ARGONNE NATIONAL LABORATORY
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DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH

Annual Report
1972

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WARREN K. SINCLAIR, DIRECTOR
JOHN F. THOMSON, ASSOCIATE DIRECTOR

Preceding Report
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I. INTRODUCTION

Warren K. Sinclair, Director

The organization of the 1972 Annual Report of the Division of Biological and Medical Research is the same as that used last year: progress reports of the various aspects of the Divisional research program, organized according to the major research groups. However, for reasons of economy, individual reports are briefer than in former years, and the format is different.

The current organization chart of the Division is shown in Figure I-1. The scope of the activities of the groups is as follows:

NEUTRON AND GAMMA-RAY TOXICITY STUDIES (JANUS)

This program, which is designed to provide information on radiation as an environmental hazard, is concerned with numerous manifestations of late effects of radiation exposure in animals subjected to doses of neutron or $^{60}$Co $\gamma$ radiation over long periods of time. Exposure to neutrons is expected to result in data which can be extrapolated to very low dose levels more confidently than is possible for $\gamma$-ray exposures. The biological studies, which encompass a broad range of test systems, primarily in the mouse, are complemented by extensive dosimetric measurements; both are supported by sophisticated techniques of data management and processing. The highlight of 1972 has been the completion of the irradiation sequences in a major experiment involving nearly 10,000 mice, in which dose additivity for neutrons and $\gamma$ rays is studied for a variety of dose fractionation patterns. The results from this experiment, still accumulating, will influence profoundly the conduct and protocols of future late-effects experiments.

CARCINOGENESIS

An important late effect of radiation and other environmental pollutants is an increased incidence of tumors in exposed populations. Thus it is highly appropriate to study the mechanisms of induction of cancer in general, whether it develops spontaneously or is induced by radiation, chemicals, or viruses. Research in carcinogenesis falls into four principal areas: the role of isozymes in carcinogenesis, hepatic tumorigenesis and its enhancement by phenobarbital, strain and species differences in susceptibility to oncogenic agents, and the effects of treatment of skin with psoralen and near-ultraviolet light.
Fig. I-1.—Organization Chart, 1972.
AGING

Another late effect of radiation and possibly of other environmental agents is that of aging, the premature death of irradiated animals not only from tumors but also from assorted degenerative diseases. Like the Carcinogenesis group, the Aging group complements the JANUS program, and also carries out research concerned with age-dependent changes in a variety of biological systems. Several aspects of the problems of the comparative biology of myomorph rodents are reported here, and a new program on the relationship between energetic efficiency and aging in insects is underway. Work continues on immunity and aging, the mechanism of erythrocyte aging and destruction, and protein synthesis as a function of age. A new development that may be of considerable importance for the treatment of heavy metal poisoning is the encapsulation of chelating agents in liposomes.

BIOCHEMISTRY

There are four programs in the Biochemistry group, represented by a variety of contributions. One report deals with progress in the isolation from mouse liver of a homogenous preparation of peroxisomes, the cytoplasmic organelle that contains enzymes that both produce and utilize hydrogen peroxide. In the course of studies on the biochemistry of yeast cells, techniques have been worked out for isolation of the vacuole. The reports from the plant radiobiology section cover a multitude of activities dealing with interactions of visible light, ionizing radiation, and gravity on an assortment of plant materials. Finally, research on the therapy of plutonium poisoning has continued to expand; studies on dogs, with particular reference to the comparative effects of monomeric and polymeric plutonium, have been one of the major new approaches to the problem.

BIOPHYSICS

Two programs in this group are concerned with different approaches to the study of large molecules of biological significance, particularly proteins and nucleic acids. Protein studies have focused on high-resolution X-ray crystallography of (a) a Bence-Jones protein, the urinary excretion product of an immunoglobulin that is pathognomonic of multiple myeloma, and (b) the phytohemagglutinin concanavalin A. A major accomplishment in nucleic acid structure studies has been the synthesis of nucleoside diphosphates with one of the nucleosides completely deuterated, to permit the unequivocal assignment of structure in solution from NMR spectroscopy. A third program is concerned with biological clocks, specifically the molecular basis of temporal rhythms in biological systems, subsumed under the title of Circadian Cybernetics. A fourth major activity is the study of radiation response and its modification in mammalian cells in tissue culture; current emphasis is on the role of sensitizers such as N-ethylmaleimide in modifying radiation response at different phases of the cell cycle. The fifth program is concerned with clinical applications of stable isotopes using $^3$H- and $^{14}$C-labeled diagnostic compounds and detection by mass spectrometry.
GENETICS

A program in mammalian genetics has as its goal the attainment of quantitative data on radiation-induced mutation rates for sex-linked lethals and detriments in the mouse by the use of double-marked X chromosomes. The programs in microbial genetics employ bacteria and viruses (including bacteriophage) to study genetic replication and regulation; lethal and genetic effects of isotope decay; and mutagenesis, lethality, and recovery processes (including photoreactivation) as a consequence of irradiation with ultraviolet and visible light.

RADIATION TOXICITY IN DOGS

To reflect the changing emphasis of this program in recent years, the name has been changed from "Toxicity and Metabolism of Radionuclides" to a more general descriptive title. Although a number of dogs injected with various fission products are still under observation, the current experiments are concerned with low-level, continuous $^{60}$Co $\gamma$ radiation at dose rates in the range of 5 to 35 R/day, both duration-of-life and terminated. The identification of dose rates that produce a high incidence of myeloproliferative disease, principally a myelogenous leukemia that clinically resembles the human disease, promises to be a most valuable experimental approach to a problem of considerable practical significance.

LABORATORY ANIMAL FACILITIES

The Animal Facilities provide essential services in support of many of the research programs in the production, care, and disease status of a variety of animal species. Much of this work consists of applied research in microbiology, pathology, and husbandry, and thus is described in this report.

EDUCATIONAL ACTIVITIES

The most significant contribution of the Division to the educational process is its postdoctoral program; the contributions of our temporary appointees in every program are self-evident from even a casual perusal of this report. The Division also participates in a variety of programs ranging from summer training of Chicago area high school students (the Neighborhood Youth Corps Program) to specialized research experience for college and university faculty members. Programs for both undergraduate and graduate students, especially in the summer, are also provided; these activities are carried out in co-operation with the Argonne Center for Educational Affairs. The Summer Graduate Student Program in Biology is now serving as a pilot program for similar activities in other divisions in the Laboratory.

Many members of the staff hold joint appointments at universities in the area and teach courses at the graduate and undergraduate level.
These appointments provide an excellent opportunity for student contacts and faculty interactions.

ADMINISTRATIVE STAFF

Sinclair, Warren K. (Director)
Thomson, John F. (Associate Director)
Anderson, Allen H. (Assistant Director)
Flynn, Robert J. (Assistant Director, Animal Facilities)
Harrison, J. William (Executive Assistant)
II. NEUTRON AND GAMMA RAY TOXICITY STUDIES

SUMMARY

Douglas Grahn, Group Leader,* E. John Ainsworth, Acting Group Leader

The major long-term experiment on the comparative toxicity of neutrons produced by the JANUS reactor and $^{60}$Co $\gamma$-rays is now well advanced, and data on late effects in mice are beginning to take definitive form. The observations on the whole animal are complemented by a broad array of studies at the cellular and systemic level.

Dosimetry studies have been restricted in this reporting period to depth-dose evaluation, in addition to normal service dosimetry and facilities operation. A greater effort has gone into development work on the computer-controlled data-management system.

All mice involved in the late-effects study have completed their radiation sequences and have been at risk for 48 weeks or longer. Interim estimates of the values for radiobiological effectiveness (RBE) indicate that, at the dose levels employed, JANUS neutrons have an effectiveness relative to $\gamma$ rays of six or greater, depending upon endpoint. For death from all causes, the value is about 1.1. The values are less for reticular tissue tumors and greater for pulmonary tumors. A high degree of dose additivity was observed in mice given 24 to 72 individual exposures to neutrons, distributed over a 24-week protraction period; i.e., there is no apparent dose-rate effect, in contrast to the extensive evidence for such effects among the $\gamma$-ray groups. This observation should permit the most economical exploitation of the JANUS reactor as a fission neutron source that is also nearly free of thermal neutron and $\gamma$-ray contamination. It is also of extreme significance in demonstrating the value of fission neutrons for extrapolating downwards to effects at very low radiation levels with much more confidence than for $\gamma$ rays.

The development of parameters for cellular and systemic injury and recovery will form the basis for models of cell injury to be correlated with pathologic manifestations of chronic radiation injury. Animals from the long-term toxicity study have been sacrificed at time intervals after the end of the fractionation exposure period to determine the loss and recovery in cell populations of blood, marrow, gut, and testis.

These studies also provide data on which to base the design of future experiments to determine the latent period and residence times of certain tumors.

Based on hematopoietic depression and repopulation of stem and peripheral cells, some sparing effect of short-term dose fractionation (1 versus 9 fractions) was observed for both neutron and \( \gamma \) radiation. The fractionation effect is greater for \( \gamma \) radiation, so the RBE increases from the single dose value of \( \sim 2.6 \) to the range of 3 to 5.

Studies with a transmissible lymphocytic leukemia, presumably of viral origin, indicate the disease may be transmitted by injection of either spleen cells from leukemic donors or cell-free materials, viz., plasma, serum, or other filtrates. The leukemia cells are characterized by a sub-metacentric translocation marker and an extra chromosome. Susceptibility studies show that both radiation history and age significantly influence the course of disease.

Work is also continuing on the techniques of evaluating, on a quantitative basis, acute and chronic microvascular injury, and on the assessment of changes with age and radiation history on the pathogenesis of respiratory infection.

NEUTRON AND GAMMA-RAY TOXICITY STUDIES STAFF

REGULAR STAFF

Ainsworth, E. John (Biologist)
Brennan, Patricia C. (Assistant Biologist)
Christian, Emily J. B. (Scientific Assistant)
Cooke, Eugenia M. (Scientific Assistant)
Frigerio, Norman A. (Biochemist)
*Grahn, Douglas (Senior Biologist)
Holmblad, Gordon L. (Scientific Assistant)
Jordan, Donn L. (Scientific Associate)
Kickels, Wayne T. (Scientific Assistant)
†Lea, Ruth Ann (Scientific Assistant)
Miller, Marietta (Scientific Associate)
Nielsen, M. Patricia (Scientific Assistant)
Stearner, S. Phyllis (Scientist)
Trier, Joseph E. (Engineering Assistant)
Williamson, Frank S. (Physicist)

TEMPORARY STAFF DURING 1972

Carrano, Anthony V. (Postdoctoral Appointee)

*On leave of absence.
†Terminated during 1972.
JANUS PROGRAM DATA MANAGEMENT

Frank S. Williamson, Gordon L. Holmblad, and Barbara Nash*

In our current fractionation experiment, JM-2, the data management emphasis has shifted to the entry of death data and to the preparation of listings and displays showing the current status of the 10,000 mouse subjects.

OPERATIONS USING THE 360 SYSTEM

Animal exits (death, sacrifice, reassignment, loss, etc.) are recorded on death tag forms, and the date of exit is entered also in a space on the cage card. This information is transcribed directly into interim files in the 360 System using a 2741 terminal which resembles a Selectric typewriter, serviced by a special interactive processor designed to meet the Division's needs for direct data entry. The interim file is batch-processed against the GENERAL and VACANCY files and rigorous cross checks are made using the redundant input data. For example, the input data must show the correct cage location for the specified exit date, as recorded for that cage in the GENERAL file. The individual number of an animal is simply its exit sequence number (1 to 5) and is checked against the recorded number of animals remaining in the cage. This procedure provides complete protection against accidental repeated processing of the same data.

Ordered listings of these files, with options to select only latest cage records, all cages, or individual animal exits with printed number of days at risk, can be obtained on request. Also available is a tabular display showing, for each group, the number of exits (by code selection) in regular time intervals, together with the decremented population. These data are presented in compact form for each replicate, and summed over replicates. Percent mortality is also given by replicate, together with a bar graph of percent mortality, summed over all replicates, against time at risk.

Since November 6, 1972, these files and programs have been running successfully on the new ANL IBM 360/195 system. We adhere to the philosophy that programs should be general and versatile with options selected at run time, and that specific questions should be addressed to an up-to-date computer file as and when answers are needed. We plan to use the Time Sharing Option (TSO), soon to be available at ANL, for interactive applications of this philosophy.

DATA ACQUISITION SYSTEM

The PDP-11/20 is operating under the Disk Operating System, version 4. Assembler language routines have been developed to store 16-bit dates

*University of Michigan.
in sortable format, and to handle interval calculations and input/output format conversion. Other routines provide for ASCII/EBCDIC translation and conversion of IBM numeric and date formats.

A dynamic module-swapping supervisor is in course of development and will later be expanded into a multi-tasking supervisor using techniques developed at the National Accelerator Laboratory.

An identification system for the load frames in the JANUS High Flux Room has been installed and the interface to the PDP-11 will be completed early in 1973. At that stage the system will be able to monitor and supervise animal irradiations.

NEUTRON DOSIMETRY FOR THE JANUS PROGRAM


PURPOSE AND METHODS

The JANUS High Flux Room is approximately 4.4 m long, 2 m wide, and 2 m high. In a volume of this size, with fission neutrons entering through one wall and undergoing scattering at all boundary surfaces, it must be expected that the neutron spectrum will vary to some degree with location in the room. Our primary concern is, of course, whether such spectrum shifts are accompanied by variations in the neutron dose-response relationship in any biological system under study.

Spectral data have been taken using a variety of techniques and were presented in a previous report in this series (1). Recently we have successfully analyzed the measured neutron spectrum at a central location into a direct and a scattered component (2). In that paper we confirmed these component spectra by using them as input into a Monte Carlo calculation of the depth-dose distribution in a 16-cm cube of water and comparing this distribution with one obtained from measurements with ionization chambers. Some features of these spectra are given in Table II-1.

Previously reported measurements by Sinclair (2) indicated that the differences in neutron spectra at various locations in the room had a small but discernible effect on the survival of irradiated asynchronous Chinese hamster cells. In order to assess the importance of differences in neutron spectrum over the array of 400 mice used in our JM-2 fractionated exposures, we must also consider the possibility of different spectra producing different depth-dose distributions in our biological material.
TABLE II-1. Characteristics of Primary and Component Neutron Spectra

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>E</th>
<th>A</th>
<th>J</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux-average neutron energy (keV)</td>
<td>1138</td>
<td>644</td>
<td>850</td>
<td>1349</td>
<td>512</td>
</tr>
<tr>
<td>Flux (n.cm⁻².sec⁻¹.watt⁻¹)</td>
<td>2158</td>
<td>614</td>
<td>1050</td>
<td>424</td>
<td>626</td>
</tr>
<tr>
<td>Average kerma/fluence (10⁻³ rads.n⁻¹.cm²)</td>
<td>1.94</td>
<td>1.37</td>
<td>1.61</td>
<td>2.2</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Points A, B and E lie in a horizontal plane at the mid-height of the reactor face from which the neutrons emerge.

*φ*<sub>B</sub> is flux at B, adjacent to reactor face (hardest spectrum).

*φ*<sub>E</sub> is flux at E, near a rear corner (softest spectrum).

*φ*<sub>A</sub> is flux at A, on midline, 100 cm from rear wall.

*φ*<sub>J</sub> is direct flux component at A.

*φ*<sub>R</sub> is reflected flux component at A.

PROGRESS REPORT

In the JM-2 fractionated study we have exposed up to 400 mice simultaneously in a curved array of 40 x 10 locations. Up to the present time we have measured the dose at the approximate center of a 30 g "muromorphic" mouse phantom made of Al50 Shonka tissue-equivalent (TE) plastic. Two ionization chambers were used, each of 0.05 ml. One chamber has Al50 plastic walls and electrodes and TE gas filling, the other magnesium walls and electrodes and argon filling. The chamber in use, and the phantom, were contained in a quartz vessel wherein the entire atmosphere consisted of TE gas or argon. Doses were obtained with five orientations of the phantom and "in air," inside a simulated polyethylene container as used for housing one mouse. Two locations in the 40 x 10 mouse array were surveyed (the eight frame locations are numbered from 5 to 12).

(1) Frame location 8, center of shelf 5 (near the center of the array, furthest from the reactor face).

(2) Frame location 5, center of shelf 10 (an upper corner of the array, closest to the reactor face).

These data are given in Table II-2.
TABLE II-2. Depth Doses at Approximate Center of 30 GM Mouse Phantom, Relative to Dose Measured "in Air"

<table>
<thead>
<tr>
<th>Angle to neutron port (Head to port = 0 deg.) (degrees)</th>
<th>Location 5, Shelf 10</th>
<th>Location 8, Shelf 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutron ratio</td>
<td>Gamma Contribution (percent)</td>
</tr>
<tr>
<td>0</td>
<td>0.76</td>
<td>3.9</td>
</tr>
<tr>
<td>45</td>
<td>0.82</td>
<td>3.4</td>
</tr>
<tr>
<td>90</td>
<td>0.85</td>
<td>3.3</td>
</tr>
<tr>
<td>135</td>
<td>0.79</td>
<td>3.5</td>
</tr>
<tr>
<td>180</td>
<td>0.78</td>
<td>3.5</td>
</tr>
<tr>
<td>Average</td>
<td>0.80</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Composition of Al50 plastic (percent by wt.)
H = 10.25, C = 77.28, N = 3.49, O = 3.99, F = 2.43, Ca = 2.57

Notes: Neutron ratio is expressed as the ratio of neutron dose within the phantom to neutron dose in the absence of the phantom.
Gamma ratio is expressed as the ratio of gamma dose within the phantom to neutron dose measured in the phantom.
The remainder of the 400 locations were filled with mouse phantoms.

These preliminary data lead us to expect that there will be no significant variation of depth-dose distribution in mice due to spectrum variation in the JM-2 experiment, and hence we do not expect to find a significant variation in dose-effect relationship. Further measurements will be made in support of JM-2, and in more extreme locations in order to characterize the facility completely.

REFERENCES
A RANDOM NUMBER SET FOR MONTE CARLO DOSE COMPUTATIONS

Norman A. Frigerio

PURPOSE AND METHODS

In the operation of large Monte Carlo dose computation programs (1) over the past few years it was found that the congruential random number generator was inadequately random. Thus, in large problems such as those involved in JANUS dosimetry and spectrometry computations, bias errors of up to 1.5% occurred, which could be compensated only by parallel runs using the number complements, at a doubling of overall cost. This is a general problem with such numbers (2), and we have approached its solution through the use of numbers generated by the output of random physical processes, such as radioactive decay (3,4). Thus the output of a high-resolution a counter was coupled through the ANL Analog-Digital computer hybrid to produce strings of count values, each count being the events occurring in a fixed time of the order of 20 msec. The parity of each such count was then extracted as a single bit, and this string of bits used to form 31-bit random numbers by sequential assembly (3,4).

PROGRESS REPORT

Over the past year the above system was used to accumulate 3,791,052 counts, containing a total of 9.2 x 10^7 events, at an average count rate of 24.315761 events per 20 msec count time. This string of counts proved to fit perfectly the appropriate Brockwell-Moyal distribution (5), as predicted by our theoretical analysis (3,4), and thus indicates proper operation of the system.

The parity bits of these counts were then assembled in sequence to form 122,292 31-bit random number words, and these transcribed to magnetic tape. This set of random numbers was then subjected to a battery of tests including 12 chi-square tests of different types, tests of moments, serial correlation tests, and Tukey spectrum analysis (6,7). The present tape set has passed all of these, where the best congruential or natural set previously presented to this test battery had passed only eight tests (7). Thus, it appears that this set is presently the best available, and is certainly superior to any congruential set, practically or theoretically (2). At the moment six other laboratories have obtained copies of this tape set, and seem to be employing it with good results. Future work will consist of utilizing the same method to increase the quantity of numbers available as a tape set until the approximately 10^7 random numbers needed for our JANUS dosimetry computations have been obtained. The present system generates about 6000 such words per hour, at a cost of about $15/hr. However, a modified system, using the JANUS PDP-11 computer system on a shared-time basis, is planned. This system should permit generation of these random numbers at the rate of about 10^5 per hour at little or no cost.
REFERENCES


4. Frigerio, N. A. Poisson and non-Poisson behavior of radioactive systems. Submitted for publication.


PROGRESS OF JM-2 AND RELATED NEUTRON- AND GAMMA-RADIATION TOXICITY STUDIES


PURPOSE AND METHODS

One of the objectives of the JANUS Program is to define basic mechanisms of chronic radiation injury and to relate mortality parameters, such as survival time, death rates, and excess risk for neoplastic and non-neoplastic diseases, to cell and tissue injury. Our first late-effects experiment, JM-2, is concerned especially with the determination of the degree of additivity of small doses of neutron or \( \gamma \) radiation delivered at different intervals and dose rates over a period of 24 weeks. Survival and pathology data are presented here, and an analysis of death and selected tumor rates is presented in a separate report (1). A sacrifice series is being conducted in connection with experiment JM-2 for the purpose of evaluating injury, the changes in cell populations and proliferative capacity of critical cell renewal systems, and their recovery. A limited effort is devoted to selected short-term studies, the objective of which is to define and compare the basic mechanisms of neutron- and \( \gamma \)-radiation injury and recovery in various tissues and organs.

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PROGRESS REPORT

Experiment JM-2. By June, 1972, all irradiations, or sham irradiations, for the 10,000 B6CF1/Anl[Anl 66] mice involved in the experiment were completed. The allocation of animals to the various experimental groups are as follows: 5,600 animals received fractionated exposures to either neutron or γ radiation; 3,600 animals received single doses of neutron or γ radiation at 110 to 120 days of age, the age at which animals in the fractionation series received their first fractionated exposure; and 800 animals have been devoted to studies in which a single exposure was given to animals at 194 or 278 days of age, corresponding to the middle and end of the 24-week fractionation sequence. Since animals surviving from the earliest fractionation replicates are now approximately 690 days old, preliminary estimates of mortality, death, and radiation-induced changes in critical cell populations provide comparative data on the effects of neutron and γ rays.

Mortality in fractionation replicates 1 to 3 is used to monitor the mortality trends in the experiment. Mortality data from these groups with the longest post-exposure history are used to predict logistic requirements and the appropriate times for programmed sacrifices in the various treatment groups. Results from replicates 1 to 3, in Table II-3, show little difference in mortality by 588 days in the various neutron-irradiated groups which received a total dose of 240 rad, but relatively larger differences exist within the γ-irradiated mice given 855 rad.* The extent

<table>
<thead>
<tr>
<th>Date (1972)</th>
<th>Days</th>
<th>Weeks</th>
<th>Neutrons</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>322</td>
<td>46</td>
<td>11</td>
<td>- nil</td>
</tr>
<tr>
<td>May</td>
<td>392</td>
<td>56</td>
<td>24</td>
<td>4 8</td>
</tr>
<tr>
<td>June</td>
<td>441</td>
<td>63</td>
<td>31</td>
<td>8 16</td>
</tr>
<tr>
<td>August</td>
<td>483</td>
<td>69</td>
<td>36</td>
<td>14 21</td>
</tr>
<tr>
<td>November</td>
<td>586</td>
<td>86</td>
<td>59</td>
<td>24 29</td>
</tr>
</tbody>
</table>

*Midline tissue doses, in rad, were computed from exposures in kerma (rad) or R using a factor of 0.80 for neutrons and 0.95 for γ radiation.
of fractionation and instantaneous dose rate per fraction appear to influence mortality rate in the \( \gamma \)-irradiated mice, but not in mice exposed to neutrons. So far, cumulative mortality is similar, 25\% and 29\%, respectively, in groups which received 80 neutron rad and 855 rad of \( \gamma \) radiation in 24 fractions; thus our current provisional estimate of RBE for life shortening is \( \geq 10 \). This estimate of 10 is more clearly evident from the actuarial analyses presented elsewhere in this report (1). The extent to which these mortality trends are sustained remains to be seen; the patterns may change with changes in the causes of death in later life.

The pathology and mortality results in Table II-4 summarize our experience, based on all replicates, as of November 1972. Almost all lung tumors detected so far have been examined microscopically to assess their characteristics, as these tumors appeared to be larger and more aggressive than found in previous experiments (2). Lung tumors are the tumors of highest incidence in the neutron-irradiated mice. Of more importance is the fact that a considerable number of these tumors have been judged to be the cause of the moribund state or death. As the pulmonary tumors, especially in the unirradiated mice, may have a long residence time, it is necessary to obtain morbidity rates before a complete description can be given of any radiation-induced increase in the incidence or appearance of these tumors. Consequently, a separate serial sacrifice series on unirradiated mice is in progress.

The JM-2 sacrifice series carried out in neutron-irradiated and control groups at 28, 36, and 44 weeks after the start of the irradiation has

<table>
<thead>
<tr>
<th>Fractionated ( b )</th>
<th>Total Deaths</th>
<th>Autopsied</th>
<th>With Tumors</th>
<th>Reticular Tissue Tumors</th>
<th>Lung Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )-240-72</td>
<td>131</td>
<td>126</td>
<td>91</td>
<td>19</td>
<td>54</td>
</tr>
<tr>
<td>( \gamma )-855-72</td>
<td>39</td>
<td>38</td>
<td>36</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>( n )-240-24</td>
<td>113</td>
<td>103</td>
<td>80</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>( \gamma )-855-24</td>
<td>49</td>
<td>45</td>
<td>36</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>( n )-80-24</td>
<td>59</td>
<td>53</td>
<td>45</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>( \gamma )-1140-24</td>
<td>81</td>
<td>76</td>
<td>53</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>( n )-240-24-6( c )</td>
<td>126</td>
<td>118</td>
<td>88</td>
<td>17</td>
<td>43</td>
</tr>
<tr>
<td>( \gamma )-855-24-6( c )</td>
<td>35</td>
<td>32</td>
<td>27</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>( n )-240-6</td>
<td>111</td>
<td>103</td>
<td>82</td>
<td>18</td>
<td>42</td>
</tr>
<tr>
<td>( \gamma )-855-6</td>
<td>62</td>
<td>56</td>
<td>43</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Controls</td>
<td>77</td>
<td>67</td>
<td>37</td>
<td>13</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>No. of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>77</td>
</tr>
</tbody>
</table>

\( a \)Pooled results for males and females.

\( b \)The group dose code indicates type of radiation, the total dose (rad), and the number of radiation fractions.

\( c \)The groups received one weekly exposure in 360 minutes; the average weekly exposure in all other groups was 45 minutes.
provided a small but informative sample for the design of an experiment to determine the latent period for the pulmonary tumors. It is clear that these tumors have been found earlier in the neutron-irradiated groups. However, unless the mortality rates are similar in the neutron- and γ-irradiated and the control groups, no conclusion can be reached about the radiation-dependent effects on this tumor type. At present, one other finding that appears to differ from previous experience is the incidence of malignant vascular tumors found in various sites.

Bacteriological examinations are made of moribund or "fresh dead" animals in JM-2 and in short-term experiments. Animals in the JM-2 experiment are cultured only when there is gross evidence of infection, e.g., pneumonia. Diagnostic procedures have recently been extended to detect *Mycoplasma pulmonis*. The organisms isolated from mice in the late effects JM-2 experiment are shown in Table II-5. The same organisms have also been isolated from acutely irradiated mice, but the incidence of *Enterobacter cloacae* and *Proteus* species is different. After acute exposure, *E. cloacae* septicemia continues to occur in high frequency, and the frequency of *Proteus* sp. septicemia is higher than was observed previously.

| TABLE II-5. Incidence of Bacteria Isolated from Heart and/or Lungs of Mice Exposed to Fractionated Neutron or Gamma Radiation |
|---|---|---|---|
| Organism                  | Neutron a | Gamma a | Unirradiated Control* |
| *Escherichia coli*        | 18.8      | 22.2     | 17.2               |
| *Enterobacter cloacae*    | 6.8       | 8.1      | 6.9                |
| Fecal streptococci       | 9.0       | 8.1      | 10.1               |
| *Proteus* species         | 4.3       | 6.5      | 4.2                |
| Other coliforms           | 2.9       | 4.6      | 4.2                |
| *Staphylococcus* species  | 4.3       | 0.9      | 17.2               |
| *Pasteurella pneumotropica* | 0.4     | 0.9      | 4.2                |
| Alpha streptococci       | 0.4       | 1.9      | 0                  |
| Lactobacillus species     | 0.4       | 0.9      | 0                  |
| Micrococcus species       | 1.3       | 0.9      | 0                  |
| Sterile cultures          | 54.0      | 45.0     | 45.0               |

*There have been 234 cultures of neutron-irradiated mice, 108 of gamma-irradiated mice and 29 of control mice.*
The viral contact status of stock B6CF₁ mice, as well as that of the parent strains, is known from routine serological monitoring for antibodies to the indigenous murine viruses. From 1967 to 1972, antibodies to Sendai virus and mouse hepatitis virus have been detected in all three strains. About 10% of the sera tested were positive for Reovirus 3; however, this finding may not be indicative of viral contact, because nonspecific hemagglutination inhibitors are present in many mouse sera. B6CF₁ mice have occasionally had titers to PVM in addition to other viruses mentioned. All strains have been negative for GDVII, K, Polyoma, mouse adenovirus, ectromelia, and LCM viruses.

During the last year we began testing sera from selected experimental mice to determine whether the contact status had changed with time or radiation experience. A group of 10 "old" B6CF₁ mice (about 800 days of age) and seven groups sacrificed from the JM-2 experiment have been examined.

The results in all the groups were the same. Sendai virus antibody was present in almost all sera tested, but there was no antibody to PVM. Antibody to Theiler's GDVII mouse encephalomyelitis was detected in all sera. This virus has been reported as the most prevalent indigenous murine virus. It is an enteric virus of low virulence; infection of the central nervous system is regarded as a "biological accident." No overt signs of disease have been observed in mice with antibody to this virus. Fourteen mice had titers to Reovirus. This observation is probably attributable to nonspecific inhibitors rather than new infection.

**JM-2 Sacrifice Series.** A sacrifice series has been conducted in support of experiment JM-2 to provide information on cell and tissue injury at various times after completion of either fractionated or a single radiation exposure, and to provide preliminary information on tumor latency. The sacrifice times for the various groups of neutron- or γ-irradiated animals are selected on the basis of cumulative mortality or death rates in particular groups; animals are sacrificed three times over a 16-week period before mortality has reached a cumulative value of 15% in the group. In addition to a complete autopsy, the following measurements are made: complete blood count, colony-forming unit (CFU) content in the femur and spleen, weight of testes (dry), dry weight of the entire intestine, and incorporation of tritiated thymidine in both gut and testes. Various oral tissues, kidneys, and eyes, as well as tumors and other macroscopically abnormal tissues, are taken for histological examination. Some of the same end points have been measured by sampling small numbers of moribund animals at later times when the death rate is comparatively high.

Between the programmed sacrifice series and the data obtained from moribund animals, we hope to establish the extent to which comparatively early tissue injury has predictive value insofar as chronic injury is concerned. Cellular studies on moribund animals may contribute to a better understanding of physiological impairments and interactions which contribute to death. The total array of data obtained thus far from the sacrifice series is beyond the scope of this report, but a few highlights based on neutron-irradiated animals exposed to a total of 240 rad and sacrificed at
28, 32, or 44 weeks after the beginning of a fractionated exposure are as follows. At 28 weeks, i.e., 4 weeks after completion of the last radiation exposure, testes weight was significantly reduced in all neutron-irradiated groups; during the succeeding 16 weeks, testes weight increased to approximately 50% that of the aged controls. Although the variability of total intestinal dry weight is marked, no significant reduction in intestinal weight was detected during the period of observation.

The hematopoietic stem cell content in femur and spleen was reduced by approximately 50%, circulating platelets were significantly reduced, but all other peripheral blood elements were found in normal numbers. Representative results from the neutron group which received 72 radiation fractions (group A) are shown in Figure II-1. This effect on stem cells is not restricted to neutron-irradiated animals as preliminary results from some γ-irradiated groups sacrificed between 50 and 54 weeks show a reduction of the same magnitude. The extent to which changes in the stem cell compartment are totally dose-dependent is not yet clear. The stem cell studies show: (a) that spleen and femur responses may vary independently, (b) a reduction in stem cell content occurs in both neutron- and

Fig. II-1.—Hematopoietic responses of B6CF1 mice after fractionated neutron irradiation. A total exposure of 300 kerma (240 rad, midline tissue dose) was given in 72 fractions over a period of 24 weeks (3 fractions per week); each fraction of 4.2 kerma was delivered at 0.28 kerma/min. The total leukocyte (WBC) and platelet counts are based on 9 animals at each sample time; colony-forming units (CFU) counts are pooled values from 3 animals (6 femurs and 3 spleens) at each of 3 sample times. Means and 95% confidence limits are plotted.
γ-irradiated animals, and (c) significant differences in femur and spleen CFU content have been detected within the γ-irradiated groups, but not within the neutron-irradiated groups.

Injury to the stem cell population has also been evaluated in 24 individual animals which were sacrificed when moribund. Peripheral hematology has been done on a total of 39 irradiated animals, but to date, the CFU and peripheral blood measurements have been completed on only 4 unirradiated, shammed controls. As this sample is comprised only of moribund animals taken randomly from our populations at risk, it is not possible to achieve large numbers from any individual group over a particular period of time. Nevertheless, it is clear that most moribund animals, irradiated or controls, show some changes in either circulating or precursory blood cells. In some cases, the femur and spleen content of stem cells is reduced to 5 to 10% of the values observed in unirradiated, normal controls. As in the programmed sacrifice series, some animals, even in a moribund state, are capable of maintaining a near-normal total leukocyte count under conditions where the stem cell content in the marrow and spleen is greatly reduced. A wide array of blood dyscrasias has been observed. Six animals from irradiated groups had evidence of leukemia based on total leukocyte counts in excess of 20,000/mm³; all had marked splenic enlargement and a marked reduction in spleen stem cell content based on colony-forming units per 100 mg (wet weight) of spleen tissue. We have no evidence for an increase in the size of the marrow stem cell compartment in leukemic animals; in the animals evaluated to date, the marrow stem cell content has been reduced to 20 to 50% of that for normal, unirradiated control animals. As the sacrifice studies continue and the hematopoietic injury parameters are correlated with the histological findings, it should be possible to establish causes of death and understand the various forms of systemic interactions which occur when different radiation-induced diseases and types of neoplasia are present. Animals from the single dose series are included in the sacrifice experiment, so protraction factors for these forms of cell and tissue injury, as well as for life shortening, will be evaluated.

**Acute Lethality Studies.** The objective of these lethality studies, comparing neutron and γ radiation in three mouse strains, is to determine the extent to which LD_{50/30} or LD_{50/6}, respectively, can be related to survival of hematopoietic or intestinal stem cells. The results show that LD_{50/30} is not predicted by stem cell D₀ or the surviving number of stem cells in the three strain comparison.

Studies in progress on aged animals extend our earlier observation that no enhanced intestinal susceptibility was observed in 300-day-old B6CF₁ mice (3). Studies of age and sensitivity to both hematopoietic and intestinal death are being done with the B6CF₁ mouse at approximately 200, 300, 500, 700, and 900 days of age. Due to limited availability of the parental strains, age and sensitivity to neutron or γ radiation will be studied over a more limited time frame.

Questions of repair-recovery or proliferation as a function of dose rate or fractionation interval of neutron or γ radiation are basic to our principal questions with regard to late effects produced by these radiations.
Fig. II-2.--The influence of dose rate on platelet counts in B6CF<sub>m</sub> mice exposed to 780 R (740 rad, midline tissue dose) at 35 or at 3.3 R/min. The values plotted are means and 95% confidence limits based on 13 to 20 animals per point.

Fig. II-3.--The influence of neutron dose rate on platelet counts in B6CF<sub>m</sub> mice given a single exposure of 360 kermo (240 rad, midline tissue dose) at 16.4 or 15. kermo per minute. Means and 95% confidence limits are based on 13 to 20 animals per point.
Injury accumulation based on split-dose lethality was evaluated in an experiment in which mice were given nine small doses of either neutron or γ radiation, and their LD$_{50/30}$ or LD$_{50/60}$ was determined 2 days after the last radiation fraction. A companion hematological study, described in the following section, was conducted to establish responses to fractionated irradiation. The purpose of this lethality experiment was to repeat and extend the results of experiment JM-1 which was reported earlier (4). LD$_{50/30}$ measurements were made 2 days after the last of nine fractions of 32 rad of neutron or 83 rad of γ radiation, respectively (total doses of 288 or 740 rad). The results confirm our earlier observation that, based on LD$_{50/30}$, injury is greater and/or recovery of neutron radiation injury between fractions is less effective than is the case with γ radiation. In the case of intestinal injury, estimated by LD$_{50/6}$, no injury was detected in either neutron- or γ-irradiated animals at 2 days after the last radiation fraction. Repair-proliferation of the intestine adequately compensates for both neutron- and γ-radiation injury sustained during the 3-week fractionation period. In these experiments the exposure time per fraction was approximately 20 min for both neutron and γ radiation, i.e., the instantaneous dose rates were comparatively low. In order to eliminate any effects of γ dose rate on these estimates of RBE for hematopoietic injury after fractionated exposure, this experiment is being repeated using a higher dose rate at which no dose-rate effect is expected for γ radiation.

Hematological Studies. An experiment has been conducted to evaluate hematopoietic injury and recovery after whole-body exposure of mice to 288 neutron rad or 740 rad of γ radiation. These total doses were given either as a single exposure in either 20 or 240 min, or in nine 20-min exposures protracted over a period of 3 weeks. The dose ratio of 2.6 (740/288) is the same as the RBE for 30-day lethality and the ratio of D$_0$'s for survival of marrow colony-forming units. Data from this experiment permit inferences pertaining to the effects of instantaneous dose rate for neutron and γ radiations, and the influence of fractionation on hematopoietic injury and recovery. As nine hematopoietic parameters and changes in body weight have been measured in six different experimental groups, the large number of potential comparisons is beyond the scope of this report.

Based on depletion and subsequent regeneration (repopulation), no instantaneous dose-rate effect for neutron or γ radiation was observed for thymus weight, erythrocyte, lymphocyte, or neutrophil counts. In the case of platelets, a sparing effect of low-dose rate was observed during the second week after irradiation for both neutron and γ radiation (Figs. II-2 and II-3); the dose-rate effect was greater for γ than for neutron radiation. A dose-rate effect on stem cell repopulation of the femur was seen only at 21 days after γ irradiation; at later sample times, between 45 and 180 days, no differences were found. A paradoxical effect of neutron dose rate on stem cell repopulation was observed. No significant differences were observed at 8 or 11 days, but at 15 and 21 days the femur stem cell content was significantly lower in the animals exposed at the lower dose rate. At 45 days the stem cell content of animals exposed at the lower....
rate did not differ significantly from the content of unirradiated controls, whereas animals exposed at the higher dose rate showed less repopulation.

Comparisons between the neutron and γ groups exposed at the higher dose rates show the following: the nadir in platelet counts was lower at 11 days and the repopulation slower before 21 days in the neutron-irradiated group; between 21 and 180 days no significant differences between groups were observed (Fig. II-4). The effects of neutron and γ radiation on neutrophil counts are shown in Figure II-5. In neutron-irradiated animals, the counts declined somewhat more slowly, did not reach the nadir observed for γ-irradiated animals, and during the fourth week, the granulocyte "overshoot" was greater in the neutron-irradiated animals. The lymphocyte counts in neutron- and γ-irradiated animals did not differ significantly during the first 45 days after irradiation, but a "normal" lymphocyte count was achieved earlier in the neutron-irradiated groups (Fig. II-6). The femur stem cell content was lower in neutron-irradiated animals at the earliest sample times, 8 and 11 days, but at 15 days the stem cell content was higher in neutron-irradiated animals; no significant differences between groups were observed thereafter. Since the depopulation and repopulation responses of various hematopoietic components vary after a single exposure to neutron or γ radiation, no single RBE is meaningful.

Fig. II-4.--Platelet counts in B6CF1 mice given a single neutron exposure of 360 kerm (240 rad, midline tissue dose) for 780 R (740 rad, midline tissue dose) of 60Co γ radiation. Means and 95% confidence limits are based on 13 to 20 animals per point.
Fig. II-5.—Neutrophil counts in B6CF₁ mice given a single neutron exposure of 360 kerma (240 rad) or 780 R (740 rad) of ⁶⁰Co γ radiation. Means and 95% confidence limits are based on 13 to 20 animals per point.

