carcinogens, and the effect of the exposure on cellular phenotype and chromatin characteristics will be determined.

In parallel studies, conducted in collaboration with C. Peraino, transplantable rodent liver tumors are examined for their patterns of cellular protein synthesis and the presence of common chromatin antigens and nonhistone chromatin proteins. Any immunological and biophysical markers that are established will be utilized in a further examination of the biochemical and immunological characteristics of tumor initiation and progression.

PLASMA ISOZYMES AS POSSIBLE INDICATORS OF MUTAGENESIS IN MAMMALS

R. N. Feinstein and E. M. Buess

The need for rapid tests for carcinogenicity of environmental pollutants has been emphasized in the preceding reports. Because of the long time and the high cost requirements of the customary search for overt tumor production in mammals by suspect agents, emphasis has been diverted to testing for mutagens, as a first step toward testing for carcinogens. The most rapid and most used tests for mutagenicity are such revertant mutant microbial techniques as the Ames test. Other tests for mutagenesis range through tissue culture techniques to studies involving whole mammals. Although the latter are the most valid, they generally suffer from the defects of long time requirements and low incidence of detectable mutations.

We have devised, and subjected to preliminary testing, a new technique for direct observation of mutagenesis in mammals. Although implementation of the concept still requires a long time period, the system can detect over a hundred markers simultaneously in single plasma samples, and thus partly compensates for the expected low mutation rate.

The technique consists of feeding male mice a diet containing the suspect agent. (Other modes of administration are equally feasible.) After some weeks on the special diet, the male is mated to normal females, and the F_1 generation is examined after weaning. The examination consists of subjecting blood plasma to polyacrylamide gel electrophoresis, then incubating the gels in mixtures containing a number of substrates for dehydrogenases and oxidases, together with the two coenzymes NAD and NADP, the electron carrier phenazine methosulfate (PMS), and the ultimate gel marker nitroblue tetrazolium (NBT). It was soon found that normal plasma contains activity for lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PD), and xanthine oxidase (XO). Each plasma is therefore electrophoresed on four gels: the first gel is incubated in a mixture of lactate plus glucose, 6-phosphogluconate, succinate, and isocitrate; the second gel is incubated in a mixture containing malate, ethanol, cytochrome C, sarcosine, kynuramine, and putrescine; the third gel mixture contains glucose-6-phosphate, urate, propionaldehyde, glycerophosphate, glycolate, and phenyllactate; and the fourth gel contains hypoxanthine, xylitol, DL-alanine, DL-proline, DL-glutamate, and uridine-5'-diphosphoglucose. All gels also contain NAD, NADP, PMS, and NBT.

If one considers (1) that many of the above substrates are acted upon by enzymes using NAD, by enzymes using NADP, and by enzymes using no coenzyme (e.g., an oxidase); (2) that, in many cases, each enantiomorph of the substrate has its own enzyme(s); and (3) that, in many cases, the enzyme in question is composed of two or more subunits, each of which is subject to mutation, it can be calculated that the system possesses well over a hundred potential markers of mutation. These markers are, moreover, biochemically identifiable.

In the limited time this system has been under scrutiny, we have tested approximately 90 plasmas from normal mice, and approximately 400 plasmas from the progeny of males fed for various periods of time on diets containing aminotriazole, phenobarbital, DDT, BHT, AAF, or dimethylaminoazobenzene, i.e., several known carcinogens and some known noncarcinogen "promoters." In these preliminary series, no truly abnormal isozyme situations were encountered. It is considered that these preliminary results are inadequate either to demonstrate or to disprove the validity of the concept.

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7. LIPOSOMES AS BIOLOGICAL CARRIERS

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This program is concerned with studies seeking to develop liposomes as biological carriers for the targeted delivery of drugs. Liposomes are microscopic lipid vesicles that are formed by mixing aqueous solutions of various electrolytes or drugs with lipid materials under appropriate conditions. Larger size, multilamellar liposomes (0.2 to > 5 μm) or smaller size unilamellar liposomes (< 0.1 μm) can be prepared by the methods of shaking or sonication, respectively. Drugs can be incorporated into liposomes either within the aqueous center space and between the lipid bilayers of the liposomes or within the lipid bilayers. In the former case the liposomes are called "aqueous phase liposomes" (APL) for convenience and in the latter case, they are called "lipid phase liposomes" (LPL).

Liposomes differing in surface properties can be made by use of either a single or a combination of two or more lipids; and selective tissue uptake of these different types of liposomes can be obtained. In addition to being an attractive and versatile tool for approaching many basic problems in the field of biological research, liposomes have the potential for being useful in future clinical applications for drug delivery. At the present time, our research is centered mainly around the use of liposomes for the delivery of two categories of drugs--metal chelating agents and antitumor drugs.

Since October 1977, the program related to the use of antitumor drugs has been supported by a grant awarded by the National Cancer Institute (NCI). Another grant, specifically supporting the work on liposome application to iron overload, has been awarded by the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). Although the program of liposome studies at Argonne is currently supported under the grant system by NCI and NIAMDD, it is also related to the mission of the Department of Energy. Causative substances for both metal poisoning and cancer are emitted into the environment in part as pollutants produced by various energy related technologies.

The current program has two specific objectives: (1) to develop the delivery of drugs to specific target tissues by using liposomes with different physical and chemical properties and (2) to define and solve possible problems before the use of this drug delivery system in clinical applications.

LIPOSOME APPLICATION TO METAL CHELATION THERAPY

Y. E. Rahman, E. A. Cerny, B. J. Wright, M. M. Jonah,[1] and R. A. Guilmette

One of the major barriers to the use of liposomes for drug transport lies in the fact that intravenously injected liposomes are largely taken up by organs rich in reticuloendothelial cells, such as liver, spleen, and bone marrow. In many diseases, the target tissues are not limited to the reticuloendothelial organs. For application to metal chelation therapy, however, liposomes are particularly suitable, because the organs where excess metals are accumulated are precisely those that are rich in reticuloendothelial cells. For a review see Y. E. Rahman, Liposomes in Biological Systems, Eds. A. C. Allison and G. Gregoriadis, John Wiley and Sons, New York, in press.

We have used liposomes to deliver ethylenediaminetriacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) to specific target organs where toxic metals are accumulated. We have also used liposomes containing these chelating agents to treat experimental metal poisoning in mice, and compared the therapeutic efficacy of the encapsulated drug to that of the conventional nonencapsulated form. These studies are being continued.

After injection of lead or mercury into animals, these metals have a tendency to bind proteins in the cell nuclei of kidneys and liver. Liposomal DTPA given 10 hours after lead injection is effective in preventing the lead deposition in the cell nuclei.

During the year we have studied the pharmacokinetics of a potent iron chelator, desferrioxamine (DF), as affected by liposome encapsulation. Both unilamellar and multilamellar liposomes are effective in delivering DF to the critical organs of iron storage, primarily the liver and the spleen. However, the plasma disappearance of DF was slower when the chelating agent was encapsulated in unilamellar liposomes than when it was encapsulated in multilamellar liposomes.

Multilamellar liposomes have been prepared with various asialoglycolipids, gangliosides, sialic acid, or brain phospholipids in the liposome membrane and with EDTA encapsulated in the aqueous compartments. Some degree of selectivity in tissue uptake of EDTA has been obtained. Liposomes made of sialic acid or sialogangliosides show low uptake in the liver (15-20% of injected amount), while liposomes made of galactocerebroside or brain phospholipids show high liver uptake (45-50% of injected

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amount). Liposomes containing sphingomyelin show high uptake both by the lung and the brain. These results demonstrate that inclusion of sialogangliosides in liposome membranes decreases uptake of liposomes by liver, thus making direction of encapsulated drugs to other organs more feasible.

Studies on the mechanisms of liposome uptake by various tissue cells are being continued by biochemical, morphological, and autoradiographic methods.

LIPOSOME APPLICATION TO CANCER CHEMOTHERAPY

Y. E. Rahman, E. A. Cerny, and B. J. Wright

Most drugs used in cancer chemotherapy are highly toxic, not only to the tumor cells, but often to rapidly dividing normal cells, e.g., those in the bone marrow and the gastro-intestinal mucosal surfaces. Therefore, in order to avoid the toxic reactions in normal tissues and thereby to increase the therapeutic index of antitumor drugs, the delivery of an antitumor drug specifically to the tumor-bearing tissues becomes one of the most important problems to be solved in the field of cancer treatment. We have used liposomes to approach this challenging problem, in the hope of developing a new technique for delivering antitumor drugs to specific target tissues in order to improve therapeutic effectiveness and to reduce drug toxicity to normal nontarget tissues.

Due to the anatomical structure of the liver sinusoids, materials administered into the blood stream are in direct contact with liver cells, and large amounts of injected particulate materials, including liposomes, are taken up by the liver. This phenomenon results in low uptake of liposome-encapsulated drugs in other organs. This undesirable high uptake of liposomes by the liver constitutes one of the most serious limitations for application of liposomes in the delivery of antitumor drugs.

Two major objectives of this project are to search for specific types of liposomes for which liver cells have low affinity, and to search for liposomes that would be selectively taken up by target organs other than the liver. Our finding mentioned above that liposomes prepared with sialic acid or sialogangliosides have a low uptake in the liver is a step toward the first objective. For the same purpose, liposomes containing antitumor drugs have also been prepared with lipids extracted from various mouse tissues. When liver or kidney lipids are used to prepare liposomes, some degree of selectivity in liposome uptake by the spleen has been obtained. We are now in the process of identifying the lipid(s) that is responsible for this tissue selectivity.

We have previously shown that Actinomycin D (Act. D) encapsulated within liposomes is less toxic to mice than nonencapsulated Act. D, but retains its tumoricidal activity. We have attempted to elucidate the mechanisms of this reduction in drug toxicity by liposome encapsulation (Rahman, Y. E., et al., Ann. N. Y. Acad, Sci., in press). Injected at a sublethal dose of 0.4 mg/kg, Act. D encapsulated in the aqueous phase of liposomes was less toxic to white blood cells and to the nucleated cells and colony-forming stem cells of the bone marrow than the nonencapsulated drug. Drug toxicity in the proliferating intestinal cells, measured by its effect on ^{3}H-thymidine incorporation, was reduced by about a factor of 4 by encapsulation in aqueous liposomes, 24 hours after Act. D administration. Effects of Act. D on antibody production by spleen cells, determined by the "limited hemolysis in agar" assay, showed that immunosuppression was most markedly reduced by liposome encapsulation when the drug was given 1 day before the antigen, either in the aqueous or in the lipid phase of liposomes.

In summary, the delivery of a given drug to a specific target tissue is one of the most challenging problems in the field of pharmacology. We are particularly interested in using liposomes to deliver metal chelating agents and antitumor drugs. We have shown that liposomes can deliver metal chelators such as EDTA, DTPA, and DF to target organs of metal storage, and are therefore now in the position to explore the feasibility of applying liposomes for use in metal poisoning. Experimental iron overload will be our first test model for this drug delivery system. Regarding possible application of liposomes in cancer chemotherapy, experiments are being carried out in the search for liposomes that have a low affinity to the liver and that can be selectively delivered to tumor bearing tissues.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

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8. RADIATION TOXICITY IN DOGS

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The objective of these studies is to determine the detailed responses of beagles subjected to continuous (22 hr/day) irradiation from a ^{60}Co gamma-ray source. This work addresses problems relating to biological effects of fission energy technology. The studies are oriented toward investigating the late effects of continuous or protracted irradiation such as might occur if radioactive substances were deposited in man by inhalation, ingestion, or through wounds due to accidents or weapon systems. The problems of late effects from radionuclides cannot be easily investigated directly because of the nonuniform distribution, complicated metabolism, and dosimetry of radionuclides deposited in the body.

The effects of three major factors associated with the irradiation procedures are being explored: (1) the daily irradiation dose rate, (2) the accumulated total radiation exposure, and (3) the influence on the effects observed of age at the time irradiation is initiated. The work compares the responses of young-adult beagles ($\sim$ 400 days of age) of both sexes, kept in the gamma-ray field until they die, with those of similar dogs that have accumulated predetermined total exposures ranging from 600 to 4000 R. In addition, the life-time effects of irradiating the

developing beagle fetus continuously throughout its gestation period are determined, and this work is being extended to include irradiation during the postnatal period.

In earlier studies, young-adult and fetal beagles were irradiated at rates ranging from 5 to 300 R/day (Norris, W. P., et al., ANL-75-30, 1974, 9; Norris, W. P., et al., ANL-76-99, 1975, 10; Fritz, T. E., et al., ANL-77-55, 1976, 34). When dogs are irradiated until death at exposures in excess of 35 R/day, they uniformly die within a few weeks of bone marrow aplasia and septicemia. Over the range of 5-35 R/day, however, there were consistent systematic, highly dose-rate-dependent responses that were limited primarily to the hematopoietic and reproductive organs.

The dose-rate-dependent nature of these responses--bone marrow aplasia, bone marrow hyperplasia (myelogenous leukemia), aspermia in the male adult and fetus, and anovular ovaries in the female fetus--provides an opportunity for interspecies comparisons of dose-rate-dependent responses and investigation of mechanisms of damage that utilize specific physiological end points other than death or incidence of malignancies (Norris, W. P., S. A. Tyler, and G. A. Sacher, Biological Effects of Low-Level Radiation, Vol. 1, International Atomic Energy Agency, Vienna, 1976, 147-155).

With terminated exposures, the importance of dose rate is essentially unknown, and the acute disorders of the bone marrow, septicemia and anemia, are infrequently related to causes of death. In these studies, dogs are exposed in the gamma 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 (Fritz, T. E., et al., Proc. Symp. on the Late Biological Effects of Low Level Radiation, March 13-17, 1978, Vienna, in press). Longevity and terminal pathology will be evaluated on the basis of total exposure and exposure rate.

As a result of the data from the exposure rates at 5 R/day and above, it became important to extend the studies to daily dose rates substantially below 5 R/day. More than a year ago we therefore began to irradiate dogs starting at 400 days of age in a new gamma-ray exposure facility that accomodates 200 beagles at exposure rates of either 2.5, 1.0, or 0.4 R/day. The three main purposes of this work were to: (1) acquire data on the mortality rates in young-adult beagles that will extend our present comparisons of responses between species, (2) determine the nature of the responses and pathologic changes that may occur, and (3) define the exposure rates that allow for continued reproduction.

An especially important aspect of the second objective is to gain an insight into the changes that lead to induction of leuke-

mia and its opposite, aplastic anemia, through alteration of control mechanisms and functions in the hematopoietic system. Comparison of these effects of chronic irradiation to those of other agents, particularly chemicals, that also have a marrow suppressive and leukemogenic effect, also is an important and relevant goal. This aspect of our research is now covered in a separate program (see the following report by T. M. Seed et al.).

Bred female beagles, irradiated continuously at rates from 5 to 35 R/day from conception to parturition or for appropriate portions of gestation, produce apparently normal litters. On further evaluation, however, we find that the irradiated pups are significantly smaller and fail to develop full dentition in adult life. Females irradiated in utero at 5 R/day and higher were sterile due to hypoplastic anovular ovaries, while male littermates similarly irradiated at 5 R/day had normal sperm and were regarded as fertile. Females irradiated only at 2.5 R/day and less had apparently normal ovarian development.

Adult female beagles irradiated at 5 R/day and above during fetal life had atypical estrous periods, more frequent estrous periods, and copulated in spite of the fact that their ovaries were afollicular. Preliminary assays of ovarian and pituitary hormones have shown aberrations in hormone levels and mechanisms of ovarian function and control.

All dogs irradiated in utero that have not been sacrificed for sequential studies of the effects on gonads are being maintained for length of life to determine late effects and allow important comparisons to adult dogs receiving terminated exposures over the same dose range at the same exposure rates.

PROTRACTED IRRADIATION OF YOUNG-ADULT BEAGLES

T. E. Fritz, T. M. Seed, C. M. Poole, D. V. Tolle, L. S. Lombard; J. M. Angerman, S. M. Cullen, D. E. Doyle, L. V. Kaspar, W. G. Keenan, N. D. Kretz, P. H. Polk, and R. C. Simkins

Responses to Continuous Exposure to ^{60}Co Gamma Rays

All dogs irradiated at exposures down to 5 R/day are dead. Three separate radiation-induced causes of death, all related to the hematopoietic system, have been defined and shown to be related more directly to dose rate than to total dose. Briefly, these were septicemia associated with granulocytopenia (17-35 R/day), anemia (10-17 R/day), and myelogenous leukemia and myeloproliferative disorders (MPD) in dogs exposed to either 5 or 10 R/day was about 50%, and the onset of the disorder was characterized by hematological changes well in advance of clinically apparent disease. At 5 R/day, there was little or no effect upon erythropoiesis. Other responses, unrelated to the hematopoietic system, were evident in the last 10 decedents exposed to 5 R/day. In this group of 10 dogs there has been a number of malignancies, including one osteogenic sarcoma, and a variety of degenerative processes.

During the past year, data (times to death and causes of death) from the completed study at doses of 5 R/day and higher were evaluated, and the ^{60}Co sources were changed in the new radiation facility to provide for a greater number of dogs at the lowest exposure rate. Previously, on the basis of incomplete data, the radiation facility was programmed for irradiation of 100 dogs at 2.5 R/day to provide a large enough group to guarantee statistically significant data on life-shortening effects at this dose rate. With the death of all dogs in the study at 5-35 R/day, it became obvious that fewer dogs were necessary to ensure meaningful data at both 2.5 and 1.0 R/day. With the change in sources, the experimental design now provides for 100 dogs at 0.4 R/day, and 50 dogs each at 1.0 R/day, 2.5 R/day, and in the control group. Thus at 0.4 R/day there is now the chance for significant data on life shortening as well as for obtaining clinical data.

Most of our effort during the past year has been concerned with entering dogs into the lower exposure rate study at 2.5, 1.0, and 0.4 R/day. As of February 1, 1978, a total of 162 irradiated and control dogs have been entered into this study. There have been no deaths to this date, but dogs irradiated for $\sim$ 600 days at 2.5 R/day are showing a depression from control levels of platelets and leukocytes in the peripheral blood. Those irradiated at 1.0 and 0.4 R/day show little if any change.

Six male dogs at each low exposure rate and six controls were selected for sequential evaluation of the irradiation effects on spermatogenesis. Before the dogs entered the gamma field, two semen samples were collected from each dog and evaluated on the basis of both quantity and quality (sperm count, motility, live-dead ratio, etc.). Subsequent samples in the gamma field are being collected at 4-week intervals. After about 250 days of irradiation, the dogs exposed at 2.5 and 1.0 R/day are showing reductions in sperm counts to approximately 25% and 50%, respectively, of preirradiation levels. There are associated increases in abnormal morphology and reductions in motility and the ratio of live to dead sperm. The dogs in the 0.4 R/day group are maintaining normal preirradiation values suggesting that spermatogenesis, as well as hematopoiesis, may be refractory to protracted irradiation damage at this exposure rate.

Influence of Radiation Dose Rate and Total Dose on Late Effects Induced by Terminated Exposure to ^{60}Co Gamma Rays

The irradiation schedule has been completed for terminated exposure to protracted whole-body irradiation at fixed values of total exposure between 600 and 4000 R, as described previously. Three hundred fifty-four dogs (~ 20 per group) were irradiated and observed for development of early hematopoietic depression, recovery, and development of late effects.

Of these 354 dogs, 102 have died, either of septicemia or anemia, during the irradiation period or within 100 days after the exposure was terminated. A total of 31 have died at times longer than 100 days. These include 11 of those irradiated for a total of 4000 R at 10 R/day and 9 irradiated for a total of 4000 R at 35 R/day. No more than three dogs in each of the other groups has yet died more than 100 days after the termination of irradiation. There were 9 malignancies, including 4 leukemias, among the 11 dogs dead at 4000 R (10 R/day) and 5 (one leukemia) among the 9 dead at 1400 R (35 R/day).

The malignancies in dogs dying after 100 days now include a total of 4 cases of myelogenous leukemia, 3 lymphocytic leukemias, and 1 monocytic leukemia. The 4 myelogenous leukemias all occurred within 1 year after irradiation, as did 2 cases of lymphocytic leukemia. The monocytic leukemia, closely related to the myelogenous, occurred at 1241 days, while the other lymphocytic leukemia occurred 2341 days after termination of irradiation. In addition to the 8 hematopoietic tumors (leukemias) there were 9 other malignancies. These include a carcinoma of the intestine, a squamous carcinoma of the buccal cavity, 2 mammary carcinomas, 1 spenic neurofibrosarcoma, 2 splenic angiosarcomas, 1 generalized mast cell tumor, and 1 transitional cell tumor of the bladder.

Other than the malignancies, which are excessive for the ages at which the dogs have died, there is no other pattern yet emerging as regards significant pathological end points or lesions specifically related to the irradiation.

Because of the schedule on which dogs were staged into the gamma field, survival time to date varies considerably. Some of the dogs have been observed for more than 3500 days after exposure. Subsequent to the early wave of myelogenous leukemias, described above, and the other soft tissue tumors, there were no other deaths that can be related to the irradiation. The minimum terminated exposure to produce myelogenous disorders still remains 1700 R, and the mean induction time is about 400 days. The incidence of myelogenous leukemia and related myeloproliferative disease decreases rapidly in dogs that survive beyond this time and (as in dogs irradiated until death) appears related to the recovery of the bone marrow, in this case after termination of irradiation.

Hematological data are being collected to evaluate the recovery pattern as a function of total dose and dose rate. Initial comparison of the various groups of dogs reveals large differences in the rates at which the cellular elements in peripheral blood return toward normal values after the exposures are terminated. Among the dogs given the same total dose of irradiation, the nadir to which the cellular elements fall and the recovery rates are related to the dose rate. The higher the dose rate, the lower the nadir for the erythrocyte, leukocyte, and platelet levels. Recovery rates become progressively slower as the exposure rate is increased.

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

T. E. Fritz, T. M. Seed, C. M. Poole, D. V. Tolle, D. L. Zeger, L. S. Lombard, W. G. Keenan, D. E. Doyle, P. H. Polk, and M. M. Sanderson

A total of 65 adult dogs irradiated during fetal life are now surviving at ages between 1600 and 2500 days of age. There have been only six deaths in dogs surviving the neonatal period. Two of these six decedents, both irradiated at 10 R/day, died of renal disease at 918 and 1168 days of age. These are the only deaths that appear related to the radiation exposure during fetal life. The other four dogs died of epileptic seizures (two cases), accidental strangulation, and endometritis. The remaining dogs appear normal except for the reproductive effects described earlier, their smaller than normal size, and the failure of a significant number of teeth to erupt in many animals.

Additional assays of serum hormone levels from groups of females irradiated *in utero* and from control animals have been conducted during the past year (in collaboration with Dr. G. Niswender, Colorado State University). Comparisons were made among pregnant, nonpregnant, and irradiated bitches. The results show that a preovulatory surge of luteinizing hormone (LH) occurred in the irradiated animals, but that ovulation did not occur. Although basal levels of estradiol were similar between normal (pregnant and nonbred) and irradiated bitches, there was no evidence in the irradiated bitches of the peak in serum estradiol levels that occurs in normal bitches prior to the peak in LH. Serum levels of follicle stimulating hormone (FSH) in the irradiated bitches were high and variable around the time of the peak in LH, and were similar to those reported in castrated bitches. These data suggest a lack of negative feedback inhibition of FSH. This suggestion cannot be fully substantiated by these data, but it is consistent with reports that the ovaries (and testes) secrete a nonsteroidal factor (inhibin) that controls FSH levels in the blood. This factor is derived from antral follicles, which are not present in ovaries of irradiated bitches.

To study further the influence of irradiation on ovarian function and hormone production, two normal adult bitches and two adult bitches irradiated *in utero* were castrated. Daily serum samples were collected for 2 weeks before and after surgery, and 4 times each day on the day after surgery. Two adult bitches irradiated *in utero* and castrated during the first month of life were similarly sampled. Assays for LH, estradiol, FSH, and progesterone will be run to determine the respective influence of irradiation and castration on the circulating hormone levels and to test further the hypothesis of the role of a follicular factor in FSH secretion.

CELLULAR EFFECTS OF MYELOSUPPRESSIVE AGENTS ON HEMATOPOIETIC TISSUE STRUCTURE AND FUNCTION: MECHANISMS OF LEUKEMIA INDUCTION

T. M. Seed, T. E. Fritz, D. V. Tolle, C. M. Poole, P. C. Brennan, L. V. Kaspar, S. M. Cullen, M. M. Sanderson, G. T. Chubb, B. J. Wright, N. D. Kretz, R. L. Devine, J. M. Angerman, W. G. Keenan, P. H. Polk, and D. E. Doyle

Under the appropriate conditions, the chemical agent, benzene, and the physical agent, protracted ionizing gamma radiation, both produce marked myelosuppression, which is often followed by either aplastic anemia (marrow aplasia) or leukemia (marrow hyperplasia). Our previous studies using the beagle showed that continuous gamma irradiation in the daily dose range

of 5-35 R/day produced three separate, dose-rate dependent causes of death--septicemia, anemia, and myeloproliferative disorders (MPD). The latter were expressed largely as myelogenous leukemia and were most common at the lowest dose rates (5 and 10 R/day). Although the causes of death were generally dose-rate dependent, individual dogs at a given dose rate did not all die from the same cause of death, indicating a wide variation of the individual response to the daily stress of hematopoietic intoxication.

The question of individual variation in response to a graded dose of a toxic agent becomes a critical one that should be addressed in low-level toxicology studies. For the individual, the specific response is all important and determines longevity. For the experimental group (population), the variation has not been adequately studied or explained, but clearly shows a wide potential for adaptation to chronic exposure to a toxicant. What then are the early indicators of the defined responses? What are the mechanisms behind each response? These are the problems that we are attempting to address.

Our approach includes (1) multiphasic longitudinal studies centered on characterization of the hematopoietic response(s) of dogs subjected to protracted exposures to low doses of myelosuppressive agents, and (2) development and application of *in vitro* techniques of hematopoietic cell cloning. In the former, major emphasis is on a series of dogs given continuous ^{60}Co gamma irradiation, but studies have also been initiated with the leukemogenic aromatic hydrocarbon benzene. In both approaches, emphasis is placed on the response of the committed hematopoietic stem cells, the suspected targets for myelosuppressive leukemogenic agents.

Radiation Leukemogenesis: Longitudinal Studies

Survival and clinical observations. To date twenty-three beagles (three irradiated groups of six animals each, plus five control dogs) are being followed. The three irradiated are receiving whole-body ^{60}Co gamma radiation at 10 R/day and as of February 1, 1978 had accumulated total radiation doses of 2330 R, 5130 R, and 6900 R. Within the latter two groups, six dogs died of marrow aplasia following a mean exposure time of 245 days and an accumulated radiation dose of about 2450 R. The six surviving dogs exhibited hematological profiles characteristic of preclinical phases of developing myeloproliferative disorders. Recently, two of these long-term survivors have died of erythroid aplasia (at a mean of $\sim$ 450 days and 4500 R). The selective ablation of erythropoiesis in the face of continuing myelopoiesis in these two dogs is an important example of the differential sensitivities of marrow components that undoubtedly play an important role in leukemia induction.

Hemopathology. Data collected over the past year continue to support and strengthen the concept that distinct phases precede the onset of radiation-induced leukemia and that these phases are readily recognized and therefore prognostic. A critical sequence of events that determines the pathological fate (i.e., aplasia or MPD) of these irradiated dogs occurs during an early period of acute hematopoietic crisis (i.e., at 200-300 days, 2000-3000 R accumulated dose). Dogs that do not die of marrow aplasia during this crisis period then show a rather dramatic, broadly based partial recovery of hematopoietic capacity. Becuase these long-term survivors have a high probability of developing MPD, the recovery period is being carefully scrutinized.

Increases in peripheral blood platelets and leukocytes observed during this partial recovery phase are accompanied by a progressive increase in marrow cellularity. Local areas of intense cellular proliferation exhibit pronounced megaloblastoid and/or monocytoid features. At the ultrastructural level there is an elevated incidence of cytologically aberrant erythroid and myeloid progenitors; nuclear lesions (i.e., clefts, sessile bodies and unusual chromatin distributions) are particularly prominent. The more distinctive cytoplasmic lesions include the massive Golgi regions of the myeloblasts and the localized dilated cisternae and ferritin-containing membranous inclusions of proerythrocytes.

The bacterial endotoxin stress assay (Marsh, J. C., and S. Perry, Blood 23, 581, 1964) has been utilized to detect specific sequential compartmental changes in the cellular reserves of the marrow throughout the course of irradiaton. During the first 300 days of irradiation there is a progressive reduction in both the size and mobility of the granulocyte reserve; this reduction appears time and total dose dependent. At later periods, i.e., 400 days, there is a partial recovery in the size of this compartment and its function (mobility) under acute enlotoxin stress.

Accompanying this increase in granulocyte reserve is a small but significant expansion of the granulocyte stem cell pool (GM-CFUa). The in vitro soft agar culture method (Marsh, J. C., et al., J. Lab. Clin. Med. 79, 1041, 1972), used to quantitate sequentially committed stem cells within irradiated marrow, has revealed marked qualitative changes in the cloning pattern of granulocytic progenitors, the suspected cellular targets of leukemogenic gamma irradiation. Preliminary cytological-ultrastructural evaluation of such aberrant granulocyte clones reveals an increased incidence of nuclear abnormalities and a generalized retardation of normal cellular differentiation.

Special ultrastructural techniques are being developed that will allow cloned granulocytic progenitors to be examined directly for altered patterns of cellular proliferation and dif-

ferentiation with special reference to cell surface markers and cellular interactions within developing colonies. Utilizing our recently developed EM techniques which allow agar embedded colonies to be topographically analyzed (Seed, T. M., et al., Micron, in press), we have observed differences between cell surface projections of normal and leukemic cell clones and in the manner in which adjacent cells within the colony interact.

The proliferation and differentiation of the hematopoietic stem cell committed to granulocyte-monocyte differentiation are regulated by a hormone-like serum factor (CSF). Using the soft agar cloning technique and isolated mononuclear bone marrow cells from normal, control dogs as the cellular targets for CSF, the serum activity of CSF has been sequentially assayed in dogs with accumulated radiation exposure ranging from 0 to 6900 R. Serum titers of CSF are elevated during phases of hematopoietic crisis and partial recovery but appear to decline later as marrow plasticity increases. In combination with the endotoxin stress assays, the 24-hour change in production of CSF in serum following endotoxin stress of control and irradiated dogs is being studied. Control dogs generally exhibit two peaks of serum CSF activity at 2 and 8 hours after endotoxin injection; these activity peaks correspond in time with the linear granulocytic response. In contrast, irradiated animals produce broad single peaks of CSF activity between 2-6 hours after injection. Up to approximately 300 days of irradiation, the serum activity curve following endotoxin stress is elevated; after exposures of over 400 days, the serum CSF response is depressed below control values, although the shape of the curve remains about the same.

We continue to describe the hemopathology of cases of overt radiation-induced MPD. In every instance, the disease complex (i.e., MPD) closely resembled the "spontaneous" disease in humans. Grown in culture, cells from an irradiated dog with monocytic leukemia showed a striking increase in the number of clonable, circulating stem cells committed to monocytic differentiation. This increase corresponded to the previously noted large number of circulating, morphologically aberrant immature monocytes (granulocytes). The increased rate of cloning was only observed after pretreatment with phytohemagglutinin (PHA) or freezing with dimethylsulfoxide. Leukemic serum had a suppressive effect on cloning of normal GM-CFUa cells, but, in contrast, a marginally enhancing effect on cloning of autologous PHA-stimulated leukemic cells. The sum of these observations of leukemic cells in culture suggest that both intrinsic cellular defects as well as extrinsic factors (e.g., plasma and tissue effects) influence pathological processes involved in myeloproliferative diseases, e.g., leukemia.

Immunopathology. We are continuing to make serial assessments of change in cellular immune competence in dogs assigned to these longitudinal leukemogenic studies. Results are presently

being assessed in terms of changing patterns of hematopoiesis. In general, function of thymus-derived lymphocytes appears to decay gradually with prolonged radiation exposures.