Fig. II-6.—Lymphocyte counts in B6CF₁ mice given a single exposure of 360 kerma (240 rad) or 780 R (740 rad) of ⁶⁰Co γ radiation. Means and 95% confidence limits are based on 13 to 20 animals per point.
When total doses of 240 rad of neutron or 740 rad of γ radiation are given in nine fractions protracted over 3 weeks, rather than as a single dose, the effects on the hematopoietic system differ markedly. In all parameters measured, there was evidence for a sparing effect of fractionation for both neutrons and γ radiation, viz., less depression and some evidence of earlier repopulation, but in many cases the sparing effect was manifested during the first 1 to 4 weeks after irradiation, with little fractionation effect later as animals approached near-normal levels of circulating cells. In the case of femur stem cell repopulation, a greater fractionation effect was observed with γ radiation than with neutrons (Figs. II-7 and II-8). With the γ radiation, the sparing effect of fractionation was quite marked during the first 15 days after completion of exposures, but in the case of neutrons, no significant differences between groups were observed after 11 days. Figure II-9 compares the effects of fractionated neutron and γ radiation on repopulation of femur stem cells and shows that 2 days after the last fraction the content in neutron-irradiated animals was significantly lower than in animals exposed to γ radiation. During the succeeding 8 days the stem cell content increased rapidly in the γ-irradiated group, but repopulation in the neutron-irradiated animals was comparatively slow. These results, indicating a greater accumulation of hematopoietic injury during fractionated exposure in the neutron-irradiated mice,

**Fig. II-7.--**Femur stem cell content in B6CF1 mice after 780 R (740 rad) of 60Co γ radiation given either as a single exposure at 35.5 R/min or in nine 87 R fractions at 5.0 R/min. Means and 95% confidence limits are based on 3 to 6 donor animals per point.
Fig. II-8.--Femur content of colony-forming units after a total exposure of 360 neutron kerma (240 rad) administered either in a single exposure at 16.4 kerma/min or in nine 40 kerma fractions at 2.3 kerma/min. Means and 95% confidence limits are based on 3 to 6 donor animals at each time point.

Fig. II-9.--Femur stem cell content in B6CF, mice which received nine radiation fractions totaling 360 neutron kerma (240 rad) at 2.3 kerma/min or 780 R (740 rad) of 60Co γ radiation at 5.0 R/min. Means and 95% confidence limits are based on 3 to 6 donor animals per point.
and consistent with the results of split-dose LD$_{50/30}$ studies in which more injury was detected in neutron irradiated mice when exposed 2 days after the last of nine radiation fractions (4). Differences in stem cell content between groups given fractionated exposures to neutron or γ radiation are not totally reflected by peripheral blood cell counts. The peripheral hematology will be presented elsewhere.

**Age and Stem Cell Content.** The effect of age on femur stem cell content was evaluated earlier in connection with studies of late injury to the hematopoietic system (3,5). Although the femur stem cell content in aged animals is more variable than the content in young adult animals, no evidence was found for a marked age-related decrease in stem cell content in the B6CF$_1$ mouse. Since an age-related decrease in stem cell content has been reported by others (6), we have broadened our sample to include the parental strains for the B6CF$_1$, namely the C57BL/6Anl[Anl 66] and BALB/c Anl[Anl 66].

The stem cell content in C57Bl mice 450 to 550 days of age was evaluated in four separate experiments in which 4 to 5 donors were sacrificed, and their marrows pooled and injected into supralethally irradiated recipients; the spleen stem cell content was also assayed. The stem cell content per femur ranged from 5,035 to 7,225, and the spleen content of stem cells ranged from 1,750 to 2,800. As was the case with the B6CF$_1$ mouse, these values for CFU content per femur and spleen indicate no age-related decrease by 450 to 550 days of age. Five different assays were performed with BALB/c mice over the same age span. The CFU content per femur ranged from 5,900 to 7,400, and the stem cell content per spleen ranged from 1,000 to 2,000; the BALB/c animal is characterized by a somewhat larger spleen and a lower spleen content of stem cells. The nucleated cell content and CFU ratio per 10$^5$ nucleated cells was quite variable in aged mice of the three strains. Thus no evidence for an age-related decrease in stem cell content has been observed in the three mouse genotypes.

**Transmissible Leukemia.** The principal effort devoted to the transmissible leukemia described previously (3) has been to test susceptibility of mice to the disease as a function of either age or prior radiation experience. In most experiments test animals were injected intraperitoneally with spleen cell suspensions from leukemic donor animals. The principal end point is mean survival time; percent mortality within a specified period of time after injection may also be a usable end point, but since most animals eventually succumb to the disease, an appropriate time end point is difficult to establish. The results in Table II-6 indicate that susceptibility is markedly influenced by age. Unirradiated young adult animals 100 to 120 days of age are comparatively resistant and show a mean survival time of in excess of 80 days. Groups of unirradiated test animals were challenged at five ages between 243 and 840 days of age, and the mean survival time decreased with increasing age.

Late radiation effects on susceptibility to the transmissible leukemia cells has been evaluated at 250 and 300 days after an acute exposure to either 740 rad of γ radiation or 288 rad of neutrons. The irradiated survivors show a marked increase in susceptibility, compared with appropriately
TABLE II-6. Susceptibility to Transmissible Leukemia: Effects of Age and Radiation History

<table>
<thead>
<tr>
<th>Challenge Cell Dose</th>
<th>Aged Controls</th>
<th>Irradiated Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 × 10^6</td>
<td>Age (Days)</td>
<td>Dead/Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>20/20</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>20/21</td>
</tr>
<tr>
<td>9 × 10^6</td>
<td>117</td>
<td>6/29</td>
</tr>
<tr>
<td></td>
<td>243</td>
<td>17/29</td>
</tr>
<tr>
<td></td>
<td>313</td>
<td>26/30</td>
</tr>
<tr>
<td></td>
<td>621</td>
<td>25/25</td>
</tr>
</tbody>
</table>

*Mean survival time and 95% confidence limits.

^b Incomplete.

^c 288 fn or 741 γ rad at 110-120 days.

Aged controls challenged at the same time with the same test suspension. Although the mean survival time of neutron-irradiated animals is shorter at both ages, the differences are not statistically significant. Although irradiation clearly influences susceptibility, the time relationships for this form of injury, presumably to components of the immune system, are not completely defined.

As described elsewhere (3), this transmissible leukemia was originally isolated from an aged, irradiated B6CF1 mouse, the same hybrid which has been used for evaluations of testing susceptibility. The observation that radiation significantly influences susceptibility in young adult mice indicates that some host defense mechanisms influence tumor cell proliferation. From this we infer that the tumor cells differ antigenically from the host animals to some extent. The transmissible leukemia cells are apparently recognized as "foreign" by the inbred parental lines (C57Bl and BALB/c) since very few deaths have occurred when unirradiated adult animals from the parental lines were injected with numbers of leukemia cells which produce significant deaths in B6CF1 mice within 100 days. In addition to the parental lines, susceptibility has been evaluated in four other inbred mouse strains after exposure to 200 R of 250 kvp X-rays, and the sensitizing effect was much greater in the B6CF1 hybrid than in any of the inbred strains. This pattern of immunogenetic responsiveness is not restricted to transplantation of leukemia cells; after injection of 0.5 ml of a 1:100 dilution of pooled serum from leukemic donors into irradiated (200 R 250 kvp X-rays) mice, 15 of 20 B6CF1 succumbed, whereas none of 16 C57Bl and two of 16 BALB/c animals succumbed to the disease within 90 days.
The time course of the disease has been studied between 3 and 10 days after the injection of leukemic cells into X-irradiated (200 R) recipient mice. During this time the spleen weight increased from ~ 45 to ~ 850 mg, and the peripheral leukocyte counts from ~ 1200 to 19,000/\text{mm}^3. The spleen colony assay for leukemic stem cell clones showed an increase from < 1000 to ~ 10^6/spleen. Over the same time period, no increase in thymus weight, compared with irradiated controls, was found in leukemia-injected animals.*

Cytogenetic analysis of spleen cells from leukemic B6CF_1 mice indicates a stem-line origin for the leukemia. The karyotype of the neoplastic cell consists of 41 chromosomes (2n=40) including a submetacentric marker. The presence of the Y chromosome has been established through the chromosomal banding techniques. This is of particular interest since the leukemia originated in a female B6CF, but has been subsequently passed to male recipients. The ease of identification of the leukemic cell at metaphase permits analysis of cellular kinetics during the course of neoplastic progression.

The increase in spleen weight is being correlated with the appearance of chromosomally marked metaphase cells in the bone marrow and spleen of irradiated animals given spleen cells from leukemic donors. The generation time of the leukemic cells as well as the length of each phase of the cell cycle at 7 days post-injection is being determined by the pulse-labeling method with \(^3\)HTdR. No chromosomally marked metaphases were detected in either marrow or spleen at 3 days after injection of leukemic cells; by 5 days after injection the spleen weight had doubled to approximately 110 mg and 49.4% of the metaphases in the bone marrow and spleen, respectively, showed the metaphase marker; at 7 days after injection the spleen weight increased to 466 mg and the percentage of marked metaphases in the marrow and spleen was 94% and 99%, respectively. The generation time of the leukemic cell population in the spleen of host mice 7 days after injection of leukemic cells is approximately 9 hr, with the cell cycle stages being estimated as follows: \( G_1 \geq 1.3 \text{ hr} \); \( S \leq 6.4 \text{ hr} \); \( G_2 \geq 1.0 \text{ hr} \); and \( M \leq 0.3 \text{ hr} \).

The disease has been transmitted using a variety of tissues described earlier (3), as well as cell-free supernatants from our routine spleen cell preparations, and by injection of either plasma or serum. Attempts to isolate the virus using various centrifugation techniques have met with some success. Preliminary collaborative studies with Dr. R. E. Ecker have shown that infectious material may be obtained by high speed centrifugation of supernatants from freshly isolated cell suspensions from leukemic spleens; in one experiment relatively more infectious material was isolated from the pellet than from the supernatant. Attempts at density-gradient separation of infectious material by Dr. J. F. Thomson have shown that a band of RNA-rich material, unique to the spleens of leukemic animals, produces evidence of leukemia, based on peripheral hematology, at 6 months after intravenous injection into irradiated recipients. Attempts to isolate and identify the virus are continuing (8).

*Lawrence C. James II, a summer student from Howard University, contributed to these studies.
REFERENCES


INTERIM ANALYSIS OF DEATH RATE STATISTICS; EXPERIMENT JM-2

By November, 1972, all mice in Experiment JM-2 had experienced at least 48 weeks at risk. Death rates for all causes of death have risen to levels of $10^{-3}$/day or greater for irradiated animals and to about $5 \times 10^{-4}$/day for the controls. A major proportion of mice have tumors at death. Therefore, preliminary analysis of the course of mortality for all deaths and for deaths with specific neoplasms can be accomplished with the reasonable expectation that these interim results will define the general expectations for the experiment.

*Departments of Radiology and Pharmacology, University of Chicago.
In order to make preliminary estimates of RBE values, the data must be corrected for differences in duration of the post-irradiation period, as the exposures were accomplished in ten replications distributed over several months. The term chosen to describe the risk is "mouse-weeks at risk." This is derived from the product of the number of weeks in a time interval and the mean number of mice alive during that interval. Summing across all intervals, one derives the total mouse-weeks at risk. The number of deaths for all causes or for any specific cause can be expressed as probability per mouse-week. Some inequities exist in the number of weeks or the number of mice which cannot be eliminated with this statistic. However, the different fractionated dose groups in the neutron- and γ-ray series only differ in number of mouse-weeks at risk by ± 15%, and therefore can be directly compared in an equitable manner with this common denominator term.

Age-specific death rates have been derived for a number of the experimental groups for all causes of death and for deaths with selected specific tumors. These have been calculated by previously defined methods (1,2). In several cases, linear equations have been fitted to these death-rate data and the slopes and intercepts derived for further comparative analysis.

Death rates have been estimated through the 80th week, and death probabilities per mouse-week have been estimated through the 88th week. In the single dose series, which has been at risk for only about 30 weeks, comparisons have been made only for the highest dose groups.

**PROGRESS REPORT**

A summary of the mortality probabilities per mouse-week is given in Table II-7. These data suggest that all the fractionated neutron groups given a total dose of 240 rad have the same general course of mortality, and that the separate dose fractions are essentially additive. This is the case for all causes, reticular tissue tumors, pulmonary tumors, all tumors other than pulmonary and reticular, and all tumors. The only possible exception is the group exposed to only six fractions of 40 rad. This series has a slightly lower death risk, but the significance of this difference cannot yet be established. For the comparative analysis with the γ-ray series in Table II-7, only groups A, B, and E have been pooled.

In the γ-ray series, the 72- and 24-fraction groups (A and B) seem identical and are pooled. Dose rate effects are apparent among the other γ-ray groups; the 24-fraction (6 hr per fraction, series E) shows the lowest mean death rate while the six fraction group has the highest rate among those given a total dose of 855 rad. The differences are in the expected direction.

The RBE value is between 11 and 15 for all causes of death, and between 6 and 18 for deaths with specific neoplasms, with the highest values related to pulmonary and miscellaneous tumor incidences. That is, neutrons are less efficient in raising the incidence of reticular tissue tumors than the incidence for all other tumors. This differential might diminish, however, as the rates for pulmonary tumors are increasing at a slower rate.
TABLE II-7. Probability of Frequency of Given Event Per Mouse-Week at Risk. "RBE" Values can be Derived by the Ratio: (n/γ) 3 for the A, B, E, H, S3 Groups and (n/γ) 12 for Group D.

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>All Deaths $\times 10^{-3}$</th>
<th>Leukemia $\times 10^{-4}$</th>
<th>Pulmonary Tumors $\times 10^{-3}$</th>
<th>Other Tumors $\times 10^{-3}$</th>
<th>All Tumors $\times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: 240-72-1/4</td>
<td>6.2</td>
<td>8.9</td>
<td>2.5</td>
<td>1.5</td>
<td>4.3</td>
</tr>
<tr>
<td>B: 240-24-3/4</td>
<td>5.5</td>
<td>9.3</td>
<td>2.3</td>
<td>1.1</td>
<td>3.9</td>
</tr>
<tr>
<td>E: 240-24-6</td>
<td>5.7</td>
<td>7.7</td>
<td>1.9</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>H: 240-6-3</td>
<td>4.8</td>
<td>7.8</td>
<td>1.8</td>
<td>1.6</td>
<td>3.5</td>
</tr>
<tr>
<td>D: 80-24-3/4</td>
<td>2.3</td>
<td>5.2</td>
<td>1.2</td>
<td>0.64</td>
<td>1.8</td>
</tr>
<tr>
<td>HBE combined</td>
<td>5.8</td>
<td>8.6</td>
<td>2.3</td>
<td>1.6</td>
<td>4.1</td>
</tr>
<tr>
<td>S3: 240-1</td>
<td>1.9</td>
<td>9.4</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
</tr>
<tr>
<td>Gamma Rays:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: 855-72-1/4</td>
<td>1.7</td>
<td>4.3</td>
<td>0.82</td>
<td>0.64</td>
<td>1.6</td>
</tr>
<tr>
<td>B: 855-24-3/4</td>
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<td>5.2</td>
<td>0.64</td>
<td>0.56</td>
<td>1.4</td>
</tr>
<tr>
<td>E: 855-24-6</td>
<td>1.4</td>
<td>4.0</td>
<td>0.47</td>
<td>0.32</td>
<td>1.1</td>
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<tr>
<td>H: 855-6-3</td>
<td>2.5</td>
<td>4.8</td>
<td>0.80</td>
<td>0.84</td>
<td>1.7</td>
</tr>
<tr>
<td>D: 1140-24-3/4</td>
<td>3.2</td>
<td>9.8</td>
<td>0.83</td>
<td>0.63</td>
<td>2.1</td>
</tr>
<tr>
<td>AB combined</td>
<td>1.8</td>
<td>4.7</td>
<td>0.72</td>
<td>0.70</td>
<td>1.5</td>
</tr>
<tr>
<td>S3: 855-1</td>
<td>5.6</td>
<td>34.5</td>
<td>-</td>
<td>-</td>
<td>3.9</td>
</tr>
<tr>
<td>Controls</td>
<td>0.74</td>
<td>1.2</td>
<td>0.18</td>
<td>0.13</td>
<td>0.35</td>
</tr>
</tbody>
</table>

in neutron-irradiated mice than among mice exposed to γ radiation (Fig. II-10). RBE values for tumor incidence can be assessed for lethal tumors when growth rates of the tumors are similar in the different irradiated groups. In the case of non-lethal tumors or tumors which contribute to death to a varying degree, RBE values can be assessed either from data obtained in a sacrifice series or from data in irradiated groups with similar mortality rates where the incidence of the tumor of interest is also independent of other diseases and tumors. As the mortality rates for
Fig. II-10.--Death rates of B6CF₁ mice with specific tumors derived for 16-week intervals. The neutron data from groups A, B, E, and H are combined. The γ-ray data from groups A, B, and H are combined (see Table II-7).
the neutron groups are greater than in the \( \gamma \) or control groups, and because pulmonary tumors are not always the cause of death, any RBE estimates for pulmonary tumors would be artifactually high.

Dose groups D, 1140 rad of \( \gamma \) rays vs. 80 rad of neutrons delivered in 24 equal weekly fractions, give tentative RBE values for deaths from all causes similar to those for the compared groups at 240 rad of neutrons and 855 rad of \( \gamma \) rays.

In the single dose series, the comparison of 240 neutrons and 855 \( \gamma \) rad at this time gives an RBE of \( \sim 1 \) for all causes and all tumors, with the reticular tissue tumors value dropping to 0.8. Single point comparisons from the present data could result in an underestimate of the RBE value because the risk of reticular tissue tumors does not increase at a constant rate over the life-span (2).

The death rate data show a clear separation of the neutron, \( \gamma \), and control groups (Fig. II-11), although the group given 80 neutron rad is superimposed upon the \( \gamma \) groups (A plus B), which received 855 rad. The apparent parallel displacement for all causes suggests that after termination of a fractionated series, the continued excess mortality reflects a fixed level of residual injury that acts upon the population in the same manner as injury induced by single doses. Generally, these data also support an RBE of about 10.

The death rates for reticular tissue tumors and other specific causes (Fig. II-10) are rising steadily for the fractionated exposure groups,

---

Fig. II-11.—Death rates for all causes of death derived for 8-week intervals in the B6CF1. Sexes are combined. The 240-rad neutron data are combined for all four fractionated series (groups A, B, E, and H in Table II-7). The 855-rad \( \gamma \)-ray data are from groups A and B (see Table II-7).
though for some the rate of increase is beginning to drop below simple linear trends. However, the pulmonary-tumor and all-tumor death rates for the the γ-irradiated mice are still rising linearly, which indicates the RBE for pulmonary and other tumors may decline with increasing age of the populations. Death rates for control mice with specific tumors have not been calculated because of the small numbers that have died so far with specific neoplastic diseases.

The single-dose 855-rad γ-ray group is plotted in Figure II-11 for reticular tissue tumor mortality only. A typical phasic response is noted, and the level of response is identical to that seen over 15 years ago with the same mouse strain after exposure to single doses of X rays in the acute-lethal dose range.

Finally, selected dose groups have been fitted with linear equations over those age intervals where the rise in death rate seemed most nearly linear. The slopes and intercepts are given in Table II-8 and the slopes are plotted in Figure II-12. Although no detailed statistical analysis of these data has been made, it seems clear that no major slope differences exist and that the post-exposure excess mortality over the controls may be described by a single dose-dependent displacement of the Gompertz function as previously described by Sacher (3), Grahn (2), and others for populations

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Slope (Per Week)</th>
<th>Intercept (X10^-5)</th>
<th>Interval (wks.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrons:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AB: 240-all</td>
<td>+0.052</td>
<td>10.5</td>
<td>28-76</td>
</tr>
<tr>
<td>D: 80-24-3/4</td>
<td>+0.071</td>
<td>1.1</td>
<td>20-76</td>
</tr>
<tr>
<td>Gamma Rays:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB: 855-72-1/4</td>
<td>+0.069</td>
<td>1.1</td>
<td>36-76</td>
</tr>
<tr>
<td>24-3/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: 855-24-6</td>
<td>+0.071</td>
<td>0.64</td>
<td>36-76</td>
</tr>
<tr>
<td>H: 855-6-3</td>
<td>+0.060</td>
<td>2.4</td>
<td>36-76</td>
</tr>
<tr>
<td>D: 1140-24-3/4</td>
<td>+0.054</td>
<td>4.8</td>
<td>36-76</td>
</tr>
<tr>
<td>Controls:</td>
<td>+0.062</td>
<td>0.54</td>
<td>36-76</td>
</tr>
</tbody>
</table>
Fig. II-12.—Linear regressions of age-specific death-rates on age for indicated neutron- or γ-irradiated groups of B6CF₁ mice. See Table II-8 for details.

exposed to single or briefly fractionated exposures. If so, then the late life RBE values can be estimated from the displacement of the intercepts.

CONCLUSIONS

A surprisingly high degree of dose-additivity is noted for the neutron exposures. Life shortening predictions are premature as there are still some non-linear changes in the rate of increase in mortality that must stabilize before more complex functions can be fitted to the data for prediction purposes. The data, however, do suggest that analysis can be made on the basis of present models of radiation injury. On the other hand, RBE values for certain specific tumors based on the incomplete data may not be consistent with presently held opinions on the mechanism of their induction. In the case of the γ radiation, the results show that a 24-week fractionation period is sufficient to bring the animals into the injury mode typical of low-intensity low-dose exposure.
REFERENCES


EFFECT OF RADIATION ON SUSCEPTIBILITY TO RESPIRATORY INFECTION

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

PURPOSE AND METHODS

Enhanced susceptibility to experimental respiratory infection following chronic exposure to low-level $\gamma$ radiation has been reported (1,2). No similar data exist for neutron-irradiated animals. Consequently, the study of altered host susceptibility to respiratory infection following neutron exposure constitutes an important aspect of the JANUS Program. Accordingly, we have developed a model system to detect such changes, using *Mycoplasma pulmonis* as the challenge organism. Preliminary results with this system in normal unirradiated B6CF$_1$ mice have been reported (3).

The mice used in the present study received either $\gamma$- or neutron-radiation exposures as outlined previously (4). Mice were challenged with a colony forming unit (CFU) concentration estimated to produce clinical pneumonia in 10 to 15% of normal unirradiated mice of comparable age at 4 days after intranasal instillation. All other methods have been reported (3,5).

PROGRESS REPORT

The results of challenge 6 weeks after acute radiation exposure or 36 to 66 weeks after the first fractionated exposure of mice in JM-2 are shown in Table II-9. The results of the acute exposure are difficult to interpret since only 1% of the $\gamma$-irradiated mice developed pneumonia. However, an experiment currently in progress in which mice received single doses of 288 rad of neutron or 740 rad of $\gamma$ radiation and were challenged 5, 11, and 21 days later suggests that the RBE for increased susceptibility to respiratory infection is $\sim$2.6.

Susceptibility to infection has been evaluated in mice from experiment JM-2 which received a 6-month exposure totaling either 240 rad of
neutron or 855 rad of γ radiation (4). In the case of γ-irradiated animals from the various groups, susceptibility was influenced by fractionation and instantaneous dose rate, but in an unexpected direction. Mice that received three 15-min exposures per week at 0.9 rad/min showed a significantly increased susceptibility to infection compared with animals given one exposure per week or one exposure per 4 weeks. One might expect that animals receiving the highest single dose per fraction (those exposed every fourth week) would exhibit the greatest injury as is the case for overall mortality among γ-irradiated animals in experiment JM-2. No difference in susceptibility to respiratory infection was observed in neutron-irradiated animals exposed three times per week as compared with animals given one exposure per week. Comparisons between neutron- and γ-irradiated animals indicate that the RBE under conditions of dose fractionation is greater than 2.6. This observation is generally consistent with our other results that indicate greater accumulation of injury to the hematopoietic system as a consequence of fractionated neutron exposure compared with fractionated exposure to γ radiation.

TABLE II-9. Susceptibility to *Mycoplasma pulmonis*-Induced Pneumonia in Neutron or Gamma Irradiated Mice

<table>
<thead>
<tr>
<th>Time of Challenge</th>
<th>Radiation-Dose</th>
<th>Challenge Dose (CFU)</th>
<th>Pneumonia Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks after acute exposure</td>
<td>fn 192-240</td>
<td>10^7</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>γ 740-920</td>
<td>10^7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>10^7</td>
<td>10</td>
</tr>
<tr>
<td>36-62 weeks after first fractionated exposure</td>
<td>fn 240</td>
<td>2.5 x 10^{11}</td>
<td>51</td>
</tr>
<tr>
<td>40-66 weeks after first fractionated exposure</td>
<td>γ 855</td>
<td>2.5 x 10^{11}</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Aged controls</td>
<td>2.5 x 10^{11}</td>
<td>14</td>
</tr>
</tbody>
</table>

^aMidline tissue dose (rad); fn, fission neutrons.

^bMice challenged 12-42 weeks after completion of 6 months of fractionated neutron or gamma irradiation.
REFERENCES


DETERMINATION OF THE RBE FOR CHROMOSOMAL ABERRATIONS DURING THE CELL CYCLE OF CULTURED HUMAN LYMPHOCYTES

Anthony V. Carrano

PURPOSE AND METHODS

The differential response of cultured mammalian cells to low LET radiation during the cell cycle is well documented. The effects of high LET radiation are not as well defined, especially with respect to the induction of chromosomal aberrations. Current evidence from X-ray data indicates that radiation-induced cellular lethality is likely mediated through chromosomal damage, at least at low to moderate doses of radiation. Thus it would be of direct concern to both radiotherapists and experimental biologists to establish the response of chromosomal damage following neutron irradiation throughout the cell cycle.

The human leukocyte system was chosen for study, as these cells can be readily stimulated to enter a cycle of synchronous growth and are easily handled during treatment and preparation. Cultures will be irradiated with JANUS neutrons (6.25 rad/min) or $^{60}$Co $\gamma$ (approximately 50 rad/min). Subsequent to an analysis to determine the length of each phase of the cell cycle, appropriate times during the cycle will be selected that will permit irradiation in unstimulated ($G_0$), stimulated ($G_1$), early $S$, late $S$, and $G_2$ cells. The required population will be monitored by pulse labeling with $\text{H}^3\text{HdR}$ immediately prior to irradiation for proper identification. For each time interval the cells will receive 25, 50, 100, or 150 rad of neutrons, or 50, 100, 200, or 300 rad of $^{60}$Co $\gamma$ rays.
PROGRESS REPORT

The neutron irradiation for the unstimulated blood has been completed. Preliminary analysis of the dose response indicates that the RBE for chromosome deletions as compared to 250 kVp X rays (X-ray data obtained from a separate analysis) is approximately 4.5. Two-hit aberrations (dicentrics, centric rings, and tricentrics) demonstrated a linear response following neutron exposure with a slope of about 1.0, wherever the slope for chromosome deletions was about 0.9.

IDENTIFICATION OF THE CHROMOSOMES IN A MURINE LEUKEMIA STEM LINE BY AUTOMATED BANDING ANALYSIS

Anthony V. Carrano and Donald Hodges

PURPOSE AND METHODS

Procedures have been developed in the past two years that permit not only the rapid identification of individual chromosomes within a species but also the deviation of each chromosome from an established norm. These chromosomal banding techniques have thus become extremely valuable in all major cytogenetic studies, especially in clinical diagnosis. This procedure normally necessitates a tedious and subjective karyotype analysis in which individual chromosomes must be cut from photographic paper and classified according to their banding patterns and size. The ALICE image processing system permits automation of this procedure and thereby offers the distinct advantages of a less subjective and a more rapid analysis.

Cells from the spleens of leukemic B6CF1 mice (described elsewhere in this report) were placed in suspension, prepared for cytogenetic analysis by hypotonic treatment and acetic-alcohol fixation and dropped on wet slides. Normal chromosome spreads were made from mouse bone marrow, testes, spleen, or 9 to 13 day embryos. Chromosomal banding patterns were obtained by either the method of Schnedl (1) or a modified trypsin procedure. Photographs of the banded chromosomes were taken and the 35-mm negatives analyzed by the ALICE system to provide density profiles of the banding patterns in each chromosome. Identification of homologous chromosomes and alterations among the chromosomes (translocations or inversions) is currently being accomplished by comparison of the density profiles in the normal and abnormal chromosomes.

*Applied Mathematics Division, Argonne National Laboratory.
PROGRESS REPORT

To date approximately 75 chromosome spreads have been examined. Not all have been satisfactory for analysis by the ALICE system, either because of insufficient contrast on the negative or because of overlapping or touching chromosomes within each spread. These problems can be overcome by further refinement of existing procedures.

The transplantable leukemic cell possesses 41 chromosomes (2n=40), including a submetacentric marker chromosome. Preliminary studies indicate two possible explanations. The first possibility is addition of an extra chromosome in linkage group XVIII, while the submetacentric marker may result from a translocation within the mouse genome. The alternative is that the submetacentric marker is an additional chromosome itself. C-banding studies, which stain specifically for repetitive DNA in the mouse, appear to support the latter hypothesis.

REFERENCE


MEASURE OF CAPILLARY BLOOD FLOW IN IRRADIATED MOUSE TISSUES

S. Phyllis Steamer and Emily J. B. Christian

The radioactive inert gas, $^{133}$Xe, dissolved in physiological saline solution, is used to measure capillary blood flow in localized tissue regions. The efficiency of the capillary circulation can be estimated from measurements of the rate at which radioactivity is lost from the region. This procedure has been used in experimental animals and in man to measure capillary blood flow in various tissues and in tumors (1-3). Xenon is a lipid-soluble gas that is rapidly distributed by diffusion throughout a tissue. It passes through the capillary endothelium into the circulating blood and is removed in the venous outflow from the region into which it was introduced. Xenon is then carried to the lungs where, it is estimated, about 95% of the injected amount passes out of the body via the expired air in the first circulation (4).

In our study of long-term radiation effects on microvascular changes in the mouse, radioxenon washout has been used to measure capillary efficiency in the lung, liver, kidney, and subcutaneous region of the dermis. Changes in local capillary blood flow are of special interest in relation to age and radiation treatment. $^{133}$Xe (Amersham/Searle), dissolved in isotonic NaCl solution, was either injected directly into the tissue or via the arterial blood. The concentration was 2 to 5 mCi/ml, and a volume of
0.02 to 0.10 ml was used for each determination of tissue clearance rate. A Packard 400-channel analyzer, equipped with a NaI crystal (1-inch diameter, 1-inch thickness), was used to count the 80-keV γ-ray emitted by $^{133}$Xe. The crystal was collimated to monitor either the entire mouse with the exception of the tail, for which the mouse was placed against the end of the collimator (6 cm from the crystal), or the entire body including the tail, for which the mouse was placed at a distance of 21 cm. The background activity was recorded before injection of $^{133}$Xe, and sequential counting was continued without interruption during the injection and the subsequent washout period. The time per channel ranged from 0.5 to 5.0 sec, depending on the washout rate of the tissue (Table II-10). After appropriate corrections for background and for dead time, the constants of the disappearance curve, fitted either to a single or to a two-exponential function, were determined for individual tissues.

When $^{133}$Xe was injected into the tail vein, the disappearance rate reflected rate of blood flow through the lungs. For determinations of capillary flow in liver and kidney, the isotope was injected directly into the parenchyma of these tissues. For determination of subcutaneous microvascular efficiency, injection was made into the loose connective tissue of the dermis. The disappearance of xenon from the dermis was usually adequately fitted by a single exponential, while from the other tissues the kinetics typically followed a two-exponential curve. The faster component is considered to reflect regional capillary blood flow, and the slower component probably reflects recirculation of the isotope. In regions of high capillary efficiency (e.g., lung) a relatively small fraction is recirculated. Preliminary control values for the faster component of the washout curve is shown in Table II-10. The initial lag that is sometimes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time per Channel (sec.)</th>
<th>Total Recorded Washout Time (min.)</th>
<th>$T_{1/2}$ Faster Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung (I.V. injection in tail)</td>
<td>0.5</td>
<td>3</td>
<td>3-6 sec.</td>
</tr>
<tr>
<td>Liver</td>
<td>2.0</td>
<td>12</td>
<td>45-60 sec.</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.0</td>
<td>19</td>
<td>60-90 sec.</td>
</tr>
<tr>
<td>Subcutaneous region of the dermis</td>
<td>5.0</td>
<td>31</td>
<td>~8 min.</td>
</tr>
</tbody>
</table>
observed may be caused by a localized effect of the injection solution. Xenon disappearance is either directly or indirectly dependent on its expiration from the lung; therefore, lung washout was determined whenever possible at each sampling time.

Vasodilatation and venular tortuosities that occurred within 3 or 4 days after a radiation exposure in the acute lethal range appeared to be associated with a slower clearance, while washout rates tended to be faster when the vasculature was constricted. Data are not sufficient for correlations between xenon washout from various tissues and the blood vessel diameters at later times after irradiation.

REFERENCES


MICROVASCULAR EFFECTS OF NEUTRON AND GAMMA RADIATIONS IN THE MOUSE

S. Phyllis Steamer and Emily J. B. Christian

Microvascular damage (e.g., increased permeability, vasodilatation, endothelial cell injury, and stasis) is a component of the acute syndrome after single, whole-body exposures to ionizing radiations. Late effects include parenchymal degeneration as a direct or indirect result of chronic inflammatory changes and microvascular fibrosis. Observations of the early microvascular changes after whole-body exposures in the acute lethal range are made in vivo in the vasculature of the mouse pinna, or external ear. Investigations of long-term microvascular damage and its relation to tissue function are also made. Principal objectives include evaluation of the comparative effectiveness of fast neutrons and $^{60}$Co $\gamma$ rays, and the influence of dose protraction over 6 months or longer.

Attention during the past year was directed toward longitudinal studies of the structural and functional effects on the microvasculature in the pinna, which can serve as an indicator of the condition in other tissues of the body. These studies are complemented by determinations of
microcirculatory efficiency (calculated from the rate of $^{133}\text{Xe}$ washout) in a subcutaneous region, usually over the hind leg or back (1). The comparative effectiveness of fast neutrons and $\gamma$ rays is of special interest in studies of long-term effects of fractionated exposures. Our initial investigations of early effects after doses in the acute lethal range will provide a basis for comparison with later effects in animals exposed at low-dose levels.

Female B6CF1/Anl[Anl 66] mice were 100 to 120 days of age at the time of exposure to either fast neutrons from the JANUS reactor or to $^{60}\text{Co} \gamma$ rays. Conditions of irradiation and maintenance were those in use for experiments in other phases of the JANUS Program (2). Exposure series include the 72-fraction groups in the JM-2 experiment and single dose exposures to 100, 300, 420 (LD$_{50/30}$), or 500 rad fast neutrons, or to 300, 900 (LD$_{50/30}$), or 1200 R $^{60}\text{Co} \gamma$-rays (exposure time 15 to 30 min). Technical details for microscopic study of the pinna in the living mouse have been described (3). In order to record the long-term changes over the life of individual animals, maps of the major blood vessels of the pinna are constructed from 2-X enlargements of 35-mm Kodacolor photomicrographs. There is a similar pattern of arteries and veins in most individuals, with only minor variations in the details of the branching. Measurements of vessel diameters are taken from three representative regions of the three or four principal branches of the central vessel. Details of the capillary endothelium and dynamic changes in the circulation are determined from direct observations and from film strips taken during the periodic microscopic examinations. The film strips provide the greatest information content, and details of the vessel wall and contained cells can be determined at relatively high magnifications.

After an exposure in the acute lethal range, a vasodilatation was present in both arterioles and venules within 1 or 2 days (Table II-11). Stasis in a few vessels, principally venules and capillaries, and tortuosities in some smaller veins and venules were also seen. Vasodilatation decreased after a week or so in most individuals that survived beyond 30 days. In contrast, petechial hemorrhages and stasis were prominent in superficial vessels for as long as a week or two before death in 30-day decedents. Occasionally, although vasodilatation was present, blood flow and heart rate appeared abnormally fast and there was an increased number of functioning vessels. In any given treatment group, individuals in which these responses were observed usually survived longer than did those with sluggish blood flow. After 30 to 60 days, vessel diameters returned to the control range. After 6 to 12 months, the capillary network appeared somewhat decreased, arterioles showed regions of stenosis, while local saccule-like dilatations and tortuosities were especially prominent in small veins and venules. Mean arteriole diameters appeared somewhat reduced, but localized changes in venules were not reflected in mean values because measurements were taken from undilated regions of these vessels. Data on larger vessels of the pinna are not yet analyzed.

In the 72-fraction groups of the JM-2 series, observations are completed on only a small number of animals at 6 months after completion of the exposures. A few venules showed localized dilatations and tortuosities;
some arteriostenosis was present, but quantitative comparisons of the various treatment groups cannot be made at this time.

REFERENCES


RESPONSE OF MICE HETEROZYGOUS FOR GENES INDUCING MACROCYTIC ANEMIA TO SINGLE AND DAILY EXPOSURE TO IONIZING RADIATION

Douglas Grahn and Katherine H. Allen

There are several loci in the mouse genome characterized by allelic series that cause macrocytic anemia. Generally, the homozygotes have poor viability or are prenatal lethals, while the heterozygotes have nearly normal red cell and hematocrit values. Some dominant effect is evident in these hematopoietic factors, and the presence of the mutant is also recognized by an effect on coat color, either as white spotting, blazing, flecking, or general dilution. Two mutants have been maintained at the laboratory for diverse purposes, and information has been accumulated on the general response to single and daily exposures to X and γ radiation. These mutants are Ames dominant spotting (wa) and Steel (Sl). Both have normal viability in the heterozygote but are homozygous prenatal lethals. They are presently maintained as a double-marker stock, though wa is also kept in a separate stock (MWA). The radiation response of wa/+ vs. +/+ has been tested with single doses of X rays and with daily exposures to γ radiation. The Sl/+ mutant and Sl/+wa/+ double heterozygotes have only been tested with daily γ irradiation. The purpose has been to ascertain the effect these mutants might have on the erythropoietic syndrome (1,2) and to establish their potential value as a means to better understanding of this subacute syndrome.

Sl and wa have similar phenotypic manifestations on peripheral blood parameters, but have clear differences in the responsiveness of their marrow stem cells to proliferation-inducing stimuli (3). wa, for example, is attributed to a subnormal stem cell number and an inadequate response to erythropoietin. W-allele anemias can be cured by marrow transplants, even with Sl marrow, so the deficit is specific to the stem cell, not the general marrow physiology. Sl is not curable in this manner and the basis of its deficit is not clear. One contrasting feature, for example, rests in the spleen colony-forming capacity of irradiated stem cells of viable homozygotes of W/W vs. Sl/Sl. The Steel mice have normal colony-forming capacity while the wa mice are extremely reduced in this capacity, though microcolonies do form.

The response to radiation stress measured in survival terms has revealed the independence of genetic action of these two mutants. The wa/+ and Sl/+wa/+ mice have an identical response and show the most severe survival deficit at daily levels between 40 R/day and 100 R/day, where the erythropoietic syndrome predominates. Survival times are only 50% to 70% of that seen in their normal sibs. The maximum survival deficit is at 56 R/day. Normal survival is between 80 and 100 days at this dose level, well beyond the time of severe hematopoietic system injury. The survival of wa/+ and Sl/+wa/+ mice is only 30 to 50 days, typical of acute to subacute marrow damage. Below 40 R/day, the wa carriers progressively improve in
survival time and move toward their wild-type sibs, although a 10 to 25%
survival-time deficit persists.

The Sl/+ mice do not show any difference from their +/+ sibs, with a
possible exception at 74 R/day where survival time is only 90% of the normal
53 days. In all other instances, the Sl/+ animals show a response that is
remarkably similar to the normal.

The single-dose LD50/30 values for the Wa/+ and their +/+ sibs are
about 450 R and 650 R, respectively. There have not been sufficient Sl/+ mice to carry out single-dose testing on that mutant. The Wa/+ mice pre­
sent a picture consistent with the concept of a stem-cell deficient marrow
that is inadequately responsive to demand for increased formation of
erthrocytes. The Sl/+ mice seem essentially normal, and there is no
synergistic effect of the two mutants in the same individual. From what
is known of the behavior of these mutants, the present data support the
thesis that there is a time/dose-dependent erythropoietic syndrome. This
is normally only evoked by continued irradiation and is maximum between 35
and 50 days after the start of irradiation (about one red-cell life
expectancy).

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III. CARCINOGENESIS

SUMMARY

R. J. Michael Fry, Group Leader

The reports of progress are concerned with four areas of investigation: the role of isozymes in carcinogenesis, hepatic tumorigenesis and enhancement by phenobarbital, strain and species differences in susceptibility to oncogenic agents, and the effects of treatment with psoralen and near-ultraviolet (NUV) light.

A systematic search for alterations in isozyme patterns in chemically and radiation induced as well as spontaneous tumors has begun. The first group of enzymes examined were the dehydrogenases; both markedly increased enzyme levels and altered isozyme patterns were found in liver tumors induced by 2-acetylaminofluorene (AAF). Neither spontaneous nor radiation-induced tumors have so far shown consistent altered isozyme patterns. The consistent changes in the liver tumors are not a reversion to fetal patterns, nor does proliferative activity induced by partial hepatectomy cause similar changes. The objective is to determine whether or not changes in the isozyme patterns in tumors are due to altered gene activity.

The mechanism by which phenobarbital enhances the incidence of liver tumors induced by AAF is still not clear, but results of experiments this year have shown that enhancement of the chemical tumorigenesis only occurs with prolonged phenobarbital treatment, and occurs if phenobarbital is given even at a considerable time after the treatment with AAF. These findings would be consistent with an immunosuppressive action, and it has been reported that phenobarbital is an immunosuppressant. However in two experiments which have been carried out (one in collaboration with B. N. Jaroslow and M. Menon) to investigate the effects of phenobarbital on the growth of two transplanted tumors in mice, no evidence of immunosuppression was found. The important question of whether the enhancement effect of phenobarbital is restricted to liver tumors or is a more general phenomenon is being investigated in a study of chemical tumorigenesis in the skin.

The third area of study is concerned with the underlying causes for age-, strain-, and species-dependent differences in susceptibility to oncogenic agents. So far these experiments have been restricted to in vivo experiments, but to complete these studies it will be necessary to use organ and cell cultures. The question of the relationship of both age and proliferative activity to susceptibility to chemical tumorigenesis is still unanswered. Findings reported this year suggest that in the case of AAF-induced tumors, susceptibility is related to the age-dependent proliferative activity. It is probable that the strain- and species-dependent
differences in susceptibility are determined by inherited factors, such as metabolic patterns and the presence of endogenous viruses. Such factors as immune competence and proliferative activity are also probably important, especially in alterations of the latent period. The alveologenic tumor is a good test system for these studies of the cell kinetics of these pulmonary tumors, and it appears that the transplanted alveologenic tumor will be a useful system for studying various aspects of systemic effects on tumor growth, as well as kinetics of tumor cells.

Perhaps the most interesting result in the studies on the effects of furocoumarins and NUV light is a negative one, namely, the lack of carcinomas in the treated mice. The question arises whether the carcinogenic effects found by others depend on lower wavelengths than 365 nm despite the fact that cellular and tissue effects, such as inhibition of DNA synthesis and erythema, occur with the ~ 365 nm light source.

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*Terminated during 1972
INCREASED TOTAL ACTIVITY AND ISOZYME COMPLEXITY OF ALDEHYDE DEHYDROGENASE IN CERTAIN RAT HEPATOMAS

Robert N. Feinstein and Erma C. Cameron

PURPOSE AND METHODS

In a systematic search for modifications in isozyme patterns in tumors, we first examined the enzyme class of dehydrogenases. As we have reported (1), we found that rat hepatomas induced by the feeding of 2-acetylamino-fluorene (AAF), followed by phenobarbital, show a greatly increased total activity of aldehyde dehydrogenase (Ald D) (EC 1.2.1.3), as well as a considerable increase in complexity of the isozyme pattern of this activity. We wish to study how general this phenomenon is, and to investigate its possible physiological and oncological significance.

The methodology is essentially twofold: (a) assay of the enzyme activity by measurement of NAD reduction, and (b) observation of isozyme patterns on polyacrylamide gels after electrophoresis, followed by incubation with NAD, phenazine methosulfate (PMS), and nitroblue tetrazolium (NBT). The NBT is reduced, by transferred electrons, to an insoluble dark formazan, which forms bands on the gel corresponding to zones of Ald D migration.

PROGRESS REPORT

Although we have observed occasional increases in total Ald D activity and isozyme complexity in other tumors, consistent changes have only been found in hepatomas induced by AAF, with or without phenobarbital. It is of importance that the phenomenon is observed in tumors induced without the use of phenobarbital, because Redmond and Cohen have shown (2) that phenobarbital alone can induce Ald D activity in normal rat liver, and Deitrich has found (3) that this inducibility is a genetic trait, observable in some rat substrains and not in others. We have attempted to develop inducible and non-inducible substrains of Sprague-Dawley rats obtained from Charles River Breeding Laboratories; however, all rats tested proved to be non-inducible.

A surprising finding has been the observation that several of H. P. Morris' "minimal deviation" hepatomas do not show an increased Ald D activity. The three Morris hepatomas thus far tested are:

(a) 9618B, average transfer time 10 months, cells well-to-highly differentiated, originally induced by 2-(4'-methyl)benzoylaminofluorene (MBAF)

(b) 7777, average transfer time 1 month, poorly differentiated cells, originally induced by N-2-fluorenlyphthalamic acid (FPA)

(c) 5123tc, average transfer time 1.2 months, cells poorly-to-well differentiated, also originally induced by FPA.
Thus three rat hepatomas, ranging from poorly to highly differentiated, do not show the phenomena of high Ald D activity and complex Ald D isozyme pattern exhibited by our own AAF-induced rat hepatoma.

CONCLUSIONS

There appear to be three possible explanations for the differences between our AAF-induced tumors on the one hand, and Morris' hepatomas on the other: (a) We use Sprague-Dawley rats, and Morris uses the Buffalo strain; (b) we use AAF for tumor induction, and Morris uses other carcinogens; and (c) our hepatomas are directly induced by feeding carcinogen, while Morris' hepatomas represent a great many generations of transplantation. We are investigating these three possibilities; it would be of particular interest if it should develop that continued re-transplantation causes loss of a biochemical ability, a phenomenon well known in cases of prolonged tissue culture.

REFERENCES


DETECTION OF OXIDASES ON POLYACRYLAMIDE GELS

Robert N. Feinstein and Ronald Lindahl*

PURPOSE AND METHODS

In a continuing systematic search for modifications in isozyme patterns in tumors, we wished to investigate the enzyme class of oxidases, those enzymes which use molecular oxygen as electron acceptor and, generally, produce $H_2O_2$. However, this enzyme group has been very little studied from the point of view of detection and localization on gels, so a necessary preliminary was the development of adequate detection methodology.

PROGRESS REPORT

After investigation of a variety of possible detection methods, we have decided that by far the most sensitive and most definitive method is the use of the electrons liberated in the oxidation to reduce nitroblue

*Summer Graduate Student in Biology from Department of Biology, Wayne State University, Detroit.
tetrazolium (NBT), either directly or via mediation by phenazine methosulfate (PMS). In the absence of PMS, the reaction is a great deal slower, but the background coloration is lighter, often permitting a sharper contrast and better delineation of isozyme lines.

Because the reduction of NBT to the dark, insoluble formazan is potentially a non-specific reaction, it was felt necessary to assure ourselves that we were truly observing only oxidases. To this end, we compared the PMS-NBT technique directly with the 3-amino-9-ethylcarbazole (AEC)-peroxidase technique, as described most recently by Robinson and Lee (1). The AEC-peroxidase technique is specific for oxidases, because it actually is totally dependent upon \( \text{H}_2\text{O}_2 \) production. Although the AEC-peroxidase technique is highly specific, it is considerably less sensitive than the PMS-NBT technique.

We have compared a series of oxidase activities in rat and mouse liver by the two techniques and have found perfect matching of mobility by use of the two detection methods in the following cases: L-amino acid oxidase, xanthine oxidase, urate oxidase, \( \alpha \)-hydroxy acid oxidase (short chain), and aldehyde oxidase. Because there was considerable variation in mobility of the various oxidases, yet the visible banding was always matched by the two techniques, we conclude that the sensitive PMS-NBT technique is specifically detecting oxidase activity.

CONCLUSION

We now have available a specific, sensitive method for the detection of a variety of oxidases on polyacrylamide gels, and after polishing details for maximal effectiveness, we plan to proceed with a survey of oxidase isozymes in fetal, normal adult, and tumorous tissues.

REFERENCE

animals are more susceptible to chemical carcinogenesis than older animals (1). In an attempt to resolve this question further, we exposed rats of two different ages (21 days and 41 days old) to dietary acetylaminofluorene (AAF) and measured tumor incidence. We also determined hepatocyte proliferative activity and carcinogen binding in rats of both ages to determine whether age-dependent changes in tumor incidence correlated with changes in these parameters. Details of the experimental procedure are as follows:

1. Tumor Incidence. Male rats, 21 days old, were divided into two groups, each containing 120 rats. Group 1 received a diet containing 0.02% AAF for 18 days followed by the control diet for the remainder of the experiment. Group 2 received the control diet for 21 days, then the AAF diet for 18 days, then the control diet. Beginning when the rats were 140 days old, representatives from each group were killed at intervals and examined for tumors. Examination of Group 1 ended when the rats were 310 days old; Group 2 was examined over an additional 265-day period.

2. Hepatocyte Proliferation. Male rats, 21 and 41 days old, were injected intraperitoneally with 0.5 μCi/g body weight of 3HdR at 6-hr intervals for 24 hr. Two hours after each injection 3 rats from each group were killed, and their liver nuclei were isolated (2) and assayed for radioactivity. Autoradiographs of both tissue sections and smears of isolated nuclei were prepared.

3. AAF Binding. This experiment tested the effects of both aging and phenobarbital treatment on AAF binding. The phenobarbital effect was studied because our previous work showed that phenobarbital given simultaneously with AAF reduced the incidence of tumorigenesis (3). Three groups of rats (3 rats per group) were used. Group 1 was 21 days old, Group 2 was 41 days old, Group 3 was also 41 days old but had received a diet containing 0.05% phenobarbital for 20 days. All rats were given a single intraperitoneal injection of 14C-labeled AAF at a dosage level of 0.1 μCi/g body weight. The AAF was suspended in cottonseed oil. All rats were killed after 8 hr; their livers were removed, and both the total radioactivity and that precipitated by trichloroacetic acid (TCA) were determined.

PROGRESS REPORT

1. Tumor Incidence. In Group 1, which received the AAF diet at 21 days of age, 21% of the rats examined before 310 days had liver tumors and 40% of the rats examined on the 310th day had tumors. In Group 2 which received the same AAF treatment as Group 1, but delayed by 3 weeks, no tumors were seen at 310 days and only 1 rat with a liver tumor was seen at the end of 575 days. These data, therefore, show a very striking age-dependent reduction in the susceptibility of rats to chemical carcinogenesis.

2. Hepatocyte Proliferation. Figure III-1 shows the uptake of thymidine liver into nuclei in rats 21 days and 41 days old. The uptake was more than ten times greater in the younger rats, indicating a much higher
level of proliferative activity in these animals. This correlates well with the much greater susceptibility of these animals to chemical carcinogenesis, as described above.

3. Carcinogen Binding. Table III-1 shows the effects of age and phenobarbital treatment, the total AAF uptake, and the amount bound to TCA precipitable macromolecules. The results indicate that the uptake and binding of AAF were increased in older animals but the percentage of AAF bound was slightly reduced. Phenobarbital treatment greatly decreased the amount of free and bound AAF in the liver.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total (^{14}\text{C} ) Labeled AAF in the Liver</th>
<th>Bound (^{14}\text{C} )</th>
<th>Percent Bound (^{14}\text{C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 days old</td>
<td>30.42(^{d}) ± 4.43</td>
<td>24.81 ± 3.01</td>
<td>81.6 ± 10.02</td>
</tr>
<tr>
<td>41 days old</td>
<td>67.81 ± 7.37</td>
<td>49.02 ± 10.02</td>
<td>72.3 ± 10.02</td>
</tr>
<tr>
<td>41 days old, 20 days</td>
<td>41.31 ± 8.53</td>
<td>24.81 ± 8.07</td>
<td>57.5 ± 10.02</td>
</tr>
<tr>
<td>phenobarbital</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Total radioactivity (dpm) per mg wet liver  
\(^b\)Cold TCA insoluble radioactivity (dpm) per mg wet liver  
\(^c\)(Bound/total) \times 100  
\(^d\)Average ± S.D. from 3 rats
The age-dependent increases in overall AAF uptake and binding do not correlate well with age-dependent decreases in susceptibility to tumorigenesis. Such a correlation might be uncovered by studies of the distribution of bound AAF among various classes of molecules.

Phenobarbital given with the carcinogen does lower both AAF levels and tumor incidence. Evidently, binding of AAF to critical target molecules is lowered by phenobarbital to the point where the molecular events leading to neoplasia are reduced.

CONCLUSIONS

Susceptibility of rats to AAF induced liver tumorigenesis is clearly age-dependent, with older animals showing a greatly reduced susceptibility. This reduction in susceptibility is not associated with any age-dependent reduction in the overall concentration of AAF in the liver. Such changes may occur however, in the distribution of AAF among various liver cell components.

The protective effect of phenobarbital on AAF hepatic tumorigenesis is accompanied by a reduction of AAF levels in the liver, indicating that phenobarbital decreases the amount of AAF able to reach its site of action.

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1. Toth, B. Cancer Res. 28, 727 (1968).

ENHANCEMENT BY PHENOBARBITAL OF 2-ACETYLAMINOFLUORENE-INDUCED HEPATIC TUMORIGENESIS IN THE RAT: CONSEQUENCES OF VARIOUS TEMPORAL COMBINATIONS OF 2-ACETYLAMINOFLUORENE AND PHENOBARBITAL TREATMENTS*

Carl Peraino, R. J. Michael Fry, Everett Staffeldt, and Walter E. Kisielweski

The enhancement by dietary phenobarbital of hepatic tumorigenesis induced in rats by the prior feeding of 2-acetylaminofluorene (AAF) was studied. A 5-day or 20-day treatment with phenobarbital produced little

*Abstract of a paper to be submitted for publication.
tumorigenic enhancement in comparison with that seen in rats receiving phenobarbital throughout the experiment. Conversely, the interposition of a 10-day or 30-day interval between the cessation of AAF treatment and the beginning of phenobarbital treatment (which was then continued throughout the experiment) had little effect on the degree of enhancement produced by beginning the phenobarbital treatment immediately after cessation of AAF feeding. These results indicated that: (a) prolonged exposure to phenobarbital was required for tumorigenic enhancement, and (b) the pre-neoplastic molecular lesion produced by AAF was relatively stable, and its expression could be enhanced by phenobarbital long after the cessation of AAF treatment. Observation of the kinetics of tumor incidence throughout the experiment showed that phenobarbital (a) decreased the latent period between the end of the carcinogen treatment and the appearance of tumors, (b) increased the growth rate of the tumors, and (c) increased the rate of appearance of new tumor foci.

INTERACTIONS AMONG REGULATORS OF RAT LIVER ORNITHINE AMINOTRANSFERASE*

Carlo Peraino

The activity of ornithine aminotransferase in rat liver was increased as the level of dietary protein was raised. Triamcinolone administration elevated ornithine aminotransferase activity in rats fed a low protein diet but depressed the enzyme in rats fed a high protein diet. At intermediate dietary protein levels the effect of triamcinolone on ornithine aminotransferase was minimal. Glucagon also enhanced the enzyme activity in rats on low protein. In rats on high protein, glucagon slightly depressed ornithine aminotransferase activity but counteracted the stronger negative effect of triamcinolone on the enzyme. The latter effect was not accompanied by a similar reversal of the positive glucocorticoid effect on serine dehydratase in the same animals.

These results suggest the existence of a homeostatic control system for ornithine aminotransferase whereby endogenous regulators (e.g., glucocorticoids) tend to maintain the enzyme at a finite "basal" level in the face of unbalancing influences of exogenous regulators (dietary constituents). The specific reversal by glucagon of the negative glucocorticoid effect on ornithine aminotransferase suggests that glucagon interferes in some manner with the interaction of glucocorticoids with the regulatory system for ornithine aminotransferase.

*Abstract of a paper to be submitted for publication.
SEPARATION OF PEPTIDES FROM CHYMOTRYPTIC DIGEST OF ORNITHINE AMINOTRANSFERASE

Carl Peraino and J. Emory Morris

PURPOSE AND METHODS

The unique adaptive and molecular properties of ornithine aminotransferase (1-4) render this enzyme a useful tool for studies of the mechanism of gene expression in normal tissue and in tissues undergoing carcinogenesis. Because a full understanding of the biological behavior of ornithine aminotransferase requires a thorough knowledge of its molecular properties, an investigation of the molecular structure of the enzyme has been initiated. This involves both the determination of the amino acid sequence of the molecule and the determination of tertiary structure of the molecule, using X-ray crystallography. The present study describes the initial phases of our work on the separation of chymotryptic peptides, which will subsequently be analyzed for amino acid sequence.

Ornithine aminotransferase is purified from rat liver and crystallized as previously described (3). The crystals are dissolved, and the solution is treated with solid sodium borohydride to reduce and thereby stabilize the linkage between the prosthetic group (pyridoxal phosphate) and the apoenzyme. This prosthetic group is tritium-labeled by injecting tritiated pyridoxine into the rats from which the enzyme is subsequently isolated. The enzyme is then denatured in urea and treated with iodoacetate in order to carboxymethylate the cysteine sulfhydryls and to prevent the formation of disulfide bonds during subsequent steps.

The reduced carboxymethylated enzyme is incubated with chymotrypsin, which cleaves the enzyme into peptides. The peptide mixture is then treated according to the procedure of Chin and Wold (5). This involves application of the mixture to a phosphocellulose column and elution of the peptides with KCl gradient. Absorbance is monitored continuously at 230 nm and an aliquot of each collected fraction is monitored for radioactivity. Individual peaks are desalted on BioGel P-2 and then applied to a TEAE column and eluted with a sodium borate gradient. Absorbance and radioactivity are monitored as described above.

The purity of the fractions is determined by high voltage paper electrophoresis. Fractions producing a single electrophoretic band are then subjected to amino acid analysis as a final criterion of purity.

*Summer faculty research appointment from State University of New York College at Brockport.
PURPOSE AND METHODS

Twenty-five peaks were eluted from the phosphocellulose column. Peak 12 was radioactive, indicating that it contained the pyridoxal binding peptide. Four of the peaks from the phosphocellulose column appeared pure by electrophoretic and amino acid analysis; other peaks contained several peptides. Separation of the peptides in these peaks on TEAE-cellulose is in progress.

CONCLUSION

The procedure outlined above appears to be suitable for the complete separation of peptides of ornithine aminotransferase, a prerequisite to the determination of their amino acid sequences.

REFERENCES


AUTOMATION OF CONTINUOUS SUSPENSION CULTURES BY MEANS OF A NEPHELOSTAT*

Carl Peraino and William J. Eisler, Jr.

Earlier studies (1) showed that the Nephelostat, originally developed for automated continuous culture of microorganisms (2), could be modified for use with suspension cultures of mammalian cells. The system has since been modified to improve both convenience and performance. These modifications are as follows:

1. General Layout. The arrangement of glassware and tubing resembles that shown in earlier studies (1), with two exceptions: first a trap is now included in the gas outflow line to prevent the sodium hydroxide solution,

*Abstract of an article to be published in Methods and Applications of Tissue Culture, Academic Press.
which is used as a contamination barrier, from inadvertently being aspirated into the culture vessel, and second, separate pumps are now used to control the inflow to, and outflow from, the culture vessel. The inflow pump is controlled by the Nephelostat control console, and the outflow pump runs continuously at a slow rate, preventing the volume of the culture from exceeding the level of the lowest open outflow port on the culture vessel. This was found to be the preferred method for maintaining the culture at constant volume. Since each pump used has two channels, the pumping capacity was doubled by connecting both channels in parallel, using silicone rubber tubing and stainless steel "Y" connectors fabricated at ANL.

2. Culture Vessel. Figure III-2 is a diagrammatic representation of the culture vessel now in use. The rotary stirrer and seal (1) have been replaced by a vertical vibrator which enters the vessel through an autoclavable Neoprene gasket. The turbulence produced in the cell suspension by this agitator is depicted by the circular pattern of arrows. The stainless steel agitator paddle has been offset from the gas stream in order to prevent excessive foaming. The gas outflow port is now sufficiently far from the surface of the culture to prevent any foam from entering the outflow tube and causing back-pressure. A pressure equalization tube now connects the sampling port cover with the main culture chamber. The vessel

Fig. III-2.—Diagrammatic representation of culture vessel.
also contains 3 culture outflow ports which are connected to a glass manifold and then via a single tube to the outflow pump. During operation at maximum capacity the tubes from ports 2 and 3 are clamped. Port 2 is opened in order to drain the main body of the culture vessel while still leaving the monitoring probe full. This operation permits long-term maintenance of the culture at low volume in order to conserve medium. Port 3 is opened in order to drain the system entirely or for partial drainage to clear the system of debris that might accumulate over a long period.

This system has been used successfully to maintain and monitor suspension cultures of HeLa cells and Chinese hamster cells (V79 line).

REFERENCES


SPECIES SUSCEPTIBILITY TO ONCOGENIC AGENTS: URETHAN DISTRIBUTION AND CLEARANCE IN 3- TO 4-WEEK-OLD AND ADULT C3Hf AND C57BL/6 MICE

Walter E. Kisielewski and R. J. Michael Fry

PURPOSE AND METHODS

Species, sex, and age differences in susceptibility to chemical oncogenic agents are well known (1) but not understood (2). The long-term objectives are to determine the bases of the differences in susceptibility. There appear to be at least two important aspects to the problem: (a) the metabolism of agents to the ultimate oncogenic agent, and (b) the spontaneous incidence of the specific tumor in the strain or species in question. Activation of viruses or even immunosuppression by the chemical agents may be possible mechanisms by which oncogenic chemical agents alter the spontaneous appearance of tumors. Factors which influence uptake distribution as well as metabolism are obviously of importance.

Clearly in vitro cell and organ culture methods can be used to study (a) whether a compound is an ultimate carcinogen or is metabolized to one in the target cells, and (b) whether the compound acts directly on the target cells. The rodent lung provides a model system for testing some aspects of these questions, though only at the organ culture level in the case of in vitro studies.

The initial experiments have been concerned with distribution and clearance of urethan as a function of age and strain.
Ethyl carbamate-\textsuperscript{14}C-labeled urethan (Nuclear Dynamics, El Monte, Calif.) was injected intraperitoneally at a dose of 1.0 mg/g of body weight. The solution used was 25.0 mg (12.5 \textmu Ci) of urethan per 0.1 ml of 1% saline.

A total of 144 animals were divided equally into four groups each containing an equal number of males and females. The first group consisted of 36 C3Hf/Anl[Anl 70] adult mice (10 to 12 wk old), 18 females and 18 males, the second, third, and fourth groups consisted of C3Hf young mice (3 to 4 wk old), C57BL/6Anl[Anl 70] adult mice (10 to 12 wk old), and C57BL/6 young mice (3 to 4 wk old), respectively. After injection of the carcinogen, 3 males and 3 females from each group were sacrificed by ether anesthesia at 1, 2, 4, 6, 24, and 48 hr. Blood samples were taken from each animal, and liver, lung, and thymus were removed, weighed and immediately frozen with dry ice. The tissues were kept frozen until prepared for liquid scintillation counting by an oxidative procedure, using a Packard Model 305 Tri-Carb sample oxidizer. The samples were then counted and their $^{14}\text{CO}_2$ content measured in a Packard Model 3375 liquid scintillation spectrometer. Counting rate data allowed determination of the specific activity of each tissue for each time interval.

PROGRESS REPORT

The results indicate no significant species, age, or sex difference in urethan distribution or clearance for 3- to 4-wk-old and adult C57BL/6 and C3Hf mice. These results agree with our earlier studies showing that urethan is taken up at the same level and cleared at the same rate in the resistant strain \textit{P. leucopus} (18 to 20 months old) as in susceptible C3Hf mice (3). There also appeared to be no difference in C3Hf mice at 85 and 282 days of age.

In addition, no species or sex difference in distribution and clearance of urethan was noted, in spite of the fact that C57BL/6 mice have a greater liver tumor and lesser lung tumor susceptibility than do C3Hf mice (4), and that males are more susceptible to hepatomas than females. Correlations between susceptibilities to spontaneous and urethan-induced lung adenomas in various mouse strains have been observed (5,6). These parallel susceptibilities to spontaneous and urethan-induced lung tumors indicate that these mouse strains differ in some parameter not peculiarly related to urethan; i.e., the parameter is unlikely to involve differences in urethan metabolism. If the same can be said of C3Hf and C57BL/6 mice, then our results bear this out.

CONCLUSIONS

Since our results tend to show no species, age, or sex differences in urethan distribution or rate of elimination for C3Hf and C57BL/6 mice at the ages studied, perhaps the question of tumor susceptibility is related to some genetic factor(s); for example, a viral agent rather than to metabolic differences. Both studies on the binding to macromolecules and the effects \textit{in vitro} on lungs in different strains and species will be necessary before any generalization can be made.
REFERENCES


CELL KINETICS OF MOUSE PULMONARY TUMOR

R. J. Michael Fry, Charles Zeller, and Anthony R. Sallese

PURPOSE AND METHODS

The spontaneous alveologenic tumor in mice grows slowly. Pulmonary tumors that occur in irradiated mice either appear earlier or grow more rapidly or perhaps both. In order to interpret the data for pulmonary tumor incidence in carcinogenesis experiments, some information about the growth and cell kinetics will be necessary. Chemically-induced pulmonary tumors have been found to be influenced by immunosuppression (1), and it is possible that radiation influences, similarly, the pulmonary tumor appearance. Therefore, a test system for investigating such aspects as immunosuppression would be useful. The objectives of the investigations are to determine (a) the cell cycle and proliferative characteristics of transplanted pulmonary tumors, and (b) the effect of immunosuppression on the cell kinetics of the tumors.

A number of pulmonary tumors from irradiated B6CF1An1[An1 70] male mice have been removed and minced; small fragments were implanted in young syngeneic hosts. In most cases, alveologenic carcinomas have developed from the implants; occasionally either a sarcoma or a mixed tumor has developed. The growth rate of the tumors increases after the first transplantation. We chose to determine the cell cycle after the third transplantation of a tumor which was a typical alveologenic tumor and retained its...

*Summer Graduate Student from Northern Illinois University, DeKalb.*
Characteristics. Twenty-eight tumor-bearing mice were injected with \(^3\)HTdR, 0.5 \(\mu\)Ci/g (S.A. 0.36 Ci/mM), and sacrificed at intervals between 1 hr and 27 hr.

**PROGRESS REPORT**

The fraction of mitoses labeled curve was obtained for the first 27 hr after injection of label. The data are shown in Table III-2. It was suspected that the tumor cells might have a long cycle time, but an estimate, useful for future experiments, could be obtained from the labeling index and the duration of DNA synthesis (S). The labeling index, 12.6% (11.5 to 14.6) shown in Table III-3, and the duration of S, 7.8 hr, give an estimate of \(\approx 43\) hr for the cell cycle. It can be seen from Table III-3 that the variation in proliferative activity for the 3 tumors sampled at 1 hr was small. Because the 28 tumors used in the experiment were similar in appearance and in proliferative activity, the system would seem to be satisfactory for studies of cell kinetics.

**CONCLUSIONS**

The murine alveologenic tumor should provide a suitable system for the study of factors influencing tumor growth. In an established transplanted pulmonary tumor the cell cycle time was estimated to be about 43 hr.

**TABLE III-2. The Fraction of Labeled Mitoses**

<table>
<thead>
<tr>
<th>Time</th>
<th>No. of Mitoses</th>
<th>Observed Fraction of Labeled Mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>311</td>
<td>0.064</td>
</tr>
<tr>
<td>2</td>
<td>202</td>
<td>0.925</td>
</tr>
<tr>
<td>3</td>
<td>203</td>
<td>0.985</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>1.000</td>
</tr>
<tr>
<td>7</td>
<td>105</td>
<td>1.000</td>
</tr>
<tr>
<td>8</td>
<td>301</td>
<td>0.973</td>
</tr>
<tr>
<td>9</td>
<td>198</td>
<td>0.585</td>
</tr>
<tr>
<td>10</td>
<td>213</td>
<td>0.280</td>
</tr>
<tr>
<td>12</td>
<td>206</td>
<td>0.085</td>
</tr>
<tr>
<td>15</td>
<td>203</td>
<td>0.035</td>
</tr>
<tr>
<td>19</td>
<td>203</td>
<td>0.080</td>
</tr>
<tr>
<td>22.5</td>
<td>225</td>
<td>0.055</td>
</tr>
<tr>
<td>25</td>
<td>213</td>
<td>0.140</td>
</tr>
<tr>
<td>27</td>
<td>208</td>
<td>0.330</td>
</tr>
</tbody>
</table>
REFERENCES


STUDIES ON THE EFFECTS OF PSORALEN AND NEAR-ULTRAVIOLET LIGHT

Donald Grube and R. J. Michael Fry

PURPOSE AND METHODS

We have chosen to use treatment with furocoumarins and near-ultraviolet (NUV) light to study skin carcinogenesis because this combined treatment produces a specific molecular lesion (1) that is mutagenic (2), and it is therefore possible to examine whether such a specific and mutagenic lesion is casually related to tumorigenesis. The long-term objectives are (a) to establish the acute effects of the combined exposure to NUV and furocoumarins, (b) to determine the carcinogenic effects of the combined treatment and of exposure to NUV alone, (c) to determine the relationship of cell proliferation and repair processes to tumor induction, and (d) to investigate the importance of systemic factors such as immune competence in skin tumorigenesis. This present report is concerned with initial studies on the carcinogenic effects and the effects of the combined treatment on cell proliferation.

Hairless mice were injected with 0.8 mg 8-methoxypsoralen (8-MOP) and exposed to $2.5 \times 10^6$ ergs/cm$^2$ NUV from Magnaflux lamps and after irradiation were injected with $^3$HtdR, 4.0 $\mu$Ci/g (S.A. 20 $\mu$Ci/mM), at 1/2, 2, 4, 8, 24, and 32 hr, and at 3, 4, and 7 days, and sacrifice 45 min later.

---

### TABLE III-3. Labeling and Mitotic Indices

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Total No. of Cells</th>
<th>Labeled Cells</th>
<th>Mitoses</th>
<th>Labeling Index</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>711</td>
<td>104</td>
<td>15</td>
<td>14.6</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>890</td>
<td>105</td>
<td>17</td>
<td>11.8</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>885</td>
<td>101</td>
<td>18</td>
<td>11.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

---
The total number of cells and the number of labeled cells were determined. The effects of increasing the NUV exposure to $1.2 \times 10^7$ ergs/cm$^2$ were also studied.

The initial studies for the selection of suitable doses for studying carcinogenesis were based on the findings of Griffin et al. (3). Female hairless mice, 4 months old, were given 0.4 mg 8-MOP intraperitoneally and exposed to $1.2 \times 10^7$ ergs/cm$^2$ NUV either two or five times per week for 6 weeks. Two groups given 0.8 mg 8-MOP were exposed to $2.4 \times 10^6$ ergs/cm$^2$ and $1.2 \times 10^7$ ergs/cm$^2$ five times per week.

Progress Report

The results of the effects of the combined treatment are shown in Figure III-3. The higher exposure level causes a brief increase in the number of labeled cells, presumably due to a greater inhibition of the S/G$_2$ transition than at the G$_1$/S interface. At the subsequent intervals, marked inhibition of the uptake of $^3$HTdR was noted. Mitoses were seen at 8 and 24 hr, but cellular and mitotic abnormalities, as well as cell debris, became prevalent. By the third day, marked edema and infiltration of the dermis by neutrophils were seen, and by the 5th day the epidermal cell population was reduced, at least, to 25% of the normal level. Within 10 days there was marked hyperplasia. At the lower exposure levels the inhibition of the uptake of $^3$HTdR was shortlived and by 32 hr there was a marked increase in the number of labeled cells, possibly attributable to an increased flux of cells from G$_1$. Evidence for unscheduled DNA synthesis has been found at 30 min after single and repeated exposures. When repeated exposures (5/week for 6 weeks) at this level were given, slight erythema but no desquamation occurred; but 2 months after termination of the treatment, focal ulceration appeared. There were marked changes in the dermis, including thickening of the small vessel walls, suggesting that the late epithelial breakdown was secondary to the vascular changes.

Fig. III-3.—The average value and ranges of the number of labeled cells per field in the basal layer of the epidermis at intervals after exposure of HR/Anl[Anl 66] mice to 8-MOP and NUV. ■ control:8-MOP but no NUV; □ 0.8 mg 8-MOP and $2.5 \times 10^6$ ergs/cm$^2$ NUV; ■ 0.8 mg 8-MOP and $1.2 \times 10^7$ ergs/cm$^2$ NUV. Up to 32 hr the cell density per field in the basal layer remains within control values and cells in DNA synthesis were found only in the basal layers.
In the groups of mice given fractionated exposures, no carcinomas have been found, although the experimental conditions are similar to those of Griffin et al. (3) who reported a 100% incidence of carcinomas or sarcomas on the ears of haired mice. Three out of 16 mice exposed to 1.2 x $10^7$ ergs/cm$^2$ have shown sarcomas, and 25% of the mice have had transient papillomas. No lesions, including erythema, have been found in mice exposed to either NUV or 8-MOP alone.

CONCLUSION

While the characteristic effects of photosensitization have been found with the combined treatment of 8-MOP and NUV light, as yet no neoplastic change that can be related to a specific lesion has been found. It remains to be seen if wavelengths other than 365 nm are important, as it has been found that topical administration of 8-MOP and exposure to light from a solar simulator produce 100% incidence of tumors (4).

REFERENCES


PHOTOREACTIONS OF 8-METHOXYPSORALEN AND DNA IN VITRO

Richard E. Ecker

PURPOSE AND METHODS

The phenomenon of cutaneous photosensitization by the furocoumarins has been known for centuries. Only recently has it been found that such sensitization can result in the induction of skin tumors, and with exposure to near-ultraviolet (NUV) light these compounds form covalent photoadducts with DNA. This has led to the suggestion that the photoaddition of the furocoumarin to DNA is the primary molecular lesion in this type of skin carcinogenesis.

In the present study, the photochemistry of DNA and the furocoumarin 8-methoxypsoralen (8-MOP) is investigated with particular reference to the kinetics of the photoaddition reaction. The binding of tritiated 8-MOP to
Escherichia coli DNA is measured. Irradiation is accomplished with a mercury vapor lamp, filtered to emit almost exclusively at a 365-nm wavelength.

PROGRESS REPORT

Because photodimerization of 8-MOP is substantial at psoralen concentrations as low as $10^{-6}$ M, photoaddition of 8-MOP to DNA cannot be treated as a simple first order reaction. The exact equation for the total reaction is complex, but workable with the aid of a computer. For the reactions:

$$8\text{-MOP} + \text{DNA} \xrightarrow{k_1} \text{photoadduct}$$
$$2(8\text{-MOP}) \xrightarrow{k_2} \text{photodimer},$$

estimates of $k_1$ and $k_2$ have been made.

$$k_1 = 7 \times 10^{-9} \text{ min}^{-1} \text{ D}^{-1} \text{ C}^{-1}$$
$$k_2 = 3 \times 10^{-5} \text{ 1-mole}^{-1} \text{ min}^{-1} \text{ D}^{-1},$$

where D is the incident dose rate in ergs/mm² sec and C is the molar concentration of nucleotide pairs in DNA. The rate constant $k_1$ also depends on the absorption cross section of the NUV-excitable molecule or complex (the exact nature of this entity is still undetermined) and on the quantum yield of the resulting photoaddition reaction. Neither of these parameters can be evaluated at this time, but there is some indication that their product, the "photoaddition cross section," is very small.

THE USE OF THE SCANNING ELECTRON MICROSCOPE FOR THE STUDY OF THE LUMINESCENCE OF BIOLOGICAL MATERIALS

Daniel G. Oldfield* and C. Theodore Chubb

PURPOSE AND METHODS

The objective is to develop the instrumentation and techniques for the use of fluorescence scanning microscopy. A successful technique has the potential of increasing by a factor of 10 to 100 the spatial resolution that can at present be obtained with fluorescence microscopy. There are obvious possible applications for such a technique, for example, the localization and partial quantitation of fluorescent or fluorescent-tagged substances on cell and tissue surfaces, in cells, and on chromosomes.

*Faculty Research Participant from DePaul University, Chicago.
A detector system consisting of (a) a focusing first-surface aluminum mirror, (b) a lucite double-prism and light pipe, (c) a glass vacuum-seal port assembly, and (d) a photomultiplier (EMI 9635QB) and amplifier (Keithley Picoammeter plus auxiliary amplifier) was designed, fabricated, and tested on the Cambridge Stereoscan II SEM. The materials used for the initial tests of the system included various types of glass (soft glass quartz glass, yellow-fluorescing glass, red-fluorescing glass); various dyes (acridine orange, hematoxylin, erythrosin), and various SEM mounting media (silver paint and several acrylic cements).

Biological specimens used for the tests were prepared in the form of cell suspensions which were spread on #1-1/2 coverslips, briefly air-dried, and then fixed in Carnoy's solution. The cell types used were chiefly mouse peritoneal leukocytes, mouse spleen lymphoid cells, and Ehrlich mouse acites tumor cells.

When comparisons of phase microscope and SEM images were desired, the coverslip preparation was first examined by phase microscopy and cells of specific type (e.g., lymphocytes), or cells in specific phases of their cycle (e.g., in mitosis) were found and mapped, and a 3 mm x 4 mm segment ("chip") of the coverslip containing these cells was cut out for further processing. Specimens (glasses, dyes, chips from one or more coverslips), after being mounted with silver paint on a standard SEM stub, were very lightly gold- or carbon-coated in vacuum. These specimens were then examined in the SEM and operated in either the conventional secondary electron mode or in the luminescence mode.

PROGRESS REPORT

The light emitted from specimens of interest (glasses, dyes, cells) produces, at present, images recordable at 1000 lines per frame with an exposure of 200 sec or less using Polaroid 55 P/N film. Replacement of the present photomultiplier and amplifier with more suitable types can be expected to decrease the exposure time somewhat.

The planar surfaces of such specimens as glasses, dye crystals, or well-flattened and dried cells are imaged by the present optical setup without shadowing effects, but when surface details in the specimen are present, the shadowing of one detail by another will occur because of the configuration of the mirror optics employed. Taking two or more photographs of the same specimen at different stage-rotation angles could resolve this problem. Alternatively, replacement of the mirror optics with 3 or more light pipes viewing the specimen from different angles might eliminate this problem.

Several materials other than the specimens used, such as aluminum oxide on the stub surface, slat crystals, Uvac Mountant, etc., luminesce under electron bombardment. Therefore, it is important that background subtraction, comparisons of stained and unstained materials, and possibly some simple form of spectral analysis be used so that fluorescence which is related to a specific dye contained in the specimen can be analyzed.
A dependence of light emission on incident beam energy (kilovoltage) is present. Thus, it may be feasible to use various depths of penetration into (and hence analyses of) cells and tissues.

CONCLUSION

The progress, so far, is encouraging; however, considerable increase in sensitivity is necessary to realize the potential of the method.

STUDIES ON A TRANSMISSIBLE MURINE LEUKEMIA

E. John Ainsworth, Rosemarie L. Devine, R. J. Michael Fry, Sharron L. Nance, M. Patricia Nielsen, Theodore N. Tahmisian, John F. Thomson and Sandra L. Tollefsen

PURPOSE AND METHODS

Among the objectives of these studies are (a) to isolate and characterize the virus which is presumed to be the agent involved in transmission of the lymphocytic leukemia originally derived from an irradiated mouse and which can be transmitted with cell-free filtrates (1), and (b) to study the sequential changes in the development of the leukemia after transmission by both cell-free preparations and spleen cells from leukemia donors.