Virological assessment. To date, budding C-type viral particles have not been observed by electron microscopy in any leukemic tissue taken at necropsy nor in any biopsied tissues taken in the preclinical development of leukemia.

We extended our electron microscopic search for C-type virus to fetal placental tissue, purportedly a privileged site of C-type virus expression. We have examined a total of 60 tissues, 44 of them from fetuses of control bitches and 16 from fetuses irradiated throughout gestation at either 2.5 or 5.0 R/day. C-type virus has not been observed in any of these tissues. For comparison, we have initiated a similar study using Sprague-Dawley rats, a strain which has a low expression of C-type virus in fetal placental tissues. Positive findings in the rat will reinforce our negative results in the dog.

Other virologic techniques (e.g., reverse transcriptase assays), which may be useful for identifying the presence of virus, are planned but not yet initiated.

Histocompatibility analysis: Bone marrow grafting. Through a collaborative study with the Radiobiology Laboratory, University of California at Davis, all the dogs presently in the longitudinal leukemogenic study were typed for histocompatible antigens (DLA) during the past year. The relationship of DLA type to the induction of a given type of hemopathology will be assessed retrospectively.

We have made two attempts to graft (transplant) allogeneic monocytic-leukemogenic cells into term canine fetuses or gamma-irradiated neonates (150 R). Both attempts, at this time, appear unsuccessful.

In summary, the results to date in our longitudinal radiation studies suggest that distinct preclinical phases precede the onset of the radiation-induced leukemia in the beagle. An early period of deteriorating hematopoiesis followed by partial restoration and equilibration of subnormal hematopoietic function appear to be a consistent sequence of events preceding the onset of leukemia. Preliminary data show a rather dramatic reversal in marrow plasticity in these preclinical periods, especially at the level of the committed granulopoietic stem cell; this is consistent with the possibility that the chronic myelosuppression induced by protracted low dose irradiation promotes a clonal selection of aberrant cell populations responsible for leukemia induction.

In this regard, much of our effort in the past year has been devoted to establishing ultrastructural-cytochemical assays, based on the soft agar cloning method, that permit us to detect cellular (clonal) aberrancy within one of the suspected cellular targets of leukemogenic ionizing irradiation (i.e., granulocyte committed stem cells). The use of such phase specific markers is intended to provide additional evidence that subpatent disease exists during these very early periods, and might provide extremely useful prognostic indicator(s) of impending leukemia.

Chemical Leukemogenesis: Benzene Intoxication

A pilot study was initiated during the past year in which graded doses of benzene were given to three dogs, by either subcutaneous, intravenous, or oral route. The primary intent was to assess the importance of administration route relative to the degree of induced myelosuppression. Because of the acutely toxic and necrotizing effect of benzene given by parenteral routes, and the related acute effects on peripheral blood values, the subcutaneous and intravenous routes proved to be experimentally unattractive. Oral administration in quantities as high as 5 ml/day for up to 6 months, on the other hand, failed to produce a pronounced effect on peripheral blood values. Inhalation, the usual route of exposure for man, is seemingly the route of choice for future evaluation of biologically significant doses delivered to experimental animals.

The direct myelotoxic effects of benzene on free hematopoietic cells are being evaluated *in vitro*. Sealable glass CO_2 culture chambers have been designed and are currently in use to test the effect of benzene atmospheres up to 15,000 ppm on cytotoxicity and the inhibition of granulocyte progenitor cell proliferation. Initial observations indicate that between the concentration range of 5,000 to 15,000 ppm of benzene, the clonogenic activity of normal granulocytic progenitors is virtually eliminated (in the presence of normal canine serum). Nonproliferating culture cells, however, appeared viable as indicated by trypan blue dye exclusion. Dose-response curves for benzene will be determined in the coming year and correlated with results of animal toxicity studies.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

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9. NEUTRON AND GAMMA-RAY TOXICITY STUDIES

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

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

An integral component of the program is the use of two types of radiation, with quantitative differences in their biological effects: gamma radiation from ^{60}Co sources, and fission neutrons

from the JANUS reactor. One of the most important differences is the relative lack of dose-rate effects with the latter; in terms of the quadratic model, the coefficient of the dose-squared term is extremely small, if not zero. Therefore, it is our intention to employ single exposures of neutrons at low doses, down to the lowest doses at which lethal effects or selected physiological changes can be reasonably expected to be observed with numbers of animals that are manageable in terms of the resources of our animal facilities. A limited number of experiments with fractionated doses of neutrons will be carried out to assess the validity of the assumptions of dose-rate independence. (Because of the regulations governing the operation of the reactor, truly protracted or continuous exposures become prohibitively expensive; however, our data suggest that fractionated exposures can serve as surrogates for continuous neutron irradiation.)

The approach to the problem of gamma irradiation will require considerable attention to dose protraction and fractionation. The constraints on continuous neutron exposures do not apply to ^{60}Co gamma irradiation; however, there is a large amount of data already available, much of it generated in this Laboratory, concerning dose-rate effects on life shortening, based largely on duration-of-life exposures. Considerably less is known about protraction and fractionation effects on tumorigenesis. However, we feel that relatively few experiments in which animals are continuously exposed will be required to bridge the gap between the present program and the earlier experiments.

The obvious choice of experimental animals for a variety of reasons is the laboratory mouse (Mus musculus), specifically the B6CF$_1$/Anl hybrid. The extrapolation of Mus data to man, however, requires at least two intermediate stages. The first of these is the use of another rodent, the white-footed mouse (Peromyscus leucopus), about the size of Mus but with more than double the life-span and with a spectrum of tumors that is distinctly different from that of the B6CF$_1$ mouse. A limited number of single exposures to the same doses of neutron and gamma radiation already employed in Mus should suffice to establish whether the life shortening per rad is an absolute value, or whether it is a percentage of total life-span.

The second stage involves the beagle, for which exposures to low-level continuous ^{60}Co gamma radiation have been under way for several years (see Section 8, Radiation Toxicity in Dogs). The problems of dosimetry, numbers of animals needed, and the long life-span of the beagle militate against studying neutron effects. We expect that reasonable predictions can be made from the gamma-ray data by means of the comparisons established for the two radiation qualities between Mus and Peromyscus.

Studies of late radiation effects on the cardiovascular system serve to complement life shortening and cancer experiments in the JANUS Program, and to provide improved definition of the role

of systemic injuries in the late effects syndrome. Little is known about long-term cardiovascular effects of exposure to any of the various environmental hazards associated with energy conversion technologies. The techniques used in the studies involving nuclear energy can also be applied to assessment of the effects of chronic exposure to products of nonnuclear energy conversion. Subclinical cardiovascular damage may reduce physiological reserves and thereby increase susceptibility to subsequent infection or other stress situations. Vascular injury contributes to radiation-induced degenerative changes via effects on the slowly dividing cell populations that comprise the blood vessels and heart. The role of this largely noncytokinetic injury in late morbidity requires further study. The $B6CF_1$ mouse is again used as the principal test animal. Studies of a more limited nature, using Peromyscus leucopus, will provide data for interspecies comparisons between two rodent species having at least a twofold difference in life-span. Such comparisons will eventually assist in human hazard evaluation.

Our studies of late damage to the cardiovascular system emphasize relationships between (1) functional deterioration, assessed by clearance of injected ^{133}Xe as an indicator of capillary blood flow, and by measurements of blood pressure; and (2) histopathological changes in the subcutaneous microvasculature, in the heart and aorta, and in the kidney. The studies include light and electron microscopic observations. Additional investigations of the microvasculature are planned with the use of microangiography. Coronary artery damage, vascular deterioration and fibrosis in essential organs, and microvascular efficiency are end points of interest in connection with therapeutic use of low- and high-LET radiation, as well as in many phases of nuclear technology. Of particular interest in our current findings is the high sensitivity of the coronary artery to fractionated neutron irradiation.

LIFE SHORTENING AFTER EXPOSURE TO NEUTRONS AND GAMMA RAYS

J. F. Thomson, K. H. Allen, R. J. M. Fry,[1] D. Grahn, J. L. Hulesch, D. L. Jordan, L. S. Lombard, V. A. Ludeman, M. Miller, A. R. Sallese, E. F. Staffeldt, and F. S. Williamson

The status of the irradiation experiments is as follows:

JM-2 involved a total of 7200 mice of both sexes (plus 2000 controls) that were exposed to single or fractionated doses of neutrons and ^{60}Co gamma rays. A number of animals were subsequently removed from the experiment, but 5869 irradiated and 1700 control mice were observed for their life-span. Preliminary analyses of life-shortening data have been published; histopathologic examinations are complete, and a detailed analysis of death is under way. In a supplementary experiment JM-YZ, single doses (80 and 240 neutron rads, 268 and 788 rads gamma rays) were given to older animals to compare the responses of 194- and 287-day-old mice with those of about 110 days of age, the age of the JM-2 mice at the time of their initial exposures; all of this group of 2400 mice are now dead.

JM-3S is a single dose exposure series, 20 to 160 neutron rads and 90 to 569 rads gamma rays, which are expected to produce 2% to 25% life shortening. Irradiations were completed (2695 animals) in October, 1977.

JM-4K is a fractionated dose series given over 23 weeks, about 20% of the life-span. The total neutron doses are the same as those used in JM-3S, whereas the gamma ray doses are higher (206 to 5110 rads total dose). The last replicate for irradiation treatment was completed in April, 1977. Four thousand seven hundred fifty mice were exposed.

JM-4L will involve the exposures of both Mus and Peromyscus to continuous ^{60}Co gamma radiation for varying periods of time (no neutron exposures). This experiment, which has been delayed pending recalibration of the Low Level Gamma Facility (see below) and construction of special animal housing, will begin in mid-1978.

JM-4W and JM-5 are a series of single and fractionated doses for both serial evaluation and sacrifice for cardiovascular, hematopoietic, and immunologic studies. All irradiations have been completed for both series (3275 mice).

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JM-6 involves irradiations of mice with both single and protracted exposures for the carcinogenesis study with pituitary hormones and isografts. Irradiations have been completed for 1890 mice.

JM-7Q consists of fractionated doses given in 60 weekly exposures over 59 weeks (~50% of the life-span) for comparison with JM-4K. As in most experiments, irradiation is begun when the mice are about 110 days old. The final component of this series was cancelled; the last of the 1045 animals committed to this experiment will receive their final exposures in July, 1978.

JM-7R is a companion experiment, in which single doses are given to mice at 535 days of age, i.e., at the end of the 59-week fractionation period used in JM-7Q. Originally, 1700 animals were assigned to this series, but after 1020 mice were irradiated, the remaining replicates were cancelled.

JM-8U employs one exposure per week for the duration of the life-span of the animals for comparison with similar total doses given over shorter intervals or as a single exposure. The original experimental design called for the allocation of 2000 mice to three dose rates of neutrons (0.667, 1.667, and 2.667 rads/exposure) and three of gamma rays (6.95, 17.4, and 31.4 rads/exposure). After 1200 mice had been entered into this regimen, the decision was made to commit no more animals.

JM-9X is a new series involving low doses of neutrons and gamma rays that will be carried out in two stages. The first part, for which irradiations were begun in June, 1977, consisted of single and fractionated exposures to 10 rads of neutrons, and single exposures to 5 rads. The second stage, involving single exposures to 1, 2.5, 5, 10, 20, and 120 rads of neutrons and 22.5, 45, and 90 rads of gamma radiation, will be initiated after the barrier modifications to the animal facilities are completed and the mouse strains are rederived. The reason for deferring the second stage is that there is concern that the present population of animals, having been raised under conditions that are somewhat less than optimal, may show the so-called "beneficial effects of radiation," i.e., an increase in mean after-survival following low doses of radiation, as a result of a decrease in early deaths from intercurrent infection.

JM-10E employs Peromyscus leucopus as the experimental animal. Neutron irradiation at single doses of 20, 40, 80, and 160 rads, along with fractionated exposures to 40 and 160 rads, were begun in November, 1977. When they are completed, gamma irradiation (90, 143, 206, and 417 rads) will begin. The reason why the neutron and gamma exposures are not carried out in parallel reflects the fact that in order to standardize the body weights as much as possible, we must reject about 40% of the animals that are bred; to obtain all of the animals needed at one time would overstress the animal production facilities.

JM-11E was designed by Dr. R. J. M. Fry (now at ORNL) to establish dose-response curves for a specific tumor system, namely, the Harderian gland. The approach is to give the irradiation in fractions--2, 4, 8, and 16 weekly exposures of 2.5 neutron rads; 24, 36, and 48 exposures of 17.4 gamma rads--and after the final fraction to implant pituitary isografts as tumor promoters. The exposures were begun in October, 1977. Experiments with single doses have previously been carried out in the JM-6 series (see above).

The "300" series (no JM designation) was planned by Dr. Douglas Grahn to evaluate various genetic end points after single exposures to essentially the same doses of neutron and gamma radiation planned for the second phase of JM-9X. The design and rationale for these exposures are described in Section 10, Genetic Toxicology. The first exposures in this series took place in November, 1977. Dr. Grahn is also studying the effects of fractionated neutron and gamma radiation on these same end points, with additional male mice added to the JM-7Q and JM-8U exposures.

Provisional analysis of the life-shortening data from the JM-2 through the JM-8 series reveals a number of general trends: (1) females are more sensitive than males to neutron but not to gamma radiation; (2) fractionation of gamma radiation produces a "sparing" effect, i.e., a longer mean survival time than after the same total dose given in a single exposure; (3) fractionation of neutron irradiation, on the other hand, produces an augmentation of life shortening at high doses (> 100 rads), but not at low doses; (4) life shortening per rad is lower in older animals; (5) projections from present data suggest that the relative biological effectiveness (RBE) for single dose JANUS fission neutrons relative to gamma rays will fall between 10 and 100 in the dose range of 1 to 0.1 rad.

The last conclusion is derived from the observation that when excess mortality rates are plotted against dose on a log-log plot, the slope (i.e., the power function exponent) for single-dose neutron exposures is about 0.5, whereas that for single-dose gamma irradiation is near unity. Preliminary analysis of some of the tumor data from JM-2 suggests that the same slopes will obtain, despite the fact that the mean after-survivals for animals dying with various tumors may vary by 20% of the life-span of the mouse. In other words, regardless of cause of death (in the cases examined), the life shortening per rad varies inversely with single doses of neutrons but remains relatively constant for gamma radiation.

DOSIMETRY AND DATA PROCESSING

F. S. Williamson, G. L. Holmblad, E. G. Johnson, Jr., and J. E. Trier

Neutron Dosimetry

Since June, 1977, the JANUS reactor has been operated only 2 days per week. This arrangement is unquestionably cost effective, but has the result that until mid-1978 no time is available in the JANUS High Flux Room for experimental neutron dosimetry.

We found that the draft report for the International Commission on Radiological Units on the International Dosimetry Intercomparison (INDI) contained errors in reporting our data. Our data have been reevaluated and checked and the discrepancies reported to the INDI Committee.

The Shonka 4-inch tissue-equivalent ionization chambers used in exposure monitoring have been refilled using improved sealing technique and show negligible gas leakage. This should reduce the need for calibration checks in the JANUS High Flux Room.

Phantom depth-dose measurements were made to support the Monte Carlo calculation of depth doses in phantoms corresponding to mice of different sizes. The data are in process of evaluation.

Gamma and X-ray Dosimetry

The High Level Gamma Facility is in use 2 days per week (fully saturated days) for the ongoing JM-4 through JM-11 series of irradiations, and receives frequent, though sporadic, use on other days.

The Low Level Gamma Facility has been recommissioned, and preliminary dosimetry calibrations and worst-case vertical scans have been made. Source attenuators designed to compensate for decay in the ^{60}Co source over the next few years have been fabricated and checked. These thick attenuators cause great perturbation of the shape of isodose contours, in the vertical plane, close to the source, so that a requirement for a wide range of dose rates combined with large numbers of animals presents a very complex design problem in the facility. Fortunately, a reduction in the number of animals planned for JM-4L will ease this problem, though it will still be complex.

We expect to use our automatic readout thermoluminescent dosimeter (TLD) system extensively in the dosimetry of the Low Level Gamma Facility. A study of TLD response in phantoms of aluminum, lead, polyethylene, and bone-equivalent plastic con-

firms that photon absorbed doses in such media can be measured using established cavity theory.

Data Processing

Gross autopsy records from 9150 JM-2 animals are now on file, and 6533 histopathology records have been processed. A special program to examine record sequence numbers identifies those few records that were overlooked. Programs have been written that display the frequency distribution of individual disease codes in either of these files in two-dimensional tables (treatment versus code). Another program permits the examination of possible combinations of disease codes--we propose to use this to establish plausible code pools for life table analysis.

The programs for life table analysis using a combination of gross autopsy data and histopathology data have been tested. At present the histopathology data are being tested against the gross autospy data as rigorously as possible so that we may have full confidence in the inferences to be drawn from analyses of these data. It is not always appreciated that pathology is fundamentally not a quantitative science, yet we must make it quantitative and rigorous to achieve our objectives.

A new program displays the status of animals in an ongoing experiment with mean and median survival times for each replicate. This is particularly useful for estimating trends before all the animals have died. Work is now in progress on developing the direct-entry pathology system for the PDP-11/20. Although this task is of high priority, the analysis of JM-2 pathology takes precedence, and data processing support for the weekly irradiations has the highest priority of all.

CARDIOVASCULAR EFFECTS

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

Long-term studies of radiation effects on the cardiovascular system in the $B6CF_1$ mouse continued to emphasize comparisons of structural and functional changes. The comparative effects of total-body exposure to ^{60}Co gamma rays or fission spectrum neutrons on subcutaneous capillary blood flow have been estimated by measurement of xenon clearance after subcutaneous injection of ^{133}Xe in a saline solution. These findings are important as an indicator of functional changes in the microvasculature that can

then be related to structural changes observed both *in vivo* and with the electron microscope. The clearance of ^{133}Xe from the subcutaneous injection site can be fitted to a single exponential. In unirradiated control mice, the mean half time ($0.693/\lambda$) in 4-month-old animals was 9.83 ± 0.72 min, and ranged between 9.48-10.70 min in 4-16 month-old animals. The half time increased to 130-140% of the initial mean value between 16 and 28 months, and then decreased to as low as 50% in very old animals. In mice irradiated at 4 months, the clearance half time of ^{133}Xe increased significantly 3-6 months after exposure to gamma rays or fission neutrons at dose levels well below the acutely lethal range (i.e., 268 gamma rads or 80 neutron rads). After exposure to higher dose levels, but still below the acutely lethal range, there was little change from mean control values. The data for both irradiated and control mice clearly indicate, however, that clearance times were shorter at ages in excess of the mean survival time, and suggest a compensatory increase in capillary blood flow. This increase occurred at an earlier age in irradiated than in control mice, and may be associated with the morbidity that contributes to life shortening. The increase in rate of blood flow may be related to a decreased efficiency in utilization of oxygen and/or to development of hypertension. The pinna vasculature of mice exposed to gamma ray doses that resulted in longer xenon clearance half times showed fine structural evidence of apparent endothelial proliferation, to the extent of blood flow obstruction in some instances. Such findings could be correlated with the increased vascular tortuosities observed with the light microscope. We have noted that early microvascular repair (less than 30 days after irradiation) is associated with proliferation of undifferentiated cell populations from the subendothelial interstitial region in the revascularizing rabbit ear chamber. Thus, mesenchymal-type connective tissue cells may also contribute to repair of endothelial damage.

Evaluation of ultrastructural changes in the heart and aorta through 24 months after irradiation is in progress, while changes in the myocardium and cardiac microvasculature through 12 months have been reported. Study of the later effects, at 18 and 24 months after the beginning of the experiment, revealed a persistent and increasing damage that was most severe in neutron-treated animals. Quantitative evaluations of degenerating capillaries and myocytes in heart tissue showed values significantly above the control level throughout the later period after single dose exposure to 788 gamma rads or 240 neutron rads. In contrast to the extensive microvascular damage in the heart at the early observation times after exposure, degenerative changes in the larger coronary arteries and the aorta first appeared 3-6 months after irradiation, and became progressively more severe at later times (12-24 months). The major changes in the arterial wall included degeneration of the smooth muscle, fibrosis, and accumulations of debris and extracellular matrix. The matrix material was periodic acid Schiff (PAS) positive, indicating the presence of proteoglycans. At 12 months, there was more focal smooth mus-

cle degeneration in the medial layer of the coronary arteries, with some accumulation of matrix and of debris, and increased amounts of collagen in the outer adventitial layer. Damage was more severe at 18 months, but little additional change was noted at 24 months. Coronary arterial plaques, found in various treatment groups at 12-18 months after irradiation, appeared to involve smooth muscle proliferation and resembled lesions associated with human cardiovascular disease, except that they contained no lipid.

Fractionation of the gamma ray dose produced a marked sparing effect, presumably due to repair of radiation injury. It may be assumed that the lesions produced by gamma radiation result from radiation-induced cell killing in postmitotic tissues, and that the initial gamma doses used, 34 or 112 rads per fraction, were sufficiently low to permit some repair. In contrast, fractionation of the neutron dose (0.83, 3.3, or 10 rads per fraction) produced equal or even greater arterial smooth muscle degeneration than did a similar or the same total dose.

A comparative evaluation of the degenerative changes in the coronary artery, 18 months after the beginning of exposure, has been made in relation to the type of radiation, radiation dose, and fractionation. It appears that coronary artery damage after 2690 rads of fractionated gamma radiation was intermediate between that seen after a fractionated neutron dose of 20 and 80 rads. Although the mechanism for apparent augmentation of damage with fractionation of the neutron dose to the vasculature is not known, this response is consistent with those observed for other biological end points that have been evaluated in the JANUS experiment. The coronary arteries and aorta have shown a higher than expected sensitivity to fission neutrons. In connection with the use of high-LET radiations for radiotherapy, the sensitivity of the vasculature or other postmitotic tissues to manifestations of radiation injury is an area where further investigations are needed.

Evaluation of late changes in blood pressure are of interest in view of the structural and functional changes that have been observed in the cardiovascular system. In collaboration with George Svihla, a method for indirect measurement of blood pressure in the mouse is being developed that involves use of a pressure cuff on the hind limb. Preliminary measurements indicate that there is no consistent change in mean values in relation to age or radiation treatment. Higher values were recorded in individual irradiated animals, however, and a fall in blood pressure was frequently correlated with a near-terminal condition. Further development of the equipment is in progress, and evaluation of treated groups will proceed.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

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10. GENETIC TOXICOLOGY

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Among the major deleterious effects of chronic exposure of living cells to pollutants is the production of genetic (DNA) lesions. Genetic lesions are responsible for the heritable disorders in man, which include some types of mental retardation and which can lead to various long-term physiological disabilities and decreased reproductive fitness. It has been estimated by the International Agency for Research on Cancer that environmental mutagens cause as many as 80% of cancers.

Because environmental mutagens act by producing DNA lesions, this genetics program is concerned primarily with the kinds of DNA lesions arising from environmental and energy-related mutagens, the mechanisms of action of these lesions, and their repair. These kinds of information are vital to the rational development of improved mutagen testing systems for assessment of human risk, and to the development of procedures for reducing mutational damage.

For studies of DNA lesions and mechanisms of mutation at the molecular level, bacteria are by far the best mutational system available because of the great amount of information from previous mutational studies, their exceptional convenience for laboratory studies, and the fact that their genetic maps are much more complete than those for any other organisms. Mammalian cells are also used, however, because these have mutagen sensitivities that are likely to be more similar to those in man. Animals are used when questions of metabolic behavior are involved.

Repair of lethal lesions induced with ultraviolet light, near-ultraviolet light, and visible light continues to receive special emphasis because human populations are exposed to large integrated doses of these radiations. Current studies are examining the nature of the DNA-chromophore interactions leading to these lesions. Mutagenesis by far-ultraviolet light continues to provide a standard of reference for testing strong and weak environmental mutagens, for probing possibilities of synergistic interactions among environmental mutagens, and for examining effects of mutagenic lesions induced by SOS repair. The latter process is one of the leading candidates for carcinogenesis.

At the eukaryotic cell level, mouse myeloma mutants are being isolated for biochemical and mutagenesis studies and for use as a testing system for environmental mutagens and carcinogens. The availability of prokyarotic repair ennzymes also makes possible the study of the biochemistry of DNA repair in this eukaryotic system.

Comparative effects of lethal and mutational lesions produced by ^{239}Pu, fission neutrons, and gamma rays in the laboratory mouse also are under study. Plutonium, present in significant quantities in the fuel rods of all light water reactors, is a potential pollutant. To estimate the genetic risk of ^{239}Pu retained in the gonads, data have been obtained for dominant lethal mutations induced in both spermatogonial and postspermatogonial cell stages and for reciprocal chromosome translocations induced in spermatogonia.

MUTATIONAL SYNERGISM

H. E. Kubitschek and D. Venters

Currently, large numbers of environmental and energy related pollutants are under examination in many laboratories for mutagenic, carcinogenic, and other toxic effects. Almost invariably the suspect agents are tested individually in one or a battery of tests. Current evidence for mutational synergism, however, indicates that these tests may severely underestimate the effects of mutagens when two or more mutagens are present simultaneously. One type of synergism that we are studying is that produced by the inducible error-prone SOS repair activity in which each mutation results from two lesions. It is expected that the successive application of two different mutagens can lead to a synergistic enhancement of mutation by generating interaction mutations in which each of the mutagens contributes one of the two required lesions. This possibility was tested by exposure of cells of *Escherichia coli* strain WP2 Hcr (*uvrA trpA*) to nitrous acid and then to ultraviolet light (254 nm). Frequencies of mutation to prototrophy after exposure to both mutagens were approximately 70% greater than the sum of the frequencies observed for separate exposures to the two mutagens, supporting the proposed synergistic mechanism.

In summary, these observations support the possibility that mutational synergism may commonly occur among environmental mutagens. If so, present testing methods will grossly underestimate mutational risk.

SYNCHRONIZED DNA LESIONS

H. E. Kubitschek and C. N. Newman

Mechanisms of action of DNA lesions were studied by a new method in which base analogs were incorporated into replicating genomes during a brief period and then DNA lesions were induced synchronously, at known intervals of DNA replication following pulse labeling of the DNA. The base analogs, either 5-bromouracil (BU) or ^{125}I-iododeoxyuracil, incorporated into DNA during a period of 2-4 minutes, provided synchronized prelesional substrates for later induction of the genetic lesions. Lesions were induced in BU-labeled regions by exposure to 50,000 J/m^2 of 313-nm radiation, and in ^{125}I-labeled regions by radiodecay during storage of samples at liquid nitrogen temperature preceding assay of survival. Cell survivals were compared with those of labeled, uninduced controls.

In all experiments, survival levels remained low when lesions were induced throughout the first generation after labeling (Figure 10.1). After replication of the labeled region, however, abrupt recovery occurred in both experiments with the wild-type strain. Three very different patterns of recovery were observed:

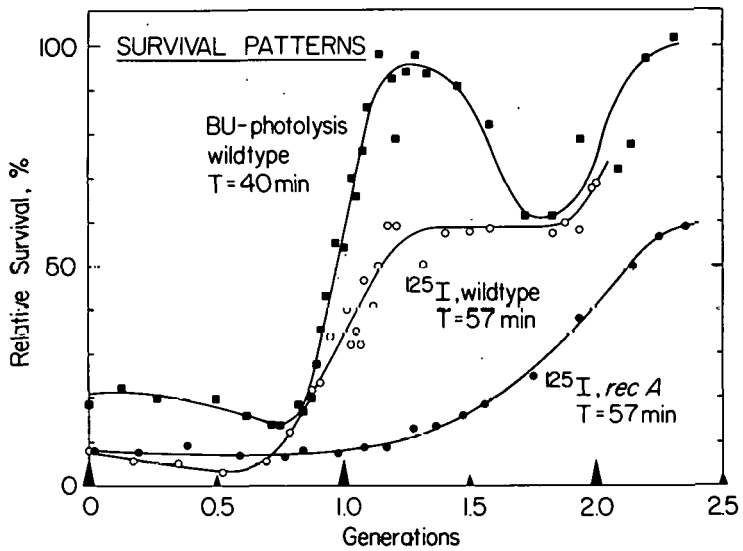

Fig. 10.1. Survival patterns of two strains of Escherichia coli B/r after induction of lesions by ^{125}I decay or photolysis of 5-bromouracil (BU) in labeled DNA regions at various times after labeling. As described in the text, the results are dependent upon the repair capability of the strain and the nature of the lesion. Generation times (T) are shown.

1) After BU-photolysis, wild-type strain: Recovery was essentially complete (100%) at the first and second replications. The results are in agreement with repair of induced single-strand breaks.

2) After ^{125}I decay, recA strain: No observable recovery occurred at the first replication, about 50% at the second replication. These results are consistent with the production of irreparable double-strand breaks that invariably lead to cell death.

3) After ^{125}I decay, wild-type strain: Half of the cells recovered after the first round of DNA replication. The results are consistent with death of cells from double-strand breaks, that such breaks are reparable when a second, unlabeled duplex is available for recombination, and furthermore, that this repair is asymmetric since only half of the cells are capable of repair.

In summary, a new method for studying mechanisms of action of DNA lesions involves the synchronous induction and maturation of DNA lesions in exponentially growing cultures. The method appears especially useful for studying effects of segregation upon repair of DNA lesions.

REGULARITY OF DNA REPLICATION IN E. COLI

H. E. Kubitschek and C. N. Newman

The accuracy of timing of bacterial chromosome replication was measured in exponentially growing cultures of Escherichia coli B/r TT by a method employing pulse labeling of DNA with 5-bromouracil (BU) and photolysis induction with 313-nm radiation. After labeling with BU for 2.5 minutes, cells were sensitized to 313-nm radiation and survival was reduced to less than 10% at a fluence of 50,000 J/m^2 (Figure 10.2). Survival continued to decrease slightly during the remainder of the DNA replication cycle until the DNA region containing the BU was replicated, at which time the survival increased abruptly to the level observed in unlabeled cultures. It is apparent that lesions induced by BU photolysis were essentially completely repaired after replication, when the lesions were paired with newly formed daughter strands free of lesions.

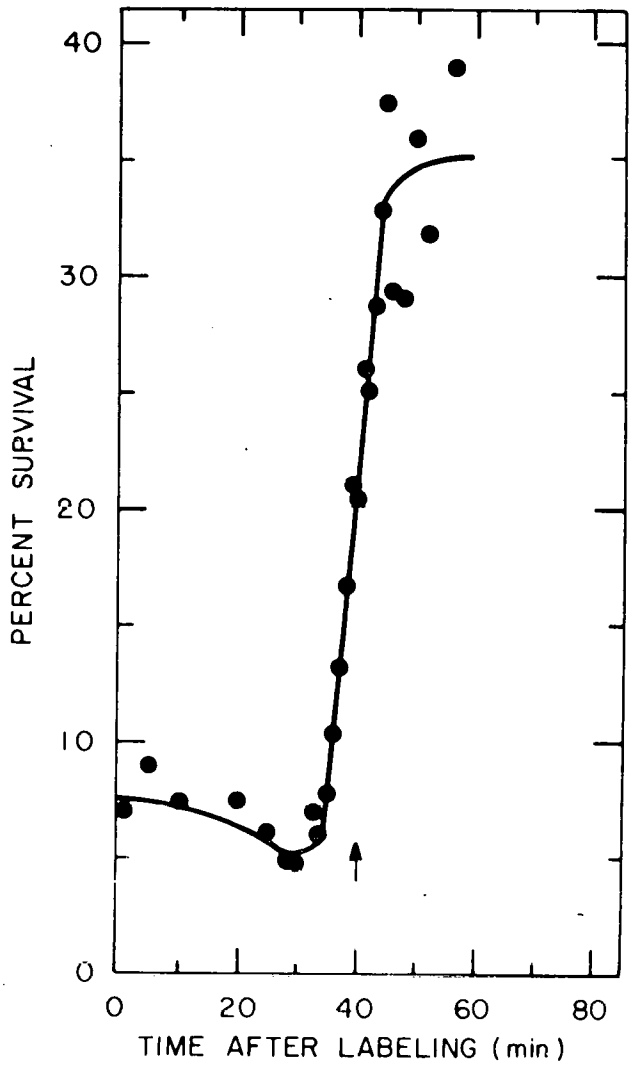

Fig. 10.2. Cell survival in an exponentially growing culture of Escherichia coli B/r TT following exposure to 50,000 Jm^{-2} at intervals after labeling the cells with 5-bromouracil. The rise at one generation after labeling (arrow) indicates decreased sensitivity after replication of DNA. The steep rise indicates the sharp control of the DNA replication period.