Leukemic cells or plasma were injected into hosts, one group of which was exposed to 200 R of 250 kVp X rays. Mice were sacrificed from these four groups at various time intervals between 1 and 60 days, and samples of lymph nodes, spleen, and thymus were fixed for electron and light microscopy. In another series, 3 donor mice were given injections of $^3$HTdR (10$\mu$Ci/mouse) at 6 hr intervals in order to label the leukemic cells. Spleen cells labeled in this way were injected into irradiated hosts ($5 \times 10^7$ cells/mouse) and samples were taken at 2, 24, 48, and 72 hr after injection. Autoradiographs were prepared from imprints, smears, and sections.

Spleens from control and leukemic mice (4.3 g in each case) were homogenized in five volumes of ice-cold Hanks' solution. The homogenates were centrifuged for 10 min at 500 rpm (2500 x g) in a Sorvall RC-2 centrifuge at 2°C. The supernatants were removed by aspiration and centrifuged for 55 min at 30,000 rpm (106,000 x g) in a Spinco centrifuge (30 rotor) at 2°C. The pellets were resuspended in 2 ml of Hanks' solution, and 1.5 ml of the suspension were layered over 35-ml linear gradients of 0.15 to 0.65 M sucrose. After centrifugation for 45 min at 20,000 rpm in an SW-27 rotor, the upper 15 ml of the gradients were removed, diluted to about 40 ml with Hanks' solution, and centrifuged for 60 min at 30,000 rpm (30 rotor). The pellets were again resuspended in 2 ml of Hanks' solution, and 1.8 ml were layered
over 35 ml-gradients of Hanks' solution and meglumine diatrizoate, USP (Reno-M-76, Squibb), 0 to 66% concentration. This gradient was centrifuged for 16 hr at 25,000 rpm in the SW-27 rotor.

PROGRESS REPORT

A band unique to the leukemia preparation was observed at a density of 1.16 g/cm³. This layer, as well as the corresponding fraction from the control tube, was removed, diluted with four volumes of Hanks' solution, and centrifuged for 150 min at 30,000 rpm (81,500 x g) in a 50-Ti rotor. The pellets were resuspended in 1.0 ml Hanks' solution; the suspensions were diluted with nine volumes of saline and 0.5 ml was injected intravenously into mice that had received 200 R of 60Co γ radiation. The presumed virus band was shown to contain RNA. An aliquot was treated with perchloric acid, the precipitate was incubated in 0.3 N KOH for 1 hr at 37°, the solution was acidified with perchloric acid and then centrifuged, and the absorption of the supernatant at 260 nm was measured. The RNA content was calculated to be about 0.7 mg/g of spleen.

After 132 days, hematologic examination revealed a lymphocytosis; white cell counts in the 15 animals ranged from 15,000 to 28,000/mm³ with 74 to 90% lymphocytes. A small number of lymphoblasts were noted in the blood smears. Three mice were sacrificed 1 week later, and, apart from slight enlargement of the spleen, no abnormalities were found. On histological examination the only finding was a marked proliferative activity of the germinal centers of the splenic nodules. These cells are being examined for evidence of virus. Further examinations will also be necessary to establish unequivocal evidence of lymphoma.

The electron microscopic findings were as follows: (a) C-type virus particles were not seen in the lymphoid tissues in either control unirradiated or irradiated B6CF1 mice (except many months post-irradiation); (b) virus particles were seen in the lymph nodes of both the unirradiated and irradiated hosts 1 day after injection. In the case of tumor cell suspensions transferred by intraperitoneal injection into unirradiated hosts, virus particles were found after the first day, but in the irradiated hosts virus particles were not found until the 8th day after injection. In the irradiated hosts aberrant formations of large cuspid-type membranes were seen within the rough endoplasmic cisternae (2). It is not clear why irradiation of the hosts results in such structures, but it appears to be related to the irradiation injury of the host cells.

Labeled tumor cells were found in most of the lymphoid tissue sampled at 24 hr after intraperitoneal injection with the exception of the thymus. The greatest number of labeled cells was seen in the renal node. The distribution and subsequent proliferation of the tumor cells is the pattern expected for a malignant lymphoma. Proliferation of the tumor cells also appeared early in the renal nodes (48 hr), whereas in the spleen, even at 3 days, the proliferation was slight. By 6 days all lymphoid tissues examined, except the thymus, were overwhelmed by the lymphomatous growth.
REFERENCES


IV. AGING

SUMMARY

George A. Sacher and Robert J. Flynn, Group Leaders

The ultimate objective of all aging research is to prolong the useful life of man. However, the attempt to prolong human life is an action with such grave implications for man's future that not even a first step should be taken toward any specific approach unless it promises the possibility of satisfying a biologically sound social ethic.

The research program of the Aging Research Group is shaped by an awareness of this requirement. It is built around the postulates that: (a) longevity is an evolved characteristic, gained by the operation of natural selection in circumstances where increased longevity has selective advantage; (b) specifically, evolutionary increase of mammalian lifespan is accomplished by natural selection for improvements in definite physiological and biochemical mechanisms for preventing or reversing deleterious aging changes; and (c) these mechanisms are present in all mammals and their quantitative expression is controlled by regulator genes. The optimum research strategy implied by this view of aging is one in which the aging process of short- and long-lived populations are carefully compared in order to discover what it is that the longer-lived population does better. This knowledge can then be turned to the development of feasible euphenic procedures for non-reproductive transfer of genetic information that will improve the effectiveness, within individual ontogeny, of those systems shown to be important for life prolongation. This evolutionary-comparative, euphenic approach is the core of our research program.

Such a program inherently requires the examination of both specific biological aspects of the aging process at the cellular and organism level, and the environment of the organism. Special account must be taken of factors that accelerate the aging process or reduce lifespan by other means, such as radiation and very probably other environmental pollutants.

Significant accomplishments during the past year include: (1) determination that the wild-type house mouse (Mus musculus) has a rate of aging (as expressed by the doubling time for the mortality rate) less than half that of the laboratory mouse (also Mus musculus); (2) confirmation that the number of neurons and nerve fibers decrease from maturity to old age in the white-footed mouse and the house mouse, but it is not yet determined if the rate of loss varies in different species; (3) determination that longevity in inbred mice is related to the ratios of kidney to liver weight.
(in which a high ratio is advantageous) and of tail length to body length; (4) determination that differences in longevity in inbred laboratory mouse strains are due to "vigor" and not to differences in rate of aging; (5) determination that antibody-forming capacity and the quality of antibody produced is the same in old and young dogs except that old dogs require more intensive antigenic stimulation; (6) determination that senescence does not significantly offset the ability of mice to recognize and clear foreign substances from the body; (7) determination that aged mice are capable of producing antibody concentrations as high as those produced in young mice; (8) determination that the density of red cells increases, the volume and protein content decrease, and the shape becomes more spheroidal with age; (9) determination that red cells become increasingly more susceptible to lysocompounds with age; (10) determination that red cells are radioreistant at doses up to 5,000 R but at doses of 20,000 R some, for reasons as yet undetermined, are rapidly removed from the circulation; (11) determination that older rats are generally less responsive to dietary and chemically-induced protein synthesis than younger rats; and (12) successful introduction of chelating agents encapsulated within liposomes into liver cells and the application of this technique to remove a significant fraction of the plutonium remaining in the liver after conventional treatment with chelating agents.

AGING GROUP STAFF

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TEMPORARY STAFF DURING 1972

Menon, Miran [Postdoctoral Appointee (Presidential Intern)]
Ross, Richard H. (Postdoctoral Appointee)
COMPARATIVE BIOLOGY OF AGING IN MYOMORPH RODENTS.
I. LIFE TABLES, AGING PROCESSES, AND PATHOLOGY

George A. Sacher, Robert J. Flynn, Peter H. Duffy, and Sylvanus A. Tyler

PURPOSE AND METHODS

This program is dedicated to understanding and making use of the fact that there is a nominal fifty-fold difference in aging rates among mammalian species. The basic experimental paradigm is the comparison of related species that differ in lifespan, with the objective of finding out what specific biochemical, physiological, and genetic mechanisms are responsible for the superior survival of the longer-lived species.

These researches are being conducted on a small group of myomorph rodent species that range in body weight from 10 to 200 g, and in lifespan from 3 to 8 years. All species breed readily, and relatively complete life tables have already been determined for most. Histopathology, organ weights, metabolic rates, and other data will be gathered to characterize these species as thoroughly as possible, as a basis for future analytical studies of differences in specific systemic and cellular homeostatic processes. These future studies will include, among other things, comparisons of primary explant cell cultures with regard to survival and susceptibility to transformation toward the neoplastic state.

PROGRESS REPORT

Provisional life tables have been determined for 7 species. These show: (a) that all the species yield good Gompertz-type life tables; (b) that the rate of aging, expressed as the doubling time for the mortality rate, varies from 150 to 400 days, and the intercepts of the log rate of mortality plots are to a considerable extent independent of the doubling times; (c) that the wild-type house mouse has a doubling time of 225 days as compared with a doubling time of little more than 100 days for inbred mouse strains or \( F_1 \) hybrids.

The finding that laboratory and wild-type mice have different rates of aging is potentially important, especially as the wild-type mouse has a low heritable variance component. An analysis of the heritability of longevity in all the rodent populations has been started.

Life expectations have now been estimated on at least a provisional basis for 13 species. Longevity was correlated with brain and body weights. The multiple regression was \( \log (\text{span}) = 1.8 \log (\text{brain wt.}) + 1.2 \log (\text{brain wt.}) + \text{constant} \). The multiple regression accounted for 92% of the longevity variance. This is a highly significant association, stronger than any found previously, and is the first time this relationship has been determined entirely from populations maintained in one laboratory under extremely homogeneous conditions.
Metabolic rates will be measured for all species by means of an open-circuit paramagnetic oxygen analyzer, which is already installed. Samples of 4 species (Mus musculus, Praomys natalensis, Peromyscus leucopus, Peromyscus californicus insignis) will be maintained at a temperature of 2°C for lifetimes, to investigate the relation between metabolic load and the aging of liver, kidney, adrenal, thyroid, and pituitary.

COMPARATIVE BIOLOGY OF AGING IN MYOMORPH RODENTS. II. COMPARATIVE STUDIES OF THE NERVOUS SYSTEM

George A. Sacher, Robert J. Flynn, George Svihla, Margaret H. Sanderson, and Peter H. Duffy

PURPOSE AND METHODS

The positive correlation of brain/body weight ratio with longevity suggests that a comparative quantitative study of the central nervous system of suitable laboratory animals may provide information on mechanisms of aging. It is generally recognized that the number of neurons and nerve fibers decreases with age, but comparative quantitative studies have not been made.

While it is desirable to compare the total number of cells in the brains of the animals to be studied, the difficulties of accurately counting the great numbers of cells and differentiating between cell types have caused us to approach the problem indirectly. A comparison of the number of myelinated fibers in optic nerves (which are, in reality, fiber-tract extensions of the brain) and vagus nerves (which are major mixed nerves that control many visceral functions) of suitable species at various chronological ages appears more readily achievable.

The two species being compared at present are Peromyscus leucopus, which has a lifespan of 7 years, and Mus musculus, which lives about 3 years. Test animals of each species are killed at approximately comparable physiological ages. Tissues are completely fixed after partial perfusion fixation and then dehydrated and embedded in Epon 812, using modifications of standard methods. p-phenylenediamine in 70% ethanol (a myelin-specific stain) is applied during dehydration. Sections are cut 240 to 320 nm thick, mounted under cover glasses with Epon, and photographed with an automatic 35-mm camera.

Counts of the myelinated fibers in the optic nerve are obtained with the assistance of ALICE, a general purpose computer-assisted image processing system. Counts of the myelinated fibers of the vagus nerve are obtained by manually counting positive photographic prints fitted into a montage.
PROGRESS REPORT

The usual methods of preparing and staining slides of Epon thick sections are unsuitable for image processing. Therefore, it was necessary to develop a method of staining the nerves *en bloc* with p-phenylenediamine and mounting with Epon 812. This technique results in nerve sections that can be counted automatically.

Optic nerve fiber counts obtained from ALICE range from 34,000 to 50,000 per nerve for *P. leucopus* and from 21,000 to 30,000 for *M. musculus*. Although the counts vary with the density and contrast of the images on the negatives and the adjustment of the instrument, counts of the same film made on different dates agree closely (<0.02%). When counts from two sets of photomicrographs of the same section made with slightly different exposure are done, the discrepancy is considerably greater (about 12%).

A microscope-image-dissector-tube station, where sections can be counted directly from the slide, is being developed as a part of ALICE.

Vagus nerve fiber counts obtained manually vary from 600 to 920 per nerve for *P. leucopus*, while those of *M. musculus* range from 510 to 783 per nerve.

Although most of the effort of the past year has been devoted to developing techniques, the counts obtained to date seem to confirm the belief that the number of neurons and nerve fibers decreases from maturity to old age. But what we have not yet determined is whether the long-lived species lose their neurons at a slower rate than the short-lived species or whether the rate of loss is the same for all species but that the long-lived species start with more.

COMPARATIVE BIOLOGY OF AGING IN MYOMORPH RODENTS.
III. LIFE SHORTENING BY CHRONIC GAMMA-RAY EXPOSURE IN MYOMORPH RODENTS

*George A. Sacher, Robert J. Flynn, Sylvanus A. Tyler, and Everett F. Staffeldt*

PURPOSE AND METHODS

Comparative study of the life shortening and disease incidence produced by chronic radiation exposure can, if carried out on an adequate scale with carefully chosen species, provide a valid basis for estimating the somatic effects of very low level radiation exposures in mammals generally and in man. It can also add to our understanding of aging.
A program of chronic \( \gamma \) irradiation of over a dozen rodent species is now moving to completion of a first phase in which all species received daily exposure at doses ranging from 12 to 125 R/day. All data were examined in terms of a recently developed relationship between the radiation-specific death rate and the daily dose rate. Almost all species showed a response like that in Figure IV-1, consisting of a two-branched function. The upper branch approaches unit slope, while the lower branch is very near a slope of two. The effectiveness per roentgen per day is therefore constant on the upper branch and proportional to dose rates on the lower branch. The species sensitivities for the two branches are in a power-function relation, such that sensitivity on the low branch increases as the \( +2.4 \) power of sensitivity on the upper branch. In other words, small differences in acute sensitivity become greatly magnified under conditions of chronic exposure.

![Fig. IV-1.](image)

Another striking outcome of this study is the finding that in almost all species the female is more radiosensitive than the male. This is also true for the single-dose LD\(_{50}\).

A mathematical model has been developed which accounts for a number of the phenomena observed, including:

(a) the two-branched function, with slope two rolling over to slope one

(b) the location of the transition at daily doses on the order of 100 R/day

(c) the 2.4-power relation between sensitivities on the two branches.

The second phase of this program is the critical one, for here we intend to expose animals at daily doses from 12 down to about 2 R/day in order to determine whether the branch of decreasing effectiveness gives
way to a third branch of constant effectiveness at low dose rates. There is evidence for this in the laboratory mouse. If the transition to the third branch can be located for a group of species, and its sensitivity for each species examined in relation to the sensitivity on the other two branches and to lifespan, the phenomenological basis will be laid for a general theory of chronic radiosensitivity in mammals.

Reprogramming of the low-level gamma room for the low-dose study will be done when the higher dose groups die out, and the low-dose exposure study can then begin full-scale.

A few species are now being irradiated on a small scale at 5 R/day.

GENETICS OF LONGEVITY IN LABORATORY MICE

George A. Sacher, Robert J. Flynn, Sylvanus A. Tyler, and Peter H. Duffy

PURPOSE AND METHODS

Inbred laboratory mouse strains differ in longevity, and so offer a convenient system for analysis of the genetic factors in longevity. A program of genetic analysis is under way, in which 5 inbred strains and their F1 hybrids are employed in a diallel design. The strains are A/Jax, C3H, C57BL/6, BALB/c, and DBA/1. One replication of the longevity study consists of 600 mice, 12 progeny of each sex from the 25 different matings. Organ weights, body and skeleton dimensions, metabolic rates, body temperatures, and biochemical data have been or will be taken. Genotypes will be compared under different lifetime environmental loads. The goals are to (a) delineate the constitutional factors associated with longevity and vigor in mice, (b) identify the unitary genetic factor involved, and (c) develop selection procedures for maximizing the multivariate vigor/longevity function.

PROGRESS REPORT

The first replication of the diallel longevity study is completed, an analysis of variance has been performed, and the genetic components of variance for longevity have been determined. Hybrid vigor is the largest component, with significant components also for inbred strains, general combining ability (additional genetic variance among hybrids), and specific combining ability (non-additive variance). Analysis of the relation of longevity to organ weights reveals an interesting association of longevity with the ratio of kidney to liver weight, in which a low ratio of kidney to liver weight is disadvantageous and a high ratio is advantageous. Another strong association is between longevity and tail length, which is interesting because tail length is correlated with metabolic rate and body temperature.
Analysis of the survival data from the diallel design tends to confirm the impression that differences in longevity between mouse strains are due to an age-independent factor which we call "vigor," and not to differences in their rates of aging. Thus far, all real differences in rate of aging have been found at the species level or above, between populations that do not interbreed.

Entry of young mice into the second replication of the longevity diallel study is now about two-thirds completed. Measurement of metabolic rates on all 25 crosses and both sexes is just beginning. A subset of the 5 x 5 diallel design will be maintained for their lifetimes at 2°C, and the comparison with the room temperature sample will pay particular attention to the functional status and diseases of liver, kidney, adrenal, thyroid, and pituitary. Development of specific-pathogen-free stocks of the 5 inbred strains is under way, and the third replication of the diallel longevity design will be done with such mice.

The finding that outbred wild-type house mice have a markedly different rate of aging from any inbred strain or hybrid (1) requires that the genetic analysis be extended to include diverse wild stocks in order to have the greatest possible genetic diversity under study.

REFERENCE


AGING OF INSECTS IN RELATION TO ENERGETIC EFFICIENCY

George A. Sacher and Richard H. Ross, Jr.

PURPOSE AND METHODS

This approach examines the effect of temperature upon the various aspects of aging in a convenient and well-defined insect species. An earlier analysis showed that the energetic efficiency of maintenance—the number of calories that can be metabolized per unit of age change—is maximal at about the midpoint of the range of viable temperatures for a species. The efficiency falls off at higher or lower temperatures. Similar relationships are also found in the growth of egg, larval, and pupal stages, and egg production.

This research project is designed to study the phenomenon and to develop a model by which aging of specific biochemical and physiological processes can be examined under various temperature regimes. Houseflies, Musca domestica L., are being used as the test organism, because they are well
adapted to laboratory rearing, they have a relatively short lifespan at room temperature, and a great volume of material has been written on their physiological and biochemical mechanisms. The larvae can be reared under aseptic conditions, or conventionally, on a mixed bran, yeast, and malt medium. Life tables will be determined at various constant temperatures ranging from about 10° to 45°C, and in periodically fluctuating regimes. Metabolic measurements of oxygen consumption will be made using a Gilson respirometer. Models will then be developed by which further studies can be undertaken on specific biochemical and physiological mechanisms of aging at optimal and suboptimal temperature regimes.

**PROGRESS REPORT**

The major problems of husbandry, equipment, and experimental procedure and design have been solved. It was found under preliminary examination that the flies could not be maintained in small plastic boxes because of a high mortality rate in the first week of adult life. It was also found that anesthetizing the adult with CO₂ for 15 to 60 min lead to a 5 to 10% increase in mortality over the first week. The lifespan and metabolic experiments have now been initiated using screen cages measuring 12" x 8" x 5". Three hundred pupae are placed in each cage, and the adults allowed to emerge at 26°C, the temperature at which the main populations are maintained. The cages are then placed in incubators at constant temperatures ranging, in the first experiment, from 17° to 32°C under a 16 hr daylight: 8 hr dark photoperiod. Each day the dead flies are removed, sexed, and recorded. Oxygen consumption is being recorded in young, middle aged, and old flies at each temperature, as it has been found that the oxygen consumption decreases slowly over most of the adult lifespan. The upper and lower temperature extremes will be examined in subsequent experiments.

**IMMUNITY AND AGING.**

I. KINETICS OF ANTIBODY PRODUCTION IN THE DOG

*Bernard N. Jaroslow and Katherine M. Suhrbier*

**PURPOSE AND METHODS**

At least two classes of disease associated with aging have an immunological component. These are autoimmune disease, which some proponents believe to be an important factor in most wasting diseases or the prime cause of age changes, and neoplastic disease. Increased incidence of these diseases with age has been related to a decreased capacity of the lymphoid-macrophage system to produce antibody-forming cells. The genesis of age-associated immunologic diseases is more likely to be associated with a
functional deficit in the immunologically reactive cells than with the inability to mount a rapid and intense immune response. Our research program is directed towards detecting and describing such changes in the immune system.

Our major effort this year involved a study of the kinetics of antibody production in dogs. Young (1 to 2 yr) and old (>8 yr) dogs were given primary and secondary immunizations with two cross-reacting bacteriophages to Escherichia coli (T2 and T4). The immunization protocols were altered to provide a comparison between the responses to intensive antigenic stimulation and to near-threshold doses of antigen. Frequent blood samples were taken and the anti-phage titers were used to determine the responsiveness and the degree of cross-reactivity of the antibodies produced in the young and the old dogs.

PROGRESS REPORT

Young and old dogs were immunized with alum-precipitated suspensions of $10^{10}$ T2 or T4 phage per injection. Intensive antigenic stimulation was achieved by giving the dogs three intramuscular injections in 5 days with a similar series given 4 weeks later. To achieve near-threshold stimulation, one injection of T2 phage was given at the beginning, and another injection was given 7 weeks later.

The kinetics of antibody production in young and old dogs are similar in the intensively immunized group. The peak titers that are reached in the primary and secondary responses are not significantly different. The only age-associated defect observed was a general decrease in the rate of production as measured by the length of the latent period and the number of days to reach peak titer. The amount of cross-reactivity between the antigenically related T2 and T4 phages was the same in both young and old dogs. (This work was done in collaboration with Drs. Thomas E. Fritz and William P. Norris).

CONCLUSIONS

Antibody-forming capacity and the quality of antibody produced is the same in old and young dogs given intensive immunization. The lowered production of antibody by old as compared to young dogs, given near-threshold antigenic stimulation, is indicative of a change in sensitivity, a qualitative change. These results suggest that old people can be successfully immunized against infectious agents, but it may be advisable to readjust the immunization protocol.
IMMUNITY AND AGING.
II. CLEARANCE OF FOREIGN RED CELLS IN AGING MICE*

Bernard N. JaroSlow and James W. Larrick†

The macrophages of the liver and spleen form the first line of defense against infectious organisms and keep the body free of malfunctioning cells. There have been several reports indicating that the efficiency of this mechanism declines with age and therefore susceptibility to disease increases. We found that the clearance rate of foreign red cells was equal in B6CF₁/An[Anl 70] mice between the ages of 150 to 800 days, but the clearance rate is higher in mice between 14 to 90 days of age. These results clarify the apparent contradictions in the literature, and show that senescence does not significantly affect the functional capacity of the blood clearance mechanism as had been widely believed.

*Abstract of paper to be published in Mechanisms of Ageing and Development.
†Student Associate from Colorado College, Colorado Springs.

IMMUNITY AND AGING.
III. SKIN GRAFT SURVIVAL IN AGING B6CF₁/ANL MICE*

Bernard N. JaroSlow and Richard Koesterer†

Studies of changes with age in the primary response involving cellular immunity have given results showing a loss with age in some mouse strains and not in others. We found that rejection of skin grafts in unsensitized mice was significantly slower (P<.05) in 700-day old than in 175-day old mice, whereas it was equally rapid in presensitized old and young mice. In both presensitized groups of mice the rejection was significantly faster (P<.05) than in the unsensitized groups of mice. These results for cellular immunity support the results from our studies of humoral immunity showing that the aged animals are capable of producing antibody concentrations as high as those produced in young animals.

*Abstract of paper to be submitted for publication.
†Resident Associate from St. Louis University.
MECHANISM OF ERYTHROCYTE AGING AND DESTRUCTION.
I. MEMBRANE MODEL FOR ERYTHROCYTE AGING

Yueh-Erh Rahman, B. J. Wright, Elizabeth A. Cerny, and Maureen Groer*

PURPOSE AND METHODS

The mechanism of red cell aging and destruction has been selected as a model for the study of cellular aging and death because (a) a phospholipid-degrading enzyme, phospholipase A, was found in rat spleen; because this enzyme readily attacks red cell membranes, we propose to use it as a tool to check the validity of the following hypothesis: "Cellular aging is the result of an interaction between external (or environmental) and internal factors;" (b) the understanding of red blood cell aging and destruction will provide a better basis for understanding some hemolytic diseases; and (c) the mechanism of red cell aging and destruction may provide a model for the investigation of the mechanisms of age changes and removal of other tissue cells.

The following methods are used in this project: (a) radioactive labeling of red blood cells with $^{59}$Fe; (b) treatment of red cells with lysocompounds and observing the effects of such treatment on the surface properties of the cells by scanning electron microscopy (for morphology), by cell electrophoresis (for surface potential changes), and by immunological methods (for surface antigen changes); (c) determination of cell volume and cellular constituents; and (d) examination of red cells by autoradiography and scanning electron microscopy for age-dependent membrane damage.

PROGRESS REPORT

We have defined some age-dependent changes in red blood cells as follows: (a) the density of red cells increases with age; (b) old red cells are smaller in volume; (c) the cell protein content decreases with age; and (d) old red cells become more nearly spherical.

By in vitro treatment of isolated young red cells with lysocompounds, i.e., reaction products of phospholipase A, we were able to reproduce the in vivo age-dependent changes described above. Red cells were found to be increasingly more susceptible to lysocompounds as they became older.

Preliminary results on attempting to combine autoradiography and scanning microscopy are encouraging. We have been able simultaneously to localize $^{59}$Fe-labeled young red cells by autoradiography and to visualize their cell shape by scanning microscopy.

*Resident Associate from University of Illinois.
CONCLUSIONS

The development of a membrane model for erythrocyte aging and death is progressing satisfactorily. We think that this model will be able to explain the programmed red cell death, which is manifested by its finite lifespan. In addition, our findings suggest that external factors play an important role in cellular aging.

Mechanisms related to the death of red cells, i.e., recognition between old red cells and the spleen reticular cells, which leads to ultimate erythrophagocytosis, still require clarification. We are currently checking two possibilities: (a) alteration of surface potential in aging red cells, and (b) exposure of new surface antigens in aging red cells.

MECHANISM OF ERYTHROCYTE AGING AND DESTRUCTION. II. EFFECT OF X-IRRADIATION ON AGING OF ERYTHROCYTES

Yueh-Erh Rahman, Maureen Groer, and David L. Elson

PURPOSE AND METHODS

The mammalian erythrocyte has generally been considered to be resistant to ionizing radiation. Discrete changes in metabolism and ion transport are observed only after very high doses. Hemolysis and gross structural damage are not apparent until doses of 2,000 rad are used. When erythrocytes are irradiated in vitro at doses ranging from 35 to 200 rad and reinjected into man, mouse, or cow, the lifespan of these irradiated erythrocytes has been reported to be significantly shortened (1-3). However, the X-ray doses used are high and the erythrocytes are usually labeled with $^{51}$Cr, which is known to give partial elution in the circulation; therefore, the results are not entirely unequivocal. For these reasons, we have used $^{59}$Fe to label the erythrocytes, and we have irradiated the cells with a relatively low dose of X irradiation.

Rat erythrocytes were labeled in vivo with $^{59}$Fe. One day after the $^{59}$Fe injection, blood of the injected rats was collected in the presence of heparin and washed three times in ice cold saline. The resultant packed cells were resuspended in saline at a hematocrit between 30 and 40%. The blood suspension in a petri dish was placed in a small ice bucket which was set on a revolving platform under the X-ray source. The radiation was delivered at 300 kV and 1039 R/min. Three doses were used: 500, 5,000, and

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Fig. IV-2.--Effect of X irradiation on the lifespan of rat erythrocytes. $^{59}$Fe activity in the blood (counts/min/mg Hb), at intervals over 60 days is expressed. The radioactivity in the first blood sample, drawn within 6 hr after $^{59}$Fe injection, was taken as 100%. 20,000 R. Irradiated blood was brought to room temperature and injected into recipient rats via the tail vein. Radioactivity of $^{59}$Fe in the blood of these rats was determined at frequent intervals over the following 60-day period. Imferon was injected intramuscularly at the time of each sampling in order to prevent reincorporation of the radioiron. Scanning electron microscopy was also used for morphological studies of irradiated erythrocytes.

**PROGRESS REPORT**

At doses of 500, 5,000, and 20,000 R, the lifespan of irradiated erythrocytes was not significantly different from the non-irradiated cells (Fig. IV-2). At the dose of 20,000 R, a peak of radioactivity was seen 4 days after injection. This peak is significantly higher than the radioactivity found in the blood sample drawn soon after the $^{59}$Fe injection (Fig. IV-2, bottom). This phenomenon can be explained by a rapid removal or sequestration of a fraction of irradiated erythrocytes from the circulation. This fraction of cells seems to have been subsequently released and destroyed. No significant morphological changes in irradiated erythrocytes were observed.

**CONCLUSION**

Using lifespan as a parameter, erythrocytes are found to be radioresistant at doses up to at least 5,000 R. At a dose of 20,000 R, a fraction of the irradiated cells
was rapidly removed from the circulation and subsequently released. The mechanism of this early and rapid removal of radiation damaged erythrocytes is not known. Short-term survival studies of irradiated erythrocytes should be useful for the understanding of this phenomenon.

REFERENCES


AGE DEPENDENCE OF PROTEIN SYNTHESIS.
I. EFFECTS OF AGE ON PATTERNS OF ENZYME ADAPTATION IN MALE AND FEMALE RATS*

Yueh-Erh Rahman and Carl Peraino

Age-dependent changes in the response patterns of serine dehydratase, ornithine aminotransferase, and glucose-6-phosphatase were studied in female and in male rats, by exposing these animals to dietary changes alone or in conjunction with glucocorticoid treatment. A general impairment of responsiveness in older animals was found. As an exception, serine dehydratase in older males showed a delayed response to glucocorticoid but ultimately attained the maximal enzyme level seen in younger animals. Patterns of age-dependent enzyme adaptation are more complex and diverse than was presumed previously. This diversity should be taken into consideration in the search for a molecular basis for the phenomenon of adaptive impairment with age.

*Abstract of paper to be published in *Experimental Gerontology.*
AGE DEPENDENCE OF PROTEIN SYNTHESIS. II. AGE DEPENDENCE OF SYNTHESIS IN ISOLATED NEURONS

Yueh-Erh Rahman and Carl K. Moy*

PURPOSE AND METHODS

One of the main interests of the Aging Research Group centers on the nervous system. In man, decline of mental abilities with age is well known although the basic mechanism of this decline has not been determined. We have demonstrated that in old rats, adaptive capacity for enzyme synthesis is significantly impaired (1); therefore, it is logical to extend our study of protein synthesis to the nervous system.

In order to study the intrinsic synthetic capacity of the neurons, it is first necessary to isolate these cells from supportive cells such as glial cells. Various gradient centrifugation techniques were used for this purpose. Ten rats were lightly anesthetized under ether and thoroughly bled from one of the jugular veins; the brains were then rapidly removed and placed in an ice-cold solution of 7.5% polyvinylpyrrolidone (PVP), 10 mM CaCl₂, and 1% bovine serum albumin (BSA). Cerebral cortices were dissected as free as possible of any underlying white matter and minced with a razor blade. Cerebral cortices from 10 rats usually weighed 8 to 10 g, and the final volume of the tissue suspension was 15 ml. The tissue mince was then forced through a truncated syringe attached at the open end with one or more layers of stretched nylon cloth. It was first forced through 333-μ mesh nylon cloth, once with a single layer, and twice with double layers. The mince was further pushed through double layers of 110-μ mesh nylon three times, and finally through double layers of 73-μ mesh nylon another three times. The tissue sample thus obtained was used for gradient centrifugation steps. The detailed procedure for various discontinuous and continuous gradients are indicated in Figure IV-3. Purified neurons and glial cells were collected after the last sucrose gradient centrifugation, and the purity of the cell fractions was checked under phase contrast microscopy. The last centrifugation was repeated if excessive contamination was present. Neurons isolated by this technique will then be incubated with radioactive amino acids so that radioactivity will be incorporated into newly synthesized proteins. A comparison can then be made of protein synthesis in neurons isolated from young and old rats.

PROGRESS REPORT

We have successfully isolated fractions of neurons from glial cells by the method described, and the purity of the fractions as checked by microscopy is highly satisfactory.

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Fig. IV-3.--Procedure for isolation of neurons and glial cells.
CONCLUSIONS

A satisfactory method for isolating neurons from glial cells has been developed. This will enable us to study the protein synthetic capacity in purified neurons and to compare this capacity in young and old rats.

REFERENCE


LIPOSOMES AND MEMBRANE TRANSPORT.
I. DISTRIBUTION AND TRANSPORT OF LABELED LIPOSOMES IN MOUSE TISSUES

Yueh-Erh Rahman, Elizabeth A. Cerny, Marcia W. Rosenthal and Betty J. Wright

PURPOSE AND METHODS

The surface active properties of lipids play an important role in the transport phenomena of various biological membranes. Simple model systems (e.g., monolayer and bilayer lipid membranes) have been developed and studied (1). A more complex and superior model system, introduced by Bangham et al., in 1965 (2), involved lipid spherules (or liposomes) prepared from synthetic phospholipids in which an electrolyte, e.g. KCl, was entrapped. In recent years, these liposomes have been used increasingly as membrane models for studies of ion diffusion and transport.

We have been able to prepare liposomes with a chelating agent, either diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA), in place of the usual electrolyte. Using either $^{45}$Ca-DTPA or $^{14}$C-EDTA, a study of the distribution, transport, and toxicity of these liposomes containing DTPA or EDTA was initiated in mice. In addition, application of liposomes containing DTPA to remove intracellular Pu has also proved successful (3). Although there are no present applications of liposomes to research in aging, it is hoped that the use of liposomes in studying various basic phenomena of biological membrane transport and fusion may one day be applicable to problems of cellular aging.

Methods used include radioactivity counting, electron microscopy, autoradiography, techniques for cellular fractionation, and determination of enzymes which require heavy metals as co-factors.
PROGRESS REPORT

From as early as 5 min up to 6 hr after an intravenous injection into mice, of liposomes containing $^{14}$C-EDTA, about 45% of the total radioactivity was found in the liver. Mice injected with liposomes containing $^{45}$Ca-DTPA had about 32% radioactivity in the liver, from 5 to 30 min after injection; but the radioactivity had decreased to 21% at 1 hr, and to 14% at 6 hr after injection. This more rapid disappearance observed with $^{45}$Ca-DTPA liposomes beyond 30 min after injection is most probably due to the rapid exchange of the $^{45}$Ca with endogenous non-radioactive Ca$^{++}$ ions.

Rapid clearance from the blood after injection of $^{14}$C-EDTA liposomes was observed: at 2 min, only 40% of the injected radioactivity was in the circulation; at 5 min, about 25%; and at 1 hr, less than 1%. Due to technical difficulties in radioactivity counting, data on $^{45}$Ca-DTPA of the blood were not reliable. The lungs contained about 20% of both $^{14}$C-EDTA or $^{45}$Ca-DTPA liposomes at 2 min after injection, but continuously lost activity with time; only 4.5% remained after 6 hr. The spleen, kidneys, and bone marrow contained a small but measurable fraction of labeled liposomes. Other tissues analyzed, such as thymus, lymph nodes, and muscle, had negligible radioactivity.

Electron microscopic studies have been initiated on liver and lungs of mice receiving either $^{14}$C-EDTA-labeled or unlabeled liposomes. Studies have also been initiated on the distribution of injected $^{14}$C-EDTA liposomes in intracellular particles of mouse liver, using cell fractionation techniques.

CONCLUSIONS

We have been successful in introducing chelating agents encapsulated within liposomes into liver cells. We now need to determine the intracellular pathway of the liposomal chelating agents and the possible toxic effects of introducing these agents into cells.

Investigation of the nature of fusion between membranes of specific cell types and liposomes of known surface properties should prove to be of basic interest in membrane recognition in biological systems. The liposome-encapsulated method of introducing drugs should be of value, not only for treating metal toxicities, but also for getting a specific drug into a specific population of target cells.

REFERENCES


V. BIOCHEMISTRY

SUMMARY

John F. Thomson, Group Leader

The research activities of the Biochemistry Group are diverse, encompassing studies on animals, plants, microorganisms, subcellular particles, and purified enzymes, and employing a variety of experimental techniques to investigate a number of physiological processes. There are four programs in this group: (a) the preparation and properties of single cells and subcellular particles, (b) biochemical manifestations of radiation damage in yeast cells, (c) hormonal and metabolic bases of radiation response in plants, and (d) therapy of poisoning by radioactive and non-radioactive metals.

The first is represented in this report by a communication dealing with an improved method of preparation of peroxisomes from mouse liver. Another contribution from this laboratory on the preliminary attempts to isolate and characterize a murine leukemia virus is reported in the Carcinogenesis section of this report.

The second program is concerned with the biochemical and structural changes that take place in X-irradiated yeast, with particular emphasis on the cell wall. A new technique for isolation of yeast vacuoles is described, in which advantage is taken of the capacity of the vacuole to accumulate S-adenosylmethionine and hence be identifiable by ultraviolet microscopy.

The plant radiobiology program has produced a series of reports on a variety of subjects in the field of plant physiology. These reports are concerned with the physiological mechanisms whereby organisms are able to amplify minute radiation energies—particularly ionizing radiation, visible light, and gravity into changes in growth and development. These reports collectively are an excellent example of the importance of students, post-doctoral appointees, and visiting scientists in one of the Divisional programs.

The fourth set of reports deals primarily with the efforts to remove plutonium from the body. Topics include the exploration of the therapeutic properties of substances related to glucan, the localization of liver plutonium by quantitative autoradiography, and the effects of the physical-chemical state of plutonium on lifetime pathological changes, comparative effects in mice and dogs, and the therapeutic use of liposome-encapsulated chelating agents.
PREPARATION AND PROPERTIES OF MOUSE LIVER PEROXISOMES

John F. Thomson, Sharron L. Nance, and Sandra L. Tollaksen

PURPOSE AND METHODS

In the previous annual report (1), we described our attempts to isolate peroxisomes from mouse liver by a combination of rate-zonal and equilibrium-sedimentation techniques. Our preliminary results indicated that zonal centrifugation (0.29 to 0.88 M sucrose gradient) followed by isopycnic centrifugation (1.46 to 2.04 M sucrose gradient) of the concentrated fraction that represented 0.27- to 0.59-μm particles produced two distinct bands, one of which was mitochondria (density 1.22 g/cm³) and the other peroxisomes (density 1.227 g/cm³).

* Terminated during 1972.
A more detailed study of this procedure indicated that the activity of urate oxidase, the enzyme that we have used as a peroxisomal marker, was spread more diffusely throughout the gradient than was visually apparent; and it was evident that our preparations contained a significant proportion of damaged peroxisomes.

Thus we have attempted other procedures to avoid exposure of the peroxisomes to high osmotic pressures. In order to maximize the separation of peroxisomes from other cytoplasmic particles with overlapping sedimentation characteristics, we have used livers from mice pretreated with Triton WR-1339 to decrease the sedimentability of lysosomes (2), and prednisolone to increase the sedimentability of mitochondria (3,4). The former was injected intravenously (100 mg/kg) 3 days before the mice were killed; the latter was given by intramuscular injections of 0.1 mg/mouse on each of the 3 days before killing. We have also, in some experiments, taken advantage of the fact that lysosomes are much more susceptible than peroxisomes to the lytic action of digitonin.

The following procedure to date has proved most promising. About 5 g of liver from mice pretreated as described above were homogenized in 9 volumes of 0.25 M sucrose. The homogenate was centrifuged for 20 min at 20,000 rpm in a Sorvall RC-2B centrifuge (40,000 x g) to sediment the nuclei and the larger cytoplasmic particles, while the microsomes remained for the most part in the supernatant. The sediment was resuspended in 10 ml 0.25 M sucrose and then introduced into a B-XIV zonal rotor containing 400 or 450 ml of a 0.29 to 0.58 M sucrose gradient. After centrifugation for a cumulative \( \omega^2 t \) of 150 x 10^7 radians^2/sec, the gradient was pumped out. The overlay, original sample volume, and the first 20 ml of the gradient were set aside (D-1), and the next 300 ml of the gradient were collected (Z-1); the remaining contents of the rotor (R-1) were also saved. The Z-1 fraction was centrifuged for 1 hr at 20,000 rpm (40,000 x g) and the sediment suspended in 10 ml of 0.44 M sucrose, either with or without 0.05 mg/ml digitonin.

This suspension (CZ-1) was then layered over another zonal gradient, 0.58 to 1.17 M sucrose, and centrifuged for a cumulative \( \omega^2 t \) of 281 x 10^9 radians^2/sec. As in the case of the first centrifugation, D-2, Z-2, and R-2 fractions were collected, and the Z-2 concentrated by centrifugation to yield the final peroxisome suspension, CZ-2.

Each fraction was assayed for two peroxisomal enzymes, urate oxidase and catalase; two mitochondrial enzymes, cytochrome oxidase and malate dehydrogenase; a lysosomal enzyme, acid phosphatase; and a microsomal enzyme, glucose-6-phosphatase.

**PROGRESS REPORT**

Table V-1 shows the results of the experiment that so far has been the most satisfactory in terms of both yield and homogeneity. The relative specific activity of urate oxidase, 34, was the same as that reported by Baudhuin (2), and the recovery was two to four times better. Contamination by lysosomes, mitochondria, and microsomal material is relatively slight;
TABLE V-1. Enzyme Activities of a Mouse Liver Peroxisome Preparation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of Homogenate Activity</th>
<th>Enrichment Factor&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urate oxidase</td>
<td>47.2</td>
<td>34.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>21.9</td>
<td>15.8</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>6.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Protein</td>
<td>1.4</td>
<td>---</td>
</tr>
</tbody>
</table>

<sup>a</sup>Enzyme activity of the preparation per unit protein relative to that of the homogenate.

We estimate the preparation to be over 90% pure on the basis of enzymatic activities.

We have varied the procedure in a number of ways to try to improve the yield, but each variation thus far has resulted in preparations of lower relative specific activity for urate oxidase, and somewhat greater contamination by mitochondria and microsomes.

A consistent problem has been the poor recovery of catalase, relative to urate oxidase. Comparison of the relative specific activities of the two enzymes is misleading, since some of the catalase in the homogenate is non-particulate (i.e., that derived from the blood entrapped in the liver); even when this amount is taken into consideration, the recovery of catalase is no better than half that of urate oxidase. The question is not resolved as to whether the poor recovery is due to extraction of the enzyme, inactivation, or assay procedure.

CONCLUSIONS

The basic features of the approach described here—pretreatment of the mice with Triton WR-1339 and prednisolone, and the use of two rate-zonal gradient centrifugations—have provided a promising technique for isolation of peroxisomes in good yield and low contamination by other cytoplasmic constituents. We are currently exploring variations in the
procedure, particularly the composition of the gradients, to try to improve the homogeneity without materially sacrificing the yield. The most troublesome contaminant is the lysosome, as measured by acid phosphatase activity.

REFERENCES


BIOCHEMICAL EFFECTS OF X IRRADIATION ON YEAST CELLS

Kenji D. Nakamura, Julia L. Dainko, and Fritz Schlenk

INTRODUCTION

In earlier reports (1,2), abnormal budding and morphology of yeast cells (Candida utilis) after X irradiation (1 to \(4 \times 10^5\) R) and continued culture have been described. The bud fails to close off and to separate from the parent cell, and it grows to an elongated form with a heavy wall structure. After about a fourfold increase in mass, the structures remain unchanged, regardless of the supply of nutrients. The events of normal and abnormal budding are schematized in Figure V-1. For simplicity, only the cell wall, membrane, and vacuoles are represented.

The separation of the bud starts with the formation of a primary septum which consists mainly of chitin (indicated by + in Fig. V-1 A). Secondary septa are juxtaposed later and form a matrix for extension and closure of the membranes of the parent cell and bud (3). The chitin-forming enzyme occurs in the cell as an inactive, membrane-bound zymogen. At the time and site of septation, the zymogen is converted to the chitin-forming enzyme by an activating factor which is a specific proteolytic enzyme. The latter is adsorbed on the surface of the vacuole and is believed to be deposited at the neck of the cell-bud aggregate when a fragment of the parent vacuole travels to the bud (4). Obviously, one or several mechanisms of this sequence are impaired in the cells after X irradiation. The defect may be a change in the adsorptive capacity of the vacuole for the activating factor and its translocation. As an alternative, a biochemical defect in the formation of chitin or the chitin septum, wall extension, or membrane
Fig. V-1.--Some features of growth and multiplication of yeast cells (Candida utilis): A, normal culture; B, growth after X irradiation ($1 - 4 \times 10^5$ R).

Fig. V-2.--Isolated vacuoles of Candida utilis. Ultraviolet micrograph at 265 nm, obtained by the procedures of G. Svihla (5).
Closure has to be considered. Experiments to explore these alternatives have been carried out.

**Isolation and Properties of Vacuoles.** The vacuole is the most elusive and sensitive organelle of the yeast cell; hence, studies of its properties have remained very limited. As a prerequisite for an investigation of the transport of the zymogen-activating factor, procedures for the production and separation of vacuoles have now been elaborated. Conventional methods for the isolation of subcellular units were found unsuitable, but osmotic lysis of spheroplasts left the vacuoles intact. A special culture regimen to load the vacuoles with S-adenosylmethionine or its homologues was required for successful conversion of the cells into spheroplasts and for the stability of the isolated vacuole. For separation of the vacuoles from spheroplasts, the tonicity of the stabilizing medium, normally 0.8 M sorbitol, was lowered to 0.4 M for a period of 3 to 5 min. More than 95% of the spheroplasts underwent lysis, and the vacuoles could be separated by differential centrifugation and sedimentation in Ficoll gradients. The high ultraviolet absorbance of the intravacuolar adenosine sulfonium compounds facilitated photomicrographic observation (Fig. V-2); ordinary vacuoles are virtually translucent.

Through microscopically the vacuoles appeared free from contaminating cytoplasmic elements, biochemical analyses indicated the presence of significant amounts of RNA, which may be adsorbed to the surface.

Our procedure of loading the vacuoles with ultraviolet-absorbing compounds proved to be the most convenient test for their integrity. On bursting, the material is discharged into the surrounding medium, and the absorbance of the latter at 260 nm is a measure of the percentage of vacuoles that have been destroyed. Some proteins were found to exert a strong surface effect on vacuoles, which leads to immediate destruction of the vacuolar membrane.

**Biochemical Experiments.** Our search for a biochemical defect in the formation of the bud-cell septum after radiation damage has permitted the elimination of several possibilities. The formation of non-septate elongated buds is not a result of the lack of growth factors and specific nutrients. Cultivation of the yeast in enriched medium prior to, or after, radiation does not alter the abnormal morphology of subsequent growth. Polyamines such as spermine, spermidine, and putrescine have been implicated in microbial morphology (6). Addition of these compounds to the post-irradiation growth medium in a wide range of concentrations did not correct the defect in bud separation. The abnormal bud-cell aggregates showed unimpeded ability to synthesize ATP; hence, there is no lack of readily available energy for biochemical syntheses. The unusually high concentration of chitin in radiation-deformed cells at sites other than the bud junction excludes a defect in the chitin-forming system: n UDP-N-acetylglucosamine + [N-acetylglucosamine]ₙ + n UDP. However, the mechanism initiating the chitin septum at the correct site and the nature of the primer remain to be explored.
HORMONAL EFFECTS ON $^3$H-THYMIDINE INCORPORATION INTO DNA OF PEAS

John E. Sherwin and Solon A. Gordon

PURPOSE AND METHODS

The majority of the research concerning hormonal effects on nucleic acid metabolism in higher plants has been upon RNA synthesis. In view of the role of hormones in RNA metabolism, it does not seem unreasonable that plant hormones such as indole-3-acetic acid (IAA) may have a role in the control of DNA synthesis. We report here some initial investigations along these lines.

PROGRESS REPORT

DNA synthesis was measured by incubating apical segments of darkgrown pea seedlings in $^3$HTdR and subsequently extracting the DNA by a modification of the Schmidt-Thannhauser procedure. The specific activity of the DNA was calculated from the DPM of an aliquot of the DNA and the concentration of the DNA as estimated by the technique of Burton. Preliminary experiments indicated that the incorporation of $^3$HTdR into DNA was linear for at least 1 hr. Therefore, 1 hr was chosen as the standard incubation period.

The results of preincubating pea apical segments in various concentrations of IAA for either 4 or 20 hr followed by incubation in $^3$HTdR

REFERENCES

are shown in Figure V-3. The inhibition of thymidine incorporation by high concentrations of IAA at both 4 and 20 hr is probably, at least in part, an effect of ethylene. High concentrations of IAA are known to stimulate ethylene formation, and Apelbaum and Burg (1) have reported an inhibition of DNA synthesis by ethylene. The lack of inhibition by $10^{-5}$ M IAA was unexpected, and is not common in plant responses to IAA. This peculiar effect could be either a direct effect of IAA, because this concentration is optimal for growth, or it could represent an effect of IAA on the uptake of $^{3}\text{H}$-thymidine. The effect of IAA on the absorption of $^{3}\text{H}$-thymidine was therefore examined. No significant effect of $10^{-4}$, $10^{-5}$, $10^{-6}$, or $10^{-7}$ M IAA, given as a 20 hr pretreatment, on the absorption was found.

These results are consistent with the hypothesis that incubation of apical segments of peas in IAA causes a shift in growth pattern from a mixture of cell division and expansion to primarily cell expansion, thus decreasing DNA synthesis. At concentrations below the growth optimum, the expansive capacity of the cells is not exhausted and DNA synthesis remains low. However, at an optimal concentration of IAA, $10^{-5}$ M, cell expansion proceeds to a point at which cell division and DNA synthesis become rate-limiting. Therefore, in order to maintain a high rate of growth, cell division and DNA synthesis must be resumed. The inhibition of DNA synthesis is probably a mixture of direct inhibition by IAA and inhibition by ethylene formed as a result of the high concentration of IAA.

REFERENCES

NUCLEAR PHOSPHORYLATION IN PEA SEEDLING NUCLEI

John E. Sherwin and Solon A. Gordon

PURPOSE AND METHODS

The inhibition of DNA synthesis and the induction of chromosomal aberrations by exposure of animal tissues to far-red light has been reported (1,2). Conceivably the action of light could be on nuclear phosphorylation directly. Nuclear phosphorylation has been reported for a variety of animal tissues (3,4), but has not been demonstrated in plants. We began to examine nuclei isolated from apical segments of pea seedlings for nuclear phosphorylation, and if it should occur, to determine the effect of light upon it. Nuclei were isolated by the method of Tautvydas (5). Examination of these preparations by light, ultraviolet, and electron microscopy indicated that the isolated nuclei were similar to nuclei in vivo.

PROGRESS REPORT

RNA synthesis ($^3$H-uridine incorporation) was linear for 30 min. DNA synthesis ($^3$H-thymidine incorporation) could not be demonstrated. Preparations of nuclei from dark-grown seedlings did not esterify $^{32}$P-orthophosphate. Nuclei preparations isolated from light-grown seedlings were contaminated with approximately 15% chloroplasts. These preparations esterified phosphate, but the presence of the chloroplasts and the lack of any proportionality between nuclei number and the esterification level leads to the conclusion that what we observed is not nuclear phosphorylation. It is probably a reaction of the chloroplasts in the preparation.

Our inability to demonstrate nuclear phosphorylation in pea seedlings may reflect either the isolation of a population of nuclei which are the equivalent of nuclei from non-radiosensitive tissues in animals, or an actual absence of nuclear phosphorylation. However, our data, correlated with the absolute requirement for exogenous ATP in nuclear RNA synthesis in many higher plants, suggest a lack of nuclear phosphorylation in peas. Nuclear phosphorylation apparently occurs in animals, but the nature of this reaction and its role in metabolism are not understood. Nuclear phosphorylation seems to be limited to radiosensitive tissues in animals (3), and therefore may be not a reaction of general distribution.

REFERENCES

ALTERATIONS OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BY LIGHT

John E. Sherwin, Adrienne Becker,* and Solon A. Gordon

PURPOSE AND METHODS

Exposure of cells to far-red light induces chromosome damage and inhibits DNA synthesis. The photon energy of this spectral region is too low to result in direct breakage of covalent bonds; it is thus presumed that these light effects are indirect. It has been suggested that in animals DNA repair is related to nuclear phosphorylation, and that this reaction, in turn, is affected by light. However, we have been unable to demonstrate the presence of nuclear phosphorylation in plants. It has previously been shown that light affects the rate of contraction of liver mitochondria and that blue light is maximally effective in this response. Mitochondrial contraction has been correlated with the integrity of the membrane and with the amount of adenosine triphosphate (ATP) in the mitochondrion. The generation of ATP by isolated plant mitochondria is inhibited by exposure to far-red light. We have therefore examined the action spectrum for mitochondrial phosphorylation in vivo to determine the nature of the photoreceptor(s) in our continuing effort to clarify the role of visible light in DNA synthesis and chromosomal damage.

PROGRESS REPORT

Etiolated 72-hour-old *Avena* seedlings were irradiated with monochromatic light at various wavelengths, obtained by interference filters (HVL 8 nm). The shoots were harvested and the mitochondria isolated in an isoosmotic medium at 2°C immediately following treatment. ATP synthesis was measured by the incorporation of $^{32}$P-phosphate. The synthesis of ATP was linear for at least 30 min.

Irradiation of seedings with far-red light (750 nm) for various times less than 2 hr resulted in an inhibition of phosphorylation. At 2 hr this inhibition disappeared and the phosphorylation level returned to that of the dark control. If these irradiations were followed by a period in the dark so that the total elapsed time was 2 hr, the phosphorylation level of

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the treated samples was the same as the dark control. Exposure of seedlings to red light (660 nm) resulted in an inhibition of mitochondrial phosphorylation. Figure V-4 shows the effect of a 2-hr exposure to increasing fluence rates of red light. On the other hand, phosphorylation levels were enhanced by exposure of *Avena* seedlings to blue light (440 nm) for 2 hr (Fig. V-5).

![Fig. V-4.---Inhibition of mitochondrial phosphorylation with increasing fluence rates of red light (660 nm).](image)

The inhibition by red light and the promotion by blue light of phosphorylation may be the result of changes in mitochondrial volume, and could thus be related to the action of light on the swelling of liver mitochondria. The relationship between DNA synthesis, oxidative phosphorylation, and mitochondrial swelling could be clarified by their action spectra.

![Fig. V-5.---Promotion of mitochondrial phosphorylation with increasing fluence rates of blue light (440 nm).](image)
THE EFFECT OF RED AND BLUE LIGHT ON DNA SYNTHESIS IN PEA SEEDLINGS

John E. Sherwin and Solon A. Gordon

PURPOSE AND METHODS

The inhibition of DNA synthesis and the induction of chromosomal aberrations by exposure of animal tissues to far-red light has been reported (1,2). The action spectra for these effects are not known. We began experiments to determine the effect of visible light on DNA synthesis in pea seedlings in an attempt, first, to determine the generality of this phenomenon, and second, to determine its action spectrum.

PROGRESS REPORT

DNA synthesis was measured by incubating apical segments of darkgrown peas in $^3$HTdR and subsequently extracting the DNA by a modification of the Schmidt-Thannhauser procedure. The specific activity of the DNA was calculated from the DPM's of the DNA and the DNA concentration as estimated by the technique of Burton. Preliminary experiments indicated that the incorporation of $^3$HTdR was linear for at least 1 hr.

Irradiation of intact pea seedlings for 10 min with red light (660 nm) does not immediately alter the incorporation of $^3$HTdR (Fig. V-6). However, if the red light treatment is followed by a 24-hr dark period before incubation of the segments with $^3$HTdR a fluence rate of 500 $\mu$W/cm$^2$ red light does enhance the incorporation (Fig. V-6). We have been unable to demonstrate an effect of blue light on the incorporation of thymidine.

Fig. V-6.—The effect of increasing fluence rates on the incorporation of $^3$HTdR into DNA of peas either immediately following light exposure (●) or with an intervening 24-hr dark period (○). Bars indicate the standard error of the mean of ten replicate samples, two experiments.
Hook opening in pea seedlings is controlled by red and far-red light via phytochrome. This control is directly attributable to an effect on ethylene formation; red light inhibits the production of ethylene in the hook region (3). It has recently been reported that ethylene inhibits both cell division and DNA synthesis in the hook region of etiolated pea seedlings (4). Although fluence rates much lower than 250 μW·cm⁻² are normally sufficient to induce hook opening, the data are consistent with the hypothesis that exposure of intact seedlings to high levels of red light induces hook opening by inhibiting the production of ethylene which had inhibited DNA synthesis and cell division. This change in the concentration of ethylene requires 24 hr to develop, and the increase in ³HTdR incorporation reflects this change.

REFERENCES


EFFECT OF MONOCHROMATIC BLUE AND RED LIGHT ON THE GEOTROPIC RESPONSE OF THE PRIMARY ROOTS OF CORN

Jane Shen-Miller and Raymond M. Miller*

PURPOSE AND METHODS

Corn roots are ageotropic when grown in the dark. In some plants light is necessary for the initiation of geotropic sensitivity in roots (1-3). Scott and Wilkins (1) reported that red, blue, and white light are equally effective in initiating the geotropic response of corn roots. The spectral bands they used were broad, and the irradiation time was extended over a period of 6 hr. The present study examines the fluence response of red and blue light on the geotropic curvature of corn roots and compares the quantum efficiency of the two wavelengths.

* Participant of the Summer Graduate Program, Illinois State University.
Primary roots of corn (Wisconsin 64A x 22R) were grown in total darkness. They were exposed to blue or red light on the day of the experiment. The irradiation source was an incandescent lamp behind infrared reflecting and interference filters ($\lambda_{\text{max}}$ 440 nm and 661 nm, HVL 8 nm). The roots were irradiated in the horizontal position. The irradiation time varied with experiments, but did not exceed 12 min. The roots were kept in the horizontal position in the dark for a period of 5 hr. The curvatures of the roots were then measured.

**PROGRESS REPORT**

Table V-2 tabulates the means of three experiments on the effect of blue and red light on the geotropic curvature of the corn roots. At equal fluence levels, the tropistic response of the root is greater in the blue than in the red. Semilogarithmic plots of the data yield linear relationships between fluence and response for both wavelengths. Least squares fits of the data indicate that the intercepts and slopes of the response curves are significantly different. On an incident equal quantal basis, the blue source was significantly more efficient than the red (Table V-3).

<table>
<thead>
<tr>
<th>Fluence (ergs·cm⁻²)</th>
<th>Blue (Curvature, degrees)</th>
<th>Red (Curvature, degrees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.23</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>8.37</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>10.8</td>
<td>2.02</td>
</tr>
<tr>
<td>500</td>
<td>10.0</td>
<td>4.00</td>
</tr>
<tr>
<td>1000</td>
<td>9.97</td>
<td>5.34</td>
</tr>
<tr>
<td>5000</td>
<td>11.0</td>
<td>8.54</td>
</tr>
<tr>
<td>10,000</td>
<td>11.7</td>
<td>8.27</td>
</tr>
<tr>
<td>50,000</td>
<td>15.8</td>
<td>10.8</td>
</tr>
<tr>
<td>100,000</td>
<td>13.2</td>
<td>14.3</td>
</tr>
<tr>
<td>500,000</td>
<td>-</td>
<td>15.5</td>
</tr>
<tr>
<td>1,000,000</td>
<td>-</td>
<td>15.5</td>
</tr>
</tbody>
</table>

| Slope a             | 2.20 ± 0.46               | 3.62 ± 0.22              |

a Derived by least squares fit of $Y = A + B \log_{10}X$
TABLE V-3. Quantum Efficiency of Incident Blue and Red Light on the Geotropic Response of Corn Roots

<table>
<thead>
<tr>
<th>Quanta ( \cdot ) ( \text{cm}^{-2} )</th>
<th>Blue (440 nm) (Curvature, degrees)</th>
<th>Red (661 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 2.2 \times 10^{13} )</td>
<td>4.02</td>
<td>3.88</td>
</tr>
<tr>
<td>( 2.2 \times 10^{14} )</td>
<td>5.97</td>
<td>4.72</td>
</tr>
<tr>
<td>( 2.2 \times 10^{15} )</td>
<td>8.20</td>
<td>5.85</td>
</tr>
<tr>
<td>( 2.2 \times 10^{16} )</td>
<td>10.3</td>
<td>8.41</td>
</tr>
</tbody>
</table>

Slope 2.11 ± 0.28 1.47 ± 0.28

In the above experiments the exposure times were kept constant and fluence rate varied. When the fluence rate was kept constant and time varied, the linear relation of log fluence to response disappeared. Reciprocity did not exist between fluence rate and exposure duration in the geotropic response of corn roots, at least in the energy range examined.

REFERENCES


EFFECT OF INDOLEACETIC ACID ON THE ORGANELLES OF THE OAT COLEOPTILE

*Stanley R. Gawlik and Jane Shen-Miller*

PURPOSE AND METHODS

In an earlier study of the effect of geotropic stimulation on the distribution and activity of the dictyosome, we found this organelle settled to the bottom of a cell and became more active upon tropistic stimulation.
In the present study we ask whether the plant hormone indoleacetic acid (IAA) is responsible for the activation of the dictyosome, and whether the kinetics of dictyosome change are compatible with those of coleoptile elongation. We also examine the effect of IAA on other cellular organelles. Oat coleoptile (Victory I) sections were incubated in a potassium phosphate buffer, 0.01 M, with or without IAA (10^{-6} M). The sections were harvested for electron microscopic examination at 6-min intervals for a period of 60 min.

**PROGRESS REPORT**

Table V-4 is the estimation of organelle number per outer epidermal cell in the 5th mm region of the coleoptile. The mean number of mitochondria and microbodies are significantly different between the control and IAA incubation. In the dictyosomes, there is an increase in number in the IAA treated segments at 18 and 24 min of incubation, an increase of 37.5% and 39.1%, respectively. Beginning at 30 min of incubation, the number of dictyosomes in the IAA-treated segments becomes significantly and increasingly less than that of the control (p < 0.005). The cisternal number per dictyosome also decreases in the IAA incubation. There is no consistent trend in the plastid number. The estimations of organelle number in Table V-4 derive from an actual count of the organelle per cell section, an actual measurement of the length of the outer epidermal cell, and approximating the diameters of dictyosome, mitochondrion, microbody, and plastid as being 0.6, 0.45, 0.4, and 1.0 μm, respectively.

Table V-5 shows the length measurements of the coleoptile segment and the outer epidermis of the segment. In the segments incubated with IAA there is an increase of 3.3% in length over a 60-min incubation. In

---

**TABLE V-4. Estimation of Organelle Number per Outer Epidermal Cell of the Oat Coleoptile (5th mm region) Averaged Over a Period from 0 to 60 min of Incubation in Control (C) and IAA Buffer.**

<table>
<thead>
<tr>
<th></th>
<th>Dictyosome</th>
<th>Mitochondrion</th>
<th>Microbody</th>
<th>Plastid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>IAA</td>
<td>C</td>
<td>IAA</td>
</tr>
<tr>
<td>No. x 10^{-3}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.1</td>
<td>8.48</td>
<td>21.9</td>
<td>2.94</td>
<td>2.39</td>
</tr>
<tr>
<td>p = 0.1</td>
<td>p = 0.025</td>
<td>p = 0.01</td>
<td>p &gt; 0.2</td>
<td></td>
</tr>
</tbody>
</table>
TABLE V-5. Length (μm) of Oat Coleoptile Segment and Outer Epidermis of the Segment Incubated in Control and IAA Buffer

<table>
<thead>
<tr>
<th>Incubation (min.)</th>
<th>Coleoptile Segment</th>
<th>Outer Epidermal Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>IAA</td>
</tr>
<tr>
<td>0</td>
<td>3000</td>
<td>3000</td>
</tr>
<tr>
<td>60</td>
<td>3000</td>
<td>3100</td>
</tr>
<tr>
<td>Increase per hr, %</td>
<td>0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

the outer epidermis, however, the increase is 13.1%. This discrepancy can be resolved if we consider that within the length of a 3 mm segment there are a maximum of 4 outer epidermal cells (2 whole and 2 cut). The cut cell does not elongate. Hence the average length-increase in a 4-cell segment would be 6.5%, an increase similar to that of the coleoptile segment tabulated in Table V-5.

In conclusion, IAA treatment increases the activity of the dictyosomes by initially increasing their formation. This effect is followed by an increase in dictyosome vesiculation, as indicated by the decrease in total dictyosome and cisternal numbers. IAA also decreases the total number of mitochondria and microbodies, but has no effect on the plastid number.

KINETICS OF STRESS-RELAXATION PROPERTIES OF THE OAT COLEOPTILE CELL WALL AFTER GEOTROPIC STIMULATION*

Jane Shen-Miller and Yoshio Masuda†

This study describes the stress relaxation of the cell wall of oat (Avena sativa) coleoptiles after different periods of geotropic stimulation. The upper and lower tissues (with respect to gravity) of geotropically

*Abstract of paper to be published in Plant Physiology.
†Faculty of Science, Osaka City University, Osaka, Japan.
stimulated coleoptiles exhibit different wall properties. The lower tissues are less resistant to deformation than the upper. The ratio of stress to strain is significantly less in the lower than in the upper tissue. Similarly, the relaxation time and the minimum relaxation time, derived from the Maxwell model which describes the physical characteristics of polymers, are also shorter in the lower tissue. However, the maximum relaxation time shows no difference between the upper and lower tissues of a geotropically stimulated coleoptile. The differences between the tissues begin at about 8 min after the commencement of stimulation, similar to the time for the initiation of dictyosome redistribution, and precede the onset of geotropism. The above responses of the cell wall of the lower tissue are similar to those induced by indoleacetic acid (IAA). The parameters of wall properties of the coleoptiles of both the control and the geostimulated fluctuate rhythmically with time. The periodic changes in wall properties of the coleoptile are compared to other cyclic physiological phenomena.

RHYTHMICITY IN THE BASIPETAL TRANSPORT OF INDOLEACETIC ACID THROUGH COLEOPTILES*

Jane Shen-Miller

$^{14}$C-indoleacetic acid was applied to coleoptiles of corn (Zea mays) and oat (Avena sativa). The coleoptiles were detached from the endosperms at 6-min intervals after IAA application, and the radioactivity was determined in successive 2-mm regions. The intensity (%/min) of basipetal transport of IAA is periodic in various regions of the coleoptile, with a period of about 20 min. The possible relation of this cyclic phenomenon to other rhythmic processes of similar periodicities is examined. A distinct acropetal transport (against the concentration gradient) from the subapical region to the apical 2-mm region of the coleoptile was detected.

The velocity of IAA transport differs in different regions of the coleoptile. Within an entire coleoptile the velocity can be divided into three classes for corn, 41 mm/hr (apical), 13 mm/hr (mid), and 34 mm/hr (base), and two classes for oats, 28 mm/hr (apical) and 14 mm/hr (remainder). An inverse relationship between the velocity of indoleacetic acid (IAA) transport and growth rate of the coleoptile is discussed. Corn coleoptiles exceed oat coleoptiles both in amount and velocity of IAA transport.

*Abstract of paper to published in Plant Physiology.
TOMATO LEAF RIBONUCLEASES: pH INDEPENDENCE OF LEAF FRACTIONS AND DEHYDROGENASE

Lewis D. Dove,* Solon A. Gordon, and Evelyn M. Buess

PURPOSE AND METHODS

Plant leaves contain several ribonucleases, some of which undergo activity changes as the leaves develop (1). A combination of tomato leaf ribonucleases with a pH optimum of 5.0 in crude leaf mixtures shows the greatest activity following leaf damage (2). Other pH peaks occur at 6.0, 6.6, and 8.0 in crude preparations dispersed in acetate-Tris or phosphate buffer. Work at Argonne National Laboratory was designed to investigate the pH dependency of these enzymes when they are separated into soluble and particulate fractions with the ultracentrifuge, and to examine changes due to leaflet dehydration.

Leaflets were weighed and dispersed in 0.05 M Tris/0.4 M sucrose/0.01 M NaCl solution which also contained 0.001 M MgCl	extsubscript{2} and 0.01 M glutathione. This solution was buffered at a pH of 7.5. The leaflet material was disrupted with 2 min of grinding in a well-chilled mortar using sea sand. A cell wall-free homogenate was prepared by filtering the leaf preparation through nylon cloth. A 10-min centrifugation at 500 x g removed the sand, debris, and unbroken cells. The supernatant fluid was centrifuged at 110,000 x g for 120 min. The fraction pelleting at 500 to 110,000 x g was called the particulate fraction and contained organelles less dense than nuclei (mitochondria, chloroplasts, etc.) as well as fragments of the endoplasmic reticulum and ribosomes. The particulate fraction was washed and and the soluble fractions were tested for ribonuclease activity over a 4.4 to 8.0 pH range. The distribution of ribonuclease activity in preparations from well-hydrated leaves was compared to the distribution of activity in dehydrated leaves. Ribonuclease activity was expressed in absorbance units per mg of protein nitrogen and also as absorbance units per fresh or rehydrated weight of the leaflets.

PROGRESS REPORT

The soluble ribonuclease enzymes showed peaks of activity at pH 4.6, 5.4, and 6.0, with possibly other peaks at pH 6.4 and 8.0. The particulate fraction has the same number of pH peaks at approximately the same location as the soluble fraction, probably indicating that the same ribonuclease enzymes are present in the two fractions. The pH peaks for the particulate fraction are 4.8, 5.2, 5.8, 6.2, and 7.6. Thus it is not possible to associate a particular ribonuclease activity with either of these gross soluble or particulate fractions. The relative contributions of each peak to the total activity (the pH profiles) are also about the

*Western Illinois University, Macomb.
TABLE V-6. Changes in the Soluble Ribonuclease Activity of Tomato Leaflets Following Dehydration

<table>
<thead>
<tr>
<th>pH optima</th>
<th>Units/g/hr&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Units/mg/hr&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dehydrated</td>
</tr>
<tr>
<td>4.6</td>
<td>116</td>
<td>84</td>
</tr>
<tr>
<td>5.4</td>
<td>100</td>
<td>112</td>
</tr>
<tr>
<td>6.0</td>
<td>103</td>
<td>112</td>
</tr>
<tr>
<td>6.4</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>8.0</td>
<td>13</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup>Based upon fresh weight of control leaflets and rehydrated weight of dehydrated leaflets, in grams.

<sup>b</sup>Considerable soluble protein N was lost during dehydration: control leaflets 2.1 mg N/g, dehydrated leaflets 1.0 mg N/g.

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TABLE V-7. Changes in the Particle-Bound Ribonuclease Activity of Tomato Leaflets Following Dehydration

<table>
<thead>
<tr>
<th>pH optima</th>
<th>Units/g/hr&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Units/mg/hr&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dehydrated</td>
</tr>
<tr>
<td>4.8</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>5.2</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>5.8</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>6.2</td>
<td>6.3</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup>Based upon fresh weight of control leaflets and rehydrated weight of dehydrated leaflets, in grams.

<sup>b</sup>Particle-bound protein N increased from 0.67 mg N/g to 1.09 mg N/g.
same for soluble and particulate fractions. The activity of the particulate fraction was found to be 13 and 18% of the total activity of tomato leaflets in two experiments.

The influence of dehydration damage on the distribution of ribonucleases in tomato leaflets was studied. The dehydration rate was very rapid, resulting in extensive protein breakdown so that the ribonuclease activity of drought-damaged leaves on a protein nitrogen basis far exceeded the activity of undamaged controls (Table V-6). This increased activity was not consistently manifest when the data were exposed on a rehydrated weight basis; some soluble ribonuclease may have been lost by the leaves during their rehydration in water. The data indicate that no single soluble ribonuclease was affected by dehydration much more than another. Particle-bound ribonuclease activity (Table V-7) was consistently enhanced by dehydration, an enhancement significant at the 1% level. There was no indication of a dehydration-induced loss of nitrogen from the particulate fraction.

REFERENCES


GRAVITY COMPENSATION AND INDOLEACETIC ACID BIOSYNTHESIS

*Metin Bara and Solon A. Gordon

PURPOSE AND METHODS

Several works have shown that gravity compensation increases the growth of dicotyledonous plants and raises their content of auxin. We have found that the activity of cellulase, an enzyme contributing to the cell-wall loosening process, is increased by gravity compensation (1) and by treatment with indoleacetic acid (IAA). This observation suggests the possibility that the activation of cellulase by compensation may be mediated by a rise in the level of IAA. The present study was therefore undertaken to explore the possibility that growth of a dicot on the horizontal clinostat would increase the activity of enzymes involved in the biosynthesis of IAA.

PROGRESS REPORT

Etiolated hypocotyls of Helianthus annuus were grown for 5 days and used as the source of the enzymes that convert tryptophan and tryptamine

*University of Turkey, Istanbul.
to auxin. The enzymes were purified by column chromatography, ammonium sulfate precipitation, and dialysis. They were incubated with substrate under conditions yielding zero-order reaction rates. The acid fraction of the reaction mixture was purified by thin layer chromatography and IAA was localized with the Prochazka reagent. In this preliminary work, areas of the spots were measured by weight.

Table V-8 shows the effect of growth on the horizontal clinostat on the relative amount of IAA produced in vitro from tryptamine and tryptophan. The data support the possibility that gravity compensation gives rise to raised auxin level by increasing the activity of enzymes involved in auxin biosynthesis. A more rigorous investigation of this possibility is warranted.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tryptamine</th>
<th></th>
<th></th>
<th>Tryptophan</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment Number</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>VR</td>
<td></td>
<td>5.2</td>
<td>3.2</td>
<td>2.7</td>
<td>4.2</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.70</td>
<td></td>
<td></td>
<td>3.79</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td></td>
<td>8.9</td>
<td>5.7</td>
<td>4.3</td>
<td>10.6</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.27</td>
<td></td>
<td></td>
<td>7.09</td>
<td></td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Incubation mixtures consisted of 0.33 mM substrate plus 0.3 μM peridoxal phosphate and 0.2 μM α-ketoglutaric acid in a total volume of 3.0 ml. Incubation time 4 hr at 30°C.

REFERENCE

THE EFFECT OF GRAVITY COMPENSATION ON GROWTH AND CELL WALL-LOOSENING ENZYMES IN *HELIANTHUS ANNUUS* HYPOCOTYLS*

Metin Barat and Solon A. Gordon

The effect of gravity compensation by the clinostat on elongation, weight, and activity of two cell wall-loosening enzymes (cellulase and β-1,3-glucanase) in *Helianthus annuus* hypocotyls was examined. Gravity compensation increases elongation (28.1%) and weight (18.3%). The activity of cellulase extracted from the apical sections is raised, but there is no significant effect on the β-1,3-glucanase. The relationship between gravity compensation changes in auxin level, and function of these two enzymes in respect to elongation, is discussed.

*Abstract of paper published in *Physiologia Plantarum.*

†University of Istanbul, Turkey.

QUANTIFYING RHYTHMIC MOVEMENTS OF *ALBIZZIA JULIBRISIN* PINNULES*

Willard L. Koukkari, † Franz Halberg, † and Solon A. Gordon

Pinnule pairs of *Albizia julibrissin* display pronounced circadian rhythmic movements in alternating (LD) or constant light (LL), and in continuous darkness (DD). In LD_{16:8} the best fit of the rhythm was 24.2 hr, whereas in DD and LL the rhythms were approximately 23.0 and 24.5 hr, respectively. Parameters of the cosinor technique, applied previously to animal organisms, are shown to provide a quantitative method for studying rhythm properties of either intact or excised pinnules. Phase shifting in *A. julibrissin*, as compared to the slower phase shifts of various circadian phenomena in the insect, bird, and mammal, occurs within 1 day. Also in contrast to the animal, rhythm adaptation in the pinnules occurs more rapidly when the lighting regimen is advanced than when it is delayed.

*Abstract of paper to be published in *Plant Physiology.*

†University of Minnesota.
Polarity and Rate of Transport of $^{3}$H-c-AMP in the Coleoptile

Solon A. Gordon, Erma Cameron, and Jane Shen-Miller

Transport of tritiated cyclic AMP in the coleoptile of oats (Avena sativa) and corn (Zea mays) is polar, with a basipetal-acroptetal ratio of three. The rate of transport is high, approximately that of indoleacetic acid. The linear velocity of transport, however, is at least five times that of the auxin. A loss in transport polarity of the nucleotide occurs in subapical tissues within several hours after decapitation of the tip, accompanied by a decrease in transport rate. The loss in polarity and decreased transport are not reversed by exogenous auxin. Exogenous cyclic AMP is metabolized rapidly by coleoptile tissue. If cyclic AMP does have a hormonal action in the coleoptile, its transport behavior is compatible with a primary as well as a "second" hormone function.

*Abstract of paper to be published in Plant Physiology.

Light-Phase Circadian Rhythms of Hepatic Tryptophan to Auxin and Tryptophan Aminotransferase Activities in the Mouse

Solon A. Gordon, Mark D. Challberg* and Evelyn M. Buess

Male CF-1 mice were maintained on alternating 12-hr periods of light and darkness. The ability of cell-free homogenates of the livers of these mice to convert tryptophan to the auxin, indoleacetic acid, was circadian, rising in the dark and falling in the light. An inversion in phase was induced by reversal of the illumination cycle. Tryptophan aminotransferase activity behaved identically; it is suggested that the periodicities of the enzyme complex derive from the periodicities of the transferase component. Although the cycle of indoleacetaldehyde dehydrogenase activity peaked in the dark, unlike the transferase it did not continue in the absence of food, and was not reversed in phase by inversion of the illumination cycle.

*Abstract of paper to be published in International Journal of Chronobiology.

†Student Aide, Bowdoin College, Brunswick, Maine.
THE INTERCELLULAR AIR SPACES IN OAT SEEDLINGS: AN INTERCONNECTED TUBULAR SYSTEM

Ray R. Hinckman

PURPOSE AND METHODS

For nearly half a century the oat seedling has been used extensively as a test organism in plant physiology. Therefore, it was somewhat unexpected to find previously unreported anatomical features of potential physiological function. This report will describe an extensive system of intercellular, interconnected, tubular air spaces that extend throughout the oat seedling. The intercellular air-space system is a common anatomical feature in plants that can range from discontinuous to continuous (1). The oat coleoptile, since it is thought to be a highly modified leaf (1), would be expected to possess the continuous type. However, no data on the air spaces of oats were found in the literature.

Entire freshly excised 72 hr oat shoots, coleoptiles, either with or without the distal 1 mm removed, and excised mesocotyls and roots, were placed in infiltration baskets and submerged in a solution of commercial India ink diluted 1:1 with distilled water plus 0.1% Tween 20. The ink solution containing the plant parts was immediately placed under aspirator vacuum for 10 min. This served to infiltrate and coat with a visible marker of carbon particles the inner surfaces of any air spaces open to the epidermal or cut surfaces of the organs. In another experiment only the cut basal ends of entire shoots, coleoptiles (with or without tips) or excised mesocotyls were placed in the India ink solution prior to aspiration. After both methods of ink infiltration the organs were rinsed, examined fresh, or fixed, and cleared in lactic acid. The organs were examined as whole mounts, split longitudinally and laid flat, or as cross sections.

PROGRESS REPORT

Entire shoots, detipped coleoptiles, and excised mesocotyls from both methods of infiltration contain long, ink-particle-filled tubular air spaces that run the entire length of the shoot (or organ) and are continuous through the node. This distance is often 25 mm or more, and a given tubular channel can often be traced from the coleoptile tip to the base of the mesocotyl. The air channels are between 2 and 6 μm in diameter (mean = 4.8 μm), varying in width along their length. The air channels number about 300 per shoot and tend to be parallel to one another, but do not follow a straight path. In cross sections of the coleoptile the air spaces are observed to be present throughout the entire parenchymal region, but are much more numerous in the regions surrounding the vascular bundles. In the mesocotyl the channels are more or less uniformly distributed through the parenchyma.
The longitudinal air channels are connected to each other by numerous transverse channels and to the substomatal air spaces to form an anastomosing network open to the ambient atmosphere. Interconnections are formed between the several tubular air spaces surrounding a given cylindrical parenchymal cell at the point where it abuts the next cell in a vertical file. At this point--where the crosswall is formed--the two cells pinch in slightly and allow several of the air spaces to fuse. The stomatal openings and the substomatal air spaces became coated with ink particles, an indication that the stomata are a site of entry to the network. The substomatal air spaces are interconnected with the channels in a manner similar to that already described.