The observed increase in survival at the first generation after labeling provides an estimate of the regularity of DNA replication. From Figure 10.2 and other similar data, an upper limit for the coefficient of variation for the interval between successive replications is 9.3%. This variability is considerably smaller than that for cell division, about 20%. The results suggest control of major cell cycle events by DNA replication rather than by cell division. The increased variability in the cell division cycle over that in the DNA replication cycle appears to be due primarily to a failure in regulation of the timing of cell division.

In summary, the timing of DNA replication in E. coli is much more regular than that of cell division. The results support the thesis that major events in the cell cycle are controlled by processes related to the DNA replication "clock" rather than by cell division.

BIOCHEMICAL STUDIES OF DNA REPAIR IN MOUSE MYELOMA CELLS

T. Matsushita, A. M. Shotola, and G. Matsushita[1]

Purification of Micrococcus luteus UV-Correndonuclease

Most mutagens and carcinogens modify DNA structure. DNA-specific enzymes are a sensitive and direct means of detecting such modifications of DNA structure. We have succeeded in purifying one such enzyme, the UV-correndonuclease of Micrococcus luteus. The purification procedure includes steptomycin precipitation, ammonium sulfate fractionation, and DEAE-cellulose, phosphocellulose, and Sephadex G-75 column chromatography.

Measurement of UV-Induced Pyrimidine Dimers

Because pyrimidine dimers are specifically attacked by the M. luteus UV-endonuclease, dimers can be detected in mouse myeloma DNA by measuring the shift to lower molecular weight DNA on the alkaline sucrose profiles after enzyme treatment. Results indicate that dimers are not removed, even after two generations of cell growth following irradiation. While measuring the dimers produced by sublethal doses of UV-irradiation, we found a nonspecific killing effect from suspending myeloma cells in phosphate buffered saline. Because nonspecific killing could mask any

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excision repair, we attempted to develop techniques for minimizing cell killing during irradiation and succeeded in minimizing the cell killing by using bovine serum albumin during the preparation and irradiation of cells. Some of the older dimer excision data found in other mouse cell lines should therefore be evaluated to determine the possibility of a contribution from nonspecific cell killing.

Isolation of Mutant Cell Lines

In order to correlate mutagenesis and DNA repair, we have isolated new mouse myeloma variants and have now isolated and characterized two types of somatic cell variants. They include various clones of ouabain-resistant mutants and 1-β-D-arabinofuranosylcytosine (Ara-C)-resistant mutants. These two markers show a great advantage over the more widely used 8-azaguanine and 6-thioguanine mutations, which require a long time to be expressed (10-14 generations), and are difficult to use as selection agents in mutation experiments. In mouse myeloma cells, on the other hand, only 1-2 generations are required for expression of ouabain resistance and 2-4 generations for Ara-C resistance, thus providing a great saving of time.

Sister Chromatid Analyses

At present, chromosome analyses are used in two ways to detect effects of mutagens and carcinogens, namely by chromosome aberrations and sister chromatid exchanges (SCE's). Although mouse chromosomes are quite small, we have succeeded in extending SCE analyses to mouse myeloma cells. Using the method of Stetten and Wolff (Mutat. Res. 41, 343, 1976), cells are allowed to incorporate bromodeoxyuridine for two generations, the chromosomes are treated with colchicine, and differently stained chromatids are observed by fluorescence microscopy or UV light-induced differential Giemsa staining. Current results show excellent UV induction of SCE's at sublethal doses. A dose of 2.5 J/m² increases SCE's by 120% over background levels.

In summary, techniques have been developed to investigate the rate of pyrimidine dimer removal in mouse myeloma cells. Although some investigators maintain that other rodent cell lines show no such capacity for excision repair, the results are still controversial. Our observation that the method of preparation of cells can result in significant cell killing may help to resolve the controversy. Since normal human cells perform excision repair, this capacity needs to be examined carefully in rodent cells if rodent data are to form the basis of mutagen/carcinogen screening tests for human populations. Studies with our mouse myeloma mutants and with the sister chromatid exchange system will provide not only genetic and cytogenetic information on DNA repair, but also the basis for new pollutant detection systems.

GENETIC EFFECTS OF DNA LESIONS INDUCED BY SOLAR ULTRAVIOLET RADIATION AND BY VISIBLE LIGHT

R. B. Webb, B. S. Hass, and M. S. Brown

Photosensitized Mutagenesis by Acridine Orange: Lesion-Specific Wavelengths

Acridine orange (AO) is known to associate with native duplex DNA in two ways: by internal binding of monomers between adjacent DNA base pairs (intercalation), and by external binding (possibly in dimeric form) in the DNA grooves. A number of different DNA lesions have been identified with AO and broad-spectrum light. These lesions include DNA single-strand breaks, alkali-labile bonds, DNA base damage, and DNA-protein cross-links. Since AO plus visible light is capable of producing mutations and chromosomal breaks, we have investigated mutagenesis and lethality in genetically related strains of *Escherichia coli* B and B/r that differ in DNA repair capability: WP2 (repair proficient), WP2s (*uvrA*), WP10 (*recA*), and Bs-1 (*lexA uvrB*). Mutation and survival were measured in the same continuous chemostat cultures. Two wavelengths were employed: 460 nm, absorbed by externally bound AO, and 500 nm, absorbed by internally bound (intercalated) AO. Acridine orange was added at 2 μM in all experiments reported here. This concentration of AO does not significantly inhibit excision or recombination repair.

DNA lesions induced in cells of *E. coli* B/r and *E. coli* K12 in the presence of AO by both 460- and 500-nm light were efficiently repaired by the recombination repair system (Table 10.1). Based on F_{37} values, cells of *E. coli* WP10 (*recA*) were 115 and 183 times more sensitive than strain WP2s (*uvrA*) with 460- and 500-nm light, respectively. Furthermore, strain WP2s was 1.6 and 2.1 times more resistant than strain WP2 (wild type). Survival curves obtained with AO and 460-nm light showed significant differences from curves obtained with AO at 500 nm. At 460 nm, all curves were either simple exponentials or exponentials with a small shoulder, whereas at 500 nm, all curves revealed two components, a sensitive fraction comprising 60 to 95 percent of the population, and a resistant fraction comprising 5 to 40 percent of the population. At 500 nm, the resistant component of the population may be capable of greater repair than the sensitive component.

The high mutation rates at 500 nm shown by the *recA* and *lexA uvrB* strains relative to the *uvrA* and wild-type strains implicate an important role for the *rec*$^+$*lex*$^+$ gene products in the error-free repair of mutational lesions induced by AO plus 500 nm. In contrast, at 460 nm low mutation rates were obtained with the *recA* and *lexA uvrB* strains. After correcting for mutation rates expected from the measured overlap between the absorption spectrum of intercalated AO and the transmission spectrum of the

460-nm interference filter, mutation rates at 460 nm are not significantly different from zero (Table 10.1). The mutational pattern shown by the four strains with AO-460 nm light is qualitatively similar to that for X-radiation. In contrast, the mutational pattern with AO-500 nm light is unique. The lethal responses at 460 and 500 nm demonstrate that DNA lesions induced by both wavelengths are efficiently repaired by the rec$^+$lex$^+$ repair system. However, the mutation data support error-prone repair of AO-460 nm-induced DNA lesions and error-free repair of AO-500 nm DNA lesions.

Acridine orange also is a moderate mutagen in the dark in all repair-deficient strains tested, although not in wild-type strains. These results suggest that repair-deficient cells may not be able to replicate or repair DNA without errors in the presence of bound intercalating agents.

Table 10.1. Effect of Acridine Orange (2 μM) and 500- or 460-nm Light on Lethality and Mutagenesis (Resistance to Phage T5) in Chemostat Cultures of E. coli

Wavelength nm	Repair Characteristics	Mutat. Rate per 10^8 per day per W m^{-2}	Relative Mutation Rate	Sensitivity $1/F_{37}$ (m^2 J^{-1})	Relative Sensitivity	Ratio 500 nm / 460 nm	Strain
500	uvrA	120	1	9.1×10^{-7}	1	3.3	WP2s
	Wild type	190	1.6	2.5×10^{-6}	2.7	2.3	WP2
	lexA uvrB	899	7.5	8.0×10^{-5}	88	5.2	Bs-1
	recA	2903	24	1.31×10^{-4}	143	4.1	WP10
460	uvrA	78	1	2.8×10^{-7}	1		WP2s
	Wild type	201	2.1	1.11×10^{-6}	4		WP2
	lexA uvrB	78 (-17[a])	1 (0[a])	1.54×10^{-5}	55		Bs-1
	recA	18 (- 5[a])	0.23 (0[a])	3.2×10^{-5}	115		WP10

[a]Mutation rate at 460 nm corrected for the 500-nm component due to the overlap of the transmission spectrum of the 460-nm filter and the absorption spectrum of the intercalated acridine orange (3.0%).

Solar-Ultraviolet-Light Effects

Radiation at 365 nm produced both oxygen-dependent and oxygen-independent DNA lesions. Both DNA single-strand breaks (oxygen dependent) and dimers (oxygen independent) probably are produced by sensitized processes. Under anoxic conditions, uvrA strains tolerated 8 times as many dimers produced by 365-nm radiation than dimers produced by 254-nm radiation, compared with reference to constant survival. This result indicates that dimers produced at 365 nm under anoxic conditions are repaired much more efficiently than dimers produced at 254 nm.

Reversion to tryptophan independence (*trp* → *trp*$^+$) is readily induced by 365-nm radiation. Tryptophan revertants were produced at 365 nm in *E. coli* strains WP2 (wild type), WP2s (*uvrA*), and WP6 (*polA*) when the assay for reversion was made on minimal medium supplemented with nutrient broth (SEM plates). The frequency of *trp*$^+$ revertants was much lower in the same strains when the assay made use of minimal medium supplemented with tryptophan only (trpM plates). Strains WP10 (*recA*) and WP100 (*recA uvrA*) did not show detectable mutants at 254 or 365 nm on any type of assay medium utilized. The relatively high revertant yields at 365 nm when assayed on SEM plates *versus* the very low yields when assayed on trpM plates suggest that recovery of the recA$^+$-dependent DNA repair system occurs on SEM plates but not on trpM plates. Selective damage by near-UV radiation to DNA repair systems is suggested by the high mutant frequencies obtained in strain WP2 with monochromatic 365-nm radiation (SEM plates), in contrast to the absence of detectable mutants with broad-spectrum near-UV radiation with this strain. Following exposure to near-UV radiation, revertant frequencies at 254 nm depend upon wavelength, assay medium, and the bacterial strain. Recent results suggest that the error-prone branch of postreplication repair may be more sensitive to damage by broad-spectrum near-UV radiation, whereas excision repair may be more sensitive to monochromatic 365-nm radiation.

FILAMENTATION OF RAPIDLY GROWING *E. COLI* K12 IN THE PRESENCE OF CASEIN HYDROLYSATE

D. M. Darby and R. E. Krisch[1]

A variety of injurious chemical and physical agents are known to inhibit cell division by *Escherichia coli* without affecting cell growth, resulting in long nonseptate forms referred to as filaments. Nutritionally induced filamentation has been shown in *E. coli* B. Our data indicate that a number of *E. coli* K12 strains form filaments when growing at a rapid rate in the presence of casein hydrolysate. Table 10.2 presents data for *E. coli* K12 AB2497. Filamentation also occurred in *E. coli* strains AB1157, AB1899, AB2487, and KL166 when growing with doubling times of 35 to 50 min in M9 minimal medium supplemented with 2.5 mg/ml casein hydrolysate. As the cultures approached stationary phase, essentially all filamenting cells returned to normal size. No filamentation was observed in strains AB1884,

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Table 10.2. Effect of Increased Concentration of Casein Hydrolysate[1] (CAA) on Filamentation of Rapidly Growing E. coli AB2497

Concentration of Decolorized CAA (mg/ml)	Doubling Time (min)	% Filamentation
0.025	No Growth	None
0.250	75	None
0.500	75	None
1.000	50	25-35
1.500	50	25-35
2.000	47	30-40
2.500	50	30-40
25	50	30-40

[1]The casein hydrolysate was added to M9 medium, a glucose salts minimal medium, supplemented with 4 µg/ml thymidine, and 1 µg/ml thiamine.

AB2500, B/r A, and B/r K, growing with a doubling time of 35 to 38 minutes in the same casein supplemented medium.

How or why filamentation occurs is unknown. It has been suggested that a small nonprotein metabolite is necessary to trigger septation. The cause of the reversion is also unknown. It may be a reflection of either a slowed growth rate, a depletion of the casein hydrolysate in the medium, or both. At a molecular level, it may result from the division promoting substance found at high cell densities.

GENETIC AND CYTOGENETIC EFFECTS OF ^{239}Pu ALPHA PARTICLES, FISSION NEUTRONS, AND ^{60}Co GAMMA RAYS IN MALE $B6CF_1$ MICE

D. Grahn, B. H. Frystak, C. H. Lee, J. J. Russell, and A. Lindenbaum

Plutonium-239 is present in significant quantities in the fuel rods of all light water reactors, and the possibility exists that small quantities could be released to the environment, become incorporated into the food chain, consumed by domestic animals and man, and be metabolically distributed to the skeleton and soft tissues. Approximately 0.05% of the systemic burden is retained in the gonads with little or no loss with time (Richmond, C. H., and R. L. Thomas, Health Phys. 29, 241, 1975; Russell, J. J. et al., Health Phys., in press). Preliminary reports relative to the potential genetic hazards of the ^{239}Pu retained in the gonads published by K. G. Luning et al. (Mutat. Res. 34, 539, 1976) and A. G. Searle et al. (Mutat. Res. 41, 297, 1976) show that plutonium is mutagenic. However, the exact magnitude of this mutagenicity in comparison with other more conventional radiations has not been firmly established.

The present report uses a limited set of genetic end points readily measured in the laboratory mouse to compare the effectiveness of ^{239}Pu, fission neutrons, and gamma rays in the induction of dominant lethal mutations in both spermatogonial and postspermatogonial cell stages and of reciprocal chromosome translocations in spermatogonia.

All radiation exposures employed young adult C57BL/6J x BALB/cJ F_1 ($B6CF_1$/Anl) hybrid males. All irradiations by ^{60}Co gamma rays or fission neutrons were whole body external exposures. Internal exposure to ^{239}Pu alpha particles followed intravenous injection of a citrate solution of monomeric plutonium at levels of either 5 or 10 μCi/kg of body weight. Gamma irradiations were given as single, weekly, or continuous (22 hours/day) exposures; neutron irradiations used the JANUS reactor and were given only as single or weekly exposures. Since exposure to the plutonium alpha emissions can only be continuous, only one direct comparison was possible, that with continuous gamma irradiation. The single versus weekly neutron exposures tested for the additivity of repeated neutron irradiations (the absence of significant recovery from injury during the course of protraction), so that the validity of comparisons between plutonium alpha and fission neutron exposures could be established. Dose rates were high for single exposures, intermediate for weekly exposures, and low for continuous exposures. For the gamma irradiations, these were in the range of 45, 0.2 to 5.0, and 0.002 to 0.004 rads/minute, respectively. Neutron dose rates

were about one-third to one-tenth of the gamma ray rates. Plutonium alpha dose rates to the whole gonad were less than 1 mrad/minute. The latter exposure rate is an average integrated whole organ dose rate which does not reflect the extreme heterogeneity of exposures. Because about 85% of the gonad is unirradiated, and because the mean free path of the alpha particle in tissue is only about 45 μm, the actual dose to the different germ cell stages being evaluated is uncertain.

Dominant lethal mutations, measured late in gestation by observing the survival rate of fetal implantations, show a linear increase in frequency with increasing dose to both premeiotic and postmeiotic germ cell stages. The data in Table 10.3 show that the mutation rate (regression of ln survival on dose) varies systematically with LET, dose rate, and cell stage. The mutation rate for alpha particle irradiation emitted internally within the gonad is 0.0064 ± 0.0011 lethals per gamete per rad to the whole gonad. In this case, the mutagenic dose is assumed to be that accumulated over the 4-week meiotic and postmeiotic period required for differentiation of the resting primary spermatocyte to the mature sperm at the time of release from the spermatogenic tubules. The cytogenetic data (see below) support the conclusion that the dominant lethal mutations are almost all induced in these postspermatogonial stages. On that basis, the alpha particle and the fission neutron have equal mutagenicity, and they are both at least 10 times more effective than low intensity gamma radiation.

The cytogenetic data (Figure 10.3) present a different picture. Weekly exposures to neutrons are more effective than single exposures, at least above ∿ 20 rads, and the response is generally linear with accumulating dose. Continuous low intensity gamma radiation has only 1/40th the effect of protracted neutron exposure. The response to plutonium alpha particles does not correlate with accumulated dose to the gonad; it is, instead, a rather flat, but varying response slightly above that seen following low level gamma irradiation, and below the response to neutrons at all dose accumulations of 20 rads or more.

Although the reciprocal chromosome translocations are detected at the first meiotic metaphase in appropriate preparations of spermatogenic elements (Evans, E. P., et al., Cytogenetics 3, 289, 1964), the pertinent mutagenic dose is that accumulated by the spermatogonia. Plutonium is deposited in the interstitial tissue and along the basement membrane of the spermatogenic tubule, and our studies of distribution and retention of plutonium suggest that the dose to the spermatogonial cells along the membrane may be 4 times greater than the estimated dose to the whole gonad. The latter is about 1 rad/week for an injected dose of 10 μCi/kg. D. Green et al. (Nature 255, 77, 1975) gave a ratio of 2.5/1 for the dose to the spermatogonia versus the dose to the whole tissue. While the exact ratio may be uncertain, and may also be both strain and species dependent,

Table 10.3. Dominant Lethal Mutation Rate (x 10^{-4}) per Gamete per Rad, Measured by Postimplantation Mortality

Radiation	Cell Stage Exposed	Exposure Pattern		
		Single	Weekly	Continuous
^{60}Co γ-Rays	Postmeiotic[1]	10 ± 1	11 ± 1	5 ± 0.6
	Premeiotic[2]	1.0 ± 0.3	0.32 ± 0.07	0.17 ± 0.09
Fission Neutrons	Postmeiotic[1]	54 ± 4	88 ± 21	Not available
	Premeiotic[2]	3.7 ± 1.1	3.4 ± 0.7	"
^{239}Pu Alpha Particles	All stages[3]	Not available	Not available	64 ± 11

[1]Based upon total dose delivered up to 5 weeks prior to mating.

[2]Based upon total accumulated dose prior to mating.

[3]Based upon total dose delivered over a 4-week period.

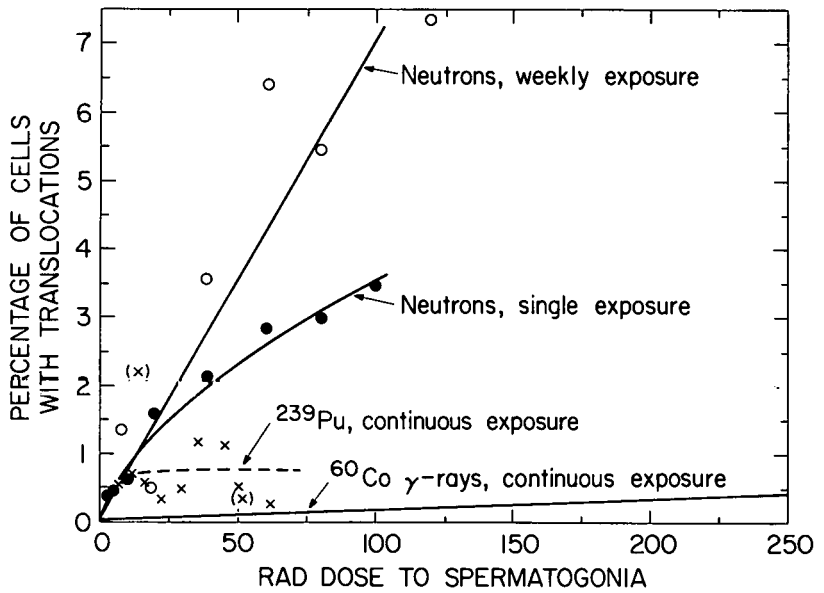

Fig. 10.3. Percentage of cells with reciprocal chromosome translocations for the indicated exposure conditions and radiation qualities. Data from ^{239}Pu exposures identified by X were derived from a comparison study of ANL B6CF$_1$ and ORNL C3H x 101 F$_1$ males; all other ^{239}Pu data were obtained concurrently with the data from neutron and gamma ray exposures. Regression coefficients: neutrons weekly, + 0.071%/rad; gamma rays, continuous, + 0.0017%/rad; neutrons, single, $D^{+0.65}$.

it is clear that the use of the dose to the whole organ in risk estimates is conservative. Because the whole-organ dose is the lowest estimate, it yields a maximum value for any estimated "response per rad." Thus the dose to spermatogonia pertinent to the induction of translocations may actually lie between about 30 and 250 rads rather than between only 7 and 60 rads (Figure 10.3). If so, then the translocation induction frequency per rad at the highest accumulation levels is no greater than that seen following comparable rad doses of low intensity gamma radiation. Use of the whole organ dose is also conservative for estimates of dominant lethal mutation rates, since the early stages of meiosis occur at or near the basement membrane.

The absence of a dose-response relationship for chromosome translocations in plutonium-exposed mice is an enigma. The effect may be due to cell killing, which is presumed to account for the observed nonlinear response to single doses of neutrons. It is possible that clones of stem cells might form in the spermatogenic tissue in radiation-free areas and steadily overwhelm the few surviving irradiated stem cells. Plutonium also has some tendency to relocate in the interstitial tissue away from the membrane, which would also reduce the rate of exposure of the spermatogonia. Some combination of the above possibilities might also occur.

In summary, ^{239}Pu injected intravenously into the mouse is retained in the interstitial tissues of the testes at a low but virtually unchanging level of 0.05% of the injected dose. The spermatogonial cells receive the highest exposure, although all gamete cell stages are irradiated. The plutonium alpha particle and the fission neutron are about equally mutagenic for the induction of dominant lethal mutations in postspermatogonial stages, and plutonium is 10-12 times as mutagenic as low intensity continuous gamma irradiation. Protracted neutron irradiation is about 40 times as effective as low dose rate gamma irradiation for the induction of reciprocal chromosome translocations in spermatogonia. Plutonium, however, is much less effective than neutron radiation, and little or no dose-response relationship between translocation frequency and accumulated dose is evident. As a result, the potential genetic hazards associated with long-term burdens of ^{239}Pu could be significantly overestimated if only the short-term dominant lethal mutation rate for mature germ cells was to be considered without regard to the declining effect of accumulating dose upon the spermatogonial stem cell population.

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11. TOXICOLOGICAL EVALUATIONS OF COAL CONVERSION TECHNOLOGIES

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The Divisional program to evaluate the toxicological aspects of coal utilization, especially those of newer, emerging technologies, was initiated in December 1976. Calendar year 1977 represented a substantial effort to become acquainted with (1) technical aspects of coal conversion and combustion processes, and (2) potential environmental and toxicological problems associated with the several processes. It was also essential to identify existing or developing bench-scale pilot development units (PDU), pilot plants, and commercial facilities that offer opportunities for meaningful toxicological studies. In the latter regard, two considerations existed: (1) the question of scalability to ensure that measures derived from PDU or pilot-plant facilities would be relevant to commercial size installations, and (2) matters relating to accessibility, since the physical layout and operating procedures of some facilities may impose limitations on peripheral toxicological studies.

Four substantially different technologies for coal utilization have been selected for toxicological evaluations: (1) conventional combustion, (2) fluidized bed combustion (FBC), (3) gasification, and (4) magnetohydrodynamic conversion (MHD). Toxicological work related to fluidized bed combustion was begun in 1977, and corresponding studies of coal gasification should begin early in 1978.

The facilities that have been examined for their suitability for toxicological evaluation are listed in Table 11.1.

Table 11.1. Coal Conversion Facilities under Examination[1]

	Type Facility	Location	Comment on Toxicological Study
A.	Conventional Combustion	ANL steam plant	Future
B.	Fluidized Bed Combustion (FBC)		
	1. 6" Pressurized FBC	CEN/ANL	Studies initiated, 1977
	2. 6" Atmospheric pressure FBC	CEN/ANL	Studies begin, 1978
	3. 18" Atmospheric pressure FBC	MERC, WV	Studies begin, 1978
	4. Pressurized FBC (CTIU)	ANL	Future
	5. Pilot-plant atmospheric pressure FBC	Rivesville, WV	Future
C.	Coal Gasification		
	1. Hi-BTU pilot plant	(HYGAS) Chicago	Studies begin, Feb., 1978
	2. MED-BTU pilot plant	MERC	Studies begin, 1978
	3. PDU for hi-BTU	Carnegie-Mellon University	Future
D.	Magnetohydrodynamics		
	1. Components development & integration facility	ANL	Facility under construction

[1]Abbreviations used: CEN/ANL, Chemical Engineering Division, Argonne National Laboratory; CTIU, components test integration unit; MERC, Morgantown Energy Research Center; PDU, pilot development units.

The process streams, effluents, and solid wastes from all these facilities represent complex mixtures of organic and inorganic materials. In addition, their compositions, in a given process, may vary significantly with operating conditions. Meaningful toxicological evaluations of these processes therefore require an appropriate advisory relationship with engineering and chemical engineering consultants in addition to the biological work. A considerable competence is also required in the chemical and physical characterization of the process streams and effluents being subjected to biological evaluation.

To satisfy the above requirements, the Division of Biological and Medical Research has established collaborative interactions with several other ANL Divisions: Chemical Engineering and its Analytical Chemistry Laboratory, Chemistry, Energy and Environmental Systems, and Engineering. Outside Argonne, we have support from staff of the Institute of Gas Technology, the operator of the HYGAS facility, as well as from members of the Department of Chemical Engineering, Carnegie-Mellon University, who serve as advisors and coordinators for the Fossil Energy Division of the Department of Energy and to HYGAS.

Toxicological evaluations of effluents from the bench scale pressurized FBC in the Chemical Engineering Division were begun in 1977. Arrangements are complete to begin work with process streams in the HYGAS hi-BTU coal gasification pilot plant in Chicago, and this work will begin early in 1978. The toxicological evaluations will emphasize the use of cell tester systems (Ames test and mammalian cells in culture) in order to obtain results quickly. Selected tests in mammalian systems will be developed to substantiate indications derived from results with cell tester systems.

DESIGN OF ATMOSPHERIC EFFECTS SIMULATORS FOR EFFLUENTS FROM FLUIDIZED BED COMBUSTION[1]

W. P. Norris and J. O. Hutchens[2]

The hot effluent stream from combustion of coal is subject to significant changes once it emerges from the smokestack. Some of these changes are important factors in evaluation of toxicity and effects on the environment. As the effluent stream cools, its volatile components are known to condense, and to adsorb on surfaces of particles also contained in the stream. Since such particles typically are of a size that, once inhaled, may be retained in the lung, the condensation and adsorption phenomena can be expected to exert an influence on the type of toxic manifestations that may be observed. There are also possibilities for gas-gas condensations that can produce respirable aerosols--the best recognized of these being condensations of SO_3 and water vapor to produce sulfuric acid aerosols. In addition, it is also known that sunlight can produce photochemically induced reactions among the gaseous and organic by-products of coal combustion. It is expected, therefore, that the combination of time and atmospheric conditions (i.e., temperature, humidity, and light intensity and quality) can modify appreciably the composition, and thus the toxicity, of coal-derived effluents.

In order to enable physical and chemical characterization of the aging effluent from fluidized bed combustion of coal, as well as to provide a system for exposure of biological test systems to such an aged effluent, we have designed an "atmospheric effects simulator" (AES). The AES will receive the diluted effluent stream of the new, 6-inch diameter, atmospheric pressure, fluidized bed combustor in the ANL Division of Chemical Engineering. This FBC was built with the primary purpose of studying corrosion in components for larger systems. Since these studies project continuous, steady-state runs of up to 1000 hours duration, the system is admirably suited for biological studies.

The behavior of smokestack plumes indicates that the effluents are diluted $\sim$ 20-fold within seconds after emission. The concentrations of oxygen ($\sim$ 3%) and known toxic gases (CO, SO_2, NO_x) in FBC effluents also indicate that a dilution of

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[1]In addition to the authors, the following persons participated in the design of the systems described: S. Gordon, K. H. Schmidt, and R. A. Gorse (Chemistry Division); P. T. Cunningham, R. Kumar, G. J. Vogel, J. F. Lenc, and H. R. Isaacson (Chemical Engineering Division); and L. W. Carlson and W. J. Kann (Engineering Division).

[2]Consultant, The University of Chicago.

∿ 20-fold is required for extended survival of mammals exposed to such an effluent. Comparisons of expected concentrations of CO and NO_x with established threshold limit values for these substances indicates that 50- to 100-fold dilutions should be made for long-term studies with mammals. The system provides for such single, but variable, dilutions. The diluted effluent is held, and aged, in a 300 cubic foot tank that allows for a mean retention time of 15 minutes (Figure 11.1). The tank is fitted with Pyrex glass windows and banks of fluorescent lights that allow the tank contents to be illuminated with light having a spectral

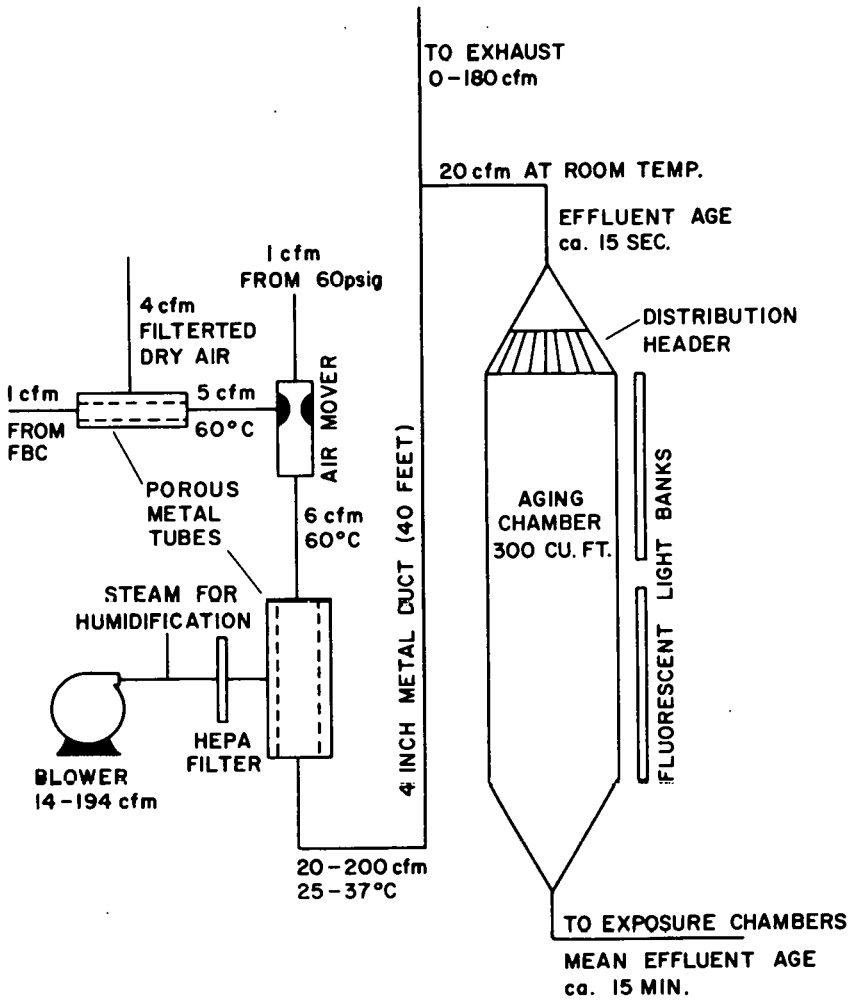

Fig. 11.1. Schematic of the aging and dilution system for FBC effluents that is presently being installed in the Chemical Engineering Division to handle the effluent of their atmospheric pressure FBC for toxicological investigations.

content similar to sunlight. The system is also designed to approximate "plug" type flow (i.e., nonturbulent flow), with an inlet header to spread the low velocity gas stream evenly across the chamber. However, we expect that energy from the illumination will heat parts of the walls sufficiently to produce local turbulence. The age distribution of the effluent at the exit point of the aging chamber will probably have to be measured using tracer pulses at the inlet.

We expect the previously filtered effluent of the FBC to have a large fraction of the particulate mass in the upper ranges of respirable dust (2-5 μm). Velocities have been kept low at the two major dilution stages, and, by introducing the diluting air through the porous walls of the conducting piping, losses through settling and diffusion are minimized. The most serious losses of particulates will probably be through thermal deposition on the walls of the 40-foot long, 4-inch diameter metal duct leading to the aging chamber. This loss will occur particularly at 20-fold dilution where the diluted effluent will be 10-15°C above room temperature. The dew point of the FBC effluent requires that an elevated temperature be maintained at the early stages of dilution. Furthermore, the rate of cooling should approximate that expected in stack effluents. The fate of gaseous components (e.g., hydrocarbons) as regards their adsorption or condensation on existing particles, or nucleation and condensing to new (probably very small) droplets will be critically influenced by dilution and cooling rates.

Our experience with the 6-inch pressurized FBC in the Chemical Engineering Division indicates that the particulate mass in the effluent is less than 100 $\mu g/m^3$ and is apt to be as low as 30 $\mu g/m^3$. Assuming that the atmospheric FBC will have a similar effluent, the total particulate mass in the 15 cfm (0.45 m^3/min) of FBC effluent is ∿15 mg/min or 22 g/day. The mass provided to the aging system is 1/15 of this, or ∿1.5 g/day. The availability of such an aged effluent is critical for inhalation toxicology on animals or for direct exposure of nonmammalian test systems to a chemically contaminated atmosphere. The concentrations of the components of the aged effluent are adequate for chemical analysis. The 1.5 g/day of particulate material, however, is very small for certain procedures, such as collection and characterization of size-classified samples, or the extraction and testing of organic compounds carried on particulates. For comparison of aged effluent with the unaged FBC output, micromethods appear to be needed.

CHARACTERIZATION OF PARTICULATES IN THE EFFLUENT FROM FLUIDIZED BED COMBUSTION OF COAL

R. Kumar,[1] H. R. Isaacson,[1] and D. A. Haugen

To gain experience and to assess problems that might occur with FBC effluent characterization, the gaseous effluent from the pressurized FBC in the Chemical Engineering Division was used as a source for sampling and analysis. Major emphasis was placed on obtaining measurements on the particles present in the effluents. We wanted to obtain the total particle mass loading, the particle size-mass profile, and the particle size-number profile; and to acquire total and size-resolved particles in sufficient quantity for various analyses.

Initially, effluent was obtained from a sample line inserted after the FBC final filter and prior to the pressure relief control valve. This effluent, still at 3 to 8 atmospheres pressure, was vented into a Plexiglass chimney, in which the pressure was at near atmospheric, and then into an exhaust hood. Sampling was carried out by pumping this effluent, via a chimney sampling port, through a filter. Total mass loadings obtained were significantly lower than those known to exist. This reduction in mass loading was partly attributed to piping wall losses but mainly to water condensation that resulted in particle removal.

To alleviate the above problems, sampling was done after the pressure had been reduced to atmospheric and the effluent had been vented into a dedicated exhaust duct. The ductwork, being under slightly negative pressure and not airtight, allowed for some effluent dilution (about 2- to 3-fold) and some cooling. As a result, no condensation problems occurred and total mass loadings of 50 to 90 mg/m^3 were obtained. The variations were due to changes made in FBC operating conditions. Background levels, i.e., when the FBC was not operating, were < 0.5 mg/m^3.

Estimates of the particle size-mass profile were made using a setup of filters, minicyclones, and various critical orifices. The orifices governed the velocity of the effluent passing through each cyclone and thus the particle removal characteristics of each cyclone. The particles not removed by the cyclones were collected on filters. The 50% cutoff values for the cyclone orifices were: 6.2, 3.8, 2.4, and 1.1 μm. A filter without a cyclone collected total particles. Sampling was done in the ductwork described above. Approximately 60% of the particle mass was less than 6.2 μm, 50% was less than 3.8 μm, 40% was less than 2.4 μm, and 35% was less than 1.1 μm aerodynamic diameter.

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[1]Chemical Engineering Division.

It was also desired to collect gram quantities of the particles in the gaseous effluent from the FBC for use in various chemical fractionations that will supply material for biotesting. A high volume air sampler (1.1 m^3/min) was connected to the ductwork from the FBC. Effluent temperatures at this point varied from 50 to 120°C with FBC operating conditions. This temperature range should provide for condensation of varying amounts of organic compounds on particles to be collected. Gram quantities of particles will be collected in a period of 1 to 3 hours. The sampler is also equipped to fractionate the particles by size.

Characterization of the effluent from a new atmospheric FBC is mainly in the design and equipment procurement stages. Total mass loading measurements have been made. These measurements, considered to be unreliable, showed the total mass loading to be very low. These low values have been attributed to particle loss through water condensation and an efficient particle cleanup system on the FBC. A sampling system is being designed to eliminate the condensation problem and allow for measurement of particles and gases.

The required instrumentation has been selected and is being procured. In addition to particle collection equipment, equipment will be required for monitoring the following variables: temperature, dew point, particle size (0.003 to > 5.0 μm), NO_x, SO_2, CO, and gas flow.

CHARACTERIZATION OF PROCESS STREAMS AND EFFLUENTS FROM COAL CONVERSION PROCESSES

P. T. Cunningham[1] and S. Bourne[1]

The Analytical Chemistry Laboratory is fractionating and characterizing crude samples from coal conversion and combustion processes, with the objective of preparing the material for mutagenicity and toxicity testing by BIM. Data on biological effects and chemical composition will be analyzed together to study the biological hazards of coal processes. Samples studied to date include: (1) condensed vapor from an FBC effluent stream, (2) fly ash from precipitators of a coal combustion plant, (3) three oil samples from the HYGAS recirculation loop and pretreater tower, and (4) two HYGAS quench water samples.

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[1]Analytical Chemistry Laboratory of the Chemical Engineering Division.

Workup techniques to date include separatory funnel, Soxhlet, and column extractions; distillation; vacuum sublimation; and chromatography. Samples have been fingerprinted by gas chromatography and infrared spectroscopy and sent to commercial laboratories for GC/MS analysis. The material from the FBC effluent appears to be mainly polynuclear aromatic hydrocarbons; the material from the fly-ash is significantly more polar; and the HYGAS stream contains a whole spectrum of organic compounds, but especially cycloparafins and phenols.

Additional separations and characterization capability is being assembled. High-pressure liquid chromatography will be used more extensively to characterize and separate the polar compounds. Expanded semiautomated chromatography equipment is on hand, and gas chromatography/mass spectrometry facilities have been acquired and will be operating in the near future. This equipment will provide for finer separations and specific identification of organic compounds.

MEASURES OF CONDENSATION NUCLEI IN EFFLUENTS FROM FLUIDIZED BED COMBUSTION OF COAL

S. Gordon,[1] K. H. Schmidt,[1] and R. A. Gorse[1]

It is generally accepted that particle size is a major factor in determining the toxic effect of airborne particulate matter (Natusch, D. F. S., and J. R. Wallace, Science 186, 695, 1974; Hatch, T. F., and P. Gross, Pulmonary Disposition and Retention of Inhaled Aerosols, Academic Press, New York, 1964; U. S. Department of Health, Education, and Welfare, National Air Pollution Board, Air Quality Criteria for Particulate Matter, Publ. No. AP 48, Washington, DC, January 1969). More than 50% of 0.01-μm particles is deposited in the alveolar region of the lung and remains there for extended periods. The deposition of still smaller aerosols in the lung has not been studied, largely because equipment for characterizing such aerosols was not readily available until the development of our Argonne Condensation Nuclei Analyzer (Hart, E. J., et al., Science 180, 1064, 1973; Schmidt, K. H., Int. J. Chem. Kinetics Symp. 1, 557, 1975). Our instrument has the unique capability of giving the complete size distribution of aerosols having particle diamters of 0.001-0.1 μm in a single operation. No other existing instrument has achieved this capability.

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[1]Chemistry Division.

Gas-gas and gas-particle condensation reactions, due to phenomena such as surface absorption, are known to occur in the atmosphere and to involve the effluent from coal combustion. Thus the condensation products are an important component in the toxicological evaluation of coal combustion effluents. There is mounting evidence that these small particles are a significant factor in biological toxicity.

Our equipment will be used to study the kinetics of condensation reactions and to monitor the quantity and quality of particles to which biological test objects are exposed.

The Argonne Condensation Nuclei Analyzer that we have developed is a dynamic cloud chamber apparatus. A block diagram of the apparatus is shown in Figure 11.2. Starting with a humidified gas mixture containing condensation nuclei (< 0.1 μm), a

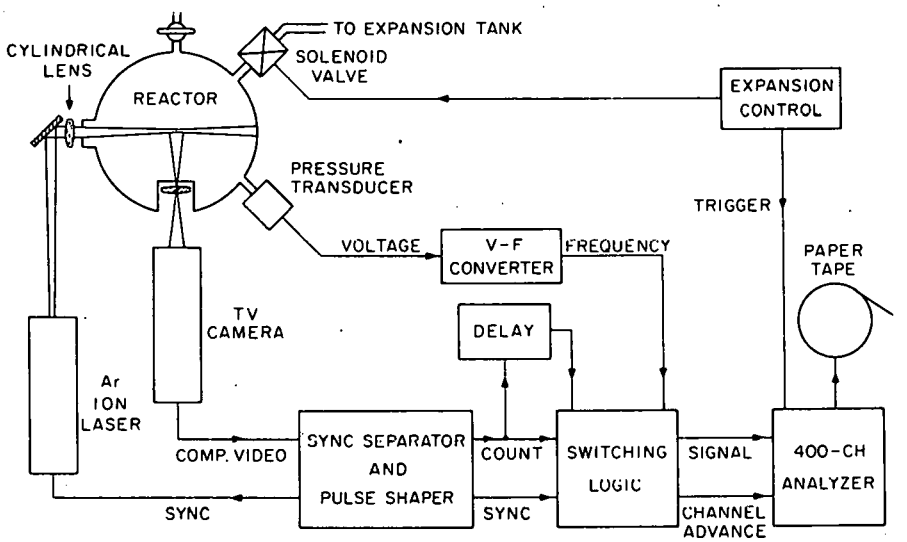

Fig. 11.2. Block diagram of condensation nucleus size analyzer. (Reprinted by permission of John Wiley and Sons, Inc. from Int. J. Chem. Kinetics Symp. 1, 557-565, 1975.)

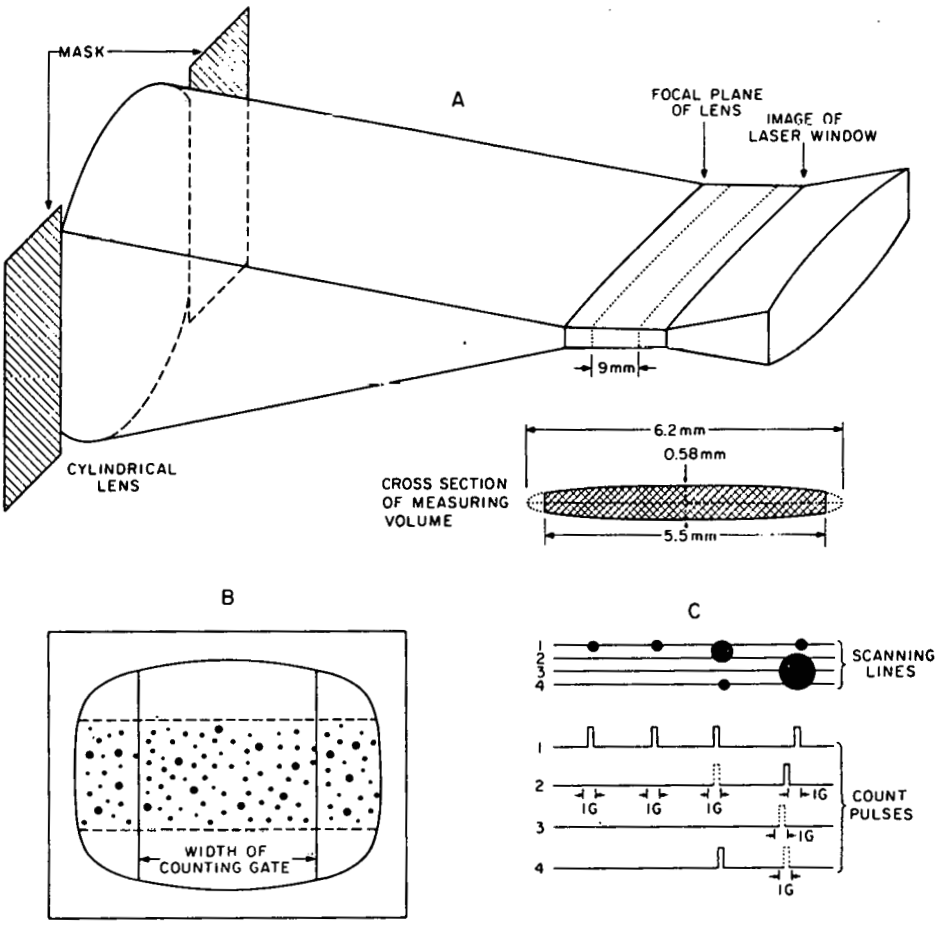

Fig. 11.3. Optical arrangement and scanning procedure for fog droplet counting. A, geometry of illuminating light beam; B, TV image of fog droplets; C, schematic diagram of scanning procedure and counting logic. (Reprinted by permission of John Wiley and Sons, Inc. from Int. J. Chem. Kinetics Symp. 1, 557-565, 1975.)

rapid adiabatic expansion is produced in a spherical flask. The supersaturation is a monotonically increasing function of the pressure drop in the flask. Adiabaticity in the central portion of the flask is maintained for up to 2 seconds by a special tetrahedral configuration of expansion outlets. This allows attainment of up to 10-fold supersaturation. The supersaturation necessary for condensational growth is a function of the radius of the condensation nuclei. The logarithm of the supersaturation is a linear function of the critical radius (Mason, B. J., Disc.

Faraday Soc. 30, 20, 1960). Therefore, as the expansion proceeds the largest condensation nuclei grow first and smaller nuclei grow later. Once the onset of growth is attained, the particles grow very rapidly via condensation and reach sizes that efficiently scatter light of 5000 Å wavelength and become visible. The central 0.007 cm^3 of the expansion flask is illuminated by a narrow ribbon of light from a pulsed argon laser with 20 microsecond pulses at 30 Hz (Figure 11.3). This central volume is synchronously viewed with an extremely sensitive TV camera. The discrete fog droplet images stored internally in the TV tube are sequentially scanned. Each droplet image results in a voltage pulse which is shaped, transferred to, and accumulated in a multichannel analyzer. The number of fog droplets in the sensitive volume is recorded and stored 30 times per second in successive channels of the analyzer. The growth time from onset of particle formation around the condensation nucleus to light scattering fog droplet is less than the counting time increment (33 milliseconds).

The shaped voltage pulses from the TV camera pass through a delayed-anticoincidence circuit to prevent multiple counting of images that cover more than one TV line. This is illustrated in Figure 11.3.

The absolute pressure in the flask is measured with a pressure transducer and stored in a multichannel analyzer every 33 milliseconds. The supersaturation as a function of time is calculated from the initial conditions and the time-resolved pressure curve. The count vs. time and supersaturation vs. time functions are then used to calculate the effective size distribution according to Mason's formulation (Mason, B. J., Disc. Faraday Soc. 30, 20, 1960):

$$\ln(p/p_\infty) = 3\, M\sigma_L / r_c \rho_L R T,$$

where M, σ_L, and ρ_L are the molecular weight, surface tension, and density of the liquid, R is the gas content, p is the pressure of the supersaturated vapor, and p_∞ is the equilibrium vapor pressure at temperature T. The "effective" size distribution may differ from the geometrical size distribution depending upon the chemical nature and charge characteristics of the nuclei. If the nature of the nuclei is known, appropriate corrections can be made to obtain the geometric size distribution. Under favorable conditions, the entire size distribution is attained in a measurement time of less than 2 seconds; some additional time is necessary for computation of final results. Repetition rate is currently limited to about 15 minutes by evacuation and refilling constraints. Computation and sampling time will be reduced in the future. Semiquantitative size distribution information is available instantaneously from the output display on the multichannel analyzer. Typical measurements for humidified laboratory air are shown in Figure 11.4.

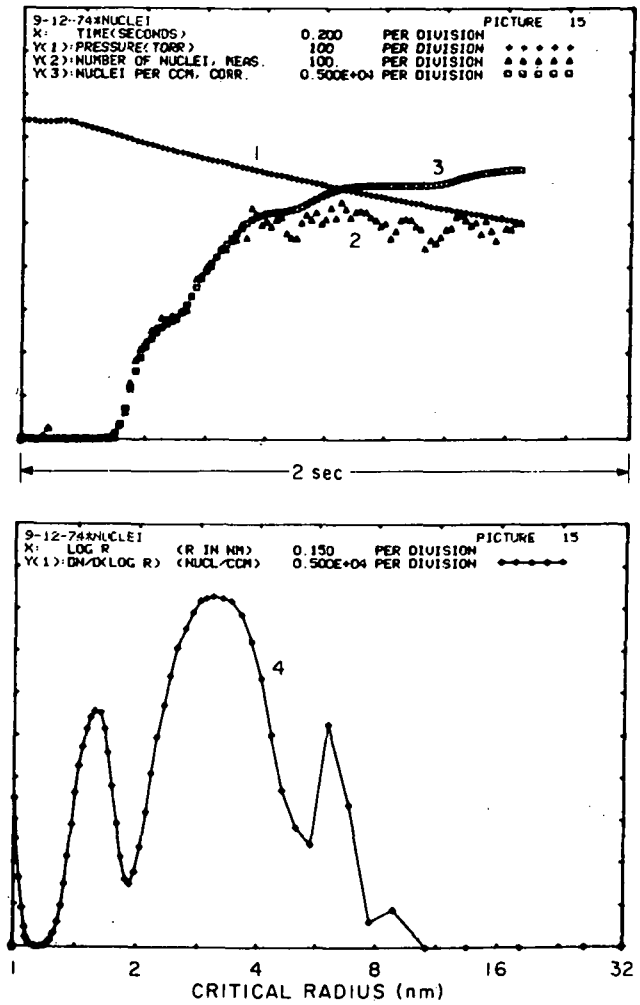

Fig. 11.4. Condensation nuclei measurement on humidified laboratory air, diluted 1:1 with filtered tank air. Curve 1, gas pressure (1000 torr full scale), showing the pressure drop during the expansion. Curve 2, measured number of fog droplets as a function of time (1000 droplets full scale). Curve 3, number of fog droplets/cm^3, after mathematical smoothing and correction for the decrease in concentration during expansion (50,000 droplets/cm^3 full scale). Curve 4, calculated size distribution of nuclei ($dn/d(\log r_c)$). Ordinate: 50,000 nuclei/cm^3 full scale. (Reprinted by permission of John Wiley and Sons, Inc. from Int. J. Chem. Kinetics Symp. 1, 557-565, 1975.)

A transportable version of the condensation nuclei analyzer apparatus is being constructed so that measurements on effluents can be made at various combustion installations. The electronic circuitry for the transportable apparatus has been completed and is being tested. The vacuum system and expansion flask have been designed and are completed. The optical system for the analyzer has been designed and is being installed.

We have submitted our requirements and recommendations for the atmospheric effects simulator to be constructed at the Chemical Engineering FBC and have participated in details of the design, construction, and necessary material procurement.

Our schedule calls for sampling from the FBC and analyzing the samples on our new instrument during FY 1979.

MUTAGENIC AND CHEMICAL CHARACTERIZATION OF FLY ASH FROM A FLUIDIZED BED COMBUSTOR

H. E. Kubitschek, D. A. Haugen, S. Bourne,[1] D. Venters, and K. M. Suhrbier

The objectives of this study are to identify the principal toxic or mutagenic components of fly ash from a pressurized fluidized bed combustor. The biological effects will be correlated with chemical composition.

Relatively large quantities (over 20 g) of fly ash were collected on a sintered steel filter at approximately 170°C from the pressurized bench scale FBC in the Chemical Engineering Division. The mutagenic activity of this material was examined with the Ames Salmonella microsomal activation assay system using strains TA98, TA100, and TA1538. Mutagenic activity without metabolic activation was detected with particulate material used directly in the cultures as an aqueous slurry, or with any of several organic extracts of fly ash. Mutation frequencies were 10-100 times as large as those observed earlier for fly ash collected from a commercial power plant (Chrisp, C. E., et al., Science 199, 73, 1978). The greater activity of our FBC fly ash may reflect lower combustion tempratures.

Dimethyl sulfoxide and dichloromethane extracts of fly ash have the greatest mutagenicity. In comparison, application of the fly ash alone gave half the mutagenicity observed with the dimethyl sulfoxide extracts. Ethyl alcohol extracts were approximately half as mutagenic; little of the activity was water soluble. The presence of a liver microsomal metabolic activation system prepared from Arochlor-treated rats caused only a minor increase in the mutagenic effect.

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[1] Analytical Chemistry Laboratory of the Chemical Engineering Division.

Parallel experiments using benzo(a)pyrene as a test mutagen gave the expected results, i.e., benzo(a)pyrene at 1-5 μg/plate was mutagenic only in the presence of the activation system. These results indicate that the principal mutagenic component(s) in the fly ash is not a simple polynuclear aromatic hydrocarbon, as represented by benzo(a)pyrene. These results are similar to those recently found in other laboratories for particulates from ambient urban air; those particulates also contain unidentified materials that are direct mutagens in the Ames assay. It thus appears that the polynuclear aromatic hydrocarbon or benzo(a)pyrene present in the particulates cannot account for the mutagenic activity.

Freshly obtained fly ash samples were compared with older samples, stored at room temperature in plastic containers for more than 6 months. There was no significant difference between the two samples. This result agrees with the stability found for the mutagenic components of urban air samples, some stored for 12 years in paper envelopes at room temperature (Talcott, R., and E. Wei, J. Nat. Cancer Inst. 58, 449, 1977). This stability of particulate mutagenicity increases the probability of long-term hazard of the material, and suggests that the particulate material may be of much greater concern than the gaseous effluents.

Two approaches are being employed to extend these studies. First, we plan to collect size-classified particulates from the FBC at a point in the effluent system where the gaseous-particulate mixture has cooled sufficiently to maximize adsorption of volatile organic chemicals on the particulates, thus more closely simulating environmental conditions. Secondly, work has begun on the chemical fractionation of the fly ash. After extraction with an aqueous solution, the remaining residue was extracted with a mixture of organic solvents, and the extract was fractionated by column chromatography on silica gel. A large proportion of the mutagenic activity was found in a chromatographic fraction containing unidentified materials that are more polar than the polynuclear aromatic hydrocarbons. Studies are in progress to fractionate these extracts further and to determine the identity of the components.

IN VITRO STUDIES USING MAMMALIAN CELLS IN CULTURE

M. M. Elkind and G. R. Lankas

Chinese hamster V79 cells grown in culture have been used to assess the effects of fly ash on mammalian cell survival and growth. Fly ash samples were obtained from the last stage of filtration of the gaseous effluent produced by the pressurized

fluidized bed combustor in the Chemical Engineering Division. The fly ash samples consisted of opaque, irregular particles up to about 10 μm diameter.

When fly ash was added at a concentration of 1 mg/ml directly to cultures of V79 Chinese hamster cells, grown in a modified Eagle's medium supplemented with fetal calf serum, the cells did not grow. When the fly ash concentration was decreased to 0.1 mg/ml of culture medium, 30% of the initial population did not form colonies, and the population doubling time was increased by 25%.

In other experiments, cell culture growth medium containing 5% fetal calf serum was used to extract the fly ash samples. Fly ash was added to the medium at concentrations up to 1 mg/ml and incubated at 37°C for either 24 hours or 2 weeks. The medium was filtered after extraction to remove the fly ash and was added directly to cell cultures.

Growth medium incubated with up to 1 mg/ml fly ash for 24 hours had little if any effect on the survival or the growth rate of the cells. However, the medium incubated with fly ash at a concentration of 1 mg/ml for 2 weeks reduced the number of surviving cells by 50% and increased the population doubling time by approximately 30%. After incubation at 0.1 mg/ml concentration of fly ash for 2 weeks, the growth medium reduced the surviving fraction by 20% and increased the population doubling time by approximately 10%.

EFFECTS OF FLUIDIZED BED COMBUSTION EFFLUENTS ON MICE

P. C. Brennan, W. T. Kickels, and S. S. Dornfeld

This research seeks to characterize the early and late effects of exposure of mice to effluents from fluidized bed combustion, at concentrations that are maximally and minimally damaging. The end points that are evaluated include: (1) immunologic competence, including susceptibility to respiratory infection; (2) respiratory function; (3) physiological response to controlled stress; (4) morphological criteria; (5) life-span; and (6) the prevalence of lung tumors at selected ages.

During this year we have had limited access to the pressurized FBC unit in the Chemical Engineering Division, and have been able to initiate a few short-term experiments utilizing effluents from this combustor diverted into a specially designed exposure chamber. The results available are preliminary but serve as guidelines for future experiments using the atmospheric FBC now being constructed in the Chemical Engineering Division.

Three experiments have been completed. In the first, $B6CF_1$ female mice were exposed from 1 to 3.5 hours to a 40-fold dilution of effluent on 3 separate days, with intervals of 1-2 days. The mice were killed 2 and 4 days after the last exposure, and the lungs were examined histopathologically. The lungs from all exposed mice showed acute to subacute pneumonitis with marked areas of collapse of alveoli and infiltration of round cells and polymorphonuclear cells. In a second experiment, $B6CF_1$ male mice were exposed for 1 hour once a week for 5 weeks to a 3-fold dilution of effluent. Histopathological examination of the lungs of the exposed mice showed polyps, round cell infiltration, lymphocyte infiltration, and large areas of consolidation with thickened and collapsed alveoli (Figure 11.5). In the third experiment $B6CF_1$ male mice were exposed 1 hour twice a week for 2 weeks to a 10-fold dilution of the effluent. Five and 15 days

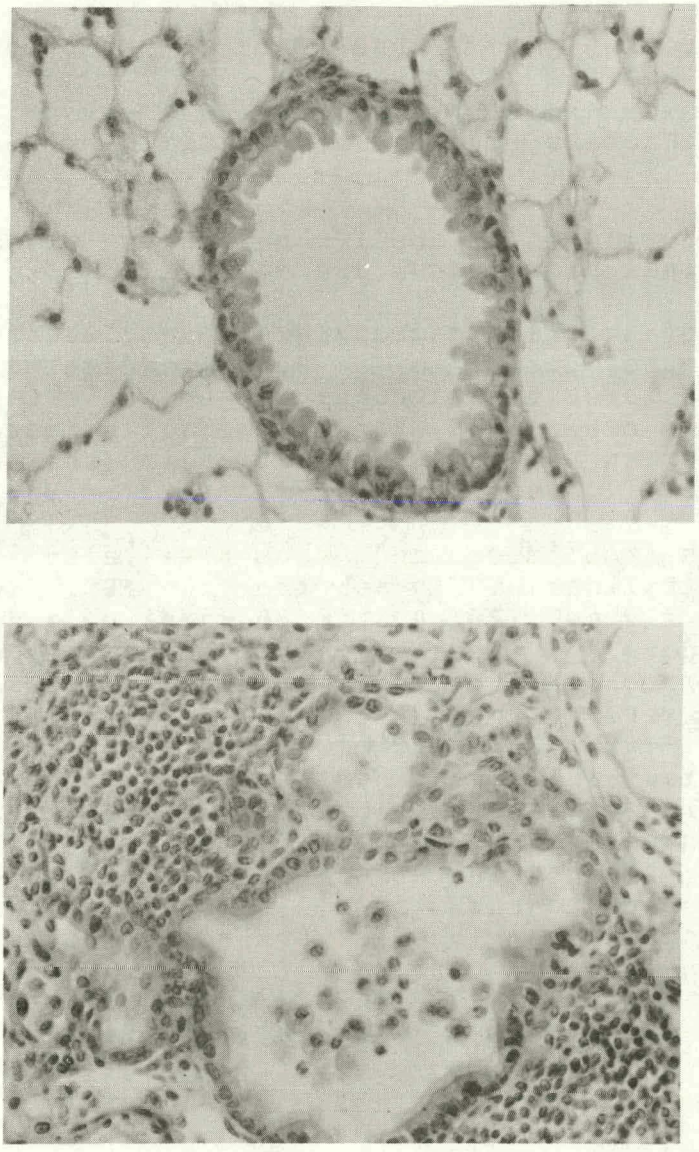

Fig. 11.5. Top: Bronchiole and surrounding alveoli from the lung of a $B6CF_1$ control mouse. Bottom: Similar area from a mouse exposed to a 3-fold dilution of an FBC effluent 1 hour a week for 5 weeks. The bronchiole contains a cellular exudate and the alveolar region shows lymphocytic infiltration and consolidation.

after the last exposure, the lungs were examined histopathologically and cultured for bacteria. Spleen cells were cultured with T- and B-cell mitogens, and the hematopoietic stem cell population was evaluated using the spleen colony forming unit assay. Marked areas of pneumonia were present in the lungs of all the exposed mice. Mice examined at 15 days had more bacteria per lung than those examined at 5 days, and four of five had B hemolytic streptococci in their lungs. The stem cell content of the femur and the proliferative response of spleen cells to mitogens were both depressed 15 days after exposure. Taken together, these data suggest that impairment of immune competence develops after termination of exposure to the FBC effluent.

EFFECTS OF FLUIDIZED BED COMBUSTION EFFLUENTS ON CULTURED PULMONARY MACROPHAGES

P. C. Brennan, W. T. Kickels, and S. S. Dornfeld

Methods have been developed to obtain pulmonary alveolar macrophages from mice, guinea pigs, rats, and dogs.

We have exposed mouse alveolar macrophages and spleen cells for 1 hour in open culture dishes to a 20-fold dilution of effluent. Eighteen hours later the macrophages showed no reduction in viability. On the other hand, the spleen cells showed a 50% reduction in viability and a 10-fold reduction in proliferative response to T- and B-cell mitogens.

Alveolar macrophages from mice and dogs have also been exposed to varying concentrations of fly ash from the FBC. Mouse macrophages cultured with fly ash (20, 40, or 80 µg/ml of medium) took up all the ash by 2 days. Viability was checked daily, and remained at about 99% throughout the culture period of 5 days. Dog alveolar macrophages were cultured for 2 days with varying concentrations of fly ash. They were checked for viability and were then examined by light and scanning electron microscopy. There was no reduction in viability at any concentration tested (up to 80 µg/ml). The number of cells containing particles was directly proportional to the concentration of fly ash in the medium. Scanning electron micrographs showed that cells exposed to higher concentrations appear to lose the typical normal "ruffled" appearance of the surface and were more flattened than control cells.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

None; this is a new program.

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12. MAMMALIAN CELL BIOLOGY

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The research of the Mammalian Cell Biology Group continues to be based upon the cultivation of mammalian cells *in vitro*. Although a series of diverse topics is being pursued, these have in common efforts to understand the cellular and molecular biology of functional changes produced by environmental agents including ionizing radiation, nonionizing radiation, and various chemicals. Although the group maintains a considerable interest in the radiobiology of ionizing radiation, its perspectives have broadened and shifted: first, to include the biological properties of various spectra of fast neutrons as well as X-rays; second, to inquire about sunlight effects and the interaction of sunlight with ionizing radiation damage; third, to include end effects such as neoplastic transformation in addition to cell killing and DNA damage; and fourth, to initiate studies of the action of chemical carcinogens by themselves and in conjunction with radiation.