Similar interconnected air spaces are observed in the root. A group of relatively large tubes form a meshwork around the central vascular tissue (stele). Several ink-filled channels extend all the way into the meristematic region in the root tip.

The air-space system in the oat seedling constitutes an internal gaseous atmosphere that is in direct contact with most of the cells of the plant as well as with the external atmosphere. The dynamics of this internal atmosphere is unknown, including how it relates to the ambient atmosphere.

The gaseous plant growth regulator ethylene is known to be synthesized within the plant and has a variety of effects on growth, development, and senescence (2). Oats and other cereal seedlings are often used as test objects for ethylene; thus, data relating to the transport and movement of this gas within the plant must now be reconsidered in terms of the internal air space network. Moreover, the mechanism of auxin transport in plants is still unresolved. Models assuming transport through the cytoplasm, vascular system, and cell walls have been proposed. The internal surfaces of the air channel network could also be considered as a possible site of auxin transport, especially if these surfaces are covered by a thin layer of liquid water as is found in leaf mesophyll air spaces. Other members of the plant group here are presently testing this possibility.

REFERENCES
A PERMANENT IODINE STAIN-MOUNTANT COMBINATION FOR STARCH IN PLANT TISSUE*

Ray R. Hinchman

The best methods for the specific staining of starch in plant tissues for light microscopy still utilize an aqueous stain containing iodine dissolved in a solution of potassium iodide (IKI or Lugol's reagent). Slides prepared with IKI are not permanent, and even when stored in a moist chamber, fade in a few weeks. In the technique described, plant material stained with aqueous IKI is mounted in sugar syrup (Karo, clear) combined with IKI to give permanent iodine stained microscope preparations for starch. Slides of cereal grain seedlings prepared according to this method remain brilliantly stained after seven years.

*Abstract of paper to be submitted for publication.

AMYLOPLAST SIZE AND NUMBER IN GRAVITY-COMPENSATED OAT SEEDLINGS

Ray R. Hinchman and Solon A. Gordon

PURPOSE AND METHODS

Clinostats are devices designed to reorient the plant continuously by rotation on a horizontal axis, so that the tropic effect of gravity direction is nullified or "compensated". In a previous report (1), increased levels of starch and soluble sugars have been observed in the shoots of gravity-compensated oat seedlings. Because the carbohydrates were determined using extraction methods, we could not determine whether the increase in starch was due to an increase in starch grain (amyloplast) size or in number.

We present here evidence that indicates the elevated starch content in compensated oat shoots derives from larger starch granules, with no increase in the number of granules. If one assumes a role in the geoperceptive mechanism for amyloplasts, these findings are consistent with reports (2,3) that clinostat rotation increases the geotropic curvature in response to subsequent stimulation.

Seeds of Avena sativa cv. Victory were soaked, cold treated, planted in moist sand in beakers, and grown in the dark either on a horizontal clinostat at 2 rpm or in a stationary vertical position. After 72 hr the
plants were harvested, fixed, and prepared for light microscopy by staining for starch with iodine (4).

PROGRESS REPORT

In the IKI-stained preparations the starch grains appear as round, uniformly dark bodies, apparently free within the cells. However, ultrastructural studies (5) of oat coleoptiles show that starch grains are always deposited within a plastid and are usually composed of 2 to 6 closely appressed polyhedral granula. Measurements of the diameter of the entire compound granules were made to the nearest 0.5 μm.

Figure V-7 shows that the starch grain diameter is significantly larger in gravity-compensated shoots as compared to stationary controls, both in the avascular tip and in the cells below the tip. In both regions, this difference is due primarily to a shift in all amyloplast size classes toward the larger classes in the compensated plants. The tip cells from both

Fig. V-7.—Starch grain diameter in parenchyma cells of gravity compensated and stationary oat coleoptiles.
treatments have a greater range of starch grain sizes than cells below the tip. No significant difference was found in the number of plastids per cell in compensated vs. control shoots in the two regions examined when counted either as starch grains (amyloplasts) per 10 μm cell section or as total plastids per cell.

A rotation-induced mixing action in the endosperm cavity causing increased starch hydrolysis (to sugars) followed by transport to the shoot could result in increased carbohydrate levels in shoot tissue as reported by Gordon (1) and, following redeposition as starch, increased amyloplast size as reported here.

Thus, an explanation can be suggested for the enhancement of geosensitivity in shoots by gravity compensation based on an increase in amyloplast size and density. Amyloplasts may function as physiologically active gravity sensors that polarize the cell by providing more starch-derived metabolites to the physically lower portion of the cell into which they settle. This non-uniform supply of carbohydrate could drive differentially such mechanisms as hormone synthesis and active transport.

REFERENCES


THE EFFECT OF LIGHT ON CELL SIZE AND MITOSIS IN OAT SEEDLINGS

Ray R. Hinchman

PURPOSE AND METHODS

In grass seedlings the coleoptile is a determinate structure present in the embryo. A brief period of scattered mitotic activity is followed by cell expansion and, eventually, cessation of growth. This report describes a light-induced acceleration of development and maturation characterized by an inhibition of mitotic activity and a transient increase in the length of parenchyma cells of the coleoptile.

Seeds of oats, Avena sativa, cv. Victory, were soaked, cold-treated, grown under conditions of continuous white light or total darkness at 25°C,
and harvested at 0, 24, 48, and 72 hr after planting. The plant organs were fixed in formalin, 5%, propionic acid, 5%, 70% ethyl alcohol, 90% (FPA) and prepared for light microscopy.

PROGRESS REPORT

Figure V-8 shows parenchymal cell lengths and mitotic indices at various times after planting. The average cell length is consistently greater in the light-grown coleoptiles when compared to the dark-grown during the first 72 hr of growth. The sharp increase in cell length between 48 and 72 hr reflects the transition from a period of cell division to one of rapid cell expansion. The two stages overlap considerably, for all of the cells expand to some extent before division commences. The average cell width also increases in light-grown coleoptiles, but because parenchymal cells exhibit polarized cell growth in the longitudinal direction, the increase in width is small compared to the increase in length.

Considerable cell expansion is observed in the light-grown organs between 24 and 48 hr, whereas in the dark there is a slight decrease in average cell length during this period (Fig. V-8). This decrease is due to the presence of a large number of small, squat daughter cells resulting from numerous anticlinal cell divisions occurring at this time. Initially in the light-grown plants, mitotic activity increases more rapidly than in the dark and reaches a relatively low peak of 4.3% around 24 hr and then rapidly drops to 0.7% by 48 hr, and to zero by 72 hr. In the dark-grown coleoptiles the mitotic index is 3.0% at 24 hr, and then increases further.

![Fig. V-8.--Cell length (o) and mitotic index (A) in oat coleoptile parenchyma cells in continuous light (—LT) or total darkness (—DK).](image-url)
to a peak of 9.3% around 48 hr, thence to zero at 72 hr. These observations demonstrate a light-induced reduction of mitotic activity, both in duration and magnitude.

Assuming a constant mitotic interval for all coleoptile cells, the lower peak mitotic index and the shorter period of mitotic activity in the light-grown organs will result in coleoptiles with a smaller total number of cells. That this is the case is shown by a comparison of the total number of parenchymal cells in a single vertical file in light-grown coleoptiles \( (98 \pm 9) \) vs. dark-grown \( (142 \pm 14) \) at 48 hr \( (p < .05) \).

At 72 hr the cells of the light-grown coleoptiles have expanded nearly to their maximum size. Between 72 and 96 hr these coleoptiles cease growth and the leaf penetrates the coleoptile tip. In the dark-grown coleoptiles at 72 hr the cells are smaller and more numerous and possess a considerable capacity for additional expansion. These coleoptiles will maintain a rapid growth rate until their cells reach a maximum size that is considerably longer than that attained by the light-grown organs \( (1) \), at which time (about 100 hr) the leaf will penetrate.

Thus the primary effect of continuous light on coleoptiles is to advance and shorten the duration of the mitotic and elongation phases of cell growth. Light hastens the transition to the next developmental stage and reduces the final number and length of the cells.

These observations may also help explain why light-grown or light-exposed coleoptiles are less sensitive to tropic stimuli than dark-grown organs; light-grown tissues have a smaller number of larger cells (at an age equivalent to our 72 hr) that may not have the potential to expand as greatly or as rapidly in response to changes in auxin concentration.

REFERENCE


METABOLIC AND THERAPEUTIC STUDIES OF PLUTONIUM AND AMERICIUM

*Arthur Lindenbaum, Marcia W. Rosenthal, David W. Baxter, Nancy E. Egan, G. Steve Kalesperis, Elizabeth S. Moretti and John J. Russell*

PURPOSE AND METHODS

The broad objective of this program is to develop new approaches to the therapy of poisoning by radioactive and nonradioactive metals \( (1) \). Plutonium-239 has received increasing emphasis during the last several years because of its importance as a radiological health hazard. The
varying tendency of compounds of the actinide series (as well as other polyvalent heavy metals) to hydrolyze and polymerize, both in solution and in vivo, results in variable particulate characteristics which influence the deposition, retention, effectiveness of therapy, and delayed pathological effects of these nuclides. Thus information obtained with plutonium and americium aids in understanding the behavior of other nuclides of the actinide, lanthanide, and rare earth series in living tissues. Plutonium compounds also provide useful information on the translocation and deposition of colloids and macromolecules in tissues.

Previous work has demonstrated the effectiveness of chelating agents, such as diethylenetriaminepentaacetic acid (DTPA), for removal of plutonium and related elements from blood, bone, and soft tissues; attention is now being directed toward other therapeutic approaches aimed at removal of that portion of the plutonium not readily removed by DTPA.

As part of the actinide program, additional studies are concerned with calcium uptake and depletion and with biochemical transformations in the calcifying matrix of preosseous cartilage. The aim of this work is to achieve a better understanding of the uptake of normal (e.g., calcium) and abnormal (e.g., plutonium and radio strontium) metals in cartilage and bone.

PROGRESS REPORT

Plutonium Decorporation by DTPA, Glucan, and Related Compounds. The removal of long-term deposits of plutonium in the liver (such as those in the dog and man) has been studied using, as a model system, the mouse liver after intravenous injection of mid-range polymeric plutonium (2,3). The polysaccharide glucan has been shown to remove a fraction of this plutonium, presumably intracellular, that is not available for removal by DTPA (3,4). Glucan and DTPA have now been tested in the beagle dog for their ability to reduce plutonium deposits. DTPA (100 mg/kg injected intravenously twice weekly for 12 weeks, starting 6 days after injection of monomeric \(^{239}\text{Pu}\) citrate) removed 50\% of the bone burden, 96\% of the liver burden, and between 50\% and 90\% of the burden of all other soft tissues assayed (except marrow). Plutonium removed from the skeleton appears to have been derived from the more rapidly remodeling trabecular bone (e.g., femurs, 3rd lumbar vertebra). There was no removal of plutonium from slowly remodeling bones (e.g., mandible, teeth) or from the marrow. When given as an adjunct to DTPA, glucan (injected intravenously three times at a dose of 15 mg/kg, at 4-week intervals beginning 6 days after plutonium) did not increase or modify the effects of the DTPA.

Monomeric plutonium in the dog liver has long been considered primarily intracellular because of its lifetime retention, analogous to hepatic retention in the mouse of polymeric plutonium, which is known to be deposited intracellularly (5) and which is not removed by DTPA. The almost complete removal of monomeric plutonium in the dog liver by DTPA, a chelating agent that remains almost entirely extracellular, suggests that there are species differences in the mouse and dog (and man?) regarding the microlocalization of plutonium in the liver (vide infra). Further studies of the action of DTPA and/or glucan in removing monomeric and polymeric plutonium from dog tissues are in progress.
In the mouse, the removal of a small amount of additional plutonium from the liver by a second course of glucan therapy, given 6 weeks after the first (2) has not been confirmed.

To elucidate the mechanism of glucan action in removal of plutonium from the liver, other related substances have been tested. Each was chosen because it was either (a) a chemical analog (isosclerotan) or a morphological analog (bacterial cell walls) of glucan, (b) an interferon-inducer and reticuloendothelial system (RES) stimulant (pyran copolymer; poly I/C; Tilarone), or (c) a disruptor of lysosomes (Triton WR 1339, a nonionic surface-active agent). Each compound was administered to mice injected intravenously with mid-range, polymeric plutonium 5 days DTPA therapy was initiated at the same time and also continued thereafter (100 mg/kg, twice weekly). All mice were sacrificed 47 days after plutonium administration. Four of the tested adjuncts to DTPA therapy removed significant (P < .01) additional plutonium from the liver (see Table V-9). Of these, the most effective (pyran copolymer) and least effective (Tilarone) are soluble interferon-inducers. Glucan and isosclerotan are insoluble, morphologically different but chemically similar glucopolysaccharide constituents of yeast and fungal cell walls, respectively. Scanning electron micrography shows glucan to retain the size and shape of the yeast cells from which it is derived. Isosclerotan, on the other hand, appears as a filamentous network in similar freeze-dried preparations. Further work with pyran has now shown that (a) another pyran copolymer is also effective in removal of hepatic plutonium, (b) removal is effected in about 1 week, and (c) hepatic plutonium removed by pyran is not translocated to bone in the absence of DTPA, as is plutonium removed from the liver by glucan (4).

We have also investigated the toxicity of these adjunct substances. Glucan toxicity, for example, depends on the dose and rate of administration and the species tested. A single intravenous injection of 6 mg/kg in the dog is almost immediately lethal, compared to 480 mg/kg in the mouse. If glucan is administered to the dog over a span of 2 hr however, 15 mg/kg may be given with complete recovery.

A new approach to the removal of intracellular plutonium from the liver is the encapsulation of DTPA within artificial lipid spherules, termed liposomes (6).
Localization of Liver Plutonium by Quantitative Autoradiography.

Further work has been done to validate the usefulness of autoradiography for the quantitative localization of actinides (7) in animal tissues. Data obtained after different autoradiographic exposure times, with livers from dogs and mice injected either with monomeric or polymeric plutonium or with polymeric americium, have been recomputed. The normalized data, expressed as tracks/dpm vs. days of autoradiographic exposure, demonstrate that plutonium concentrations based on track counts obtained after varying exposure times, and under a wide range of experimental conditions, are in reasonable agreement with gross radiochemical determinations. Quantitative autoradiography, in addition, permits assay of plutonium or americium deposited at specific hepatic microloci. Two new findings have been obtained with this technique: (a) in dog liver, 6 days after injection of polymeric plutonium, there was a ten-fold increase in the centrilobar concentration of plutonium, as compared with peripheral regions; (b) species differences in liver function are indicated by a lower concentration of both monomeric and polymeric plutonium associated with liver parenchymal cells of the dog, compared with those of the mouse.

Effects of Physical-Chemical State of Plutonium on Lifetime Pathological Changes in Mice. An experiment to compare the effects of a range of skeletal levels of a highly polymeric and a monomeric form of plutonium in female CF#1/Anl[Anl 66] mice has been reported previously (8), and interim progress has been described in subsequent annual reports. All 380 plutonium-injected mice allotted for lifetime study, and 58 non-injected controls, are now dead. After injection of polymeric plutonium, the mean survival time of the mice receiving the lowest dose level (1.1 μCi Pu/kg) was the same as that of controls; at higher doses, survival decreased progressively with increasing dose. After injection of monomeric plutonium, the mean survival time was not changed after the four lower doses levels (0.07 to 0.5 μCi Pu/kg) and was decreased only after the highest dose (0.96 μCi Pu/kg). Up to 600 days there was a higher survival of mice given lower levels of monomeric or polymeric plutonium compared to uninjected controls. This suggests that the internal deposition of plutonium may have delayed the onset of the lymphomas which were a primary cause of death in many of the control mice.

Preliminary histopathological observations in the uninjected control mice of this experiment, compared with observations made on an earlier series of 100 control female CF#1/Anl mice (studied in conjunction with Dr. H. Lisco of Harvard Medical School) (2), have now been extended. There is a lower incidence of bone lesions in the newer series of controls: 10% vs. 41% of the mice with osteomas; 0% vs. 4% with osteosarcomas.

Terminal roentgenograms of all lifetime mice have now been examined, all skeletal areas requiring histopathological examination have been removed, and the bones of 7 of the 10 groups have been prepared for histological examination. The bone sections of plutonium-injected mice are being compared with those of controls. The objectives of these microscopic studies are to correlate bone tumor incidence with skeletal dose and to compare histopathological sequelae in bone as correlated with dose, time,
and form of plutonium (monomeric vs. polymeric) administered. (Histopathological studies are in collaboration with T. E. Fritz of this Division.)

**ICRP Activities.** A report to ICRP Committee 2 on "Metabolism of Plutonium and Related Elements and Their Compounds" prepared by a task group which includes two of us, M.W.R. and A.L. (Chairman), has been completed and will be published soon as an ICRP "blue book".

**REFERENCES**


**INTRACELLULAR PLUTONIUM REMOVAL BY LIPSOME-ENCAPSULATED CHELATING AGENT***

*Yueh-Erh Rahman, Marcia W. Rosenthal, and Elizabeth A. Cerny*

The chelating agent diethylenetriaminepentaacetic acid (DTPA) was encapsulated within artificial lipid spherules (liposomes) prepared with egg lecithin and cholesterol. Encapsulated DTPA, given intravenously to mice 3 days after injection of plutonium, removed a significant fraction of the plutonium in the liver, presumably intracellular plutonium, that

*Abstract of paper to be published in Science.*
was not removed by non-encapsulated DTPA. Liposome-encapsulated DTPA also caused a significant increase in urinary excretion of plutonium, derived, at least in part, from tissues other than the liver.

The encapsulation of chelating agents by liposomes and the resulting introduction of the drug into the cell should be of potential use for therapy of poisoning by other radioactive and nonradioactive toxic metals (1).

REFERENCE


COMPARISON OF MONOMERIC AND POLYMERIC PLUTONIUM IN THE DOG AND MOUSE*

David W. Baxter, Marcia W. Rosenthal, John J. Russell, Elizabeth Moretti and Arthur Lindenbaum

Monomeric or polymeric plutonium was injected intravenously into beagle dogs and CF\#1 Anl[Anl 66] mice for the purpose of comparing early total-body distribution of two forms of this actinide in these species. For the dogs, the blood clearance rate, intralobar liver distribution, and urinary and fecal excretion were also determined. Monomeric plutonium was cleared from dog blood less rapidly than polymeric plutonium. During the first 15 min only 20% (vs. 99%) had left the circulation. At sacrifice, 6 days postinjection, significantly more monomeric plutonium remained in the blood although the amount of each form was less than 1%. Monomeric plutonium was deposited chiefly in liver and skeleton of both species. Polymeric plutonium was deposited in liver, spleen, and lungs, with a comparatively small amount deposited in the skeleton. Autoradiographic measurements of plutonium deposition within the dog liver lobe showed a relatively homogeneous distribution of monomeric plutonium, whereas polymeric plutonium tended to be associated with sinusoidal (phagocytic) cells at the center of the liver lobe. The roles of phagocytosis and protein binding in plutonium transport and retention, with possible implications for man, are discussed.

*Abstract of paper to be published in Radiation Research.
INVESTIGATION OF THE PROTEINPOLYSACCHARIDES OF CALF SCAPULAR CARTILAGE*

Nancy E. Egan

Proteinpolysaccharide complexes together with collagen comprise most of the intercellular matrix or "ground substance" of cartilage. Since it is within this matrix that the first crystals of bone mineral appear during biological calcification and subsequent ossification or bone formation, the proteinpolysaccharides are thought to either initiate or regulate this important process. A thorough understanding of the biochemical events occurring when cartilage tissue is replaced by osseous tissue is of fundamental importance to studies on aging, wound healing, bone and connective-tissue abnormalities and diseases, the therapeutic removal of bone-seeking radioisotopes, and electrolyte homeostasis.

Previous hypotheses have attributed to the proteinpolysaccharides of cartilage either a calcification-inhibiting or a calcification-promoting function. Both of these views were based on interactions presumed to occur between the proteinpolysaccharide complexes in the intact mineralizable cartilage and the calcium and phosphate ions present. These interactions were examined in the present study with proteinpolysaccharides obtained from four zones representing stages in the transformation of cartilage to bone. The calf scapula is particularly suitable for this study because it is readily available tissue from which nearly unlimited amounts of mineralizable cartilage in any desired stage of transformation may be obtained.

Two methods, differing in the amount of shear employed, were used for proteinpolysaccharide extraction. Presumably, the product of low-shear (dissociative) extraction would be more likely to resemble its state in intact tissue than would the product of high-shear (degradative) extraction. The isolated proteinpolysaccharides were compared and analyzed for hexuronic acid, hexosamine, protein, and equivalent weight. The affinity of these isolated proteinpolysaccharides for calcium ion was determined by a cation-exchange method. The highest affinity for calcium ion, as well as the highest equivalent weight, was found in the proteinpolysaccharides isolated from scapular cartilage nearest the cartilage-bone junction. Proteinpolysaccharides isolated from all areas, but especially from the area of the cartilage-bone junction, were found to inhibit the precipitation of calcium phosphate from metastable ionic solutions. The measurement of this inhibition was facilitated by a double-labeled liquid-scintillation technique developed in this study.

*Abstract of a dissertation describing work performed during tenure of an AUA-ANL Predoctoral Fellowship and submitted to the Faculty of the Graduate School of Loyola University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
The findings of increased calcium-ion affinity and inhibition of solid-phase separation of calcium phosphate as the cartilage-bone junction is neared, followed by a decrease in the new bone area, provide evidence for a "storage-and-release" function for proteinpolysaccharides in endochondral ossification.

REMOVAL OF PLUTONIUM FROM MOUSE LIVER BY GLUCAN AND DTPA*

Marcia W. Rosenthal, Helen Brown, † Dorothy L. Chladek, Elizabeth S. Moretti, John J. Russell, and Arthur Lindenbaum

Glucan from yeast is additive with DTPA (diethylenetriaminepenta-acetic acid) in effecting removal of polymeric plutonium-239 from the mouse liver. At 47 days after administration of plutonium, the net removal was 8.5% of the injected dose by glucan alone, 11.5% by twice-weekly DTPA treatments, and 19.5% by combined therapy. A 60 mg/kg dose of glucan, given intravenously, was equally effective when given (a) in a single injection, (b) divided into 3 daily injections, (c) given 5 days or 3 hr before plutonium, or (d) 5 days after plutonium. A 15 mg/kg dose of glucan was almost as effective as higher doses (30 to 120 mg/kg). Hepatic plutonium removed by glucan was excreted in the urine in the presence of DTPA, or was apparently translocated to the skeleton without DTPA.

As shown earlier for monomeric plutonium in the mouse, liver deposits of polymeric plutonium are excreted primarily through the feces, both with and without DTPA, and skeletal deposits removed by DTPA are excreted through the urine. Twice-weekly injections of 100 or 500 mg/kg of DTPA are equally effective in removal of polymeric plutonium from mouse bone and liver, also confirming results with monomeric plutonium.

*Abstract of paper to be published in Radiation Research.
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VI. BIOPHYSICS

SUMMARY

Steven S. Danyluk, Group Leader

The past year has seen continued vigorous development of Biophysics Research programs along four broad lines: studies with mammalian cells in culture, circadian cybernetics, biomolecular structure determinations, and clinical applications of stable isotopes. A governing principle behind each of these programs is the development and application of sophisticated biological, physical, and chemical techniques such as scanning electron microscopy, gas chromatography-mass spectrometry, X-ray diffraction, nuclear magnetic resonance and electron paramagnetic resonance spectroscopy, and large scale continuous culture systems, to the quantitative solution of important biological problems. Although much of the work is fundamental in nature, the focus continues to be on those systems which relate directly to cellular and organismic functioning, particularly in response to external stimuli such as radiation, protective agents, chemicals, and drugs.

An indication of the vitality of the Biophysics Group Research programs is given by the number and scope of achievements reported in 1972. Among some of the more notable are: evidence that N-ethylmaleimide inhibits repair of lethal radiation damage in oxygenated Chinese hamster cells, evidence for a chronotypic induction of tyrosine aminotransferase by theophylline in rats, characterization of key cellular processes in the infradian growth mode, calculation of an electron density map for a Bence-Jones dimer to 3.5 Å, extension of the concanavalin A structure to 2.4 Å resolution, and location of the myo-inositol binding site in concanavalin A, the first synthesis of a dinucleotide with a fully labeled (\(^{2}\text{H}\)) nucleotidyl unit, a complete three-dimensional structure determination in solution for the key antimalarial chloroquine, synthesis of several stable isotope labeled (\(^{2}\text{H},^{13}\text{C}\)) metabolites essential in clinical testing procedures, and development of a collaborative program for clinical applications of stable isotopes.

Many aspects of the individual programs are now at the point where major developments can be foreseen with confidence. Hopefully, the coming year will see fruition of this work.

BIOPHYSICS STAFF

REGULAR STAFF

Ainsworth, Clinton, F. (Scientific Assistant)
Danyluk, Steven S. (Senior Chemist)
The objectives of this program are to use mammalian cells in vitro to examine responses to ionizing and ultraviolet radiation and to determine basic biochemical properties of mammalian cells, particularly those relating to growth and regulatory mechanisms. Cell properties that are involved in radiation responses and that may relate to primary radiation damage or its repair in cells are of special significance. In general, cultured cells are used to conduct studies difficult or impossible to perform on cells in vivo, and an important part of the program is to examine the extent to which responses in cells in vitro occur also to cells in vivo.

During the last year, studies have concentrated on the relationship between radiation survival and the changing biochemistry of the cell during the cell cycle. Studies have continued with the thiol-binding sensitizer, N-ethylmaleimide (NEM), in fully oxygenated synchronous Chinese hamster cells. This agent not only produces a differential effect, sensitizing most those resistant cells near the end of the S period, but also has a

*Terminated during 1972.
pronounced post-irradiation effect, in addition to its sensitizing effect when administered before or during irradiation. The results obtained so far suggest strongly that the agent inhibits repair of radiation damage, most probably via its binding to some particular form of the intracellular sulfhydryl. However, the actual mechanism by which the inhibition is accomplished is still open to question, since various other effects of the agent must also be considered. We have already indicated in last year's report that NEM inhibits DNA synthesis, and further studies on this inhibition are reported here. The agent also binds to DNA and this binding may also be significant in its effect on radiation response. As a further avenue to understanding the mechanism of NEM inhibition of repair, similar agents such as N-ethlysuccinimide are also under investigation.

A total of five summary reports are presented on the work relating to survival. In addition, two reports are included on cytological studies on responses to JANUS neutrons and on the scanning electron microscopy of chromosome aberrations.

STUDIES WITH MAMMALIAN CELLS IN CULTURE.
I. N-ETHYLMALEIMIDE AND THE CYCLIC RESPONSE TO X RAYS OF SYNCHRONOUS CHINESE HAMSTER CELLS*

Warren K. Sinclair

In addition to DNA synthesis, at least one other factor appears to exert a controlling influence on the cyclic dependence of the lethal radiation damage in mammalian cells. Experiments designed to identify this factor have been conducted with the sulfhydryl binding agent, N-ethylmaleimide (NEM), in synchronous Chinese hamster cells. Low concentrations of NEM (0.75 μM) sensitize oxygenated Chinese hamster cells most in the late S period. The effect is primarily on the slope of the survival curve, rather than its shoulder, and the maximum sensitization factor for late S cells is about 1.6. Experiments with NEM and cysteamine (MEA) indicate that NEM forms a stable complex in the cell, and thus exposure to NEM before irradiation sensitizes the cell equally well as when NEM is present during irradiation. The sensitizing effect is less the longer the interval between pretreatment with NEM and irradiation. NEM also has a pronounced post-irradiation effect; when given immediately after irradiation the effect is the same as for pretreatment or during irradiation. The effect reduces in magnitude with time for up until 2 hr after irradiation in late S cells and for longer times in the case of G₁ cells. NEM also suppresses the rise in survival normally seen late in the cell cycle of cells inhibited by hydroxyurea from synthesizing DNA and thus presumably modifies a factor

*Abstract of paper to be published in Radiation Research.
which controls cell survival in the absence of DNA synthesis. Late S cells irradiated in the presence of NEM have the same capacity to recover from sublethal damage as do untreated cells.

These results suggest that when oxygenated cells are exposed to a low concentration of NEM repair of lethal damage is inhibited. The mechanism by which NEM accomplishes this inhibition is not known, since the evidence as to whether -SH binding is involved is conflicting and other actions of NEM also take place.

STUDIES WITH MAMMALIAN CELLS IN CULTURE.  
II. SENSITIZATION OF CHINESE HAMSTER CELLS TO X RAYS BY SUCCINIMIDE, N-ETHYL SUCCINIMIDE AND PARA-CHLOROMERCURIPHENYL SULFONIC ACID

Warren K. Sinclair

In order to investigate further the mechanism by which N-ethylmaleimide (NEM) sensitizes cells to X irradiation, some similar molecules such as succinimide and N-ethylsuccinimide are being tested for toxicity and sensitization. In addition, a reversible sulfhydryl binding agent, p-chloromercuriphenyl sulfonic acid (PCMBS), is also being tested to determine whether differential cyclic and pre- and post-irradiation effects similar to those of NEM occur.

Preliminary experiments have been conducted in asynchronous cells to establish concentrations of these agents which are effective as sensitizers without being excessively toxic. Studies with succinimide established that a concentration of the agent of the order of 750 nM sensitized and was not toxic, but repeat experiments gave results which were extremely variable from batch to batch of succinimide. The effects were probably due to a much lower concentration of an impurity.

N-ethylsuccinimide (NES) showed a marked sensitization effect at about 2 mM in phosphate buffered saline (PBS) with only a slight toxicity of the agent. Studies at about this concentration are now being carried out in synchronous cells to determine whether NES has differential sensitization and pre- and post-irradiation effects similar to those of NEM. The question of impurities in NES will also have to be investigated.

Studies with PCMBS have established that concentrations in the range 0.05 mM to 0.10 mM in PBS sensitize without being too toxic. Investigations in synchronous cells are about to begin.
STUDIES WITH MAMMALIAN CELLS IN CULTURE.
III. EFFECTS OF N-ETHYLMALEIMIDE ON DNA SYNTHESIS IN
CHINESE HAMSTER CELLS*

Warren K. Sinclair, Eleanor A. Blakely,‡ and Joy Archer

The effects of N-ethylmaleimide (NEM) on DNA synthesis in asynchronous
and synchronous Chinese hamster cells were studied using autoradiography
and liquid scintillation counting techniques. NEM was found to inhibit
the incorporation of tritiated thymidine (³HTdR) into synchronized cells,
the extent of inhibition being essentially independent of the stage of the
cell cycle the cells were in when treated. Most cells were capable of
recovering about 70% of their rate of incorporation after post treatment
incubation in culture medium at 37°C for 1 to 2 hr. The inhibition of the
incorporation of ³HTdR into whole cells and into the DNA subsequently iso­
lated from treated cells was dependent on the concentration and length of
exposure to NEM. NEM also inhibited the incorporation of orotic acid and
cytidine-5'-diphosphate into asynchronous cells.

Studies with diamide [diazenedicarboxylic acid bis-(N, N-dimethyl-
amine)], a glutathione binding agent, showed some inhibition of thymidine
incorporation but recovery was much greater than with NEM, which also binds
glutathione.

‡1972 Thesis Parts Student, University of Illinois, Urbana.

STUDIES WITH MAMMALIAN CELLS IN CULTURE.
IV. BINDING OF N-ETHYLMALEIMIDE TO DNA

Joy Archer

The possible binding of N-ethylmaleimide (NEM) to DNA was investigated
because (a) NEM has an inhibitory effect on thymidine incorporation into
Chinese hamster cells (1,2), and (b) binding of NEM to DNA can be induced
by X irradiation (3).

The binding of NEM to calf thymus DNA (Sigma) and to DNA isolated
from Chinese hamster cells was studied using several techniques.

(a) By use of membrane filtration and liquid scintillation counting
it was shown that the binding of ¹⁴C-NEM to calf-thymus DNA was about
9 x 10⁻⁶ μM/μg DNA. When Chinese hamster cells were incubated with ¹⁴C-
NEM for 30 min, and the DNA isolated, the binding was found to be similar, about $3.3 \times 10^{-6}$ μM/μg DNA.

(b) Calf thymus DNA was incubated with $^{14}$C-NEM and run on a Sephadex G25 column to remove the unreacted NEM. Chinese hamster cells were incubated with $^{14}$C-NEM, and the DNA was isolated and dialyzed against buffered 0.1 M NaCl to remove all traces of phenol, etc. The DNA was then applied to a methylated albumin coated kieselguhr column, and eluted with a linear gradient of buffered NaCl, 0.1 to 1.6 M. Five ml fractions were collected and monitored for absorbance at 260 nm and for radioactivity. Unlabeled DNA was also run.

(c) The binding of NEM to DNA bases was followed by ion-exchange chromatography on Dowex 50 columns. Binding to free bases, hydrolyzed calf thymus DNA, and Chinese hamster cell DNA was studied.

(d) The effect of NEM on the melting profiles of calf thymus and Chinese hamster cell DNA was studied. This reagent was found to decrease the melting temperature.

From these experiments it is concluded that NEM binds to DNA. It is suggested that the binding is at NH$_4$ groups on the bases, as NEM has been shown to react with these groups in proteins (4). In the cell an average of $10 \times 10^{-10}$ μM of NEM are bound to sulfhydryl and $6 \times 10^{-11}$ μM of NEM are bound to DNA. This level of DNA binding may be sufficient to affect DNA synthesis significantly, and represents one possible mechanism whereby NEM sensitizes these cells to X irradiation.

REFERENCES


STUDIES WITH MAMMALIAN CELLS IN CULTURE.
V. SULFHYDYL CONTENT OF SYNCHRONOUS CHINESE HAMSTER CELLS

Joy Archer, Melvin D. Long, and Warren K. Sinclair

The accurate determination of total sulfhydryl (SH) and non-protein SH (NPSH) in synchronized Chinese hamster cells is fraught with difficulties.
Many SH reagents are not specific except under controlled conditions; the sensitivity of many reagents is decreased in a cellular system, and steric factors hinder the binding of some reagents to SH groups in large molecules.

DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] (1), and CPDS [6,6'-dithio-bis-dinicotinic acid] (2) were found to give suitably accurate measurements when large quantities of cells were available, i.e., 5.0 x 10^5 to 1 x 10^6 cells. The Saville method (3) involving a nitrous acid-diazo compound reaction has been found to give consistent results with samples containing 2 x 10^5 cells. A fluorescence technique (4) can yield reliable results with samples containing as few as 6 x 10^4 cells. These latter two methods have been used extensively for studying the changes in total SH and NPSH in synchronized Chinese hamster cells. The correlation between the two methods is good. The differences in total SH during the phases of the cell cycle are small. Total SH was highest in M-phase, decreasing slightly at the end of G1, gradually increasing to mid S-phase and falling again in G2 phase. In comparison, studies using 14C-N-ethylmaleimide show no significant changes in total SH during the cell cycle. However, this reagent is known to be unreactive with some SH groups in proteins because of steric hindrance. It also reacts with NH2 groups.

The SH groups in the plasma membrane and nuclear membrane isolated from Chinese hamster cells have been studied using a spin-labeling technique. Membranes were labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, and then were dialyzed against phosphate buffer to remove free label. EPR spectra were then recorded. The spectrum contained both strongly immobilized spin-label and weakly immobilized spin-label, indicating that the label was attached to SH groups in two different environments in the membranes. This study is being continued with X-irradiated cells to try to demonstrate membrane damage caused by irradiation.

The isolation of peptides containing SH from the membrane and nucleo-proteins of these cells is in progress. The method uses the selective binding of SH-peptides to a mercurial resin, followed by carboxymethylation and separation by column chromatography.

REFERENCES

CHROMOSOMAL ABERRATIONS INDUCED BY JANUS NEUTRONS AND X RAYS IN CHINESE HAMSTER CELLS

C. K. Yu and Warren K. Sinclair

Our preliminary experiments (1) indicated that neutron radiation and X rays produced a similar response in the induction of chromosomal aberrations in Chinese hamster cells in vitro. Most chromatid aberrations were induced in the later part of cell cycle, and chromosome aberrations were mainly induced in G1. Because only two low doses were used, a quantitative relative biological effectiveness between these two radiations or its dose dependence was not established. This report emphasizes the effectiveness of neutrons and X rays with respect to chromatid breaks induced over a range of doses. The culture technique, experimental methods, and the preparation of metaphase chromosomes are essentially the same as described earlier (1).

The total chromatid breaks obtained from asynchronous cells irradiated by neutron doses of 60, 100, 150, 200, and 250 rad and by X-ray doses of 250, 375, 500, and 625 R are plotted in Figure VI-1. The response to neutrons is essentially linear while that for X rays increases more rapidly with dose (dotted curve). Thus the RBE is a function of dose, as has been noted before (2), ranging from about 5.0 at low doses to about 3.5 at high doses. The RBE obtained if a straight line (dashed line) is drawn through the X-ray points is about 3.7; however, this is evidently a crude approximation only.

![Figure VI-1](image_url)

Fig. VI-1.—Effect of X and neutron irradiation on total chromatid breaks in asynchronous Chinese hamster cells. In the case of X rays, the dotted line is an empirical curvilinear fit; the dashed line is the best-fitting straight-line relationship.

Some initial work on the frequency of breaks induced by neutrons at different stages of the cell cycle of synchronous Chinese hamster cells has been undertaken but not yet completed.
REFERENCES


CHROMOSOME LESIONS VISUALIZED BY SCANNING ELECTRON MICROSCOPY

C. K. Yu

X-ray induced chromosomal aberrations were examined by both light microscopy and scanning electron microscopy in an attempt to clarify the true nature of aberrations by morphological methods. What does a chromosome break really look like? How do the broken ends of chromosomes join together? Are both DNA and protein syntheses necessary for chromosome rejoining?

Cultured Chinese hamster cells of subline V79 were irradiated with 1000 R X rays. The aberrant metaphase chromosomes were arrested by colcemid, stained with acetic orcein, and examined by light microscopy. Later, these same chromosomes were isolated for scanning electron microscopy. The technique for the preparation of chromosomes was essentially the same as previously described (1).

The chromosome aberrations studied included breaks, gaps, exchanges, and rings. In some aberrations both DNA and protein syntheses were involved, while in others only one form of synthesis, or no synthesis was involved.

The unstained chromosome gap observed by light microscopy appears to be a clean break. However, scanning electron microscopy showed that a single chromosome strand, probably a chromonema, connects the two chromosome segments across the gap.

A detailed study on chromosome aberrations by scanning electron microscopy is in progress.

REFERENCE

CIRCADIAN CYBERNETICS

Charles F. Ehret

Circadian Cybernetics is the discipline of Chronobiology concerned with biological regulation that includes time constants within the ultradian-infradian domain. Eukaryotic cells are incapable of endogenous circadian oscillations during the ultradian (fast exponential) growth mode; their capacity for circadian outputs is unmasked in the infradian (slow exponential or quasi-stationary) mode (1).

The objective of this program is to improve our understanding of the mechanisms that determine ultradian-infradian time constants, with particular emphasis upon the circadian biological clocks of higher organisms. In the past year circadian studies continued at the cellular and molecular level in two principal areas: characterization of the ultradian mode, with especial attention to transitional properties of late ultradian-early infradian cells; and characterization of the infradian growth mode, with especial concern over the role of oxygen. At the organismic level, a study of properties of the mammalian clock through the application of cellular and molecular level strategies was continued.

REFERENCE


CIRCADIAN CYBERNETICS.
I. PROPERTIES OF THE ULTRADIAN GROWTH MODE IN FREE-LIVING EUKARYOTIC CELLS

A. IDENTIFICATION AND CHARACTERIZATION OF THE TRANSITION MODE

Gregory A. Antipa, Charles F. Ehret, John C. Meinert, and Alexander Sutherland

PURPOSE AND METHODS

As cultures of Tetrahymena pyriformis W switch from the relatively aerobic ultradian growth mode to the relatively anaerobic infradian growth mode, the form and composition of these cells change dramatically. This episode of change or the "transition mode" persists for at least a day, and in terms of cell count, 12 hr during the late linear exponential of the ultradian and 12 hr after the switch towards infradian. During this 24-hr episode some molecular and corresponding ultrastructural transitions have been identified.
Samples were taken from a well-aerated 10 l batch culture at 6 hr intervals and processed for electron microscopy, nucleic acids, protein, and glycogen. Nucleic acids were determined by a modification of the Schmidt-Thannhauser procedure as suggested by Munro and Fleck (1), protein by the biuret method of Koch and Putnam (2), and glycogen by the anthrone reaction (3).