MECHANISMS OF LETHALITY IN MAMMALIAN CELLS

M. M. Elkind, J. L. Dainko, M. P. Hagan,[1] A. Han, C. M. Liu, P. Q. H. Ngo, W. K. Sinclair,[2] and H. Utsumi

This research is directed at the elucidation of mechanisms of mammalian cell killing by radiation (ionizing and nonionizing) and by chemicals, and the relationship between such mechanisms and those involved with other functional changes such as mutation, neoplastic transformation, and altered differentiation. Cell killing is important in itself but, in addition, cell viability is required for functional changes to be expressed. Many agents capable of producing functional changes in cells are known, or suspected, interactants with the cell's genetic substance, DNA. The critical role of DNA in inheritance amplifies its influence in specifying and controlling the properties and functions of cells. Since inheritance of effects of environmental pollutants requires, first, that the content and/or the expression of the genetic substance of the cell be changed, and, second, that one or more target cells remain viable, this research focuses on the changes induced in DNA, and on whether and how these changes are related to toxicity and functional changes. A further point of interest is the ability of cells to repair potentially lethal damage and whether such repair processes are manifest at the level of DNA integrity and function.

Comparative Neutron Radiobiology

The relative lethal effectiveness of the following three fast neutron sources, with different average energies, E_n, has been compared. (1) Fission spectrum neutrons produced by the JANUS reactor at Argonne National Laboratory (E_n = 0.85 MeV); (2) cyclotron-produced neutrons at Franklin McLean Institute of The University of Chicago (E_n = 3.6 MeV); and (3) linear accelerator-produced neutrons at the Fermi National Laboratory (E_n = 25 MeV). V79 Chinese hamster cells were used. With increasing E_n, the survival curves become progressively more like those for X-rays. The repair capabilities of cells exposed to the JANUS or Fermi neutron beams have also been examined. The results of these experiments are summarized as follows.

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The dependence of the surviving fraction S on dose D may be expressed by

$$S = e^{-aD}[M(D)], \quad (1)$$

where a is a constant and M(D) stands for a damage accumulation mode of cell killing. Two hypotheses are current for the change in the shape of a survival curve as a function of neutron energy. In the first, a increases as E_n decreases (or as the relative biological effectiveness increases), and the contribution to cell killing from M(D) is assumed to be independent of radiation quality. In the second, the proportion of the dose applicable to the two modes of cell killing in equation (1) changes. Thus,

$$S = e^{-a(fD)} \cdot M[(1-f)D], \quad (2)$$

where $0 < f < 1$, and is close to zero for X-rays and close to unity for a maximally effective radiation. In this second model, damage accumulation contributes less and less to lethality as E_n decreases.

Since the contribution to cell killing from sublethal damage (i.e., the second factor) varies with neutron energy only in equation (2), it follows that, at the same dose, the repair of sublethal damage should be independent of neutron energy if equation (1) applies, and should decrease with decreasing E_n if equation (2) applies. Cells repaired sublethal damage after exposure to Fermi neutrons as effectively as after an equal dose of 250-kV X-rays, whereas after JANUS neutrons, the repair was clearly less than after X-rays. Thus the notion is supported that with increasing biological effectiveness (decreasing E_n) a decreasing fraction of the energy deposited by neutrons is sublethal (i.e., X-ray like); and therefore a decreasing fraction of the damage is modifiable by dose fractionation, dose protraction, or reduced dose rate.

Repair of Potentially Lethal Damage: Its Independence of Repair of Sublethal Damage

The repair of potentially lethal damage is usually observed as increased survival after a postirradiation treatment. On the other hand, the repair of sublethal damage is demonstrated by increased survival after dose fractionation. It has been hypothesized that the shoulder on a survival curve reflects the repair of potentially lethal damage at low doses followed by substrate saturation of the repair process, or inactivation of the repair process, at high doses (Alper, T., Br. J. Radiat. 50, 459, 1977). This, and related hypotheses, have been examined using anisotonic buffer solutions to enhance the expression of potentially lethal damage in cultures of V79 Chinese hamster cells. Adding such solutions after X-irradiation ensures that the enhanced single-dose killing that is observed results from an

inhibition of the repair of potential damage as opposed to the alteration of the structure of the sensitive site before irradiation. Two important observations from this work are: (1) The sensitizing action of oxygen in solution is independent of the amount of potentially lethal damage expressed. This result signifies that the spectrum of radiochemical lesions is not affected by changes in damage expression. (2) The repair of sublethal damage (observed with dose fractionation) is independent of the aforementioned influence of anisotonicity.

To test for possible connections between these two forms of damage related to cell killing and other radiation-induced functional changes, anisotonic buffer treatments will be applied to mutation induction and neoplastic transformation.

Anisotonicity and Survival after Nonionizing Radiation

Following the experiments with X-rays described above, the influence of hypo- and hypertonic buffered saline solutions was examined after exposure of normal cells to UV light (254 nm) or a near-UV sunlight-simulating source (313-315 nm maximum emission). Cells made photosensitive to longer near-UV radiation by first growing them in 5-bromodeoxyuridine (BUdR) were also subjected to the same protocol. In no case was a significant reduction in survival effected by the anisotonic salt treatment given after radiation exposure. However, as we have shown (see Han, A., and M. M. Elkind, Int. J. Radiat. Biol. 31, 275, 1977), sublethal UV damage in cells synthesizing DNA is equivalent to sublethal X-ray damage since a preexposure to UV light removes the shoulder of the X-ray survival curve. The latter result further supports the idea that potentially lethal X-ray damage is distinct from sublethal X-ray damage. Further, since BUdR/near-UV cell killing results in about 50,000 single-strand DNA lesions per D_o dose (i.e., per unit of dose that reduces survival by 1/e) and yet anisotonic treatment does not produce a comparable change in survival after UV irradiation as in the case of X-rays, we infer that cell killing by ionizing radiation involves damage to, or the misrepair of damage in, a structure containing DNA that is different from the long regions of the duplex molecule in which lesions are produced by BUdR + near UV. This conclusion illustrates how results with different radiations or cytotoxic agents can cross-illuminate mechanisms of action even though the mechanisms may differ in detail.

Repair of Single- and Double-Strand Breaks in DNA in X-radiation Survivors

The commonly used sedimentation technique of examining the linear integrity of single strands of DNA from mammalian cells--as applied in a number of laboratories including ours--has clearly shown that irradiated cells rapidly repair damage. In

some studies, it has also been possible to infer, within the resolution available, that the repair is complete. Essentially all of the measurements--as interesting as they are--are deficient in that they do not indicate if repair is complete or, in fact, occurs at all in surviving cells. Large doses of radiation (e.g., 10,000 rads or more) are usually used in order to produce enough DNA breakage to be readily measureable with sedimentation techniques. Even if the sensitivity of these techniques allowed measurement of DNA breaks at somewhat lower doses, the number of surviving cells would not be large enough to make certain the fate of the damage in the survivors. For example, only about one mammalian cell in a hundred survives the relatively low dose of 1,000 rads. A similar problem pertains to the repair of double-strand breaks.

Because of the foregoing, an experiment was devised to inquire about the fate of DNA breaks in surviving cells. Between successive X-ray doses of 1,000 rads, cells were incubated for a few days to allow killed cells to lyse and surviving cells to repopulate the culture. This was repeated until a total dose of 50,800 rads was accumulated. It was found that the survivors showed no evidence of break propagation since the sedimentation properties of the DNA were the same as those of control cells.

Our data indicate that a surviving cell contains about 1,000 single-strand breaks per D_o, or per 1/e survival dose; 80 of these breaks are due to 40 double-strand breaks. The examination of the survivors of 50.8 krads suggests the inference that, in mammalian cells, a double-strand break as such is not a lethal lesion because most, if not all, of them are repaired.

Pyrimidine Dimer Production and Cell Killing by "Sunlight"

Sun lamps are an inexpensive source of near-UV radiation in the wavelength region of $\sim$ 290 nm and above (maximum intensity at 315 nm). They produce photoproducts in DNA, particularly pyrimidine dimers, in significant numbers. The dimers produced with the Westinghouse Sun Lamps were compared to dimers induced by the irradiation of low pressure Hg germicidal lamps (UV, 254 nm). Consistent with the DNA action spectrum and the spectral emission of the Sun Lamps (which starts at about 290 nm, and has a maximum intensity at 315 nm), the rate of dimer induction was found to be 3.3 to 3.5% of that produced by germicidal lamps. However, the UV and near-UV survival curves of several cell lines are not relatable by a 3.3 to 3.5% dose factor because the curves are of different shape and cannot be interconverted by a constant modification dose factor. We conclude, therefore, that projections of the biological properties of sunlight cannot be made with confidence from measurements made with UV light (254 nm).

The Relationship between DNA Synthesis and Cell Photolethality Due to 5-Bromodeoxyuridine

When Chinese hamster cells are grown in BUdR for several generations, cells may then be killed by exposure to near-UV light (NUV; Westinghouse Sun Lamp, described above). The survival curve after uniform template labeling with BUdR is close to exponential; dose fractionation does not result in an increase in survival. Since BUdR/NUV cell killing very likely results from DNA lesions, a knowledge of the mechanism(s) involved is helpful in understanding cytotoxicity due to other agents thought to be DNA interactive, even though their mechanisms of cell killing may be different.

Cells were pulse labeled with BUdR early in the DNA synthesis or S phase and, using synchronized cells, their survival properties after NUV irradiation were traced, as a function of cell age, through the remainder of the first cycle and the S phase of the next cycle. Cells are least sensitive--and have a shoulder-type survival curve--in the remainder of the first S phase and in the next S phase after the replication of the BUdR pulse. At the time of replication of the BUdR pulse, cells are appreciably more sensitive and have a close to exponetial survival curve. Caffeine, after irradiation, enhances cell killing significantly only where the cells are resistant; that is, in the first S phase and after the replication of the pulse in the second.

Thus, sensitivity due to BUdR/NUV treatment is associated with the proximity of the lesions induced by BUdR/NUV to the DNA growing fork. Such lesions induced at a distance from the fork may be repaired by a postreplicational process.

DNA Maturation in Chinese Hamster Cells

To a significant degree, the ability of scientists to relate DNA damage to functional changes expressable at the whole cell level is limited by their incomplete understanding of the organization of eukaryotic DNA. This study was initiated to help supply some of the missing information.

The maturation of DNA pulse labeled early in S phase of synchronized Chinese hamster cells was traced using sedimentation techniques. The modal molecular weight grows to $\sim 2 \times 10^8$ daltons within 90 minutes after the pulse. In late S, it increases further to $4\text{-}6 \times 10^8$ daltons and then the DNA undergoes a conformational change to a rapidly sedimenting species. The latter material is also recoverable in the G_2 phase from newly labeled or template-labeled DNA in G_2 phase, and from template-labeled DNA in G_1 phase. Template-labeled DNA isolated in S also sediments as a $4\text{-}6 \times 10^8$ dalton species.

These data suggest that in G_1 and G_2, the DNA in Chinese hamster cells is in a conformation that does not readily release 4-6 x 10^8 dalton DNA under the lysis conditions used. During the latter part of S, new DNA matures to this size. The X-ray dose dependence of the transitions to a different sedimenting species as reported earlier, and also observed in this work, is consistent with the rapidly sedimenting material being $\sim$ 5 x 10^9 daltons. This size unit in turn is consistent with a superhelical structure of the type recently proposed by P. R. Cook and I. A. Brazell (J. Cell Sci. 19, 261, 1975).

In summary, the progress of the Mammalian Cell Biology group involves a number of diverse but related topics. We are concerned with how the DNA in eukaryotes is organized, replicated, and segregated, and how DNA-interactive agents interfere with these activities. In the case of exposure to ionizing radiation, DNA strand breaks are registered in considerable excess per unit of biological effect. Since even double-strand breaks appear to be completely repaired in surviving cells, the likelihood must be recognized that gross changes in the lineal continuity of DNA in the genome (e.g., single-strand recombination or sister chromatid exchanges) may be compatible with viability and associated with altered cell function.

INDUCTION OF FUNCTIONAL CHANGES IN MAMMALIAN CELLS

M. M. Elkind, J. L. Dainko, M. N. Gould, A. Han, G. R. Lankas, C. M. Liu, and H. Utsumi

Cultured mammalian cells may be used to measure changes caused by environmental pollutants. The counterparts of these changes in humans are among the more important long-term environmental hazards. In particular, somatic mutation and neoplastic transformation are amendable to study using *in vitro* techniques. These techniques include measurement of colony formation, population growth, mutation production, neoplastic transformation, and various forms of change in DNA, using radioactive labeling and sedimentation techniques. Since polycyclic aromatic hydrocarbons are derived from fossil fuels and therefore may constitute some environmental pollutants, since they include classical mutagens and carcinogens, and since as a group they are generally photochemically active, we use near-ultraviolet, sunlight-simulating light to study the cell biology of the interaction of these compounds and "sunlight." In addition, the mechanisms of the induction of functional changes by ionizing and nonionizing radiations by themselves are being pursued.

Sites of Photodynamic Action on 7,12-Dimethylbenz(a)anthracene (DMBA)

In earlier work, we found that the coal-derived carcinogen DMBA acts synergistically with near-UV light on Chinese hamster, mouse, and human cells when, in the presence of oxygen, they are exposed to DMBA and near-UV light simulating sunlight (Westinghouse Sun Lamps, maximum intensity at 315 nm). Measurements relative to mutation and cell transformation are in progress; additional results in respect to cell killing have also been obtained.

In an effort to learn what portions of the four-ring, planar DMBA molecule are photoreactive, analogs of DMBA were tested for photodynamic cell killing. The analogs were obtained from Dr. Ronald G. Harvey, Ben May Laboratory, The University of Chicago,, and Dr. Melvin Newman, Chemistry Department, Ohio State University. Examples of the results thus far are the following. The 2-hydroxy-, 3-hydroxy-, 4-hydroxy-, 2-methoxy-, 3-methoxy-DMBA compounds were all more effective than 7,12-DMBA. 4-Methoxy-DMBA was slightly less effective, but since we know that these materials are readily photooxidized, and that DMBA-endoperoxide is not active (with or without light), the small reduction of the potency of this 4-position substitution could reflect a contamination of the compound by its oxidation products. 5-Fluoro-DMBA was equally as effective as DMBA.

In a number of instances, the sample size of an analog was large enough to permit the measurement of its absorption spectrum. While not precisely the same, these were similar to that of DMBA. The altered photoactivity of an analog did not appear to reflect altered absorption.

The foregoing indicates that substitutions close to the bay region of DMBA in general enhance its activity. In respect to the metabolic activation of DMBA, current evidence supports the penultimate form as being the 1,2-epoxy, 3,4-diol of DMBA, and it has been shown (Moshel, R. C., et al., Biochem. Biophys. Res. Commun. 76, 1092, 1977) that a metabolically induced addition product to DMBA has a fluorescence spectrum similar to DMBA which in turn is similar to that of anthracene. Thus, the metabolic activation of DMBA appears to result from the formation of a bond from the benzene ring to DNA--since the unsaturated anthracene structure appears to be maintained. The benzene ring is the same region of DMBA where substitutions, in general, result in enhanced photoactivity.

Photo-Induced DNA Damage from DMBA + "Sunlight"

The photodynamic activity of DMBA relative to cell killing prompted a search for concomitant DNA damage. We used the alkaline-sucrose gradient method to examine changes in the sedimenta-

tion of labeled DNA from Chinese hamster cells exposed to DMBA and near-UV light from the Westinghouse Sun Lamp.

Results thus far indicate the following: (1) Single-strand breaks are produced. (2) At high levels of survival, about as many such breaks are produced as would be produced by X-rays (without DMBA) and appreciably more than by UV or near-UV light alone. (3) Cells repair single-strand breaks induced by DMBA plus near-UV light in a similar fashion to breaks induced by X-ray. This is in contrast to the increase in number of unrepaired single-strand breaks following UV light alone and the persistence of these breaks following near-UV light alone. (4) At the same level of X-ray survival, the DNA from cells treated with DMBA + near-UV light is broken down endonucleolytically more rapidly. This process occurs in cells that start to detach from their substrate and, in fact, cell rounding and detachment sets in much sooner following DMBA + near UV light than after X-irradiation, for the same level of survival.

Although only qualitative and semiquantitative aspects of DNA damage induced by DMBA + near UV light have been worked out thus far, it is clear that DNA is damaged quite effectively. The association made between DNA breakdown and cell detachment suggests that a prominent mode of cell death involves the activation and/or release (e.g., from the lysosomes) of enzymes capable of DNA endonucleolytic attack.

Exposure of cultured cells to DMBA plus "sunlight" significantly enhances cell killing in cells known to be deficient in the aryl hydrocarbon hydroxylases required for the metabolic activation of polycyclic aromatic hydrocarbons. While the possible connection between the light-induced and metabolism-induced activities have not as yet been worked out, the fact that DNA damage is associated with both suggests that they may both induce other functional changes. What role, if any, repair processes play in the functional changes induced by DMBA plus "sunlight" remains to be explored.

Neoplastic Transformation: Comparative Effectiveness of Fission-Spectrum Neutrons and 50-kV X-Rays

A study is in progress of the induction of neoplastic transformants in C3H mouse-derived 10T1/2 cells based upon colonial morphological changes. For both JANUS reactor neutrons and X-rays, the induction curves per cell rise to a maximum and then drop off indicative of the killing of induced cells. With both radiations, the effect of dose fractionation (two doses, separated by increasing intervals) was examined. For fission-spectrum neutrons little change in survival and the frequency of transformations per cell occurs when the total dose is fractionated over a 24-hour period. For X-rays, a typical increase in net survival, by a factor of 6, occurs in 24 hours. The trans-

formation frequency per cell increases 1.5 to 2.0 times in the first 9 hours but is back to the single, total dose level by 16 hours. These data indicate that cycle-dependent variations and repair processes play a minor role after neutron irradiation. After X-irradiation, however, differences in cycle dependencies and differences in the repair of subeffective damage in respect to transformation and survival are important.

Cells from transformed colonies induced by JANUS neutrons were found to produce fibrosarcomas in appropriate recipients.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

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13. ENVIRONMENTAL PHYSIOLOGY

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Everett F. Staffeldt (Scientific Associate)

The analysis of lifetime effects of ionizing radiation was at first directed almost exclusively toward effects on disease incidence and life-span, because these kinds of cumulative damage were the most prominent and serious late effects of ionizing radiation. The experimental program of our team on the effects of life-time gamma-ray exposure on the survival of mice and other rodent species was completed several years ago, but important analysis and modeling efforts continue. A report of one aspect of this analytical effort is included here.

A life is measured better by a lifetime productivity score than by years alone. The importance of productivity measures of the toxic action of energy by-products is increased now that fossil fuel products are receiving attention, because the inhaled combustion products, in particular, have their major effect on work performance and low-level chronic disease, instead of on life shortening by terminal cancer. The first stages of a program to develop simple measures of performance capacity in rodents, based on indices of energy metabolism, motor activity, and body temperature, are described below.

ENVIRONMENTAL INFLUENCES ON LIFE-SPAN, LIFETIME PERFORMANCE, AND DISEASE MORBIDITY

G. A. Sacher and P. H. Duffy

In the past, long-term bioeffects programs have concentrated on life shortening and disease incidence as end points. The programs discussed here continue to be concerned with these measures, but in the context of the overall lifetime performance or productivity of the individual or population. The reason for this redirection of effort is that many physical and chemical products of energy generation, distribution, and use have their most serious health consequences in decreased well being and work performance, rather than in increased life-threatening disease and mortality.

Our approach to the evaluation of the effects of environmental pollutants on lifetime performance is based on three instrumentalities that are used in various combinations in specific experiments.

1) Genetic and constitutional differences between genotypes and species. We employ a reproducible set of laboratory mouse genotypes consisting of the male and female progeny of the 25 mating pairs of 5 inbred mouse strains. This diallel model has been used in our experimentation for the past 10 years, so it is probably the most thoroughly characterized mouse genetic design in use today. In addition, we have developed an interspecies model system consisting of the wild-type genotypes of two small rodent species, Mus musculus and Peromyscus leucopus, that differ in life-span by a factor of 2. This model has also been thoroughly characterized and used in experiments for the past 10 years. These two small rodent species differ in regard to life-span, aging rate, and certain important aspects of cancer susceptibility, and we believe that these differences are valid models for the differences between short-lived laboratory species and the human species.

2) Controlled environments. The conventional laboratory animal facility provides a poor environment for assessing effects on performance, for it is characterized by constancy of conditions and minimum stress on the animal populations. In such impoverished environments, the variability of behavior within genotype increases. We therefore propose to use environments that introduce mild stress or controlled variation. One example is a cold room that can be operated down to 0°C while providing a healthy environment for long-term maintenance of small rodents. Cyclical variation of ambient temperature is another option that we can employ with small groups of animals.

3) Measures of physiological performance. At present we have the capability to measure three variables--oxygen consump-

tion, motor activity, and body temperature--in quasi-continuous fashion for a week or longer on four mice simultaneously, and to log the data automatically on paper tape for subsequent computer analysis. These data are obtained in a programmable environment chamber. The automatic monitoring system and the data it generates were described earlier (Sacher, G. A., et al., ANL-77-55, 1977, p. 81).

Diel Patterns of Motor Activity, Energy Metabolism, and Body Temperature

Prior to the present study, little was known about the relation of life-span to constitutional factors in mice.

In a recently completed investigation, we examined some genetic and constitutional factors related to mean life-span of laboratory mice. Metabolic rates of male mice from the 25 genotypes of the diallel design were measured for 48-hour periods. From these data we estimated the minimum, or resting rate, the maximum rate, and the average rate over the 48-hour period. These measurements were made at two ages, 6 months (two mice per genotype) and 24 months (two to eight mice per genotype). Body weights were measured at the time of the metabolism measurement. Mean life-spans had been determined on separate samples of 20 to 50 per genotype.

The analysis carried out thus far has consisted of examining the dependence of genotype life-span on the mean genotype metabolic and body weight variables measured on young and old mice. Life-span has a significant negative regression on all three metabolic rate measures, and a significant positive regression on body weight. These two relationships confirm the existence within species of the same rate-of-living factor in aging that had previously been shown to exist between species. However, we also have evidence suggesting that there is a positive association of genotype life-span with the amount of energy metabolism in excess of the resting rate. This finding is of great potential value, for we postulate that an environmentally induced increase of the activity index (AI, defined as the ratio of average to resting metabolism) should be associated with increased survival expectation, and a decrease of AI should predict decreased survival. Tests of this hypothesis are now under way.

An interesting finding is that the measurements of metabolic variables made at 24 months of age have higher correlation with life-span than measurements made on 6-month-old mice.

These and further findings about the relation of life-span to constitutional variables will help us design more sensitive and more reliable experiments on the long-term toxic effects of energy pollutants. These results may also have application to the assessment of human populations.

All of the above studies are part of the baseline phase of our research. Application to the effects of energy pollutants has now begun, and pilot experiments are under way on the effects of ionizing radiation, lead ingestion, and high-voltage electric fields.

Environmental Influences on Aging and Longevity

Under this heading we group the experiments in which environmental perturbations are applied to groups of animals in order to determine effects on life-span and disease incidence. Two such survival studies are in progress. In one of them, samples of six mice of each sex and genotype in the diallel design are being followed in the cold room at 12°C. In the other, samples of 85 Mus musculus and 85 Peromyscus leucopus are being maintained in the 12°C environment. The ultimate purpose is to determine whether the increased metabolism of mice maintained in the cold has a promoting action on the induction of tumors by chemical carcinogens. The experiments are in midcourse and there are no results to report at this time.

THE QUADRATIC RELATION OF SURVIVAL TIME TO DAILY DOSE FOR FIFTEEN MAMMALIAN SPECIES

G. A. Sacher, S. A. Tyler,[1] and E. F. Staffeldt

The dose-effect relation for mammalian populations given duration-of-life exposure to ionizing radiations is extremely complex and has not yet been adequately characterized. As part of a long-term program addressed to this problem, we determined the dose-survival relationship for 13 species of rodents given daily exposure to ^{60}Co gamma rays at daily doses ranging from 12 to 125 R/day (Sacher, G. A., and E. F. Staffeldt, Proceedings of the Third International Symposium on Radioecology, Oak Ridge, TN, D. J. Nelson, ed., USAEC Report CONF-710501-P1, 1042, 1973), and in addition utilized published data on guinea pigs, dogs, and goats given duration-of-life exposure (Lorenz, E., et al., Biological Effects of External X and Gamma Radiation, R. E. Zirkle, ed., Part 1, McGraw-Hill, New York, 1954, 24; Thomson, J. F., et al., Am. J. Roentgenol. 69, 830, 1953; Norris, W. P., et al., Biological and Environmental Effects of Low-Level Radiation, International Atomic Energy Agency, Vienna, Vol. 1, 1976, 147; Hupp, E. W., et al., Int. J. Radiat. Biol. 20, 475, 1971).

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The present report describes the mathematical analysis of those data. The outcome of the analysis is the demonstration that: (1) the relation of life shortening to dose rate for mammalian species has a characteristic quadratic form; (2) the data are well fitted with a two-parameter mathematical model in which the parameters are the dose-effect constant, λ, and a rate constant, γ; and (3) the wide range of radiosensitivities among the mammalian species tested is due in only a minor degree to variation in the dose-effect term, and primarily is due to the variation in the rate constant. A mechanistic interpretation of this rate constant is presented below.

The data from the rodent series consist of mean after-survival times of small groups of animals at each of 6 to 12 daily dose levels. In most cases the series consisted of eight daily doses, from 12 to 125 R/day. The species employed are listed in Table 13.1.

Table 13.1. Species Used in the Present Study[1]

Taxonomic Name	Common Name	Family	Body Wt g	Life Expect. Days	Total Sample N
Tamias striatus	Chipmunk	Sciuridae	100	∿1500	125
Chinchilla laniger	Chinchilla	Chinchillidae	440	∿2500	66
Sigmodon hispidus	Cotton rat	Cricetidae	130	510	149
Mus musculus (wild type)	House mouse	Muridae	17	610	192
Praomys natalensis (two series)	Multimammate mouse	Muridae	48	630	391
Rattus rattus	Black rat	Muridae	186	∿730	162
Oryzomys palustris	Rice rat	Cricetidae	50	730	47
Peromyscus californicus parasiticus	California mouse (northern)	Cricetidae	60	1070	74
Peromyscus californicus insignis	California mouse (southern)	Cricetidae	60	1300	193
Peromyscus floridanus	Florida mouse	Cricetidae	36	1740	31
Peromyscus gossypinus	Cotton mouse	Cricetidae	31	1830	56
Peromyscus leucopus	White-footed mouse	Cricetidae	23	1420	394
Reithrodontomys humulis	Harvest mouse	Cricetidae	9	710	28
Canis familiaris	Beagle dog (domestic)	Canidae	11,000	4200	--
Capra hircus	Goat (domestic)	Bovidae	65,000	∿4400	--
Cavia porcellus	Guinea pig	Caviidae	750	1250	--

[1]The body weights and life expectations for the rodents are our unpublished data. Data for the beagle dog are from Norris, W. P., et al. (Biological and Environmental Effects of Low-Level Radiation, IAEA, Vienna, 1976, Vol. 1, pp. 147-155; and personal communication from W. P. Norris). The body weights are averages from males and females. Total number of exposed rodents is shown in last column (sexes combined).

The procedure of modeling the relation of survival time to dose directly is unsatisfactory because survival time has a non-linear relation to the radiation damage. In earlier studies in this Division (Sacher, G. A., Advances in Radiation Research. Biology and Medicine, J. F. Duplan and A. Chapiro, eds., Gordon and Breach, London, Vol. 3, 1973, 1425; Norris, W. P., et al., Biological and Environmental Effects of Low-Level Radiation, International Atomic Energy Agency, Vienna, Vol. 1, 1976, 147), it was discovered that the rational approach is to examine the relation of the rate of mortality in excess of control to the daily dose. The mean radiation-specific death rate, ρ_x, at daily dose x can be estimated by the relation

$$\rho_x = \frac{1}{M_x} - \frac{1}{M_o},$$

where M_x and M_o are the mean after-survival times for the exposed and the control group, respectively. For the purposes of this analysis, ρ_x can be estimated with adequate precision by a sample of 10 animals, and the species dose-effect relations described below were obtained with fewer than 100 animals of each species in the majority of cases. This number compares very favorably with the large numbers needed to obtain satisfactory dose-effect curves with single dose exposure.

Some representative death rate vs dose rate plots are shown on log-log scales in Figure 13.1 for six rodent species. We have shown (Sacher, G. A., et al., Proceedings of the International Atomic Energy Agency International Symposium on the Late Biological Effects of Ionizing Radiation, March 13-17, 1978, Vienna, in press) that the log-log death rate-dose rate plots for the 13 rodent species, for eight laboratory mouse inbred or hybrid genotypes, and for dog and goat can be fitted with a mathematical function consisting of two straight-line branches, with slope 2 at low dose rate and a transition to a second branch with slope 1 at higher dose rates. The fits are notably better for the laboratory mice, for which the sample sizes were larger. Nevertheless, only one of the 15 species departs significantly from this two-branch function.

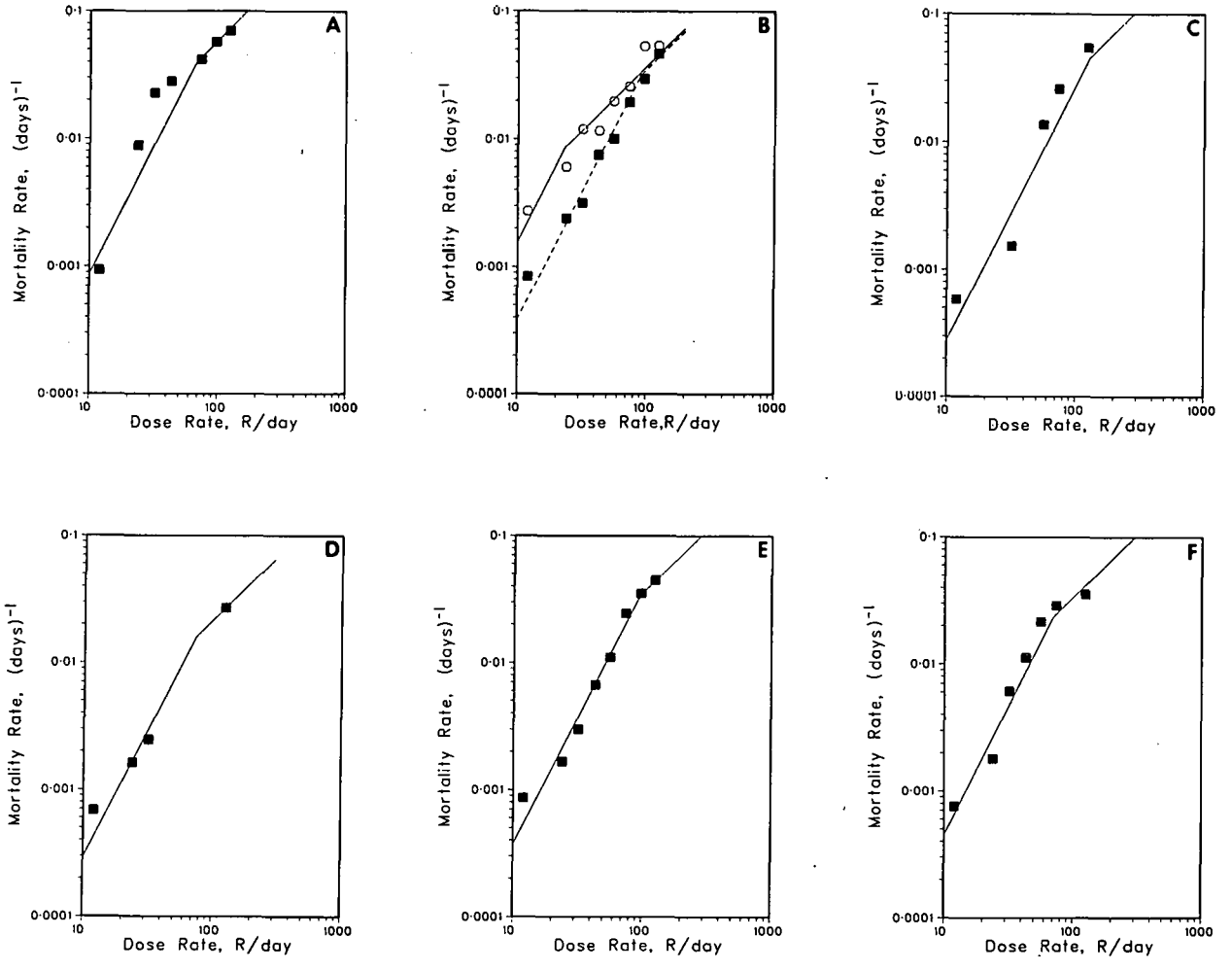

Fig. 13.1. Death rate-dose relations for six rodent species. Two-branched lines are fitted by least squares. The taxonomic names are given in Table 13.1. The species shown are: (A) rice rat: (B) California mouse, northern (o) and southern (■); (C) Florida mouse; (D) cotton mouse; (E) white-footed mouse; (F) harvest mouse.

The x and y coordinates of the intersection of the two branches specify the response for each population. Values of the x and y coordinates are given for all species tested, and also for eight laboratory mouse genotypes, in Table 13.2. The specifically quadratic nature of the dose dependence for these species led to the formulation of a mathematical model based on the assumption that life shortening results from cellular injury produced by abnormal chromosome rearrangements (Sacher, G. A., et al., ibid.). This model predicts a hyperbolic relation of log cell death rate to log dose rate, and the lower and upper

branches of the hyperbola have slopes of 2 and 1, respectively. This model has two parameters, a dose-effect coefficient, λ, with dimensions $\underline{R}^{-1}$, and a rate constant, γ, with dimensions $\underline{t}^{-1}$. The λ and γ values for each species can be estimated from the intersection coordinates $\underline{x}$ and $\underline{y}$ in Table 13.2, by the formulas

$$\gamma = y$$
$$\lambda = y/x.$$

The λ and γ^{-1} values are given in Table 13.2, which gives γ^{-1} rather than γ because a time is a more understandable quantity than a rate.

Table 13.2. Coordinates of Breakpoints, Accumulation Times (γ^{-1}), and Dose-Effect Coefficients (λ) for All Species and Genotypes Discussed Here[1]

	$Y=\gamma$ $(Days)^{-1}$	X R/Day	γ^{-1} Days	$\lambda \times 10^6$ $R^{-1} \times 10^6$
C57L/J (L)	0.0273	109.0	36.6	251
A/J (A)	0.0193	67.0	51.8	288
C57L x AF_1 (LAF_1)	0.0196	85.7	51.0	229
C3Hf/J	0.0348	119.7	28.8	289
BALB/cJ (C)	0.0219	71.6	45.7	306
C57BL/6J (B6)	0.0168	68.6	59.5	245
C57BL6 x BALB/cF_1 ($B6CF_1$)	0.0136	60.2	73.6	225
C57BL6 x BALB/cF_2 ($B6CF_2$)	0.0196	75.6	51.0	259
Chipmunk	0.0153	75.1	65.2	204
Chinchilla	$\leq$0.0023	$\leq$ 5	$\geq$435	400
Cotton rat	0.0243	92.7	41.1	262
House mouse	0.0194	92.0	51.5	211
Multimammate mouse (two series)	0.0246	94.1	40.6	262
Black rat	$\geq$0.033	$\geq$125	$\leq$ 30.0	$\geq$280
Rice rat	0.0398	67.9	25.1	586
California mouse (northern)	0.0087	23.6	115.3	367
California mouse (southern)	0.0317	91.0	31.5	348
Florida mouse	$\geq$0.0458	$\geq$128.9	$\leq$ 21.8	$\geq$355
Cotton mouse	0.0160	73.9	62.5	216
White-footed mouse	0.0340	95.6	29.4	355
Harvest mouse	0.0235	70.1	42.5	335
Beagle dog	0.0380	40.0	26.3	950
Domestic goat (female)	0.0077	22.5	130.0	342
Domestic goat (male)	$\leq$0.0025	$\leq$ 7	$\geq$400	330
Guinea pig	0.0047	9.5	212.8	495

[1]Breakpoint coordinates were estimated by least squares, as described in Sacher, G. A., et al. (Proc. of an IAEA Symposium on the Late Biological Effects of Ionizing Radiation, Vienna, 1978, in press). Sexes are combined unless otherwise noted.

It can be seen that species vary by a factor of 4 in λ values, and by at least a factor of 20 in γ^{-1} values. It must be concluded, therefore, that most of the species variation in radiosensitivity is due to variation of the time constant γ^{-1} rather than to variation of the dose-effect parameter λ.

The actual γ^{-1} values are in the range from 20 to 500 days. These are unusually long for biological time constants, so it is important to determine why γ^{-1} is so long and why it is so variable between species.

The hypothesis we propose is that γ^{-1} is not a repair or recovery time, but rather an accumulation time, i.e., the mean time over which damage to chromatin can accumulate in a critical cell population before being expressed in terms of actual chromosome breakage and reunions. The γ^{-1} value estimated by the duration-of-life exposure procedure is, moreover, the longest such accumulation time for the species. Shorter accumulation times are presumed to exist also, but their estimation would require different experimental procedures.

A plausible hypothesis about the biological basis of the accumulation time is that it is the intermitotic interval in the most slowly dividing hematopoietic stem cell population. This hypothesis deserves careful examination, for it may be noted that: (1) radiosensitivity at low daily doses increases as the square of the accumulation time; and (2) accumulation time tends to increase with body size and/or life-span (compare Tables 13.1 and 13.2). The latter raises the possibility that the human species is more radiosensitive at low dose rates than the most sensitive experimental animals yet examined. However, even this inordinate sensitivity of the human species to the quadratic component of damage would not be a matter of concern in regard to exposure levels on the order of natural background (Sacher, G. A., et al., ibid.).

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

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Sacher, G. A. Quadratic dose dependence for life shortening in mammals: Experimental data and a mechanistic model. Summary and Proceedings of a Biology Workshop on Biological Repair Mechanisms and Exposure Standards, Oak Ridge, TN, June 28-30, 1977. Ed. D. Billen. Institute for Energy Analysis, Oak Ridge Associated Universities. ORAU/IEA 78-2(R), 1978, pp. 158-176.

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Sacher, G. A. Stochastic mortality theory and the mortality potential: A biophysical model for certain competing risks. Proceedings of a Workshop on the Evaluation of Environmental Biological Hazards and Competing Risks, Institute of Energy Analysis, Oak Ridge, TN, September 7-8, 1977 (in press).

Sacher, G. A., and P. H. Duffy. Age changes in rhythms of energy metabolism, activity, and body temperature. Aging and Biological Rhythms. Eds. H. Samis and S. Capobianco. Plenum Press, New York (in press).

Sacher, G. A., and R. W. Hart. Longevity, aging and comparative cellular and molecular biology of the house mouse, Mus musculus, and the white-footed mouse, Peromyscus leucopus. Genetic Effects on Aging. Eds. D. Bergsma and D. E. Harrison. Birth Defects:Original Article Series, The National Foundation-March of Dimes, Alan R. Liss Inc., New York, 1978, pp. 71-96.

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14. BIOMOLECULAR STRUCTURE STUDIES

Clinton F. Ainsworth (Scientific Assistant)
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The research activities in biomolecular structure studies are comprised of two major projects; the first deals with the structure and function of biological molecules in solution, while the second deals with X-ray crystallographic studies of the structure and function of immunoglobulin molecules. The highlights of the work in these areas during the past year are described in the two following reports.

STRUCTURE AND FUNCTION OF BIOLOGICAL MOLECULES IN SOLUTION

S. S. Danyluk, M. MacCoss, C. F. Ainsworth, E. K. Ryu, R. Tewari, A. W. Czarnik,[1] E. F. Duhr,[2] S. H. Gray,[3] and P. W. Maffuid[4]

This research program deals with (1) the determination of quantitative structural and conformational properties for biologically important molecules, (2) translation of these properties into overall three-dimensional topographic models for biomolecules, and (3) elucidation of the fundamental relationships between molecular topology and biological function. Emphasis is placed on structural/conformational aspects because these properties underlie the mechanisms of key biological reactions such as transcription, replicaton, mutagenesis and carcinogenesis, and membrane transport.

Since the basic problem of biological structure/function is multifacted in scope, the program has developed along three closely connected lines: chemical synthesis and characterization of selected biological molecules, spectroscopic determination of structural and conformational properties, and theoretical calculations of biological structures.

A variety of biomolecular systems are currently under investigation. Among these the nucleic acids and short-chain segments thereof are of primary interest. The latter are particularly useful for quantitation of conformational flexibilities in sugar rings and ribose-phosphate backbone bonds, and serve as excellent models for properties of parent molecules. The baseline conformational data for these segments also serve as a useful starting point for evaluation of structural changes produced by covalent coupling of various environmental mutagens and carcinogens to

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[1]Spring 1977 participant in the Undergraduate Honors Research Participation Program, University of Wisconsin, Madison.

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[3]Guest Graduate Student, University of Illinois Medical Center.

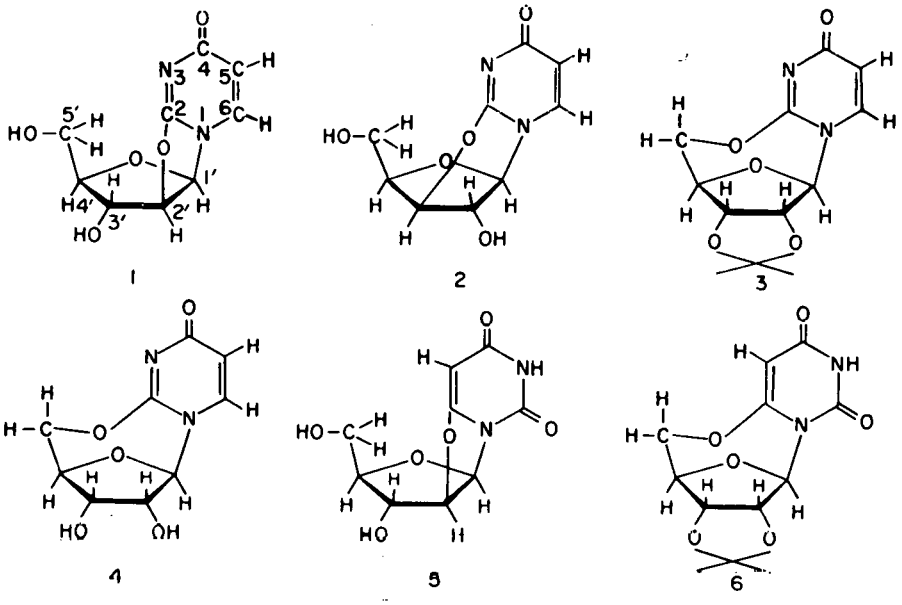
[4]Spring 1977 participant in the Undergraduate Honors Research Participation Program, St. Michael's College.

nucleic acids. Other biomolecular aspects deal with synthesis and structural characterization of membrane-targeted antitumor agents, development of selectively labeled (^{2}H, ^{13}C) probes of complex biomolecular structures, and adaptation of quantum theoretical methods to nucleic acid conformation calculations. Various aspects of these topics are detailed further in the following sections.

Chemical Synthesis of Novel Compounds

This activity encompasses a variety of projects dealing with synthesis of model compounds for conformational studies, development of cell-specific phospholipid derivatives, preparation of nucleic acid adducts of carcinogens, and adaptation of synthetic procedures for preparation of selectively labeled molecules. Each of these projects interfaces with parallel studies of biophysical properties and biological activity. As with most bioorganic synthetic studies, a significant fraction of the effort is devoted to development of new synthetic procedures and reactions.

Synthesis of conformationally "rigid" model compounds. Synthesis of model nucleoside/tide derivatives is required for definition of specific nucleotide conformational features by nuclear magnetic resonance (NMR) spectroscopy. Since nucleic acid constituents invariably exist as a dynamic equilibrium among several conformers (Davies, D. B., and S. S. Danyluk, Biochemistry 13, 4417, 1974; Davies, D. B., and S. S. Danyluk, Biochemistry 14, 543, 1975), a conformational analysis requires NMR parameters (chemical shifts, spin coupling constants) for "conformationally pure" states. Such data can be obtained by synthesis of appropriate derivatives in which one or more of the flexible bonds are locked into a rigid conformation, typically by intramolecular cyclization between functional groups. We have used this approach to sort out properties for two of the most important nucleic acid conformational features, torsion angles of glycosidic bonds and sugar ring puckers. In the former case, a series of bicyclo pyrimidine derivatives was synthesized with the pyrimidine ring linked via the C-2 or C-6 position to any one of three sites on the sugar ring, C2'-O2', C3'-O3', C5'-O5'. These conformationally rigid bicyclo anhydro compounds cover a range of χ_{CN} values spanning both syn and anti domains, as shown in Figure 14.1 for uridine anhydro nucleosides. Measurements of undecoupled ^{13}C NMR spectra for each compound enabled an evaluation of vicinal ^{13}C-^{1}H couplings across the glycosidic bond, and quantitative derivation of coupling magnitude-vicinal angle ($J_{^{13}CH}$ vs. χ_{CN}) correlations therefrom (vide infra).



	χ (deg.)
1. 2,2'- Anhydro-1-(β-D-arabinofuranosyl)-uracil	175
2. 2,3'- Anhydro-1-(β-D-xylofuranosyl)-uracil	225
3. 2,5'- Anhydro-2',3'-o-isopropylideneuridine	235
4. 2,5'- Anhydro-1-(β-D-ribofuranosyl)-uracil	235
5. 2',6- Anhydro-1-(β-D-arabinofuranosyl)-6-hydroxyuracil	125
6 5',6- Anhydro-2',3'-o-isopropylideneuridine	65

Fig. 14.1 Chemical structures and glycosidic bond torsion angles for a series of bicyclo anhydro uridine nucleosides.

Model nucleoside cyclic phosphates have also been synthesized with the particular objective of investigating the effects of phosphate ring size, type of phosphate ring-sugar ring fusion, i.e., cis or trans, and anomeric configuration upon overall ring conformational properties. Among compounds synthesized were α-nucleoside-3',5'-cyclic monophosphates, xyloadenosine-3',5'-cyclic phosphate, arabinoadenosine-2',5'-cyclic phosphate, lyxouridine-3',5'-cyclic phosphate, lyxouridine-2',5'-cyclic phosphate, and α-arabino-adenosine-2',5'-cyclic phosphate. As a typical example, the α-cyclic nucleotides were synthesized by procedures described earlier in connection with studies of anomeric effects (Robins, M. J., and M. MacCoss, J. Am. Chem. Soc. 99, 4654, 1977; Robins, M. J., et al., J. Am. Chem. Soc. 99, 4660, 1977). Following purification by DEAE-cellulose column chromatography, the compounds were dissolved as ammonium salts in D_2O solution for NMR study.

Synthesis and characterization of mutagen/carcinogen-nucleic acid adducts. Certain types of chemical carcinogens/mutagens, e.g., polycyclic diol epoxides, alkyl and aryl sulfates and halides, and alkyl diazo compounds have the capability to alkylate base residues of nucleic aicds at specific positions. Lawley and co-workers (Lawley, P. D., Screening Tests in Chemical Carcinogenesis, International Agency for Research on Cancer, 1976, p. 181) have shown that alkylation can produce a multiplicity of reaction products dependent upon factors such as nature of the base ring, electrophilicity of the alkylating agent, and presence of activating enzymes. Thus, under appropriate conditions, alkylation of adenine nucleotides can occur at six sites, as illustrated in Figure 14.2.

As part of a comprehensive study of alkylation reactions and their impact on nucleic acid conformation, we have examined the effect of methylation of N-1 of the adenine moiety in a series of mono- and dinucleotides. Such specific alkylation requires careful control of reaction conditions. For example, the choice of alkylating agent, reaction temperature, and pH of the medium are factors of critical importance for selective alkylation. The synthesis of the N-1 methylated dimers containing adenine (^{Me}ApA, Up^{Me}A, and ^{Me}ApU) was successfully accomplished following procedures described earlier (Brinacombe, R. L. C., et al., Biochemistry 11, 2452, 1965). Figure 14.3 shows a schematic illustration of the overall process. In a typical reaction, dimethyl sulfate was added slowly to an aqueous solution of the dimer under conditions of strict pH control (pH = 7.0). Following ether extraction of the mixture, the products were separated by column chromatography and characterized further by electrophoresis, UV, and NMR spectra. Yields of the N-1 methylated dimers ranged from 20-35%. These methylated compounds are currently being studied by high-resolution proton NMR spectroscopy to evaluate the impact of methylation on conformational structures.

Synthesis of new membrane-targeted molecular depots of nucleoside analogues. The use of nucleoside analogues as chemotherapeutic agents is severely hampered by their rapid catabolism to ineffective products. One approach to circumvent this problem utilizes prodrugs that protect the parent compound from catabolic enzymes and then release the drug either by enzymatic or chemical hydrolysis. This approach enables use of a lower dosage and hence fewer side effects. We have recently been interested in developing a new class of prodrug that utilizes phospholipids as the carrier molecule. Such molecules have the added advantage that the potential exists for targeting the derivatives to specific tissues, thus making possible the location of a cytotoxic agent in the immediate environs of a tumor cell. To this end, we undertook the synthesis of ara-CDP-L-dipalmitin,

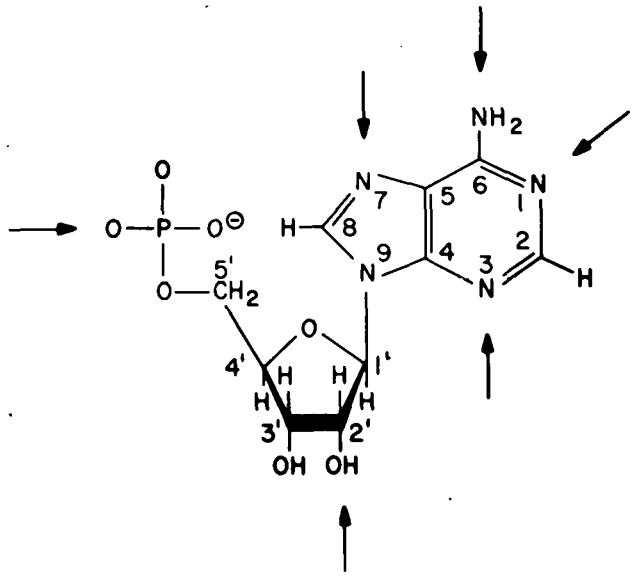


Fig. 14.2. Alkylation sites on an adenosine nucleotide.

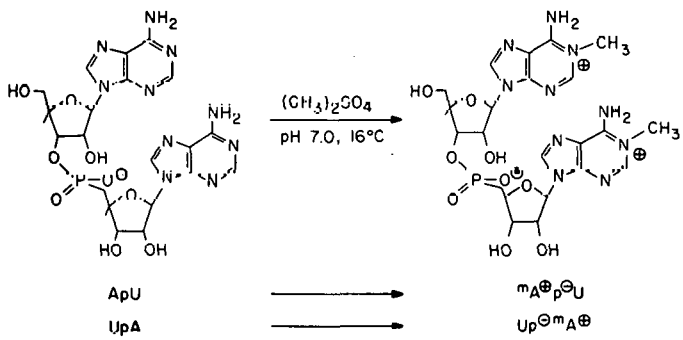


Fig. 14.3. Reaction scheme for synthesis of N-1 purine alkylated dinucleoside monophosphates.

ara-ADP-L-dipalmitin, TuDP-L-dipalmitin as phospholipid derivatives of arabinocytidine (ara-C), arabinoadenosine (ara-A), and tubercidin (Tu). After evaluation of several synthetic pathways, the phosphomorpholidate procedure of Moffat and Khorana (Moffat, J. C., and H. G. Khorana, J. Am. Chem. Soc. 83, 649, 1961)

was selected with appropriate modifications of the work-up in order to facilitate the handling of these detergent-like molecules with lipophilic head-groups and hydrophobic tails. Our procedure is illustrated in Figure 14.4. In addition, we have devised (1) a novel method of sample preparation that facilitates in vitro biological evaluation of these new chemotherapeutic agents (MacCoss, M., E. K. Ryu, and T. Matsushita, submitted for publication, 1978), and (2) a modified spray reagent that enables detection of phospholipid derivatives at extremely low concentrations on thin-layer plates (Ryu, E. K., and M. MacCoss, submitted for publication, 1978).

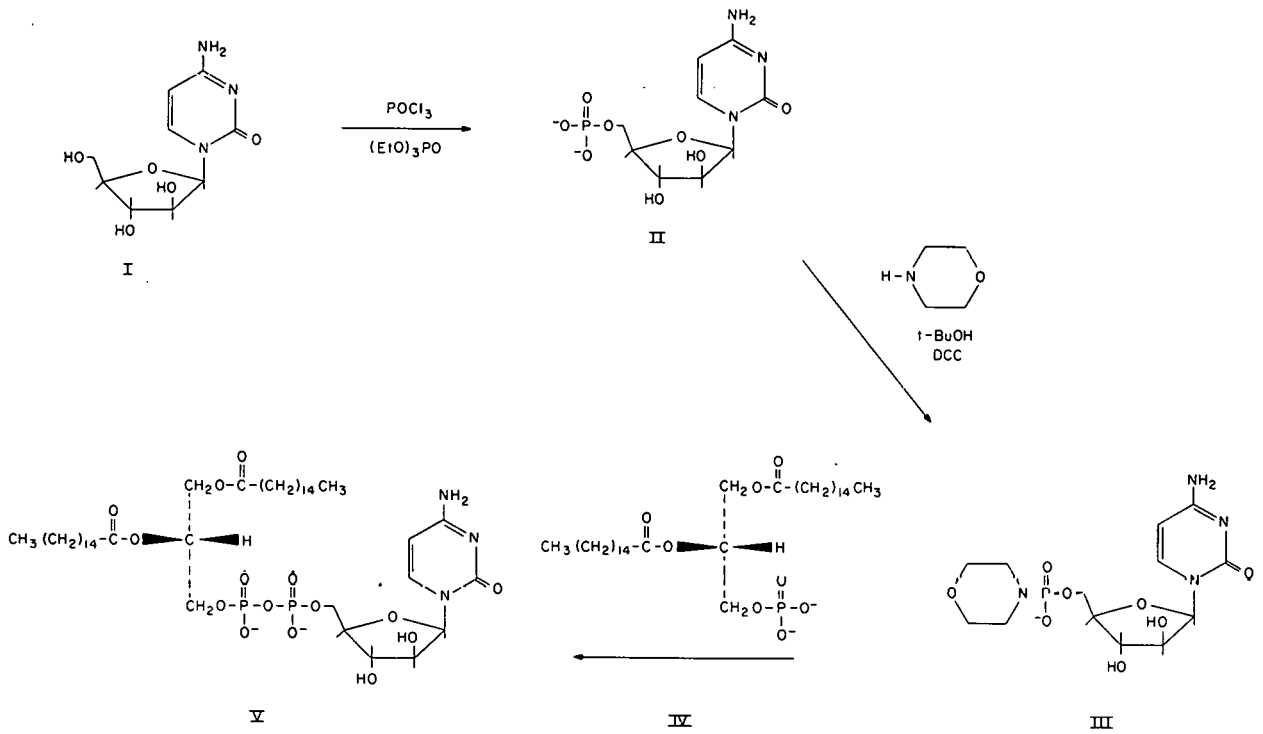


Fig. 14.4. Reaction scheme for synthesis of the prodrug, ara-C-dipalmitin.

Selective labeling of biomolecules. Selectively labeled (^2H, ^{13}C) nucleic acid derivatives are extremely useful in NMR studies of nucleic acids. Their utility has been demonstrated recently for spectral assignments of complex adenylate di- and trinucleotides (Kondo, N. S., and S. S. Danyluk, J. Am. Chem. Soc. 94, 5121, 1972; Kondo, N. S., et al., FEBS Letters 53, 213, 1975). The method is also of potential value in sorting out the factors influencing proton and ^{13}C spin-lattice relaxation parameters in these molecules. In the latter instance, deuterium atoms are introduced in place of protons at known positions in the base and/or sugar rings, as distinct from replacement of an

entire protio nucleotidyl fragment by its deuterio counterpart in oligomers. As part of a project dealing with unequivocal assignment of phosphorus resonances in nucleotide coenzymes, we undertook the synthesis of adenine nucleosides/tides and adenine coenzymes in which the two protons at the C5' position are substituted by deuterium atoms. Synthesis of selectively deuterated nicotinamide adenine dinucleotide (NAD) was carried out by a modified Michelson-Todd procedure outlined in Figure 14.5. Excellent yields of deuterium-labeled NAD were achieved, and work is currently under way to prepare NADP. Assignment of ^{31}P NMR resonances for the latter coenzyme complexed in enzymes will enable an *in situ* study of conformational changes at active sites.

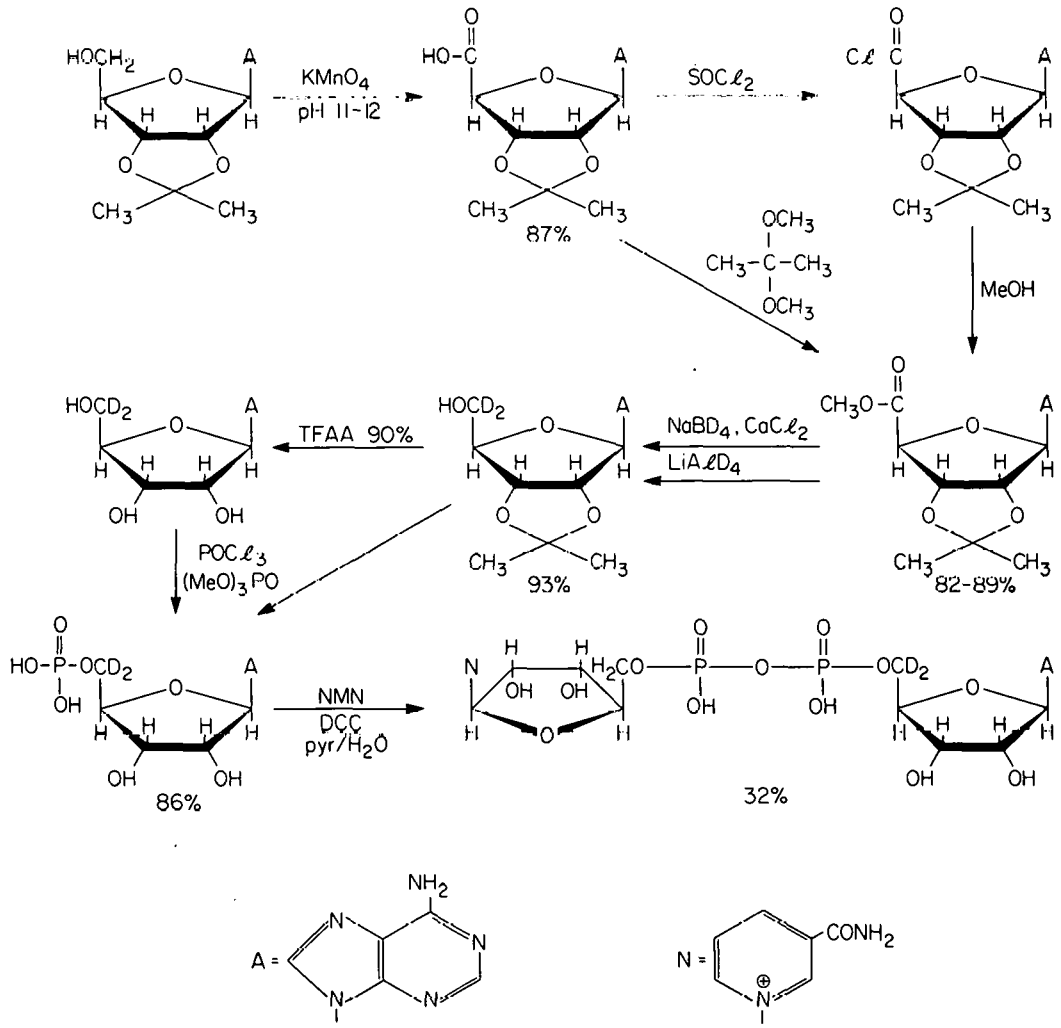


Fig. 14.5. Synthetic steps for preparation of selectively deuterated nicotinamide adenine dinucleotides.

Structural and Conformational Analyses of Biological Molecules

Conformational bonds are the "linchpins" of three-dimensional biological structures. The degree of torsional or rotational flexibility about these bonds, coupled with specific intragroup interactions (H bonding, $\pi - \pi$ interactions, van der Waals effects), determines overall shapes of biological molecules in solution and perhaps more importantly is responsible for specificities of biomolecular interactions, i.e., nucleic acid-enzyme recognition processes. For these reasons, knowledge of conformational properties is imperative. We obtain such information by a variety of spectroscopic methods, with high-resolution NMR having a special utility. This method has been developed and adapted in the biomolecular structure studies program to resolve major structure/conformation questions for nucleic acid constituents (for pertinent examples see Davies, D. B., and S. S. Danyluk, Biochemistry 13, 4417, 1974; Davies, D. B., and S. S. Danyluk, Biochemistry 14, 543, 1975; Kondo, N. S., and S. S. Danyluk, Biochemistry 15, 756, 1976; Lee, C. H., et al., Biochemistry 15, 3627, 1976). Several of the aspects covered in the past year are outlined below.

Time-average conformations of dinucleoside monophosphates. A substantial commitment of our effort in structure/conformation of nucleic acids has been made to the quantitative definition of conformational properties of diribonucleoside monophosphates, XpX, XpY, etc., where X and Y represent various combinations of purine and pyrimidine bases A, U, G, C. Interest centered on these molecules because they are the simplest repeating units of an RNA molecule incorporating all conformational bonds present in the polymer. This study encompassed proton NMR measurements, signal assignments, and spectral analyses for sixteen dimers in aqueous solution. NMR parameters (chemical shifts and spin coupling constants) were used to derive conformational properties and overall conformational structures. In the past year, the second phase of the study dealing with hetero dimers pu-py, py-pu was completed, and the results can be summarized as follows:

1) As with the homo-dimers, the hetero-molecules exhibit a flexible conformational framework in solution, with preference for a limited number of conformers.

2) Base sequence has a profound effect upon overall conformation. Whereas the conformational properties are consistent with the existence of two folded structures (a compact base-stacked right helical form, A, and a more loosely stacked loop structure, B) in equilibrium with one or more extended forms, the percent stacked population and the blend of A and B are determined by base sequence. Thus, purine-pyrimidine dimers show a greater degree of stacking than pyrimidine-purines; moreover, the ratio of right-handed/loop structures is larger in pu-py dimers.

3) Conformational interconnections exist among the conformational bonds in much the same pattern as found for homo-dimers, viz., a χ_{CN} change alters the ribose ring $^3E/^2E$ populations which in turn perturbs the g^+/g^- populations about C3'-O3'.

4) A composite of the homo- and hetero-dimer data reveals that percent stacking (A + B) increases in order py-py < py-pu < pu-py ≃ pu-pu; a similar trend is observed for the ratio A/B. Rather surprisingly, the highest stacked population was observed for a pu-py dimer, GpC.

Publication of the hetero-dimer results (Ezra, F. S., et al., Biochemistry 16, 1977, 1977) brings to completion the phase of our nucleic acid conformational studies dealing with dimer conformations. From this work have emerged fundamental information and new insights into the nature of conformational properties of nucleic acid segments, the effects of sequence thereon, and the relative intramolecular forces governing these structures. A solid basis has been laid for evaluation of the effects of base modifications (e.g., by carcinogens) upon nucleic acid conformation, for extension of the conformational analytical methods to higher oligomers, and for correlation of dimer conformational trends with nearest-neighbor structural properties in tRNA.