RESULTS AND DISCUSSION

Each of the molecular properties assayed increased exponentially with the increase in cell titer to approximately 200,000 cells/ml. At this point there was a drastic reduction in the rates of increase for both RNA and protein (Figure VI-2). This was accompanied by a fusion of nucleoli, followed by a general clearing of areas in the cytoplasm. The cells continued to divide at a 4 hr generation time (GT) for about two divisions in the absence of significant RNA and protein synthesis.

![Fig. VI-2. Growth curve for 9.5 l culture with molecular properties of glycogen Δ - Δ; RNA o - o; and protein □ - □, plotted as mg/ml of culture.](image)

Glycogen on the other hand continued to increase at an ultradian doubling time for 12 full hours after cell division had nearly stopped. It is during this infradian portion of the transition mode that cells fill up with glycogen and get their reputation for high glyconeogenic capability (4).

Following the transition mode, which may be related to anaerobic adaptation in yeast, cellular constituents are apparently modulated in a fashion to be discussed (5) for the infradian mode. Prior to the
transition mode, cellular constituents apparently follow the rate constants dictated by the ultradian mode.

REFERENCES


B. SYNCHRONIZATION BY TRANSITION MODE AUTOPHASING IN TETRAHYMENA PYRIFORMIS

Gregory A. Antipa, Charles F. Ehret, and John C. Meinert

PURPOSE AND METHODS

Many kinds of zeitgebers have been used in circadian studies to 'set' biological clocks. In this sense feeding stimuli and light-dark cycles in particular have been effective in establishing synchronous circadian populations. In these experiments we use another line of logic, and ask the question, can the transition mode alone act as a zeitgeber? We cultured cells in such a way (1) that the switch from ultradian to infradian would be quite rapid. Thus, we reasoned, ultradian cells with 4 hr GT's would suddenly become infradian (with very long average GT's) and all cells would enter the Circadian Cell Cycle (P1+T+P2) (2) "simultaneously," i.e., with phase differences of no more than 4 hr, a small fraction of the circadian period.

RESULTS AND DISCUSSION

Our circadian assay was that well known mammalian circadian element--glycogen. Although it is not yet clear what aspect of the transition mode plays a dominant role, circadian synchronization was established. Figure VI-3 shows the free running circadian output of such an experiment. An overall downward trend of the glycogen reserve is observed in such a batch
Fig. VI-3.--Eleven days of growth of a 9.2 l culture. Glycogen content in mg/10^6 cells (Δ − Δ) is shown.

culture; within the trend minor apparently circadian ripples are later followed by oscillations of significant amplitude with a circadian period of ~18 hr. The relationship between eukaryotic transition mode autophasing and stationary phase synchrony (3) as seen in prokaryotes is under consideration.

REFERENCES


CIRCADIAN CYBERNETICS.  
II. PARAMETERS OF THE INFRADIAN GROWTH MODE OF FREE-LIVING EUKARYOTIC CELLS

A. CIRCADIAN PARAMETERS OF THE INFRADIAN GROWTH MODE IN CONTINUOUS CULTURES: NUCLEIC ACID SYNTHESIS AND OXYGEN INDUCTION OF THE ULTRADIAN MODE*

Charles F. Ehret, James H. Barnes, † and Kenneth E. Zichal**

Circadian rhythms of DNA and RNA synthesis, as measured by pulse labeling with tritium labeled thymidine and uridine, were observed in photo-entrained continuous cultures of *Tetrahymena pyriformis* W. During infradian growth, the circadian S phase of active DNA synthesis is about 12 hr long, and continues into the cell division phase. Curves for uridine incorporation are like those for thymidine, except that uridine troughs and peaks occur later by several hours. Infradian cells show a burst of cell division about 3 hr after aeration; this oxygen induction of the ultradian growth mode is itself a circadian function, and is blocked by classical inhibitors of aerobic respiration, forcing the cells to remain infradian. The latter effect may explain the until now paradoxical failure of respiratory inhibitors to stop the circadian clock, since manifestation of a circadian oscillation requires the infradian growth mode.


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B. QUANTITATIVE METHODS TO COUNT CELLS AND TO MONITOR DISSOLVED OXYGEN CONCENTRATIONS IN LONG-TERM INFRADIAN CULTURES: A TRIGGER ROLE OF OXYGEN

Gregory A. Antipa, Charles F. Ehret, William J. Eisler, and Jeanne Blomquist

PURPOSE AND METHODS

In long-term circadian-infradian studies, the methods of acquisition, storage, and processing of data on cell counts are costly and timeconsuming. An expeditious semi-automatic solution to the general problem is presented
The output of a Model B Coulter counter is transformed and entered into an IBM 026 card punch through a three-channel cell counter (Figure VI-4).

One of the signal differences between ultradian and infradian cultures is the low oxygen tension during the latter mode. In order to monitor continuously any change in oxygen content, and to relate oxygen concentrations and transients to population titers and kinetics, we have introduced autoclavable oxygen probes (New Brunswick Scientific) into 2.5 and 20 l growth vessels; the latter are connected to a continuous recorder by means of switch relays, thereby facilitating the simultaneous monitoring of a number of cultures.

**PROGRESS REPORT**

The relationship between cell count, growth mode, and dissolved oxygen content is given in Figure VI-5. Duplicate culture samples are automatically collected and fixed in 0.075 N perchloric acid (PCA) at preset intervals (1). These samples are then counted or diluted with 0.075 N PCA and counted on a Coulter counter Model B. Triplicate counts (from 0 to 40,000) of each sample are directly entered into a three-channel cell counter. Counts are scrutinized, replaced if fallacious, and read out along with identifying information (run, flask, and sample identification) onto IBM punch cards (Figure VI-4). Information on sample dilution and time of sample is

![Three Channel Cell Counter Diagram](image-url)
Fig. VI-5.--Tracing of a computer printout of growth curve with dissolved oxygen data superimposed.

simultaneously read onto each card by the autoduplication mode of the IBM 026 card punch. Cards prepared in this fashion are analyzed by the IBM 360/195 computer, and graphs in the form of Figure VI-5 are generated by the Calcomp 780 plotter.

The precipitous drop in dissolved oxygen content, heralding the ultradian-infradian transition, is evident as it is plotted along with the growth curve. These results from continuous monitoring resemble those obtained by batch-assay microgasimetry (2); the rapidly accelerating decline in oxygen diminishes at about 5%, or about 2 cell divisions before the cell counts indicate a transition from the ultradian to the infradian growth mode. The low oxygen asymptote is << 0.5% and remains there throughout the infradian mode.

REFERENCES


C. THE INFLUENCE OF AERATION AND OXYGEN TENSION ON INFRADIAN GROWTH

Gregory A. Antipa and Charles F. Ehret

PURPOSE AND METHODS

Significant differences in early infradian generation times (GT) have been observed in either batch or continuous cultures of *Tetrahymena pyriformis* W that range from 30 hr to those immeasurably long. A number of factors lent suspicion to the idea that the oxygen availability on a per cell basis during ultradian growth influenced the slope of the early infradian growth curve. These included shape of culture vessel, method of agitation, amount of aeration, and size of inoculum.

The effect of aeration and oxygen tension was therefore examined in stirred 2.5 l low-form batch cultures (1). Rates of air delivery or partial pressures of oxygen were varied and the resulting cell counts were made during growth of the cultures.

RESULTS AND DISCUSSION

Figure VI-6 shows that regardless of the air delivery rate ultradian GT's remain 3 hr in all cases. However, GT's vary as the cultures pass into the infradian with the lower delivery rates giving shorter infradian

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Fig. VI-6.--Growth curves for four 2.5 l batch cultures at air delivery rates of 20, 40, 80, and 160 ml/min.
GT's. On day 3 and beyond, all rates are the same, that is, immeasurably long GT's.

The results of differing partial pressures of oxygen are given in Figure VI-7. Ultradian GT's are suppressed by low oxygen in a chemostatic fashion, the cell titer directly reflecting the available O₂ as the growth limiting factor. However, as cells switch to the infradian mode at day 2, their O₂ dependence appears to become less, and by day 10 each of these three cultures reaches a titer of ~100,000 cells/ml, at which level they persist for another 2 weeks or more. We have not yet examined the influence of CO₂ on the infradian mode, but suspect that CO₂ effectively limits infradian growth rates as O₂ limits ultradian rates. In all seven growth curves presented in Figures VI-6 and VI-7, only the one for air at 160 ml/min would not show a concomitant increase in titer if more air was applied to the culture. This indicates that in these experiments other nutrients are not growth-limiting.

A decline in the cell titer by washout had earlier been observed in shaker continuous cultures of T. pyriformis following the switch from ultradian to infradian growth (2). This "near-zero growth transit period" lasted

![Growth curves for three 2.5 l batch cultures at partial pressures of O₂ of 4.10, 2.10, and 1.05, respectively.](image-url)
several days until the cells resumed division at infradian rates, sufficient to match the imposed feeding rates. In those 16 l cultures the inoculum was generally large (~10,000 cells/ml). We now observe that when the inoculum is small (i.e., <1000 cells/ml) this phenomenon does not occur and conversely, that whereas it had previously been limited to large (>10 l) shaker cultures, we may now achieve the same effect in small (2.5 l low-form), stirred, continuous cultures through the use of a large inoculum. A low-form continuous culture inoculated with a starting titer of 35,000 cells/ml is shown in Figure VI-8. Such cultures go through the normal 3 hr GT ultradian to a titer of 600,000 cells/ml, then wash out at a reduced GT for 4 days (near-zero growth transit) before accepting the imposed 48 hr GT at a titer of 350,000 cells/ml.

Fig. VI-8.—Growth curve for 2.3 l continuous culture, Δ – Δ, cells/ml in culture; • – •, computed growth of culture.

REFERENCES


D. ULTRACYTOCHEMICAL OBSERVATIONS OF GLYCOGEN SYNTHESIS AND STORAGE DURING INFRADIAN GROWTH OF TETRAHYMENA PYRIFORMIS*

Alexander Sutherland, Gregory A. Antipa, and Charles F. Ehret

As cells progressed in their growth in a defined medium from the ultradian to the infradian mode, their ultrastructural appearance in electron microscopy of thin sections was that of cells becoming progressively emptied

*Abstract of paper submitted for publication.
of their endoplasmic constituents. Suspecting the synthesis and storage of glycogen, we applied an ultracytochemical technique for the demonstration of α and β glycogen particles, the periodic acid/thiosemicarbazide/silver protein method of Thiery to the sections. In early ultradian cells, the endoplasm is full, and no glycogen particles are visible with conventional uranyl acetate lead staining. Following treatment by Thiery's technique, the cells show small islands of glycogen in both α and β configurations. Infradian cells look very different. Few mitochondria, peroxisomes, ribosomes, and food vacuoles are seen, and these are either around the inner periphery of the cell or adjacent to the macronucleus. About 85% of the endoplasm appears empty. After treatment by Thiery's method it is shown that the apparently empty endoplasm is largely filled with glycogen. Close examination of this infradian glycogen reveals an internally branching form of α-sized particles different from the classical ultrastructural appearance of either α or β particles, and resembling the partially interconnected β particles of the embryonic chick glycogen body or of McArdle's disease (type V glycogenesis).

CIRCADIAN CYBERNETICS.
III. CIRCADIAN CYBERNETIC STUDIES OF THE MAMMALIAN CLOCK

A. CHRONOTYPIC INDUCTION OF TYROSINE AMINOTRANSFERASE BY THEOPHYLLINE IN THE RAT

Charles F. Ehret and Van R. Potter*

PURPOSE AND METHODS

The circadian clock of any eukaryotic organism is characterized by molecular and physiological parameters that oscillate with a circadian period. Appropriate zeitgebers can reset such a biological clock in free-living cells in a day or two. On the other hand resetting the circadian system in a mammal requires a week or more.

Certain theoretical considerations about the nature of circadian clocks (that the organismic and organ outputs depend upon the circadian state of gene-action systems at the cellular level) lead us to propose that we should be able to reduce considerably the time required to phase-shift the whole mammalian organism. This we are attempting in the rat with light, drugs, and hormones as the principal zeitgebers.

Protocols of program-feeding were compared by intraperitoneal telemetry of body temperature at the Biotron controlled environment research facility; on bicircadian feed-fast cycles (FS 7 + 41) the circadian component remains

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previously injected 7 + 41 rats; the latter show bicircadian hypothermia diurnally entrained rats; a!S, 2 + 17 rat (top line) is compared with

Fig. VI-9.--Telemetry tracings of deep body temperatures over 5 days in diurnally entrained rats; a FS, 7 + 17 rat (top line) is compared with 3 FS, 7 + 41 rats; the latter show bicircadian hypothermia (<36°C) superimposed upon the daily rhythm.

CHRONOTYPIC INDUCTION BY THEOPHYLLINE

Fig. VI-10.--The relationship between hepatic tyrosine transaminase concentrations and time of day. Uninjected controls are compared with rats previously injected 2 or 4 hr earlier; injection time given by arrows above.
dominant even though a strong bicircadian hypothermia appears (Fig. VI-9), reminiscent of a phenomenon also seen in pocket mice (1) and in man (2). In experimental groups at the McArdle Laboratory, comparable protocols (FS, 8 + 40) were employed on nontelemetered rats. After feeding on day 1 (Fig. VI-10), rats remained unfed and in darkness throughout the experiment. All diets contained 30% protein. Rats were injected subcutaneously with 7.5 mg/100 g theophylline in saline; controls received saline alone. Livers were removed several hours after injection and assayed for glycogen and tyrosine aminotransferase [TAT] (3).

PROGRESS REPORT

Control rats show a strong and precocious (early ~3 hr) rise in TAT on day 2 (Fig. VI-10) characteristic of FS, 8 + 40 rats (4); a small but significant rise also occurs on day 3 (and possibly again on day 4, in a quite separate group). Theophylline injected rats present an even more striking pattern of chronotypic modality, with acrophases for TAT induction occurring on days 2, 3, and 4 around noon or as early as 0900, and minima around 2100, or as early as 1700. Glycogen shows similar oscillations (Fig. VI-11) with minima near noon or earlier, and chronotypic depletion nearly to ground state by theophylline. In a parallel study employing telemetered rats the role of theophylline as inducer is being compared with its efficiency as zeitgeber as a function of circadian phase of the rat at time of injection.

Fig. VI-11.--The relationship between hepatic glycogen and time of day in the rat. Closed circles, un.injected controls; open circles, saline injected controls; closed triangles, ~2 hr after theophylline injection; open triangles, ~4 hr after theophylline.
REFERENCES


B. THE SENSE OF TIME: EVIDENCE FOR ITS MOLECULAR BASIS IN THE EUKARYOTIC GENE ACTION SYSTEM*

Charles F. Ehret

The biological time sense is a general property of higher organisms, based ultimately upon their capacity to detect introspectively the molecular outputs of cells whose life is programmed at the level of gene action by a mandatory circadian cycle. This most general form of the cell cycle always occurs in infradian growth (quasistationary phase), and is represented by the terms pretranscriptional ($P_1$), transcriptional ($T$), and post-transcriptional ($P_2$) in the manner:

$$P_1 \rightarrow T \rightarrow P_2$$

Every cell in an infradian population makes its way through this basic algorithm of RNA synthesis once each day; such cells as may divide ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) are locked into phase with it. Circadian systems display circadian molecular chronotypes, including circadian transcriptotypes (temporally characteristic RNA), chronotypic enzymes, and chronotypic cell ultrastructures. These results, from molecular hybridization studies with protozoa, and from enzyme induction studies in the rat (in which hepatic tyrosine aminotransferase is chronotypically induced by theophylline) are compatible with the Chronon Theory of eukaryotic regulation.

In perspective, Circadian Cybernetics stands as an integrating discipline alongside of the traditional integrating disciplines of Chronobiology (development, genetics, evolution) that cut across the customary levels of biological complexity. Following new understanding about mechanisms, the control of biological clocks by pharmacological and other means appears close at hand.

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BIOMOLECULAR STRUCTURE DETERMINATION.
I. X-RAY CRYSTALLOGRAPHIC STUDIES OF IMMUNOGLOBULINS

Allen B. Edmondson, Marianne Schiffer, Rowland L. Girling, and Kathryn R. Ely

PURPOSE AND METHODS

Both the serum IgG immunoglobulin and the urinary Bence-Jones protein from a patient (Mcg) with multiple myeloma have been crystallized in forms suitable for X-ray diffraction experiments. IgG immunoglobulins in both myelomatous and normal sera consist of two light chains (MW 22,000 to 23,000) and two heavy chains (MW 50,000 to 55,000) linked by interchain disulfide bonds. Bence-Jones proteins represent excreted light chains, and their presence in urine is pathognomonic of multiple myeloma. The isolation of crystalline IgG and Bence-Jones proteins from one patient is particularly significant, because the amino acid sequence of the IgG light chain constituent is identical with that of the Bence-Jones protein only when the proteins are obtained from the same individual. This combination provides a unique system for the determination of the three-dimensional structure of an antibody molecule. The high resolution study that is possible with the Bence-Jones protein can complement and extend the low resolution study attainable with the myeloma protein.

Preliminary results of the crystallographic study of the myeloma protein have been reported (1), and the structure of the Bence-Jones protein at 6 Å resolution has been described (2). In the past year the structural study of the Bence-Jones protein was extended to 3.5 Å.

Three-dimensional data were collected with an automated Picker diffractometer by procedures discussed in previous Annual Reports. The Bence-Jones protein crystallized from ammonium sulfate in the space group P3121 with unit cell dimensions of a = 72.3 and c = 185.9 Å. The crystallographic asymmetric unit was the dimer (MW = 46,000), six of which were present in the unit cell. Each Bence-Jones dimer was represented by two globular modules of electron density. Since the modules differed in size and structure, they could not be identical monomers. Instead, the modules formed in the manner observed because each polypeptide chain was divided into variable (amino) and constant (carboxyl) regions having strong affinity for the corresponding regions in the second chain. The antigenic specificity of any light chain is associated only with the variable part of the molecule.

It was necessary to prepare additional isomorphous heavy-atom derivatives to extend the resolution from 6 to 3.5 Å. One of these, the S-Hg-S derivative, was formed by the insertion of a mercury atom between the sulfur atoms in the interchain disulfide bond linking the penultimate residues of the two polypeptide chains. The interchain bond was reduced with 2-mercaptoethanol and the resulting cysteine residues were coupled with mercuric acetate. The reactions could be carried out in crystals or in solution.
Other derivatives were prepared by diffusion of methyl mercuric chloride and p-hydroxymercuribenzenesulfonate into the crystals.

**PROGRESS REPORT**

The S-Hg-S derivative formed in solution crystallized in the same space group as the native protein, but required less ammonium sulfate. The crystals were less susceptible to radiation damage than the other mercurial derivatives or the native protein. On the scale model of the Bence-Jones protein the mercury atom was located between the smaller module of one dimer and the larger module of an adjacent dimer. The mercury atom was near the surface of the larger module but did not interrupt segments of electron density, as might be expected from the insertion of a mercury atom into the chain. In contrast the chains at the extremities of the small module did appear to be disrupted. The placing of the mercury atom defines the interchain bond and therefore the C-terminal or constant parts of the dimer. The variable or amino parts must consequently be represented by the larger module.

In the 3.5-Å electron density map, part of which is shown in Figure VI-12, long segments of the polypeptide chains can be traced, and the amino acid side-chains can usually be seen. The segments of density for the variable parts of the dimer appear in the upper left, while the constant parts are depicted in the middle of Figure VI-12. A series of β-pleated sheets are the most notable features which can be observed in the structure of the constant parts. Sharp turns are found in abundance in both the variable and constant halves. There is no indication of α-helices in the molecule.

![Figure VI-12](image-url).--Superposed sections representing part of the 3.5-Å electron density map of the Bence-Jones λ chain dimer. Note the sections of parallel extended chains (β-pleated sheets). In the middle of the figure is the central cavity partially separating the variable domains (on the left) and the constant regions (on the right).
Identification of the side-chains requires knowledge of the amino acid sequence, of which only the constant half is known. The sequence of the variable half is being determined at the University of Wisconsin in the laboratory of Dr. Harold F. Deutsch (with whom we are collaborating). We are in the process of fitting molecular models to the electron density to determine the course of each polypeptide chain and identify the side-chains in the constant halves. Simultaneously, we are trying to designate the segments connecting the variable and constant regions and study the interactions responsible for stabilization of the dimer. Light chain dimers are believed to show some of the binding capacity of the parent IgG antibodies. We are attempting to bind molecules to our Bence-Jones dimer in the crystalline state in an effort to determine possible modes of its biological activity.

CONCLUSIONS

An electron density map of a Bence-Jones dimer has been calculated at 3.5 Å and a molecular model is being constructed. The carboxyl-terminal parts in the maps have been identified by placing a mercury atom between the sulfur atoms of the interchain disulfide bond linking the monomers.

REFERENCES


STRUCTURE OF CONCANAVALIN A AT 2.4 Å RESOLUTION*

Karl D. Hardman and Clinton F. Ainsworth

An electron density map of concanavalin A at 2.4° Å resolution has been produced by X-ray crystallographic methods with five heavy atom derivatives. The molecule is a tetramer with all subunits identical and each containing a single polypeptide chain of 238 amino acid residues. The course of the entire backbone has been traced and three different regions of β-structure involve about 57% of the residues. One of these β-structure regions contains six strands of polypeptide chain and is related by a crystallographic twofold rotation axis to an additional six strands of the second subunit. These two regions are linked across the twofold axis by hydrogen bonds to form a continuous 12-strand β-pleated sheet. Concanavalin A contains no helix. The Mn²⁺ and Ca²⁺ ions are hexa- and penta-coordinated, respectively, are 4.3 Å apart, and occupy what is described as a double site,

since two aspartyl residues are common ligands to both ions. The symmetry of the ligands bonded to the Mn$^{2+}$ is very nearly octahedral. The double ion site is 23 Å from the carbohydrate binding site, which is a distinct cavity and contains residues of tyrosine, serine, threonine, and a number of hydrophobic amino acids.

**MYO-INOSITOL BINDING SITE OF CONCANAVALIN A**

* Karl D. Hardman and Clinton F. Ainsworth

A three-dimensional difference Fourier map was calculated to a nominal resolution of 3.6 Å with structure factor amplitudes from a crystal of the myo-inositol-concanavalin A complex minus native amplitudes and phases derived from native protein phases. The binding site appears as a cavity and is formed entirely from the polypeptide chain of one subunit. The largest opening from the surface to the inositol position is approximately 4 x 6 Å and through this opening, the center of the inositol ring sits about 8 Å from the molecular surface. The region below is formed by a small portion of the total β-structure of the subunit. The observed positive and negative peaks near the inositol are consistent with small conformational changes in concanavalin A, produced when the complex is formed. Although Mn$^{2+}$ and Ca$^{2+}$ are required for saccharide binding, they are located more than 20 Å from the inositol site.


**BIOMOLECULAR STRUCTURE DETERMINATION. II. MAGNETIC RESONANCE STUDIES OF STRUCTURES AND INTERACTIONS OF BIOLOGICALLY IMPORTANT MOLECULES**

* Steven S. Danyluk and Norman S. Kondo

Detailed structural knowledge at the molecular level is essential for a basic understanding of biological organization and functioning. With this in mind the principal objectives of our program are (a) to determine three-dimensional structures of key biological molecules *in solution* as accurately as possible, (b) to show how these structures arise from an interplay of molecular forces and interactions, and (c) to relate unique structural features of these molecules to their biological activity.
Of the variety of physical methods available for structural studies, the technique of high-resolution nuclear magnetic resonance (HRNMR) has the greatest potential for biomolecular structure determination in solution. When combined with structural probes such as paramagnetic ion chelates, HRNMR is capable of yielding three-dimensional structural data approaching in detail that obtainable by X-ray diffraction measurements in the crystalline state. Following the HRNMR approach, extensive and systematic studies have therefore been undertaken of important classes of biomolecules, including nucleic acids, proteins, and selected tumorstatic and anti-malarial drugs. Various aspects of this work have been reported in the literature and in earlier annual reports.

In the past year our efforts have been concentrated on further development of paramagnetic ion chelates as structural probes of biological molecules (antimalarials); synthesis of oligonucleotides selectively labeled with stable isotopes ($^2$H, $^{13}$C); and studies of conformational isomerism in oligopeptides. An accomplishment of significant importance to the field of nucleic acid structure research was the first synthesis of a dinucleotide containing a fully deuterated nucleotidyl unit at a known position (1). A brief summary of this work and other aspects of our studies is given in the following sections.

REFERENCE


ENZYMATIC SYNTHESIS OF SELECTIVELY LABELED DINUCLEOSIDE MONOPHOSPHATES

Norman S. Kondo, Amy Leung, and Steven S. Danyluk

PURPOSE AND METHODS

The preparation of oligonucleotides with fully deuterated nucleotidyl units in known positions is essential for meaningful nucleic acid structure determinations in solution. As the first step in this direction we have synthesized the selectively labeled dinucleotide, 2',1',2',3',4',5',5'-heptadéuteroadényl-(3'-5')-adénosine, *ApA (1), in which the 3'-nucleotidyl unit *Ap is fully deuterated. Comparison of the 220 MHz proton spectrum for *ApA with the spectrum for the protio analog, ApA, allowed complete and unambiguous assignment of signals for the latter. From the resultant NMR parameters it has been possible to derive a conformational model for ApA in aqueous solution.

†1972 Summer Honors Research Participant, University of Illinois, Urbana.
Although extension of the above approach to other dinucleotides is feasible in principle, the classical chemical synthesis method suffers from several drawbacks. Preparation of properly blocked starting compounds is lengthy, yields are low because of side products, and the synthesis must be carried out under stringent anhydrous conditions. An alternative less troublesome preparative procedure would be highly desirable and we have, therefore, explored the enzymatic methods of Thach (2) for this purpose.

PROGRESS REPORT

A primary activity of ribonuclease T₁ is the selective cleavage of ribonucleic acids at 3'-GMP loci. However, under appropriate conditions the reaction can be partially reversed, and use has been made of this property to synthesize GpX-type dinucleoside monophosphates in good yield. The enzymatic synthesis is a relatively straightforward one-step process, and we have accordingly adapted it for the preparation of *GpX-type dinucleotides.

The actual synthesis involves preparation of fully deuterated 2',3'-cyclic-*GMP, binding of the latter to highly purified RNase T₁, and reaction of the enzyme-substrate complex with an appropriate nucleoside. In the present work the reactions were carried out in aqueous solution at ~0°C and required approximately 90 min for completion. The dinucleotides were isolated by paper chromatography, eluted with water, and further purified by passing through a DEAE cellulose column. Following these procedures it was possible to prepare ²H-labeled *GpC, *GpA, and *GpU in sufficient quantities for NMR studies in a relatively short period of time. Moreover, since the reaction proceeds cleanly, an essentially complete recovery of unreacted labeled starting nucleotide is possible, an important consideration when dealing with small amounts of deuterated nucleotides. A drawback of the enzymatic approach is its limitation to 3' guanyl dimers; however, use of RNase A (3) may well permit synthesis of 3'-*U and 3'-*C dimers.

CONCLUSIONS

The present work shows that dinucleoside monophosphates containing a deuterated nucleodidyl unit at a known position can be prepared successfully by biosynthetic procedures in amounts suitable for spectroscopic studies.

REFERENCES

A STUDY OF THE 220 MHz PROTON RESONANCE SPECTRA FOR A SERIES OF BIOLOGICALLY IMPORTANT ACRIDINE-TYPE HETEROCYCLES*

Neil S. Angerman and Steven S. Danyuk

The high resolution proton magnetic resonance spectra of acridine, phenazine, phenoxazine, and phenothiazine have been measured at 220 MHz in various solvents and at several temperatures. Spectra for all four molecules were analyzed iteratively and good agreement was obtained between observed and calculated spectra.

Significant differences are noted between the coupling constants and chemical shifts of acridine and phenazine, on the one hand, and phenoxazine and phenothiazine on the other. These differences are attributed to a nonplanar heterocyclic ring conformation for phenoxazine and phenothiazine in solution. Moreover, it is concluded from the temperature measurements that the heterocyclic rings of phenoxazine and phenothiazine are conformationally flexible in the range covered. Possible mechanisms are suggested for the chemical shift and coupling constant changes.


SYNTHESIS AND PROTON MAGNETIC RESONANCE SPECTRUM OF A SELECTIVELY DEUTERATED DINUCLEOSIDE MONOPHOSPHATE, ADENYLYL-(3'-5')-ADENOSINE†

Norman S. Kondo and Steven S. Danyuk

Proton magnetic resonance (PMR) studies have yielded much valuable information about key conformational features in a wide variety of monomeric nucleic acid constituents (1,2). It is of considerable importance to extend detailed PMR analyses to higher oligonucleotides in order to establish the influence of chain length upon the nucleotide conformation. The usefulness of PMR measurements in this direction has been hampered, however, by extensive overlapping of signals that prevents complete assignments of the spectra (3). An approach which can circumvent this difficulty involves the synthesis of oligonucleotides in which one (or more) of the nucleotides is replaced by its fully deuterated analog.

We have recently developed such an approach and have successfully synthesized adenylyl-(3'-5')-adenosine (*ApA), a dinucleotide in which the

Ap- unit is fully deuterated (4). This compound allowed us to make an unequivocal assignment of all non-labile protons in this important dimer. Chemical shift and coupling constant values, as well as temperature and concentration studies, yielded detailed information about the conformation in solution. This work establishes the feasibility of synthesizing oligonucleotides with fully deuterated nucleotidyl units at known positions for structural studies.

REFERENCES


A DEVICE FOR THE REMOVAL OF TEMPERATURE GRADIENTS IN NMR SPINNING TUBES*

Neil S. Angerman, Steven S. Danyluk, and Andrew P. Kramer

A simple paddle device is described for the removal of temperature gradients in liquids contained in high-resolution NMR spinning tubes. Experiments show that this accessory reduces temperature gradients to less than 0.10°C along the length of the liquid without any impairment in spectrometer resolution or sensitivity. The device is readily adaptable to most conventional high-resolution NMR spectrometers.

THE ARGONNE PROGRAM FOR CLINICAL APPLICATIONS OF STABLE ISOTOPES: A COLLABORATIVE MULTI-FOCAL DEVELOPMENT OF METHODS AND APPLICATIONS

Peter D. Klein, Alan F. Hofmann, William L. Nyhan, Irwin H. Rosenberg, and John B. Watkins

Stable isotopic tracers offer the clinician the unique ability to trace metabolic pathways, to measure pool sizes and replacement rates of body metabolites, and to compare key metabolic conversion rates in normal and diseased states, all without subjecting the patient to the hazard of administered radioactive compounds.

Because of their safety, stable tracers offer the first real opportunity for clinical metabolic research and diagnosis in the pregnant woman, the newborn infant, the young child, and large population groups. Exploitation of this opportunity is being carried out in a collaborative program involving eight clinical centers and the Division of Biological and Medical Research at Argonne National Laboratory. A central facility for the synthesis of labeled compounds and for the development and use of instrumentation of the required versatility and sensitivity serves the needs of individual clinical groups in their establishment, validation, and routine use of nonradioactive tracer methodology.

The systematization of this exploitation will be illustrated in four examples: (a) use of deuterium-labeled folic acid to measure folate absorption during pregnancy, (b) use of deuterium-labeled cholic acid to measure bile acid kinetics and pool size in newborn infants, (c) use of $^{13}$C-labeled glycine to diagnose and differentiate inborn errors of amino acid metabolism in young children, and (d) use of deuterium-labeled chenodeoxycholic acid to monitor changes in bile acid pool size in patients receiving chenodeoxycholate therapy to bring about gall stone dissolution.

The experience gained in this program to date suggests that a regular recognizable pattern of development is present in each clinical application and that until inexpensive instrumentation and catalogs of $^2$H, $^{13}$C, and $^{15}$N-labeled compounds become generally available, a close collaboration between organic chemist, analytical chemist, and clinician is vital to the success of the application.


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Fig. VI-13.—Comparison between the mass spectra of authentic methyl 3α,12α-diacetocy-7-keto-5β-cholanate and of the compound isolated from bile (B).

A small amount of 3α,7α-dihydroxy-12-keto-5β-cholanoic acid was found in one of the samples. Oxidation of deoxycholic acid was indicated by the presence of trace amounts of 3α-hydroxy-12-keto-5β-cholanoic acid. No attempt to quantitate the keto bile acids was made in this study.
THE IDENTIFICATION OF KETO BILE ACIDS IN HUMAN BILE

Patricia A. Szczepeanik, John B. Watkins,* David L. Hachey, and Peter D. Klein

PURPOSE AND METHODS

It has long been known that bacterial 7α-dehydroxylation of cholate and chenodeoxycholate in the intestine gives rise to secondary bile acids in the bile and feces. Moreover, the fact that intestinal microorganisms are capable of oxidation of the hydroxyl groups at C-3, C-7, and C-12 is evidenced by the presence of keto bile acids in the feces (1,2). However, the occurrence of uncommon secondary bile acids in the bile suggests that oxidation of primary bile acids can also take place in the duodenum.

Samples of human infant duodenal bile containing a large amount of uncommon secondary bile acids were separated by thin-layer chromatography and the fractions containing potential hydroxy-keto acids were examined in this laboratory by gas chromatography-mass spectroscopy. The samples were analyzed on a Perkin-Elmer 270 mass spectrometer. A glass column (6 ft x 1 mm) packed with 0.25% SP-525 on 100/120 mesh Gas-Chrom Q was used. The column temperature was 260°C; the injector and ion source were maintained at 300°C. The operating conditions of the mass spectrometer were the same as previously described in Analytical Chemistry (3).

PROGRESS REPORT

By comparison with reference compounds, four keto bile acids were identified. The major keto bile acids in each sample were 3α,12α-dihydroxy-7-keto-5β-cholanoic acid formed by the oxidation of cholic acid and 3α-hydroxy-7-keto-5β-cholanoic acid formed by the oxidation of chenodeoxycholic acid. Figure VI-13 shows the comparison of the mass spectrum of the methyl acetate derivatives of authentic 3α,12α-dihydroxy-7-keto-5α-cholanoic acid and the compound isolated from infant bile. The base peak is at m/e 269 [M-(115 + 2 x 60)]. A pronounced peak at m/e 251 [269 - 18] and smaller peaks at m/e 329 [M-(115 + 60)], m/e 384 [M-(2 x 60)] and m/e 444 [M - 60] also characterize this spectrum.

The mass spectrum of authentic methyl-3α-acetoxy-7-keto-5β-cholanate is shown in Figure VI-14. The compound gives a small molecular ion peak at m/e 446, with pronounced peaks at m/e 271 [M-(115 + 60)], m/e 253 [271 - 18], and m/e 386 [M - 60]. Presentation of the mass spectra of this compound in the bile sample was not possible due to the overlap of chenodeoxycholate present in the sample. However, the distinguishing peaks were present in the combined spectra, making the identification of the compound possible. Another attempt to reproduce these mass spectra will be made on samples further purified by thin-layer chromatography.

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CONCLUSION

Evidence for the oxidation of primary and secondary bile acids in the duodenum is presented. Four keto bile acids were identified in human infant bile.

REFERENCES


Fig. VI-14.--Mass spectra of authentic 3α-acetoxy-7-keto-5β-cholanate.
FOLIC ACID MALABSORPTION. APPLICATION OF STABLE ISOTOPE LABELED FOLATE IN THE CLINICAL DIAGNOSIS OF VITAMIN B DEFICIENCY

David L. Hachey, Irwin H. Rosenberg,* and Peter D. Klein

PURPOSE AND METHODS

Folic acid, one of the B vitamins, is an important intermediate in the biosynthesis of thymidylate from 2'-deoxyuridylate. Folate obtained from dietary sources is actively absorbed by the intestinal mucosa, more specifically in the ileum. In the presence of certain pathologic states, however, folate absorption decreases from a normal 70 to 75% to as low as 4% of the available vitamin. One of the current diagnostic procedures to test for folic acid malabsorption involves administering a tritium-labeled folate to the patient. The present procedure can not be used in pediatric and obstetric patients owing to the radiation hazard involved.

In this work we have suggested using a deuterium labeled folic acid in which the label is placed in the para-aminobenzoic acid portion of the molecule. This part of the molecule has the advantage that it can be easily labeled by existing techniques (1), is biologically inert, can be easily recovered, and can be readily analyzed by gas chromatography-mass spectroscopy (GC-MS).

PROGRESS REPORT

Para-aminobenzoic acid (PABA) can be analyzed as the methyl trifluoroacetate on a 6 ft x 2 mm 0.75% DEGS-0.25% EGS column. Figure VI-15 shows the mass spectra obtained by combined GC-MS of derivatized PABA and the corresponding PABA-3,5-d2. The base peak occurs at M/e 216 and results from loss of a methoxyl radical from the molecular ion (M+). This spectrum has the advantage of an intense peak at a relatively low mass. Such an ion is highly desirable in isotope ratio work.

The dilution curve shown in Figure VI-16 indicates that the lower limit of detection for the M+2/M ratio (218/216) is about 5 x 10^-4% labeled folate. Similarly, the M+2/M+1 ratio (218/217) will allow us to measure down to 1 x 10^-4% labeled folate. Since, in the second case, the comparison is between an ion of 100% normalized intensity and one of 10% normalized intensity, the line is displaced by a factor of 10. This sensitivity is well within the limits required by the experiment.

CONCLUSIONS

Deuterium labeled folic acid should provide a sensitive test for folate malabsorption, which should be applicable in the general population.

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Fig. VI-15.--Mass spectra of p-aminobenzoic acid (top and p-aminobenzoic acid-3,5-d$_2$ (bottom) as the methyl ester, N-trifluoroacetate derivatives.
As soon as the details for isolation and analysis have been completed, clinical evaluation of the procedure can begin.

REFERENCE


SYNTHESSES WITH STABLE ISOTOPES: SYNTHESIS OF DEUTERIUM AND $^{13}$C-LABELED BILE ACIDS

David L. Haquey, Patricia A. Szczepanik, Otto W. Berngruber, and Peter D. Klein

A series of 5β-cholanic acids labeled with deuterium in the A ring were prepared by exchange labeling of the corresponding ketone by column chromatography on deuterated alumina. Factors affecting yield and labeling efficiency are discussed.

5β-Cholanic acids labeled with $^{13}$C in the carboxyl position were prepared by treatment of the corresponding 23-chloro-24-nor-cholane with sodium cyanide-$^{13}$C followed by alkaline hydrolysis of the nitrile. The intermediates in the synthesis were characterized by high resolution NMR spectroscopy. Mass spectra are also reported for the $^{13}$C-labeled products.

*Abstract of paper to be published in the Journal of Labelled Compounds.
†Postdoctoral appointee, 1969 and 1970.
TRIKETOCHOLANOIC (DEHYDROCHOLIC) ACID: HEPATIC
METABOLISM AND EFFECT ON BILE FLOW AND BILIARY LIPID
SECRETION IN MAN*

Roger D. Soloway,† Alan F. Hofmann,† Paul J. Thomas,† Leslie J.
Schoenfield,† and Peter D. Klein

Dehydrocholic acid-24-14C (triketo-5β-cholanoic acid) was synthesized
by oxidation of cholic acid-24-14C, mixed with 200 mg of carrier, and ad­
ministered intravenously to two patients with indwelling T-tubes designed
to permit bile sampling without interruption of the enterohepatic circula­
tion. More than 80% of infused radioactivity was excreted rapidly in bile
as glycine- and taurine-conjugated bile acids. After deconjugation, ra­
dioactivity was present entirely as partially or completely reduced deriva­
tives of dehydrocholic acid. By mass spectrometry, as well as chromato­
graphy, the major metabolite (about 70%) was a dihydroxy monoketo bile
acid (3α,7α-dihydroxy-12-keto-5β-cholanoic acid); a second metabolite
(about 20%) was a monohydroxy diketo acid (3α-hydroxy-7,12-diketo-5β-
cholanoic acid); and about 10% of radioactivity was present as cholic acid.
Reduction appeared to have been sequential (3 position, then 7 position,
and then 12 position) and stereospecific (only α epimers were recovered).