Evaluation of glycosyl torsion angles. With the availability of model anhydro-cyclo nucleosides, Figure 14.1, prepared as part of the synthetic program described above, we had the opportunity to develop an unambiguous method for quantitation of glycosidic bond torsion angles, χ_{CN}, in nucleosides and nucleotides. This bond plays a critical role in establishing helical dimensions in RNA and DNA.

The method requires (1) accurate measurement of vicinal ^{13}C-proton couplings across the glycosidic C-N bond (C2-H1' and C6-H1') for bicyclo pyrimidine nucleosides in which rotational and/or torsional flexibility about C-N is foreclosed (Figure 14.1); (2) empirical correlation of vicinal $J_{^{13}CN}$ magnitudes with χ_{CN} values; and (3) utilization of these vicinal correlations to determine χ_{CN} values from measured couplings in nucleosides/tides. Vicinal couplings C2-H1' and C6-H1' were measured from natural abundance ^{13}C spectra for the compounds in Figure 14.1, recorded in the proton undecoupled mode on a Varian XL100 spectrometer. A typical spectral measurement with adequate S/N ratio required accumulation of 40-50,000 free induction decays, representing 24-36 hours of spectrometer time. Following analysis of the spectra, empirical plots of the type shown in Figure 14.6 were constructed of $J_{^{13}C-H}$ versus ϕ_{CN}. These curves can be represented by analytical expressions of the type $J_{C6H1'} = 4.5 \cos^2\phi - 0.3 \cos\phi - 0.4$, and $J_{C2H1'} = 4.9 \cos^2\phi - 2.3 \cos\phi - 0.1$, where ϕ represents the dihedral angle < C6H1' and < C2H1'; the expressions are expected to have a general applicability to pyrimidine nucleosides and nucleotides. (Curves of similar analytical form could in principle be derived for purine derivatives, with couplings C8H1' and C4H1'.)

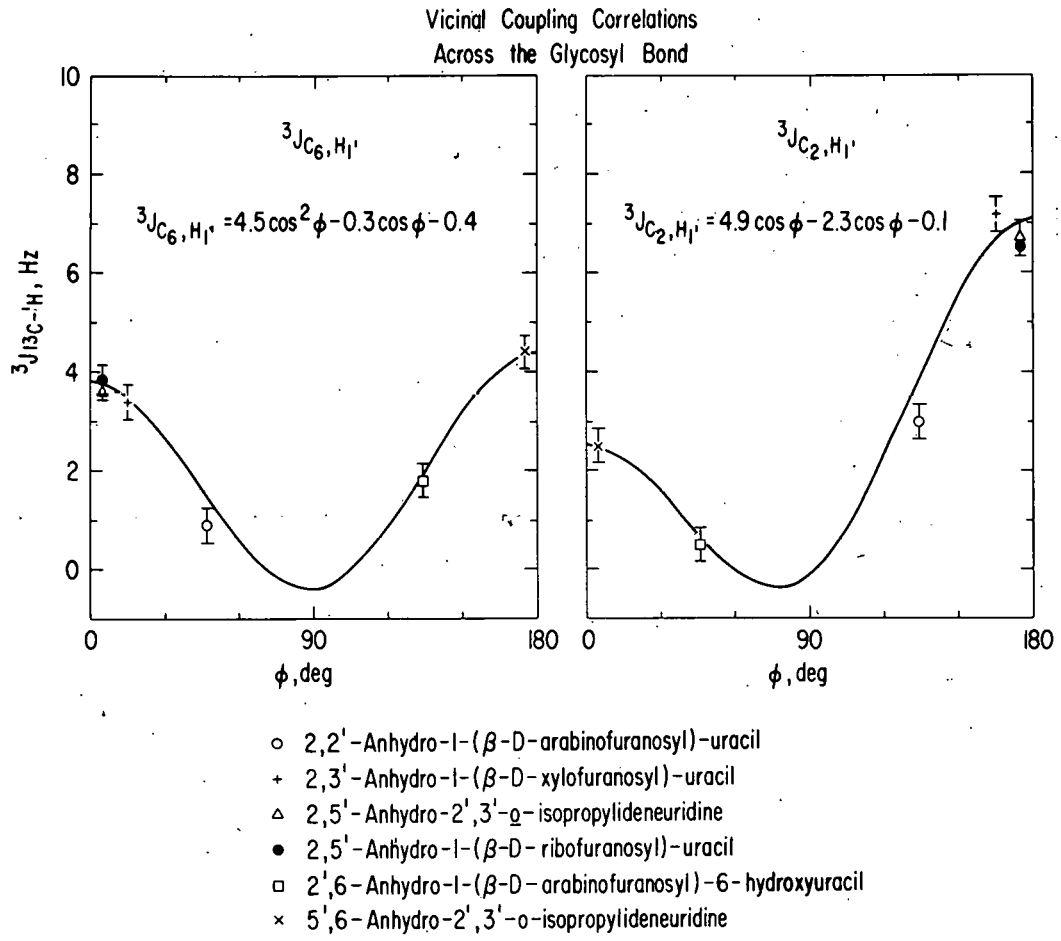


Fig. 14.6. Empirical correlation of ^{13}C-proton coupling constants with glycosidic bond torsion angle of anhydro cyclo uridine derivatives.

With these correlations established, it becomes possible to calculate χ_{CN} values for a wide variety of pyrimidine derivatives from relevant ^{13}C-H1' couplings. A brief listing of results is given in Table 14.1. Several important conclusions follow from these data. In all instances the data were consistent with the presence of <u>one</u> favored conformer as opposed to a blend of <u>syn</u> and <u>anti</u> forms. Furthermore, comparison of χ_{CN} solution values with available crystallographic data showed extraordinary agreement between the two for all compounds. This supports the contention that for pyrimidines, at least, the glycosidic bond is relatively invariant to conformational perturbants. The results of this study were reported in an invited presentation at the Symposium on Biomolecular Structure, Function, and Evolution held in Madras in January, 1978.

Table 14.1. Comparison of Glycosyl Torsion Angles in Crystalline and Solution States

Compound	Solvent	^3J(C2-H1')	^3J(C6-H1')	χ (deg.) Solution	χ^a (deg.) Crystalline
U[b]	D_2O, pD = 7.5	2.3	3.6	30, 70	16.8, 23.8[c]
U[d]	D_2O	2.4	3.7	35, 65	16.8, 23.8[c]
U[e]	D_2O	2.4	-	35, 65	16.8, 23.8[c]
dU[d]	D_2O	2.1	3.7	30, 70	24, 28[c]
5BrU[d]	D_2O	2.4	3.6	35, 65	51[c]
5BrdU[d]	D_2O	1.0	3.7	35, 65 220	47.2[c]
C[d]	D_2O	1.8	3.3	25, 75	18.4[c]
6MeC[f]	DMSO	6 ± 1	-	205, 255	104.6, 106[c]
T[d]	D_2O	2.0	3.9	30, 70	39.1, 39.3[c]
2'-CMP[d]	D_2O	2.4	3.9	35, 65	-
3'-CMP[d]	D_2O	2.3	3.5	30, 70	39.3, 41.8[c]
5'-CMP[d]	D_2O	1.3	3.0	20, 80	40, 47[g]
5'-CMP[b]	D_2O, pD = 0	1.6	3.2	25, 80	40, 47[g]
5'-CMP[b]	D_2O, pD = 6.3	1.5	3.6	25, 80	40, 47[g]
5'-CMP[b]	D_2O, pD = 12	1.8	3.4	25, 80	40, 47[g]
2',3'-CMP[d]	D_2O	4.4	4.0	190, 280	242.9, 254.9

[a] Estimated from vicinal correlation.

[b] This work.

[c] Sundaralingam, M., Conformations of Biological Molecules and Polymers, E. D. Bergman and B. Pullman, eds., Israel Academy of Sciences and Humanities, 1973, p. 417.

[d] Davies, D. B., Stud. Biophys. 55, 29, 1976.

[e] Lemieux, R. U., et al., Can. J. Chem. 50, 773, 1972.

[f] Schweizer, M. P., and G. P. Kreischman, J. Mag. Res. 9, 334, 1973.

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Examination of Configurational and Conformational Effects in Nucleic Acid Derivatives

Synthesis of nucleoside/tide configurational isomers (loc cit) has enabled a study of the interplay between configurational structures and conformational properties in nucleic acids by NMR spectroscopy. These investigations are needed to refine further the empirical correlations derived for couplings and chemical shifts (see previous section), and to assess the impact of base and hydroxyl group position in conformational features such as sugar ring pucker, glycosidic bond orientation, and exocyclic carbinol and phosphate group rotamer preferences. Several facets explored in the past year are outlined below.

A detailed ^1H 220-MGz NMR study of 9-(β-D-xylofuranosyl)adenine 3',5'-cyclic monophosphate (3',5'-xylo-cAMP, I) and 9-(β-D-arabinofuranosyl)adenine 2',5'-cyclic monophosphate (2',5'-ara-cAMP, II) in D_2O solution was carried out with the purpose of establishing the effect of phosphate ring size and site of ring fusion on sugar-ring conformation (MacCoss, M., et al., Carbohydr. Res. 62, 203, 1978). The sugar-ring conformations in I and II were found to be ^3E and ^2E, respectively, while the phosphate rings exist in a chair form. An unusual $^4J_{P,H}$ of 30.8 Hz exists between H5' and phosphorus in II. This latter coupling verifies a similar value found previously in the ara-cytidine analogue of II. An important outcome of this study was the finding that the vicinal P-H coupling-dihedral angle correlation for bonding configurations of type P-O-C-H is strongly influenced by small bond angle changes along the coupling path. Thus the use of vicinal correlations developed for a six-membered cyclic phosphate ring system leads to serious conformational errors when applied to other ring systems.

The first detailed study has been made of the 220-MHz NMR spectra of α-adenosine 3',5'-cyclic monophosphates (III, α-cAMP), α-uridine 3',5'-cyclic monophosphate (IV, α-cUMP), α-cytidine 3',5'-cyclic monophosphate (V, α-cCMP), α-deoxyadenosine 3',5'-cyclic monophosphate (VI, α-cdAMP), α-5,6-dihydrouridine 3',5'-cyclic monophosphate (VII, α-cDHUMP), and β-5,6-hydrouridine 3',5'-cyclic monophosphate (VIII, β-cDHUMP) in D_2O solution (MacCoss, M., et al., J. Am. Chem. Soc. 99, 7495, 1977). Analyses of the spectra of III-V were aided by the use of europium chloride as a shift reagent. A conformational analysis showed the sugar moieties of III-V to exhibit a conformation in the range $_2T^3$ to $^3T_2$ with an unusually high distortion from planarity, in contrast to the β anomers which prefer ^3E to $_4T^3$ and the acyclic mononucleotides which show a $^2E \rightleftharpoons {}^3E$ equilibrium (Figure 14.7). This change in the preferred conformation is

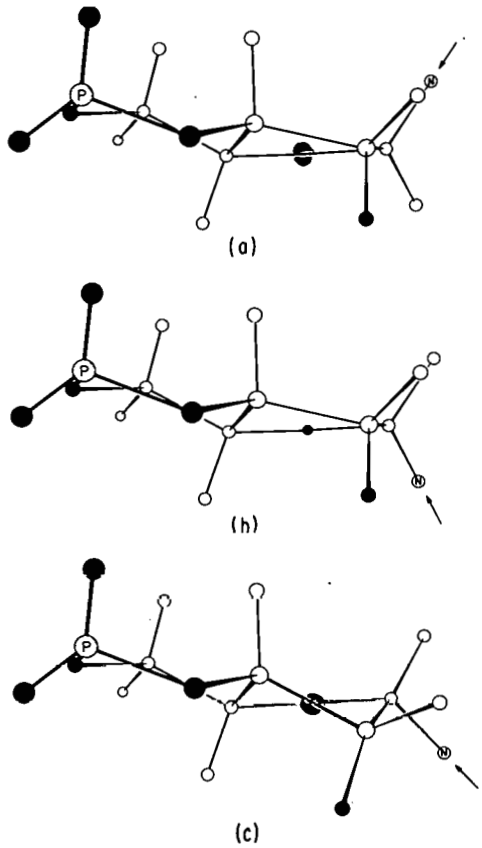

Fig. 14.7. Possible conformations of the ribose and cyclic phosphate rings in nucleoside 3',5'-cyclic monophosphates; (a) β anomers, ^{3}E sugar pucker; (b) α anomer, ^{3}E sugar pucker; (c) α anomer, $_{2}T$ sugar pucker. In all cases, the cyclic phosphate ring is shown in a chair form.

attributed to a repulsive interaction beween the 2'-hydroxyl and the base. Removal of the 2'-hydroxyl group eliminates this interaction and causes a relaxation to a less strained system. This is clearly demonstrated in the sugar ring conformation of VI which exhibits a ^{3}E to $_{4}E$ pucker and a puckering amplitude that is less than in the ribo series. Hydrogenation of the pyrimidine ring of IV and β-cUMP gave the 5,6-dihydro products VII and VIII. VIII exhibits preference for a ^{3}E to $_{4}T^{3}$ ribose ring conformation and in the case of VII, the 2'-hydroxyl-base interaction is markedly reduced owing to the increased flexibility of the aglycon. This results in a relaxation of the sugar ring conformation from the $_{2}T^{3}$ to $^{3}T_{2}$ in III-V back toward the ^{3}E to $_{4}T^{3}$ conformation found in the β anomers. Saturation of the base is not as effective as removal of the 2'-hydroxyl group in relieving the strain in these rigid systems. The phosphate ring is found to be a flattened chair form in all cases.

Theoretical Computations of Biological Structures

The application of sophisticated quantum mechanical methods to biological structure problems is relatively recent and derives its impetus from the work of Pullman (Pullman, B., and A. Saran, Progress in Nucleic Acid Research and Molecular Biology. Ed. W. Cohn. Academic Press, New York, 1976, Vol. 18, p. 215). Theoretical calculations of biological conformations are an important adjunct of spectroscopic measurements, often providing insights into structural features for molecules not accessible to experimental measurement (because of reactivity) or whose synthetic preparation is not feasible.

An approach with particular utility for conformational energy calculations of nucleic acid constitutents is PCILO (perturbated configuration interaction with localized orbitals), originally developed by Malrieu and co-workers (Diner, S., J. P. Malrieu, and M. Gilbert, Theor. Chim. Acta 13, 1, 1969) and applied by Pullman to nucleic acids (Saran, A., H. Berthod, and B. Pullman, Biochim. Biophys. Acta 331, 154, 1973). Essentially, it calculates molecular energies as a function of conformational coordinates, i.e., variations in bond torsion angles for flexible bonding arrangements. Using a computer program adapted for the ANL IBM computer, a comparative study was undertaken of configurational effects on conformational properties of α- and β-anomers of purine and pyrimidine nucleoside 3',5'-cyclic monophosphates and their 2'-arabino epimers. One important outcome of this study was the demonstration that hydrogen bonding between the 2' OH group and polar centers on the base ring is a major determinant of syn-anti preferences of bases in α-nucleoside 3',5'-cyclic monophosphates (Tewari, R., and S. S. Danyluk, Biopolymers 17, 1181, 1978).

This method has now been extended to calculations of conformational energies for selected dinucleoside monophosphates with the objective of supplementing the NMR results and assessing the importance of intramolecular interactions on structure. Using the PCILO method, preferred conformational structures and conformational energy maps were calculated for UpU and ApA. Although the results are preliminary, they indicate that the conformational preference about P-O5' (ω) and P-O3 (ω') bonds in the phosphodiester backbone is signficantly influenced by inclusion of bases and by ribose ring pucker, but not by the precise nature of the base (A or U). Similarly, the conformational preference about C3'-O3' (ϕ') is affected by ribose pucker but not by base type. An especially surprising result was the global minimum location in the ω,ω' conformational energy maps at ω,ω' values of 120° and 300° respectively for both UpU and ApA. These "favored" structures differ markedly from right-handed helical forms (structure A vide infra, with ω = 300, ω' = 300) predicted by previous calculations involving ribose-phosphate backbone segments alone; nor do they agree with NMR solution conformations (see above dimer section). On the other hand, the calculated UpU

conformation (ω/ω', 120°/300°) is stabilized by intramolecular hydrogen bonding between O3'-H of -pU and O2 of the uracil in the 5' terminal position (Up-). A similar interaction occurs between O3'-H (-pA) and N3 (Ap-) to stabilize an ω/ω', 120°/300° structure in ApA. An interaction of this type may be responsible for the specificity of recognition of 3'-terminal ends of ribonucleic acids by exonucleases. However, it should be noted that hydrogen bonding with solvent and/or changes in ionization state of the phosphate group may alter the base stacking properties of these dimers in solution.

CRYSTALLOGRAPHIC AND CHEMICAL STUDIES OF THE STRUCTURE OF IMMUNOGLOBULINS

M. Schiffer, F. A. Westholm, and N. Panagiotopoulos

Immunoglobulins are part of the body's defense mechanisms against invading foreign agents such as pollutants and pathogens. Their function is to recognize and bind foreign agents and to trigger the body's immune response. To understand how the immunoglobulins function, and what accounts for their high degree of specificity, X-ray diffraction studies of single crystals of Bence-Jones and myeloma proteins are carried out. X-ray diffraction is the only technique by which the detailed three-dimensional structure, i.e., the positions of all the atoms within the molecule, can be determined.

IgG immunoglobulins in myelomatous and normal sera consist of two light chains (mol wt 23,000) and two heavy chainns (mol wt 50,000). Bence-Jones proteins represent excreted light chains and their presence in urine is pathognomic of multiple myeloma. The light chains belong in two main classes (λ and κ), based on their amino acid sequence. The proportion of κ and λ light chains is 3 to 2 in human serum.

The study of the Mcg λ-type Bence-Jones dimer is being completed by the crystallographic refinement of its atomic positions. Structure determination has started on the first complete κ-type Bence-Jones protein (Fin) ever crystallized. Further attempts are being made to prepare and crystallize other immunoglobulin fragments, with special emphasis on κ-type Bence-Jones proteins and fragments derived from IgM proteins. Characterization of crystals of ornithine aminotransferase is also in progress.

Crystallographic Refinement of the Mcg λ-Type Dimer

The principal objective of the refinement is to obtain the most accurate coordinates for the more than 3,000 nonhydrogen atoms permitted by X-ray diffraction data for this Bence-Jones dimer obtained from patient Mcg. Accurate 3-D structures are essential for intercomparison of structural features, e.g., immunoglobulin fold, with other immunoglobulin fragments. The refinement will also facilitate the use of the Mcg protein as a search structure to determine structures for other immunoglobulin fragments by molecular replacement methods.

The refinement is the last phase of the structural study. The method of "constrained crystallographic refinement" is used. The resulting molecular model has been compared with the calculated electron density map, and manual changes were made with the aid of an interactive computer graphic system GRIP at the University of North Carolina. The refinement is progressing well; the crystallographic R factor decreased from 43 to 31%. The refinement improved the quality of the electron density map so that electron density for ∿ 140 atoms that were not seen in the map calculated with isomorphous phases became observable.

Crystallization and Characterization of Bence-Jones Proteins in Solution

We are collaborating with Dr. A. Solomon, University of Tennessee, who has a large inventory of human Bence-Jones proteins and myeloma proteins which he has carefully characterized both clinically and immunochemically. His clinical and immunochemical data will be correlated with the structural information obtained from our X-ray diffraction studies.

We have now characterized fifteen Bence-Jones proteins and purified them by chromatography on Sephadex G-200 at pH 8. Our crystallization attempts were successful for seven of the proteins, and we have started structure determination on the κ-type Bence-Jones protein Fin. Crystals of two λ-type Bence-Jones proteins, Cle and Ree, are also approaching the size where they will be useful for X-ray diffraction measurements.

Our characterization of the Bence-Jones proteins by chromatography on Sephadex G-200 at pH 8, and by SDS gel electrophoresis, in the presence and absence of mercaptoethanol, also furthered our understanding of the different forms of Bence-Jones proteins that can be found in different patients. Whereas the predominant species in excreted λ-type proteins is the disulfide bond dimer, the state of the excreted κ-type proteins varies.

Monomers and disulfide bonded dimers occur in different proportions depending on the patient. At neutral pH, some of the monomers associate to form noncovalently linked dimers while others remain as monomers. Based on the association properties of the monomers, two types of dimers can be postulated. In the first, exemplified by the Mcg protein, the monomers are held together in a compact form by noncovalent interactions, or are bound by these and also by a disulfide bond. The second type of dimer is held together mainly by the disulfide bond and probably has a less compact structure. The antigenic properties of these two postulated forms of dimers would therefore be expected to be different.

Structure Determination of the κ-Type Bence-Jones Protein, Fin

A complete human κ-type Bence-Jones protein (Fin) has been isolated and crystallized. Immunochemical and physicochemical characterization of protein Fin indicates that it is of the κ-chain subgroup κII, and that it consists of two noncovalently bound intact monomers each having a molecular weight of ∿ 23,000 daltons. Crystals of Bence-Jones protein Fin obtained from ammonium sulfate solutions (Figure 14.8) have the orthorhombic space group $\underline{P}2_12_12_1$ with cell dimensions $\underline{a}$ = 132.0 Å, $\underline{b}$ = 93.3 Å, and $\underline{c}$ = 42.3 Å. The asymmetric unit consists of a dimer of molecular weight ∿ 46,000 daltons. The diffraction pattern extends to 2.7 Å spacing. Diffraction data to 4 Å resolution have been collected for the native crystal. Our initial attempt to determine the structure of protein Fin will utilize the molecular replacement method using as search structures the λ-chain dimer Mcg and the variable fragment dimer of the κ-chain Rei.

Solving the structure of Bence-Jones protein Fin is of special interest because this particular protein is a complete κ-chain consisting of two noncovalently bound monomers and because it is a light chain of a κ-chain subgroup, κII, that is characterized by proteins possessing an insertion of up to six amino acid residues between positions 29 and 30 in the first hypervariable region.

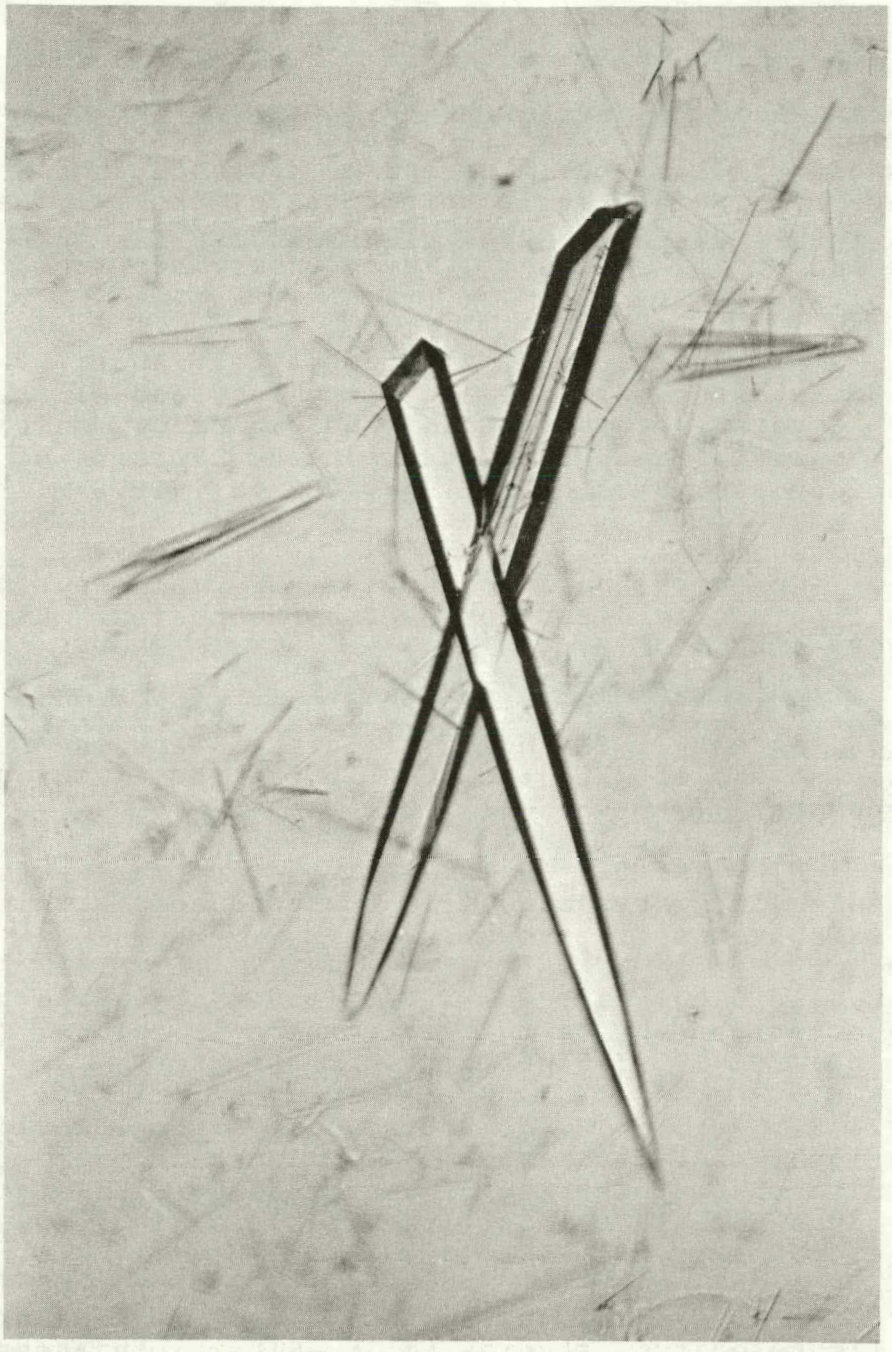

Fig. 14.8. Crystals of a κ Bence-Jones protein Fin obtained at 1.7 M ammonium sulfate (length is ~ 0.45 mm).

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15. CIRCADIAN CYBERNETICS AND CHRONOTYPIC ORGANISMIC SENSITIVITY TO ENVIRONMENTAL FACTORS

Charles F. Ehret (Senior Biologist)
Kenneth F. Groh (Scientific Assistant)
John C. Meinert (Scientific Assistant)
George Svihla (Biologist)

During the past year, studies on circadian regulation at the organismic level in the rat have focused upon the influence of food, drugs, and toxic metals upon the maintenance of circadian synchrony, the induction of phase shifts in minimum time, and the induction of circadian dyschronism, i.e., the absence of a strong daily rhythm where one normally is found.

First, in programmed feeding experiments, the challenges of phase advances or of phase delays of 7 hours (simulating a flight to Vienna and return) are best met by rats fed high protein breakfasts and low protein suppers; the inverse pattern (high carbohydrate breakfasts, high protein suppers) most often leads to circadian dyschronism ("jet lag") for 5 or more days.

Second, the influence of the circadian phase time-of-administration of L-dihydroxyphenylalanine (L-DOPA) upon the circadian rhythm of deep body temperature was also studied. Consumption of the drug before the daily thermal acrophase caused circadian dyschronism; consumption of the drug after the acrophase permitted maintenance of synchrony (Figure 15.1). The chronotypically correct (orthochronal) phase in which to give L-DOPA coincides with the time at which synthetic activity in the catecholamine ("awake") pathway normally (chronotypically) diminishes (Figure 15.2). To reduce the deleterious side effects and maximize the beneficial effects of L-DOPA, orthochronal therapy is indicated.

Third, a pilot study to determine the usefulness of induction of irregularities in circadian metabolic events as indicators of the toxicity of metals associated with energy storage has given very promising results. Lithium, lead, cadmium, and mercury administered in the drinking water of the rat each cause circadian dyschronism at remarkably low dose levels (5-1000 ppm). Thus, this test is at least as sensitive as the "behaviorally" oriented toxicity tests now in use elsewhere. Our observations also indicate that the essential metabolic pathways for circadian clock chemistry and for neurochemistry appear to be closely coupled. Therefore, our demonstration of metal toxicity at low lev-

els, using disruptions of circadian metabolic function as end points, may have profound significance in the development of short-term testing systems that are relevant to long-term behavioral and neurophysiological toxicities and dysfunctions.

Details of these three studies are described below. We propose to exploit the advances in each of the three areas by parallel molecular studies at the cellular level.

A DIET PLAN FOR TRANSMERIDIANAL TRAVELERS AND SHIFT WORKERS

C. F. Ehret, K. R. Groh, and J. C. Meinert

We have developed a diet plan for humans to assure swift adjustments to phase shifts. The diet is based upon (1) the chronobiotic action of the methylated xanthines theophylline (in tea) and caffeine (in coffee); (2) the action of food as a zeitgeber in feed-starve (FS) cycles, presumably efficacious because of glycogen depletion during a prolonged starvation (S) phase; and (3) the tendency of a high protein meal to favor synthesis of the catecholamines norepinephrine and epinephrine, and of a high carbohydrate meal to favor the synthesis of serotonin. Norepinephrine and epinephrine should be relatively high during the active phase of the animal, and serotonin relatively high during the inactive phase.

Based on the above, a procedure to help humans adjust more readily to the phase shifts associated with transoceanic travel is as follows, using an eastbound flight with a phase advance of 7 hours as a model: Remain on local time before the shift, but modify eating habits for 4 days beforehand. Assuming a Wednesday evening departure, then Sunday and Tuesday are "Feast Days," and Monday and Wednesdays are "Fast Days." "Feast" means to eat fairly large meals in order to build up large glycogen reserves. "Fast" means to eat lightly in order to encourage depletion of glycogen reserves; fructose or fruit-juice supplements are recommended to avoid becoming overly famished and hypoglycemic. Thus, the pattern is Feast-Fast-Feast-Fast. On Wednesday evening, between 1900 and 2300 local time, consume three to five cups of black coffee (no sugar, no cream) or strong tea to deplete glycogen reserves further, and also to induce the desired phase advance. (Earlier on Wednesday, and especially at breakfast, avoid caffeinated beverages entirely, as they would encourage the undesired phase delay.) Retire to rest on the plane as early as conditions permit on Wednesday night ("old time"), and avoid snacks, movies, bright lights, or lively discussions until breakfast. At about 0730 Thursday morning, destination breakfast time, move about, and break the fast with a high protein breakfast, usually eaten on the aircraft just before landing. This replenishes the energy reserves of what should be fairly well-depleted livers, in approximate synchrony with the breakfast of the destination populace. Lunch and supper should also be heavy, and on destination time, that first day. Retire early, and continue in phase with "new" time. To date we have collected only subjective results on the outcome, but trials with human subjects have apparently been highly successful. These guidelines should also be useful for shift workers, who frequently have to adjust to time changes.

The results of programmed feeding tests with rats support the expectations of the travelers' diet for humans presented

above. Rapid phase shifts in either direction were assured or encouraged by high-protein breakfasts and high carbohydrate suppers; sluggish phase shifts and/or total dyschronism for as long as 5 days were assured by low-protein breakfasts and high-protein suppers.

THE CHRONOPHARMACOLOGY OF L-DOPA: IMPLICATIONS FOR ORTHOCHRONAL THERAPY IN THE PREVENTION OF CIRCADIAN DYSCHRONISM

C. F. Ehret, J. C. Meinert, and K. R. Groh

The influence of the circadian phase time-of-administration of L-DOPA upon the circadian rhythm of whole animals, as judged by measures of core temperature, was studied in 36 male rats. Programmed feeding and watering protocols were entirely automated. Core temperatures were continuously monitored by implant telemetry, and were statistically analyzed to enable accurate determination of select circadian variables (time of acrophase, period length, phase shift, and degree of dyschronism) during "free run." Following a minimum of 8 days entrainment (see legend of Figure 15.1 for entrainment procedure), animals were placed in "free run" (continuous dim light, and food ad libitum), during which time they were given a diet containing 30 mg L-DOPA per g chow either preceding or following the ongoing thermal acrophase. If L-DOPA was given an animal preceding the acrophase, then that animal received the control (DOPA-free) diet during the 12-hour time interval that food was available after the thermal acrophase, and vice versa. The experiments showed a strong correlation between circadian dyschronism and the consumption of L-DOPA prior to the acrophase (Figure 15.1). On the other hand, animals fed L-DOPA either after the acrophase or ad libitum showed little or no dyschronism.

In another series, we tested the animals' abilities to respond to food-induced phase shifts of 90, 180, and 270 degrees and found that orthochronal administration of L-DOPA, that is, administration at the most advantageous time in the circadian cycle, permits a more rapid and complete phase shift than heterochronal administration. In some instances, however, when heterochronal administration did not result in circadian dyschronism, it was seen that the animal had phase shifted his circadian rhythm to achieve a harmonious relationship between the application of the chronobiotically active L-DOPA and the phase of the oscillation. The chronotypically correct (orthochronal) phase in which to give L-DOPA with least disturbance to the circadian regulatory system coincides with the time at which synthetic activity in the catecholamine ("awake") pathway normally (chronotypically) diminishes (Figure 15.2). We conclude that orthochronal therapy is indicated in order to minimize the deleterious side effects and maximize the beneficial effects of L-DOPA.

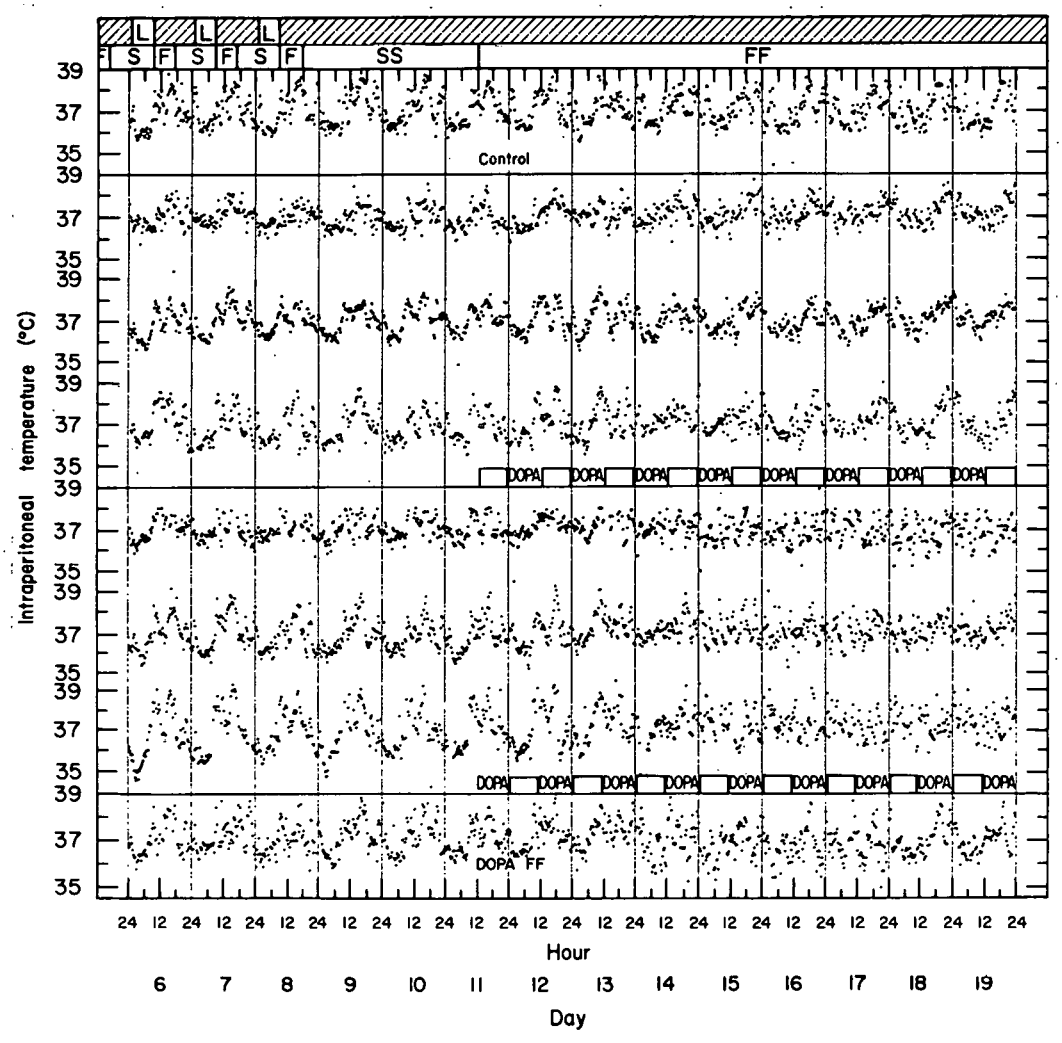

Fig. 15.1. Telemetry tracings of intraperitoneal temperatures measured every 20 minutes over 14 days are shown for eight rats. During days 1 through 8 (1-5 not shown), the animals were entrained by daily programs of feeding (FS 8:16; i.e., food available for 8 hours, none for 16 hours) and illumination (DL 16:8; dim light for 16 hours, light for 8 hours). Following 2 days starvation (SS) in continuous dim light (DD), the animals were fed ad libitum (FF) for the next 8.5 days (see top protocol bars). During days 11-19, the top animal was maintained as a control receiving only the standard 24% protein control diet. Animals 2, 3, and 4 from the top received a diet containing 30 mg L-DOPA/g chow (other dietary constituents remained the same) from midnight to noon (see protocol DOPA ▭). Animals 5, 6, and 7 received an inverse of this protocol; L-DOPA was present in the diet from noon to midnight each day (see protocol ▭ DOPA). In both cases, the animals received the control diet for the 12-hour interval of each day when DOPA was not administered. The bottom animal received the L-DOPA diet ad libitum (DOPA FF).

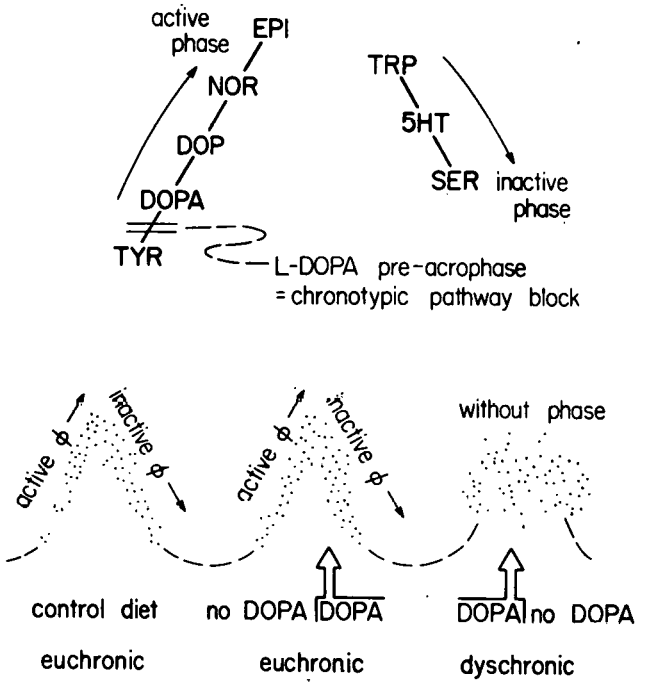

Fig. 15.2. The consequences of feeding L-DOPA during early active and late active phases of the circadian cycle are shown graphically. The administration of L-DOPA after the thermal acrophase (bottom middle) results in the maintenance of a good circadian rhythm (euchronic condition) as is conventionally seen in control animals (bottom left). However, when L-DOPA is given before the thermal acrophase, a dyschronic (nearly phaseless) condition results (bottom right). The figure's upper portion graphically displays the catecholamine pathway (to the peak of the active phase) from tyrosine (TYR) to dihydroxyphenylalanine (DOPA) to dopamine (DOP) to norepinephrine (NOR) to epinephrine (EPI); and the indoleamine pathway (inactive phase) from tryptophan (TRP) to 5-hydroxytryptophan (5HT) to serotonin (SER). When DOPA enters the metabolic pathway during the early active phase, the catecholamine pathway is shut down (chronotypic pathway block) resulting in circadian dyschronism.

DYSCHRONOGENESIS AND THE CIRCADIAN TOXICOLOGY OF METALS IN THE MAMMAL

C. F. Ehret, J. C. Meinert, K. R. Groh, and G. Svihla

Risk estimates for neurophysiological and behavioral effects of toxic metals have been difficult to make for a number of reasons, but are of particular importance in that they are more germane to the ultimate assignments of responsible threshold limit values and tolerance dosage guidelines for the protection of the general population than are any other toxicological measures. Because the essential metabolic pathways for circadian clock chemistry and for neurochemistry are so closely coupled, circadian toxicological measures have profound and fundamental relevance to long-term behavioral and neurophysiological toxicities and dysfunctions.

Using a mammalian system, we studied the influence of lithium, lead, cadmium, and mercury upon the circadian rhythm of core temperatures of 36 rats, continuously monitored by implant telemetry. Following an initial interval of entrainment by means of programmed illumination (DL 16:8) and feeding (FS 8:16), half the animals were placed in free run, in continuous dim light and with food continually available (DDFF). During this free run period they were also chronically exposed to low concentrations of metals in the drinking water (Li, as lithium chloride, 200 ppm; Pb, as lead acetate, 50 ppm; Cd, as cadmium chloride, 10 ppm; and Hg, as mercuric chloride, 10 ppm). In each case, within 3 to 7 days following exposure to the metal in the drinking water a significant loss in circadian synchrony was observed. In another series, the remaining animals were placed in constant dim light without food (DDSS), but again with low levels of lithium, lead, cadmium, and mercury in the drinking water. In this group very little water was consumed, and there was little or no indication of dyschronism with the possible exception of the rats exposed to lead; however, as soon as food was made available (DDFF), normal consumption of water resumed, and the animals became dyschronic.

The induction of circadian dyschronism ("dyschronogenesis"), detectable within 1 week at low levels of administration of toxic metals, makes the test at least as sensitive as any current behaviorally oriented toxicity tests. These observations should be significant in the development and application of short-term testing systems that are relevant to long-term behavioral and neurophysiological toxicities and dysfunctions, including mental dysfunction. The validity of a basic new toxicological index for neuropathogenesis, the "dyschronogenic potency index," will be examined by correlating circadian dysfunction with molecular and neurochemical disturbances in catecholamine and indoleamine metabolism in the rat brain and at the cellular level. Alteration of the circadian chronotype [the temporal phenotype of the organism

that biochemically relates enzyme levels (when most or least active), and ultimately cellular and organismic response to the environment] is known to have far-ranging effects, from physiological and performance failures, of the sort common to shift-workers and transmeridianal travelers, to general malaise and life shortening.

PERTINENT PAPERS, JANUARY 1977 THROUGH JULY 1978

Dobra, K. W., and C. F. Ehret. Circadian regulation of glycogen, tyrosine, aminotransferase, and several respiratory parameters in solid agar cultures of Tetrahymena pyriformis. Proceedings of the XII International Conference of the International Society for Chronobiology, Washington, DC, August 10-13, 1975. Publishing House "Il Ponte," Milan, Italy, 1977, pp. 589-594.

Ehret, C. F., and K. W. Dobra. The infradian eukaryotic cell: A circadian energy-reserve escapement. Proceedings of the XII International Conference of the International Society for Chonobiology, Washington, DC, August 10-13, 1975. Publishing House "Il Ponte," Milan, Italy, 1977, pp. 563-570.

Ehret, C. F., and K. W. Dobra. The oncogenic implications of chronobiotics in the synchronization of mammalian circadian rhythms: Barbiturates and methylated xanthines. Proceedings of the Third International Symposium on the Detection and Prevention of Cancer. Ed. H. E. Nieburgs. Marcel Dekker, New York, 1977, pp. 1101-1114.

Ehret, C. F., K. R. Groh, and J. C. Meinert. Circadian dyschronism and chronotypic ecophilia as factors in aging and longevity. Aging and Biological Rhythms. Eds. H. Samis and S. Capobianco. Plenum Press, New York (in press).

Ehret, C. F., J. C. Meinert, and K. R. Groh. The orthochronal pharmacology of L-DOPA: Implications for orthochronal therapy in the prevention of circadian dyschronism. Proceedings of the International Symposium on Clinical Chronopharmacology (in press).

Ehret, C. F., J. C. Meinert, K. R. Groh, and G. A. Antipa. Circadian regulation: Growth kinetics of the infradian cell. Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells. Eds. B. Drewinko and R. M. Humphrey. Williams and Wilkins Co., Baltimore, 1977, pp. 49-76.

Sargent, M. L. (Rapporteur), I. E. Ashkenazi, E. M. Bradbury, V. G. Bruce, C. F. Ehret, J. F. Feldman, M. W. Karakashian, R. J. Konopka, M. Mergenhagen, G. A. Schütz, H. G. Schweiger, and T. E. A. Vanden Driessche. The Role of Genes and Their Expression: Group Report. Report of the Dahlem Workshop on the Molecular Basis of Circadian Rhythms, Berlin, Germany, November 3-7, 1975. Eds. J. W. Hastings and H. G. Schweiger. Abakon Verlagsgesellschaft, Germany, 1976, pp. 295-310.

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16. SUPPORT FACILITIES

COMPUTER SUPPORT FACILITIES

Frank S. Williamson, Jeanne A. Blomquist, and Carol A. Fox

Computer support is centered on the Remote Access Data Station (RADS), which is equipped with a 1000 lpm printer, 1000 cpm reader, and a 300 cps paper tape reader with 500-foot spools. The RADS is located in a data preparation room with four 029 key punches (two of which interpret), a storage vault for archival magnetic tapes, card files, and a 30 cps interactive terminal principally used for job inquiry and routing. An adjacent room provides work space for users, with a documentation library and a consultant's office, plus file storage for programs and their documentations. sp

Approximately 50 users in the Division continue to depend on these facilities and the consultation on job management, programming, and data management that we provide.

Highlights of activities in 1977 include:

1) A flexible data base was established for G. A. Sacher for pathology and risk assessment, using our own BIOFILE-2 data management system. Records from a data acquisition system are stored by key (species/ animal identification/date). With this system, selections of time-dependent data may be made for statistical analysis, modeling, or display.

2) A foundation has been laid for several data acquisition systems using LSI-11 microcomputers. Programming is in PDP-11 Assembler Language, using structural programming macros and many service routines already developed for the JANUS PDP-11/20 Minos System.

At present, program development and debugging will be done on the JANUS PDP-11/20 so that the LSI-11 systems can be dedicated entirely to their production tasks and need have only the absolute minimum hardware configurations. We plan to acquire a separate development system in the future.

An operating system for these LSI-11's has been devised. It supports a single "event" clock, which is replenished from a

queue of dated and timed events, and an arbitrary number of totally independent interval timers that are fed from queues of timer blocks. This concept provides extreme flexibility. For example, an interval timer may repeat its cycle a programmed number of times, then be replaced by a different interval (and number of repeats), then another, and so on back again to the first. Changes between interval timers can be programmed using the "event" queue to implement these at predetermined dates and times. Experimental parameters are drawn from similarly organized parameter block queues. Data are stored in a first-in/first-out queue as acquired, and are removed by an output routine as the output device can handle them. Thus, a considerable amount of data can be stored while the printer is occupied with command dialogue.

In support of this plan, an informal course on PDP-11 assembler language with structured programming was given.

ELECTRON MICROSCOPE CENTER

Thomas M. Seed, G. Theodore Chubb, Rosemarie L. Devine, Margaret M. Sanderson, and Betty J. Wright

The facility has approximately 2,600 square feet of working laboratory space, and includes two fully equipped photographic darkrooms, sectioning and autoradiographic facilities, six microscope cubicles, and five transmission electron microscopes and one Cambridge scanning electron microscope equipped with an X-ray energy dispersive analytical system. Ancillary specimen preparative equipment includes vacuum evaporators, freeze-drying and freeze-etching equipment, ultramicrotomes, and assorted photographic and light microscopic equipment.

The Electron Microscope Center and its personnel provide specialized electron microscope services to both the Division and the Laboratory as a whole. G. T. Chubb continues to maintain EM-related equipment and to provide needed technical assistance to all users of the Center. R. L. Devine, M. M. Sanderson, and B. J. Wright assist staff members in the preparation and examination of various biological preparations. Their contributions are recognized by their coauthorship of several of the sections in this report.

During 1977 there were some 25 researchers who made active use of the available facilities. They included 8 regular staff members, 1 postdoctoral appointee, 2 graduate students, and 6 visiting scientists. The remainder of the users were from other Argonne divisions, including Radiological and Environmental

Research, Environmental Impact Studies, Chemistry, Physics, Solid State Science, and the Center for Educational Affairs.

LABORATORY ANIMAL FACILITY

Thomas E. Fritz, Patricia C. Brennan, William G. Keenan, Calvin M. Poole, Richard C. Simkins, and David V. Tolle

Laboratory animals have always played an important role in the research of the Division. Since long-term and lifetime studies have predominated, the Division has had a policy of maintaining the highest possible standards for its physical plant, equipment, and animal care programs. The Facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

The extensive physical plant of the animal facilities includes provisions for holding all species of laboratory animals under controlled conditions of temperature, humidity, and lighting. More than forty rooms are available for studies of the smaller species. These have a potential capacity of more than 75,000 mice, or smaller numbers of larger species and those requiring special housing arrangements. There are also six dog kennels to accommodate approximately 750 dogs housed in runs that consist of heated indoor compartments and outdoor exercise areas.

In addition, there are numerous supporting facilities for the husbandry and clinical monitoring of the resident animals. The supporting facilities and responsible personnel include:

1) automated cage and bottle washing and filling machinery (Animal Care Specialists);

2) steam and gas autoclaves for sterilization of equipment, instruments, and media;

3) diagnostic X-ray facilities and darkrooms (C. M. Poole, W. G. Keenan);

4) clinical pathology laboratory (D. V. Tolle, R. C. Simkins);

5) hematology laboratory (D. V. Tolle);

6) diagnostic microbiology laboratory (R. C. Simkins, P. C. Brennan);

7) necropsy laboratory (T. E. Fritz);

8) surgical suite with inhalation anesthesiology equipment (C. M. Poole, W. G. Keenan); and

9) gnotobiotic isolators for germ-free technology (L. O. Bibbs).

An important aspect of the success of any animal research program is the health of its animal population. To assure a supply of acceptable high quality animals, the Facility has concentrated on breeding its own healthy, disease-free animals. Most of the rodents, particularly mice, and all dogs are bred in the Facility. The rodent breeding is managed by LeRoy O. Bibbs and Jane M. Angerman. The beagle breeding is supervised by Calvin M. Poole and William G. Keenan, who are also responsible for the clinical and surgical care of the dogs. The beagle colony has been a closed colony for more than 18 years, and extensive computerized records are maintained on all aspects of the colony, including reproduction, genetics, hematology, pathology, and disease incidence.

The need for continued improvement and better facilities to hold and monitor animals in long-term studies has led to an active and continuing program in renovation of existing space and construction of new space. Except for installation of sterilizers and related equipment, two new "barrier facilities" for secure containment and isolation of rodents were completed during the past year. These include one renovated facility (E-wing), consisting of 26 rooms for holding experimental animals, and a new facility (QA-wing), consisting of three rooms for breeding of pathogen-free (disease-free) rodents.

During the past year plans for renovating the kennels were approved and funded. These renovations will provide new run dividers, gutters, doors, and gates. The design of the new dividers and gates will enable the use of automated cleaning equipment and will result in more efficient cleaning procedures and better sanitation.

As important as the equipment and physical plant are the staff available to manage, monitor, treat, and evaluate the animals. The personnel provide a complete range of services to users of the experimental animals. Care of the animals is performed by a group of Animal Care Specialists, listed below, under the supervision of Durward D. Banister and William H. Hart.

The Animal Care Specialists during 1977 were the following:

Earl R. Allen
Susan L. Bromberek
Roberta R. Buller
Mose Burrell
Carl C. Colegrove[1]
Claude C. Colegrove[1]
Lucille E. Daley
Leo C. Farcus[1]
Charles J. Fowler
Bonnie J. Gilbert
Carrey R. Herringer
Edward W. Jackson[1]
James Johns, Jr.
James L. Johns
Janine M. Johns
William G. McDade, Jr.[1]
Ann L. Mize
Kenneth R. Muller
Cathleen L. Nelson
William O. Robinette
Bernard A. Royer
Richard M. Santarelli
Dianna M. Snapp
Leon L. Stewart
Diane M. Thomas
Rudolph H. Tiedt
Joseph N. Wilson

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[1]Group Leader.

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17. EDUCATIONAL ACTIVITIES

POSTGRADUATE TRAINING

During 1977, a total of 33 postdoctoral appointees and research associates contributed to the research programs of the Division. Eight of these were new appointments in 1977, seven less than the number who finished their assignments during the year.

The temporary appointees, their schools, and the staff members with whom they were affiliated were as follows:

N. Leigh Anderson[1]	Cambridge University, Cambridge, England	S. S. Danyluk
Erik Boye	Norwegian Radium Hospital, Oslo, Norway	R. E. Krisch
Charles D. Brown[2]	University of Illinois Medical Center	D. Grahn
David A. Crouse	University of Iowa	J. F. Thomson
Diana Dixon-Davis[2]	University of California, Berkeley	D. Grahn/ L. J. Hoover (EES)
Jesse J. Edwards	University of Alabama, Birmingham	N. G. Anderson
Fouad S. Ezra	University of Rochester	S. S. Danyluk
Michael N. Gould	University of Wisconsin, Madison	M. M. Elkind
Raymond Guilmette	New York University	A. Lindenbaum
Wayne R. Hanson	University of Iowa	C. A. Reilly
Bruce S. Hass[3]	Texas A & M University	R. B. Webb
Gunnard Jacobson[1]	University of Chicago	C. A. Reilly
George R. Lankas	University of Cincinnati	M. M. Elkind
Chung Hee Ryu Lee[1]	University of Illinois, Urbana	D. Grahn
Karin A. Mede	University of Illinois Medical Center	P. D. Klein
Joel R. Mitchen[1]	Life Sciences, Incorporated, St. Petersburg	C. A. Reilly
Chester N. Newman	Indiana University Medical Center, Indianapolis	H. E. Kubitschek

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[1]Research Associate.

[2]Research Associate, now Scientific Assistant.

[3]Research Associate, now Assistant Microbiologist.

Frank Q. Ngo[1]	Wayne State University	M. M. Elkind
Hsien-Chi Niu	University of Missouri, Kansas City	P. D. Klein
Nicolas Panagiotopoulos	University of Pittsburgh	M. Schiffer
Meyrick Peak[1]	Rhodes University, South Africa	H. E. Kubitschek
Eung K. Ryu	Michigan Cancer Foundation Detroit	M. MacCoss
Bobby Scott	University of Illinois, Urbana	J. F. Thomson
Surendra T. Shenoy	University of California, Davis	C. Peraino
Elsie M. Sorensen	University of Texas, Austin	A. Lindenbaum
Frans Stellaard[1]	University of Technology, The Netherlands	P. D. Klein
Fred J. Stevens	Michigan State University, East Lansing	M. Schiffer
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
Diane L. Zeger	University of Illinois, Urbana	T. E. Fritz

In addition, there were eleven 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 1977, their schools, and their staff sponsors were as follows:

Jay B. Best	Colorado State University	G. A. Sacher
Richard Bockrath	Indiana University School of Medicine, Indianapolis	H. E. Kubitschek
Frederic Giere	Lake Forest College	N. G. Anderson
Ellen H. Lau	College of DuPage	C. Peraino
Faiza M. Mariy	Ain Shams University, Egypt	T. M. Seed
Eugene W. McArdle	Northeastern Illinois University	C. F. Ehret
William Millington	Marquette University	J. Shen-Miller
Daniel G. Oldfield	DePaul University	C. A. Reilly

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[1]Research Associate.

John F. Schneider	University of Chicago	P. D. Klein
Thomas A. Victor	Northwestern University	S. S. Danyluk
Ruth L. Willey	University of Illinois, Chicago Circle	R. J. M. Fry

SUMMER RESEARCH INSTITUTE IN CELL BIOLOGY

Ten students from ten different universities were enrolled in this graduate level program offered jointly by the Division of Biological and Medical Research and the Radiological and Environmental Research Division in cooperation with the Argonne Center for Educational Affairs during the Summer of 1977. Dr. Mortimer M. Elkind of the Division of Biological and Medical Research served as organizer and coordinator, with the assistance of S. Bechtold. The program, which ran for 12 weeks, featured a lecture series covering a variety of topics, including the biochemistry of DNA replication and repair, enzymology, electron microscopy, immunology, microbiology, molecular structure, radiobiology, and virology. The lectures were given by Drs. N. G. Anderson, N. L. Anderson, E. Chan, T. E. Fritz, A. Han, D. A. Haugen, C. K. Lee, M. MacCoss, T. Matsushita, Y. E. Rahman, C. A. Reilly, and T. M. Seed of the Division of Biological and Medical Research, and Dr. E. L. Lloyd of the Radiological and Environmental Research Division.

The lectures were supplemented by informal discussions and visits to laboratories as appropriate. Each student spent the remainder of his time working in a laboratory of a staff member.

The students, their schools, and their staff sponsors were as follows:

Stephen Brandt	Emory University	T. E. Fritz
Charles Daniels	Dalhousie University, Nova Scotia	Y. E. Rahman
Helen L. Drwinga	University of Texas	A. Han
Edward F. Duhr	University of Wyoming	M. MacCoss
James I. Mullins	University of Minnesota	D. A. Haugen
James G. Rose	University of Maine, Orono	C. A. Reilly
Walter Scott	Western Michigan University	T. Matsushita
Samuel Semoff	University of Arizona	T. M. Seed
Barbara Sina	University of Minnesota	N. G. Anderson
Karen Willard	Virginia Polytechnic Institute and State University	E. L. Lloyd (RER)

OTHER GRADUATE PROGRAMS

Four graduate students were Laboratory Graduate Participants working in the Division on research for their PhD degrees in a program administered by the Center for Educational Affairs. The Laboratory Graduate Participants, their schools, and their staff sponsors were as follows:

Avrom M. Brendzel	University of Illinois, Chicago Circle	Y. E. Rahman
Thomas Cunningham	University of Illinois Medical Center	D. Grahn
Michael P. Hagan	University of Illinois, Urbana	M. M. Elkind
Bruce Hammer	Northwestern University	S. S. Danyluk

In addition, Steven H. Gray, University of Illinois Medical Center, held a Guest Graduate Student Appointment, under the supervision of M. MacCoss, and Aaron D. Simms, New York University, worked under the supervision of Dr. T. Matsushita, under the sponsorship of the Affirmative Action Program.

UNDERGRADUATE TRAINING

During 1977, a total of 21 college undergraduates received training in the Division of Biological and Medical Research through the CEA-sponsored Spring, Summer, and Fall Undergraduate Research Participation Programs. The students, their schools, and their staff supervisors are listed below:

SPRING PROGRAM

Anthony W. Czarnik	University of Wisconsin, Madison	S. S. Danyluk
Jeffrey A. Goodman	University of Vermont	T. B. Borak
Paul W. Maffuid	St. Michael's College	S. S. Danyluk

SUMMER PROGRAM

Jamshed Bharucha	Vassar College	B. N. Jaroslow/ Y. E. Rahman
Deanna Loney	Northeastern Oklahoma A&M College	N. G. Anderson
Paul W. Maffuid	St. Michael's College	M. MacCoss
Edward Maytin	Clarkson College	P. D. Klein
Roger Rodby	Western Illinois University	P. D. Klein
Howard Turner	Rust College	T. M. Seed
Terry Watson	Rust College	M. MacCoss
Russell D. Yang	Trinity College, Hartford	D. A. Haugen

FALL PROGRAM

Bethann Bonner	Lawrence University	N. G. Anderson
Heath Carney	College of William and Mary	B. S. Hass
Mary Closson	Humboldt State University	P. D. Klein
Nancy Fay	Lawrence University	C. A. Reilly
Travis Gambill	Eastern Mennonite College	B. S. Hass
Timothy Howe	Pennsylvania State University	H. E. Kubitschek
Paul W. Maffuid	St. Michael's College	M. MacCoss
Carol S. Ramsay	Millikin University	C. Peraino
Steven Roth	New York University	M. MacCoss
Sam Tacke	Carroll College, Helena	A. Han

JOINT ARGONNE-UNIVERSITY APPOINTMENTS

During 1977, 21 staff members held a total of 31 faculty appointments at universities in the Chicago area. These appointments usually comprise limited teaching activities, generally of a specialized nature, at the graduate level, which involve regular contact with students. They have led to cosponsorship of graduate students and to collaborative research efforts with faculty members, some of which are described in this report.

The affiliations with Chicago area universities were as follows:

University of Chicago

Mortimer M. Elkind
Robert N. Feinstein
R. J. Michael Fry
David L. Hachey
Peter D. Klein
Timothy E. O'Connor
George A. Sacher
Warren K. Sinclair
Patricia Szczepanik

University of Illinois, Circle Campus

Douglas Grahn
Bernard N. Jaroslow
Herbert E. Kubitschek
Carl Peraino
Jane Shen-Miller
Warren K. Sinclair
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 N. Jaroslow
Herbert E. Kubitschek
Carl Peraino
Y. E. Rahman
Christopher A. Reilly, Jr.
Warren K. Sinclair
John F. Thomson
Robert B. Webb

Northwestern University

Peter D. Klein

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Distribution for ANL-78-90

Internal:

R. G. Sachs
W. K. Sinclair (3)
M. V. Nevitt
R. V. Laney
R. M. Adams
D. P. O'Neil
R. E. Rowland (2)
P. F. Gustafson
L. S. Markheim
C. F. Ainsworth
K. H. Allen
L. Anderson
N. G. Anderson
J. R. Benson
M. H. Bhattacharyya
C. S. Borso
P. C. Brennan
C. D. Brown
M. S. Brown
E. M. Buess
E. A. Cerny
E. J. Christian
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J. L. Dainko
P. J. Dale
S. S. Danyluk
R. L. Devine
D. Dixon-Davis
S. S. Dornfeld
D. E. Doyle
P. H. Duffy
C. F. Ehret
M. M. Elkind
R. N. Feinstein
M. P. Finkel
T. E. Fritz
B. H. Frystak
D. Grahn
D. D. Grube
I. L. Greco
K. R. Groh
D. L. Hachey
A. Han
B. S. Hass
D. A. Haugen
B. J. Hickman
G. Holmblad
J. S. Hulesch
L. A. Kaspar
W. G. Keenan
W. T. Kickels
P. D. Klein
N. D. Kretz
H. E. Kubitschek
C. K. Lee
A. Lindenbaum
J. L. Linsley
C.-M. Liu
L. S. Lombard
V. A. Ludeman
R. T. Lundy
M. MacCoss
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T. E. O'Connor
V. A. Pahnke
D. P. Peterson
C. Peraino
P. H. Polk
C. M. Poole
A. M. Prapuolenis
Y.-E. Rahman
C. A. Reilly
R. J. Robertson
G. Rockus
M. W. Rosenthal
J. Russell
G. A. Sacher
A. R. Sallese
M. Sanderson
M. Schiffer
B. A. Sedita
T. M. Seed
M. A. Shotola
R. C. Simkins
E. F. Staffeldt
S. P. Stearner
K. M. Suhrbier
G. Svihla
P. A. Szczepanik-Van Leeuwen
J. F. Thomson
S. L. Tollaksen
D. V. Tolle
D. Venters
R. B. Webb
F. A. Westholm
D. M. Williams
F. S. Williamson
B. J. Wright
J. M. Angerman
J. A. Blomquist
G. T. Chubb
S. M. Cullen
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F. A. Giere
S. Gordon
R. A. Gorse
S. H. Gray
W. Harrison
H. R. Isaacson
A. A. Jonke
R. Klein
R. Kumar
M. S. Matheson
G. Matsushita
M. Petrick
K. H. Schmidt
D. A. Schoeller
C. B. Shelman
S. A. Tyler
R. W. Wehman
F. M. Gentille
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