Bile flow, expressed as the ratio of bile flow to bile acid excretion,
was increased after dehydrocholic acid administration. It was speculated
that the hydroxy keto metabolites are choleretics. The proportion of
cholesterol to lecithin and bile acids did not change significantly after
dehydrocholic acid administration. In vitro studies showed that the hy­
droxy keto metabolites dispersed lecithin poorly compared to cholate; how­
ever, mixtures of cholate and either metabolite had dispersant properties
similar to those of cholate alone, provided the ratio of metabolite to
cholate remained below a value characteristic for each metabolite. These
experiments disclose a new metabolic pathway in man, provide further
insight into the choleresis induced by keto bile acids, and indicate the
striking change in pharmacologic and physical properties caused by replace­
ment of a hydroxyl by a keto substituent in the bile acid molecule.

*Abstract of paper to be published in the Journal of Clinical
Investigation.
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Minnesota.
A CONSIDERATION OF ISOTOPE EFFECTS IN THE QUANTITATIVE ANALYSIS BY MIXTURES OF THE METHYL-\textsuperscript{d3} ISOMERS OF ORTHO- AND PARA- METHYL ANISOLES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY*  

Jerome T. Adams, † Peter D. Beak, † Patricia A. Szczepaniak, and Peter D. Klein

A method of quantitative analysis for isotopically substituted isomers which takes into account isotopic fractionation on gas chromatography and allows correction for isotopic effects in mass spectrometry is demonstrated by analysis of a mixture containing the unlabeled, methyl-\textsuperscript{d3} and dimethyl-\textsuperscript{d3} isomers of both ortho- and para-methyl anisoles I-IV by gas chromatography-mass spectrometry-accelerating voltage alternation (GMA). It is found that the errors introduced by correction for the small isotope effects in the mass spectra of the ortho- and para-methyl anisoles override any advantage gained by their consideration and it is suggested that such corrections will be necessary only in cases which show appreciable isotope effects.

*Abstract of paper submitted for publication.
†Department of Chemistry, University of Illinois, Urbana, Illinois.

BILE ACID FORMATION IN MAN: METABOLISM OF 7α-HYDROXY-4-CHOLESTEN-3-ONE IN BILE FISTULA PATIENTS*

Russell F. Hanson, † Peter D. Klein, and Gale C. Williams†

7α-hydroxy-4-cholesten-3-one is thought to be an intermediate in human bile acid synthesis. This conclusion is based on in vivo experiments in animals and in vitro studies in which homogenates of animal and human liver were used. To further establish that this compound is an intermediate in human bile acid synthesis its metabolism was studied in subjects with complete bile fistulas. After administration of \textsuperscript{3}H-labeled 7α-hydroxy-4-cholesten-3-one by single intravenous injection approximately 85% of the administered isotope was recovered in the bile during the first 12 hr. Greater than 96% of the radioactivity recovered in the bile was identified as either chenodeoxycholic acid or cholic acid with only a trace amount of

†Department of Medicine, University of Minnesota, Minneapolis, Minnesota.
of the radioactivity present as neutral sterols. This study gives support to the hypothesis that 7α-hydroxy-4-cholesten-3-one is a natural intermediate in human bile acid synthesis.

BILE SALT METABOLISM IN THE NEWBORN.
I. MEASUREMENT OF POOL SIZE AND SYNTHESIS BY STABLE ISOTOPE TECHNIQUES*

John B. Watkins, † David Ingall, † Peter D. Klein, and Roger Lester †

Bile salt synthesis and bile salt pool size were determined in five normal newborn infants by isotope dilution using nonradioactive deuterium labeled bile salts. Bile salt isotope ratios were determined by a gas liquid chromatography-mass spectroscopy-acceleration voltage alternation (GMA) system.

The results establish that the newborn infant is capable of bile salt synthesis. The average cholate synthesis rate was 110 mg/M²/day with an average pool size of 290 mg/M², and both values are reduced when compared to values obtained from normal adults. This suggests that "immaturity" of the mechanisms which control bile acid metabolism and turnover may be one of the factors responsible for the inefficient fat absorption observed in normal newborn infants.

† Boston University.
VII. GENETICS

SUMMARY

H. E. Kubitschek, Group Leader

The activities of the Genetics Group consist of one program in mammalian genetics and four in microbial genetics.

Research in mammalian genetics is presently concerned with estimates of the radiation-induced mutation rate for sex-linked lethals and detrimentals in the mouse. These rates are not adequately established by the genetics research efforts at other facilities, and are, therefore, estimated directly by the use of a single-marker system. Inherent limitations of this system should be overcome by the future use of a double-marked X chromosome. Studies of the basic genetics of X chromosome activation/inactivation are also in progress.

Research in microbial genetics is directed toward an integrated understanding of the nature of genetic material, its organization, function, regulation, and replication in the living cell. Such studies are essential to the unravelling of radiation effects and to understanding other biological phenomena. It is generally known that most of the information required for cellular processes is encoded in chromosomal DNA, that chromosomes must duplicate coordinately with other cellular events during the cell cycle, and that the cell must, in turn, retrieve information from the chromosome in an orderly way.

Because bacterial and bacteriophage chromosomes are the simplest known, they provide the best systems for fundamental studies of genetic effects. Cells are frequently grown in synchronous or continuous culture in studies of mutagenesis, control mechanisms, cell growth, and the uptake of materials, because these techniques provide more information or better control of experimental conditions. These approaches have led to concepts such as linear cell growth, alternative modes of control of initiation of DNA synthesis, and a possible master strand controlling DNA replication.

The decay of radioactive isotopes incorporated into the DNA of living organisms leads to genetic alterations and cell death. The nature of this damage is studied in bacteriophage and in bacteria with isotopes such as $^{32}$P and $^{125}$I. Radiation-sensitive and -resistant mutants allow experiments which are not yet technically feasible for eukaryotic cells or multicellular organisms.
Ultraviolet (UV) and visible light also induce lethal and mutagenic lesions. Study of their effects in continuous cultures provides biological information on the nature of the DNA-chromophore interaction, the kinds of DNA lesions produced, and the mechanisms of repair of those lesions. Notable differences also are observed in repair processes of repair-resistant and repair-sensitive strains of bacteria after exposure to near UV, far UV, or visible light.

The most important advances made during the last year included the effects of near UV upon the genetic and repair apparatus of bacteria. Earlier indications that DNA lesions induced by near UV were pyrimidine dimers were substantiated by the first direct chemical evidence. Furthermore, the number of dimers was in good agreement with those expected from cell inactivation.

Another important discovery was the finding that the enzymes for photoreactivation and for dark repair of lethal lesions are sensitive to near UV at doses comparable to those for cell inactivation. In addition to accounting for many of the differences in bacterial response to near and far UV, this finding has broader implications for reduced repair of damage in human populations exposed extensively to the near UV component in sunlight.

GENETICS GROUP

REGULAR STAFF

Allen, Katherine H. (Scientific Associate)
Brown, Mickey S. (Scientific Assistant)
*Copeland, James C. (Geneticist)
**Grahn, Douglas (Senior Biologist)
Hulesch, Jane S. (Scientific Assistant)
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TEMPORARY STAFF DURING 1972

Colaianne, James J. [Postdoctoral Appointee (Presidential Intern)]
Ley, Ronald D. (Postdoctoral Appointee)
Peak, Meyrick J. (Postdoctoral Appointee)
Rinehart, Karl V. [Postdoctoral Appointee (Presidential Intern)]
†Thomas, Julian E. (Postdoctoral Appointee)
Tyrrell, Rex M. (Postdoctoral Appointee)

* Terminated during 1972.
** On leave of absence
†Now in Carcinogenesis
EFFECTS OF LEUCINE STARVATION ON CONTROL OF RIBONUCLEIC ACID SYNTHESIS IN STRAINS OF *Bacillus subtilis* DIFFERING IN DEOXYRIBONUCLEIC ACID REGULATION

Julian E. Thomas and James C. Copeland

PURPOSE AND METHODS

In a study of chromosome replication after leucine starvation, it was noted that different strains of *Bacillus subtilis* 168 synthesize different but characteristic amounts of DNA. Genetic analysis on these strains showed that chromosomes did not replicate to their termini before DNA synthesis stopped. These observations formed the basis for proposing a second control system (modulation control) distinct from initiation control for DNA. It is the purpose of this study to examine relaxed-stringency control of stable RNA and guanosine tetraphosphate (ppGpp) accumulation during leucine starvation in these strains that are known to differ in DNA control. Relaxed-stringency control of stable RNA and ppGpp accumulation is known to be affected by amino acid (leucine) starvation and may be candidates for DNA modulation control.

PROGRESS REPORT

Growth curves indicate that diluting, filtering, and resuspension of cultures have no appreciable effect on the growth of cells. Our incorporation studies of $[^3H]$ uridine and $[^3H]$ arginine into cold TCA-precipitable material indicate the following: strain BC29, which makes 15 to 20% DNA after leucine starvation, makes 7% RNA and 8% protein; strain BC252, which makes 30 to 40% DNA after leucine starvation, makes 10% RNA and 10% protein; and strain BCW503, which makes 30 to 40% DNA after leucine starvation, makes 4% RNA and 7% protein. Sucrose gradient profiles of these strains indicate that they are stringent and do not make any stable RNA during leucine starvation. The percent increase in RNA as indicated by cold TCA-precipitable material must, therefore, be attributed to mRNA synthesis. Accumulation of ppGpp after leucine starvation was 27% for BC200, 24% for BC252, and 32% for BCW503. This assay has not been made on the BC29 strain.

CONCLUSIONS

The results indicate that RNA synthesis is under stringent control in these strains, and that stable RNA synthesis stops when leucine is removed. Sucrose profiles suggest that the slight increases in RNA synthesis after leucine starvation are due to mRNA turnover. Both observations indicate the modulation control of DNA synthesis is not coupled with the relaxed-stringent response. The accumulation of ppGpp in starved cells is not correlated with the amount of DNA made after leucine starvation. Thus the modulation control system for DNA synthesis appears to be independent of controls for RNA synthesis.
THE BIOCHEMISTRY OF CHROMOSOME REPLICATION IN
ESCHERICHIA COLI

Tatsuo Matsushita

PURPOSE AND METHODS

The bacterial chromosome replicates its DNA in a complex process involving various enzymes, structural proteins, and the cell membrane. DNA replication appears to involve two subprocesses: (a) the initiation of chromosomes to start a new replication cycle, and (b) the continuation of existing DNA replication forks. The two subprocesses are reflected in the two types of temperature-sensitive DNA mutants found: gradual-stop mutants which gradually stop DNA synthesis at non-permissive temperatures are initiation mutants, whereas immediate-stop mutants are chain elongation mutants. The initiation mutants appear to map in at least two locations on the chromosome while the immediate stop mutants map in at least four locations (1). Therefore, at least six proteins are directly or indirectly involved in the replication process. However only one gene product, polymerase III (2), has been shown to be essential for DNA replication. In a general sense we wish to investigate the functional proteins necessary for DNA replication and to identify their biochemical functions. This report is an explanation of the combined biochemical and genetic approach to be followed.

In order to study the functional proteins, an in vitro system (toluenedized cells) (3) has been developed in Bacillus subtilis (4) so that the DNA product can be studied under defined conditions. Because genetic transformations are performed easily in B. subtilis, the in vitro product can be analyzed for both its biological activity (via transforming activity) and its chromosomal location (by well-mapped genetic markers).

Another advantage of this system is that in vitro replication can be distinguished from repair synthesis by use of polymerase I (the Kornberg enzyme) mutants in which repair synthesis background is low. In addition, in vitro density transfer studies result in a DNA product which has replicated semiconservatively and is, therefore, physically separable from any repair product.

PROGRESS REPORT

Since studies of complementation of DNA replication rely upon isolated proteins being able to substitute functionally on the replication complex in vitro, work is in progress to determine what size proteins can penetrate toluenedized cells. Thus far it has been shown that pancreatic DNase I (molecular weight 31,000 daltons) does penetrate into the cell (3,5). We are now purifying B. subtilis DNA polymerases and testing the permeability of toluenedized cells to these proteins. The proteins functional for DNA replication are being isolated on the basis of their temperature sensitivity. The toluenedized system will be particularly useful for complementation
of initiation of DNA replication since this function is absent in the system.

REFERENCES


EFFECTS OF RADIOISOTOPE DECAY IN MICROORGANISMS

Robert E. Krisch and Ronald D. Ley

PURPOSE AND METHODS

Radioactive isotopes ($^{32}$P, $^{33}$P, $^{125}$I) are incorporated into the genetic material of viable bacteria or bacteriophage, where they cause death or genetic damage in an increasing number of organisms as radioactive decay proceeds. The goal of these experiments is to relate such biological effects to specific physical and chemical changes of decaying atoms, as well as to physiochemical damage to the genetic material.

In some of these experiments, the lethal and genetic effects of $^{32}$P and $^{33}$P decay in bacteria and bacteriophage are compared. Each isotope is a β emitter with an identical decay scheme, except for the emission of β particles of different mean energies (0.70 MeV and 0.093 MeV) and different mean recoil energies of the daughter nuclei (20 eV and 1.7 eV) (1). The hypothesis that the lethal effects of $^{32}$P decay in microorganisms are primarily due to its high nuclear recoil energy (2) is being tested by comparing the rate of killing by these two isotopes and by comparing their effects on the transmission to recombinant progeny of genetic markers widely scattered over the phage chromosome (marker suicide experiments). These genetic experiments are carried out with mutants of Bacillus subtilis phage SP82G, known to transfer its genome in a unique linear sequence (3).
In a recently initiated part of this program, the effects of radioactive decay on the physical integrity of phage DNA are being studied by the use of sucrose gradient sedimentation techniques. In this way physical and chemical damage to DNA can be directly compared with the lethal effects of radioisotope decay.

Another isotope being studied is iodine-125, which is incorporated into DNA by bacteria and bacteriophage as 5-iododeoxyuridine (IUDR), a thymidine analog. $^{125}$I decays by electron capture. Nearly every decay gives rise to a vacancy cascade with consequent sudden multiple ionization. It has been reported that 99% of small organic molecules are fragmented as a result of the decay of incorporated $^{125}$I. We have previously determined the lethal efficiency per decay of $^{125}$I in the DNA of bacteriophage T1 as well as in four different strains of E. coli with varying DNA repair capacities (4). Recent efforts have concentrated on accurately determining the lethal efficiency per decay from $^{125}$I in bacteriophage T4 and correlating these data with measurements of the production of double-strand breaks (DSB’s) in phage DNA by parallel experiments using the same sedimentation techniques as in the radiophosphorus experiments.

**PROGRESS REPORT**

We had previously demonstrated (5) that viability of phage SP82G (B. subtilis) shows about half the sensitivity to $^{33}$p decay as to $^{32}$p decay when storage is at -196°C, implying that nuclear recoil is an important cause of $^{32}$p suicide, in agreement with earlier findings for several coliphages. Cross-reactivation (marker suicide) experiments have been carried out with widely spaced conditional mutants of SP82G, stored at both -196°C and +4°C. When storage was at -196°C, a marker transferred very late (E14) showed only a slightly increased sensitivity to either isotope when compared with a marker transferred very early (H177). The inheritance of either marker shows substantially lower sensitivity than does phage survival. When storage was at +4°C, there was a great increase in the sensitivity of the "late" E14 marker to either isotope, with little change in the sensitivity of the "early" H177 marker. There were significant differences in sensitivity to $^{32}$p and $^{33}$p in all parameters measured, but these differences were independent of marker position and storage temperature. These results are not consistent with earlier predictions that there would be a strong position-dependent marker sensitivity to $^{32}$p decay but not to $^{33}$p decay when storage was at -196°C. The similarity of the results for the two isotopes suggests that nuclear recoil does not play an important role in these genetic effects, the origins of which remain undetermined.

In the newly initiated part of this program $^{32}$p and $^{33}$p were compared with regard to the induction of lethals in phage T4 and the induction of DSB’s in T4 DNA. When intact T4 phage are stored at -196°C during decay, the breakage efficiency per decay is 0.059 for $^{32}$p and only 0.014 for $^{33}$p. DSB’s can, therefore, account for nearly all $^{32}$p lethals at -196°C ($\alpha = 0.060$), while such breaks from $^{33}$p can account for 2/3 of the smaller number of $^{33}$p lethals at -196°C ($\alpha = 0.021$). Sensitivity of T4 stored at -196°C to killing by either isotope is unaffected by the presence of 2-aminoethylisothiouronium bromide (AET) during storage, in contrast to a
marked protective effect from AET for phage stored at +4°C. Efficiency of induction of DSB's in DNA by $^{32}$P decay is independent of storage temperature (+4°C vs. -196°C) and also independent of whether storage during decay is as intact phage or extracted DNA.

Preliminary results from experiments measuring the production of both lethals and DSB's in phage T4 by $^{125}$I decay indicate one DSB per decay and 0.5 lethals per decay, with considerable variation between individual experiments. The production of DSB's is again independent of whether storage during decay is as intact phage or free DNA.

CONCLUSIONS

Radiophosphorus decay in phage SP82G does inhibit the transmission of genetic information into the host cell. This effect is much greater when the phage are stored at +4°C than at -196°C and does not appear to be related to nuclear recoil. The mechanism is not understood.

When T4 phage are stored at -196°C, at least 75% of DSB's in DNA, as well as most lethals, from $^{32}$P decay are induced by nuclear recoil.

Preliminary results indicate that every $^{125}$I decay in phage T4 causes a DSB, but that only half of all decays are lethal, suggesting a substantial capacity for repair of DSB's in DNA by the phage or the host cell (E. coli B/r).

REFERENCES


POSTREPLICATION REPAIR IN AN EXCISION-DEFECTIVE MUTANT OF *ESCHERICHIA COLI* ULTRAVIOLET LIGHT-INDUCED INCORPORATION OF BROMODEOXYURIDINE INTO PARENTAL DNA

*Ronald D. Ley*

Ultraviolet (UV) light-induced incorporation of bromodeoxyuridine (BrdUrd) into parental DNA of an excision-defective mutant of *Escherichia coli* has been observed by the selective photolysis of bromouracil-(BrUra)-containing regions in the parental DNA. It appears that the BrUra-containing regions occur only in that DNA which has served as a template for normal
semiconservative replication. After an exposure at 254 nm which results in one pyrimidine dimer per $45 \times 10^6$ daltons, incubation in BrdUrd resulted in BrUra-containing regions $\approx 1.5 \times 10^4$ nucleotides in length at intervals of $\approx 55 \times 10^6$ daltons in the parental DNA. Thus approximately one BrUra-containing region has occurred for every 1.2 pyrimidine dimers in the parental DNA. The observed incorporation of BrdUrd is interpreted in terms of a proposed model for postreplication repair in which genetic exchanges are assumed to produce single-strand gaps in the parental DNA.


THE INDUCTION OF PYRIMIDINE DIMERS IN BACTERIAL DNA BY 365 nm RADIATION*

Rex M. Tyrrell

The induction of pyrimidine dimers in bacterial DNA both in vivo and in vitro by wavelengths as long as 313 nm is well established, but little is known about the chemical nature of the damage induced by longer wavelengths. Oxygen dependent lethal effects of 365-nm radiation have been demonstrated (1) and indirect biological evidence has been obtained to suggest that at least part of the lethal damage is in the form of cyclobutane-type pyrimidine dimers (2). We now have direct chemical evidence that pyrimidine dimers are induced in DNA by 365-nm radiation.

Significant yields of photoproducts which were co-chromatographed with the thymine-thymine (TT) and uracil-thymine (UT) dimers by two established descending chromatography techniques were obtained after 365-nm irradiation of intact bacterial cells. To confirm the nature of the 365-nm photoproduct, the dimer region of the chromatogram was eluted to concentrate the photoproduct and subjected to 235-nm radiation. Essentially all the photoproduct was converted to thymine, as would be predicted for the isolated thymine dimer from the data of Wulff and Fraenkel (3). Sufficient intensities of monochromatic radiation were obtained using a mercury-xenon lamp in combination with a monochromator. Control experiments established that the results were not an artifact of scattered light of shorter wavelengths.

In order to calculate the absolute dimer yield from the radio-assay of dimers, it was necessary to measure the relative yields of the various types of pyrimidine dimer. The ratio of TT to UT was 5:1 over a tenfold change in dose. Full dose response curves have been obtained with *E. coli* B phr and B/r hor at 0°C, which indicate that the dimer yield (TT + UT) is approximately $5.5 \times 10^{-6}$ dimers per genome per erg. The ratio of yields of dimer per unit dose after 254- and 365-nm radiation is approximately

7 \times 10^5. This value corresponds closely with the ratio of 10^6 obtained with the double mutant K12 AB2480 for inactivation at the two wavelengths. Thus, at least in this extremely sensitive strain, the data are consistent with the pyrimidine dimer being the primary 365-nm induced lesion.

REFERENCES


THE DESTRUCTION OF THE PHOTOREACTIVATING ENZYME BY 365 nm RADIATION

Rex M. Tyrrell and Robert B. Webb

PURPOSE AND METHODS

In a study of the induction of thymine-containing photoproducts in bacterial DNA by 365-nm radiation, it was observed that the damage was not photoreactivable in vivo, as assessed by a chemical assay. Since we have found evidence that the 365-nm induced lesion is primarily the thymine-thymine dimer (1), it seemed unlikely that this result could be explained on the basis of a new photoproduct. In addition, it was noted that while the sensitive K12 mutant AB2480 showed a large sector of photoreactivatable damage (after 365-nm radiation) consistent with pyrimidine dimers as the primary lethal lesion, more resistant strains showed a reduced or negligible photoreactivation of 365-nm damage (as compared with 254 nm).

The total energies of lethal doses of 365-nm radiation are much greater than at 254 nm, and some consideration must be given to the contribution of protein destruction to cell death. We therefore investigated the possibility that the photoreactivating enzyme is destroyed at biologically significant doses of 365-nm radiation.

For in vivo studies, cells of Escherichia coli B/r harb were grown to late log phase in a supplemented medium containing tritiated thymidine. Procedures for harvesting, irradiation, and dimer analysis have been described previously (1). For these studies, two-dimensional paper chromatography was always used in the dimer assay. The first dimension was run in n-butanol and water (86:14, V/V) and the second in saturated ammonium sulphate, 1 M sodium acetate and isopropanol (80:12:2, V/V).
For *in vitro* studies, DNA was extracted from cells of B/r *har* labeled overnight with tritiated thymidine. To obtain crude DNA, cells were treated precisely according to the Marmur technique (2) up to the end of the first chloroform-iso-amyl alcohol deproteinization. The DNA was then "spooled" out and dissolved in saline citrate. To obtain photoreactivating enzyme, a crude yeast extract was first prepared according to the method of Lebedew (3). The extract was then treated with 55% saturated ammonium sulfate and the precipitate discarded. The supernatant was salted out with 65% saturated ammonium sulfate and the precipitate, containing partially purified photoreactivating enzyme (4), was dialyzed against 0.01 M phosphate overnight.

**PROGRESS REPORT**

A series of experiments were performed which established the following:

(a) Dimers induced in bacterial DNA by 365-nm radiation *in vitro* are completely photoreactivable and are removed from the DNA as an exponential function of the illumination time.

(b) The activity of the photoreactivating system in *E. coli* is destroyed *in vivo* as a function of the dose of 365-nm radiation. The repair activity was assessed by measuring the ability of 365-nm damaged cells to photoreactivate 254 nm induced damage (see Fig. VII-1).

(c) The activity of the yeast photoreactivating enzyme is destroyed *in vitro* as a function of the dose of 365-nm radiation at a rate comparable to the destruction of the *E. coli* enzyme activity *in vivo*. This activity was assessed by measuring the ability of 365-nm irradiated photoreactivating enzyme to remove 365-nm induced dimers from bacterial DNA.

![Graph](image_url)

**Fig. VII-1.**--The decline in the efficiency of the photoenzymatic repair system in *E. coli* B/r *har* as a function of the dose of 365-nm radiation. Each sample was treated with the stated dose of 365-nm radiation and then irradiated with 1200 ergs/mm² at 254-nm to provide substrate for the PRE. After 30 min, each sample was illuminated with photoreactivating light for 60 min at 1500 ergs/mm² sec⁻¹ and then assayed for thymine-containing pyrimidine dimers.
CONCLUSIONS

It is apparent that photoreactivating ability is destroyed by 365-nm radiation. Furthermore it is clear from the in vitro experiments that the 365-nm damage is potentially photoreversible, and we would predict that this reversal would be observed in vivo in the absence of repair enzyme destruction. This observation is biologically significant since the destruction occurs over the observed 365-nm inactivation range of several bacterial strains (5).

REFERENCES


THE DESTRUCTION OF DARK REPAIR SYSTEMS BY 365 nm RADIATION

Rex M. Tyrrell and Robert B. Webb

PURPOSE AND METHODS

Differences between the survival curves of strains irradiated with 254-nm radiation have frequently been explained on the basis of the dark repair characteristic of these strains. For example the uvr rec and uvr rec strains are approximately 40 times as resistant as the double mutant and ten times as sensitive as the wild type. At 365-nm the difference between survival curves is very much smaller (1). One possible explanation became apparent when it was observed that at doses equivalent to those necessary to measure dimer excision chemically at 254-nm, no excision could be observed after 365-nm inactivation. Since it is now known that the lesions are similar at both wavelengths (2), a study has been initiated to investigate the inactivation of dark repair systems by this near ultraviolet wavelength.

Irradiation procedures, survival curve determinations, and dimer measurements have been previously described (1,2).
Fig. VII-2 shows the survival curves of two strains of *Escherichia coli* after 365-nm radiation. There is little difference between the survival curve of the wild type which is competent in excision repair and the survival curve of the excision minus strain.

The effect of 365-nm radiation on the ability of a repair competent strain to excise dimer was assessed by dimer measurements. A range of low doses of 365-nm radiation was applied to a labeled cell culture. Each sample of 365-nm irradiated cells was then subjected to a similar dose of 254-nm radiation to provide substrate for the excision system. The cells were then held at 37°C for 90 min in a fully supplemented minimal medium. After this time, they were assayed for pyrimidine dimers. Figure VII-2

![Graph showing survival curves and dimer measurements.](image)
shows the percentage of potentially excisable dimers excised during this period of time as a function of the dose of 365-nm radiation. Clearly the excising ability of this strain is rapidly destroyed by this wavelength.

Indirect evidence, consistent with destruction of the "rec" repair system by 365-nm radiation is provided by comparing the survival curves of B/r hcr after 254- and 365-nm radiation. The ratio of the slopes at survival curves greater than 50% is approximately 8 x 10^5, which corresponds very closely with the dimer ratio of 7 x 10^5 (3). However, over the exponential portions of the curves (i.e., between surviving fractions of 10^-1 and 10^-3), the inactivation ratio falls to 1.3 x 10^5. Thus it would appear that over this range of biological inactivation, cells irradiated at 254-nm can tolerate 5 1/2 times as many dimers as cells irradiated at 365-nm. Since, the "rec" repair system is the only known dark repair system operative in this strain, this result is entirely consistent with the progressive inactivation of the "rec" repair enzyme system.

CONCLUSIONS

Fairly strong evidence for destruction of the excision repair system over a biologically significant dose range has been provided by chemical measurements in in vivo systems. More indirect evidence exists for destruction of the "rec" repair system by comparing inactivation and dimer ratios at 254 and 365 nm. We intend to investigate this destruction in both systems in greater detail, using physical and chemical techniques. At this time, it would appear that the dimers induced by 365-nm radiation are effectively more lethal than far ultraviolet dimers induced in repair competent strains, since radiation of this wavelength is extremely damaging to DNA repair systems.

REFERENCES


ULTRAVIOLET LIGHT INDUCED MUTATION IN CHEMOSTAT CULTURES OF ESCHERICHIA COLI WP2 hcr

Herbert E. Kubitschek

PURPOSE AND METHODS

As reported last year, in slowly growing glucose-limited chemostat cultures of Escherichia coli WP2 hcr, most of the latent mutants for T5 resistance induced with ultraviolet light (UV) disappear from culture,
declining by a factor of two with every passing generation. Because this pattern of decline is one of two patterns predicted by a master strand model of DNA replication, further investigations of the kinetics of mutant accumulation were undertaken to characterize the nature of the mutational process in this system. Mutational frequencies were determined both for phenotypically expressed mutants and for latent mutants carrying the UV-induced mutational potential. Very slowly growing chemostat cultures were exposed to UV, and the cultures were partitioned into several other chemostats immediately after irradiation, usually at faster growth rates, and grown in the dark. Only small UV doses were used, less than 25 erg/mm², to avoid appreciable cell death.

RESULTS

In slowly growing cultures, latent mutant frequencies decreased by a factor of two with every passing generation even when cultures were exposed to added glucose, casamino acids, or caffeine. This decrease was independent of the duration of cessation of cell division that occurred after UV irradiation. Although the cell division block was variable from experiment to experiment, recovery usually occurred at about the end of the first generation after irradiation.

Kinetics of mutation were examined in more than 60 chemostat cultures. Average latent mutant frequencies followed a dose-squared response. In addition, latent mutant frequencies increased exponentially with final growth rate. Yields of expressed mutants also increased in the same exponential manner with final growth rate, except in very slowly growing cultures. At very slow growth rates there was a slow loss of latent mutants by a dark repair mechanism with a half-life of approximately 10 hr. In rapidly growing cultures, mutant yields were approximately 60 to 80% of the latent mutant frequencies.

CONCLUSIONS

The results are in good agreement with those obtained by Bridges and Munson (1) with flask cultures of the same strain, and support Bridges' model of mutation induction (2) as due to two events, one of which is a thymine-like dimer and the other the "true" mutational event. In particular, the results lead to the following conclusions.

(a) In very slowly growing cultures, latent mutant frequencies decreased at the rate of dilution expected for nonreproducing dimers, that is, decreased by 50% per generation.

(b) A slow dark repair process also diminished latent mutant frequencies.

(c) Mutation frequencies increased with the final growth rate of the culture.

(d) Mutagenesis was a 2-hit process at all cell growth rates.
Since cells failed to divide for about one generation after UV, about half of the mutated cells were washed out of the chemostat cultures. Thus, if only one strand of each duplex was mutated, then the final mutant yield of approximately 60% in rapidly growing cultures indicates that the actual mutant yield was about twice this value or 100%; and initial latent mutant frequencies and final expressed mutant frequencies were the same. Thus, both daughters of each mutated cell carried the mutational potential.

This final result, also in agreement with a similar observation by Bridges and Munson, requires one of two alternatives.

(a) UV-induced mutagenesis in this strain is due to a (presumably recombinational) mechanism that affects both strands of the DNA duplex, or

(b) UV-induced mutagenesis in this strain is due to mutation of a single strand and DNA replication proceeds via a master strand mechanism.

REFERENCES


LEUCINE TRANSPORT IN ENERGY UNCOUPLED BACTERIA

Herbert E. Kubitschek

PURPOSE AND METHODS

Recent studies with bacteria show that there are highly specific binding proteins for each kind of nutrient taken into cells, including sugars, amino acids, inorganic ions, and vitamins. These results suggest that the binding proteins provide a major form of control of cell growth and response to environmental changes. Although progress has been rapid in studies of the chemical nature of the binding proteins, which are usually extracted and tested in vitro, there is as yet little knowledge of their action in vivo. We propose to examine binding and energy-independent transport in whole cells so that we can examine the properties of binding proteins in the environment in which they function. The cells must, however, be poisoned so that the results are not overshadowed by active transport.

Part of this approach involves testing a new method for examining binding proteins in cells. In order to establish that procedures are valid in
this approach, we have begun our studies with leucine because of the availability of results from chemical binding studies done with this amino acid.

RESULTS

Preliminary experiments on leucine influx and efflux were performed with cells of Escherichia coli B/r from overnight cultures in minimal medium. The cells were poisoned with azide (0.1 M). Steady state internal concentrations followed a response similar to that obtained by Winkler and Wilson (1) for galactosides. Approximate values for the association constant, $10^{-6}$ M, and the number of leucine binding proteins per cell, about $10^4$, are in agreement with those determined for isolated leucine binding proteins by Penrose et al. (2).

Efflux of leucine from preloaded cells decreased exponentially with time, was temperature dependent, and increased in osmotically shocked cells. Our results support Winkler and Wilson's conclusion that transport occurs by facilitated diffusion in poisoned cells.

Isoleucine and valine strongly inhibited uptake and binding of radioactive leucine in poisoned cells. These amino acids are known to adsorb to the leucine binding protein.

Leucine binding to whole cells also was found to be sharply decreased in the presence of 4 M urea, Figure VII-3, in agreement with the observation by Penrose et al. (2) that this concentration of urea reversibly denatures the leucine binding protein.

CONCLUSIONS

The results reported above are consistent with observations in the literature for extracted and purified binding proteins and for other kinds

![Fig. VII-3. Binding of $^3$H-leucine to bacterial cells as a function of urea concentration. Azide-treated E. coli B/r were exposed to $^3$H-leucine for 15 min at 37°C in the concentration of urea shown.](image)
of transport in poisoned cells. The methods appear promising for more complete studies of binding of materials to cells and of the subsequent energy uncoupled transport.

REFERENCES


MAMMALIAN GENETICS - ANNUAL REPORT

Douglas Grahn, Jane L. Hulesch, Katherine H. Allen, James J. Colaianne, and Ruth A. Lea

PURPOSE AND METHODS

The mammalian genetics program activity has been limited to two principal research areas, both concerning the X-chromosome of the mouse. These are: (a) determination of the radiation induced mutation rate for sex-linked lethals and detrimentals, and (b) genetic and pathologic analysis of an allelic series on the X-chromosome characterized by phenotypically observable effects on pigment production and a subtle effect on connective tissue development. Reduction of effort during the past year to provide manpower support for the JANUS Program and deficiencies in reproductive performance in several key stocks or matings have jointly reduced our ability to screen or evaluate X-chromosomes in the several experiments. The screening systems are being re-examined to determine if efficiency can be improved or if some studies should be discontinued. This report, therefore, will briefly describe the results of a number of activities related to these efforts.

DESCRIPTION AND RESULTS

A. Determination of the Radiation Induced Mutation Rate for Sex-Linked Lethals and Detrimentals in the Mouse. The present test series, described in detail in previous reports (1), is a three-generation system that basically evaluates the occurrence (or non-occurrence) of a mutant event in the spermatogonia of the grandsire by observing the sex ratio and marker-gene segregations among his grandprogeny. In this test, the X-chromosome exposed to 500 R of 250 kVp X rays in the 'gonial stage is marked with the Greasy (Gs) mutant. The male is outcrossed to a Mottled-brindled (Mo^br) and the F_1 daughter, + Mo^br/Gs +, is in turn outcrossed to a wild-type male to produce the third generation. Appropriate statistical criteria
are employed to permit classification of an F₁ dam either as a "suspect-carrier" of a new mutation or as a "reject" of noncarrier. A minimum of 20 progeny are required for a dam to be "at risk." If a dam dies or ceases reproduction before 20 progeny are produced, she is "discarded."

A summary of the results to date are presented in Table VII-1. There is not clear evidence of induced genetic detriment with the possible exception of the "discard" class. In this test, the discard class has been categorized in a slightly different manner than previously (2), and the demand for 20 progeny is more rigorous than prior methods. This is acceptable regarding the desired improvement of classification of dams by the criteria of sex-ratio and marker segregation, but there may be some attendant loss of subsidiary genetic information. Since the discard class would contain a mixture of fates (death, loss of fertility, disease, low fecundity), no simple interpretation can apply to explain the basis of the greater frequency of discards in the irradiated line.

| TABLE VII-1. Summary of X-Chromosomes Screened for Mutation |
|----------------|-------------|-------------|-----------|---------|---------|
| Dose (R)        | Chromosomes at Risk | Discard Sterile | <20 Progeny | Reject  | Suspect |
| 0              | 178         | 14          | 24         | 126     | 14      |
| 500            | 194         | 14          | 42         | 123     | 15      |

B. Evaluation of X-Chromosome Linkage Relationships. Although the past (2) and present studies on induced sex-linke mutation have used linkage relationships with both sex and a specific locus marker to increase statistical efficiency, the screening procedure could be markedly improved with an appropriate pair of mutant marker genes. This gene pair must have nearly normal viability, be easily classifiable and be separated by a linkage distance of 15 to 20 centimorgans or greater to make the procedure feasible and efficient. The X-chromosome of the mouse has not generally been characterized by mutants that meet these criteria, but several possibilities have been explored in the past year or so. These all involve the mutant **sparse-fur (spf)**, which is located toward the centromere. This mutant seems to have little influence on viability and is reasonably easy to classify in the hemizygous male and homozygous female by about 4 days of age. It is almost impossible to classify in the heterozygous female, though that is not critical.

Distant mutants that offered some hope are **Tabby (Ta)**, **Greasy (Gs)**, and **Bent-tail (Bn)**. These are, respectively, 45, 44, and 33 units from **spf**.
**spf-Ta.** This combination had to be abandoned owing to an unexpected interaction between the two mutants in the male. Quite simply, it was a lethal interaction; the \( spf-Ta \) male was a runt, appeared anemic, showed poor growth and died by 10 to 14 days of age. It might be noted that none lived long enough to provide positive classification of the existence of the double mutant. Judgment is based on inference.

**spf-Gs.** The \( spf-Gs \) combination also was discontinued because of a genetic interaction that tended to inhibit the expression of Greasy in the heterozygote. In the hemizygous male, the combination was not phenotypically distinct from \( spf \) alone until several months of age, at which time the pelage appeared shortened and assumed a mahogany color that was unequivocal in its appearance. However, genetic classification cannot be deferred that long without the intercurrent loss of considerable data.

**spf-Bn.** The \( Bn \) mutant has a recognized effect on viability in the male, but this mutant pair is being evaluated on the chance that the \( Bn \) detriment will be highly consistent. When that prevails, reasonable expectations can be established and probability criteria for significant deviations can be reliably used (as was the case for the \( To \) mutant in an earlier study) (2). The data to date give the following segregations from the parental genotypes: \( spf + / + Bn \times spf + / Y \).

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There is a deficiency of \( Bn \) males, but this does not seem to be interactive with \( spf \). There may be a small effect of \( Bn \) on viability among female carriers, but there is also an unexplainable deficiency of wild-type recombinants. The recombination frequency is between 21% and 25%, depending upon whether one includes or excludes the \( Bn \) segregants. These results are significantly different from the expected 33%. Both \( spf \) and \( Bn \) can be classified by about 4 to 5 days of age. This combination appears promising for more effective screening of induced sex-linked mutants.

Three approaches are being statistically and genetically evaluated. These are: (a) screen an unmarked \( X \) opposite the \( spf-Bn \) chromosome, (b) screen the \( spf + \) chromosome opposite \( + Bn \), (c) screen the irradiated \( + Bn \) chromosome opposite \( spf + \). The \( spf-Bn \) double-marked chromosome is not being considered owing to production and viability problems. The \( + Bn \) male could also be a problem, but there is a statistical advantage to the use of \( Bn \) on the chromosome to be screened. It would be expected to appear in the segregating generation at a frequency of 1:1 (\( spf-Bn \)) or less (\( \geq 3:1 \), \( spf: Bn \)); therefore, induced losses of the \( Bn \)-marked \( X \) would be more efficiently detected because the \( spf:Bn \) ratio would shift to higher values. This can only be reliably done, however, if, as noted, the \( Bn \) viability decrement is consistent among families in the generation at risk.
C. Further Genetic and Biochemical Analysis of the Mutants of the Mottled Allelic Series. A genetic and pathologic analysis of the Mottled (Mo) allelic series on the X-chromosome of the mouse was summarized earlier (3,4). This locus seems to be rather complex, as evidenced by the diversity of pathologic and lethal manifestations, so a test was set up to determine if genetic recombination might occur within the limits of the locus.

The alleles BLo (Blotchy) and MoBR (Brindled) were chosen. The BLo mutant shows severe cardiovascular lesions while also being viable in the hemizygous male. MoBR does not show vascular lesions and is a male-lethal. It is possible, therefore, to mate MoBR/+ females with BLo/Y males to obtain the MoBR/BLo F1 daughters. These are viable and fertile, though they experience some deficiency in milk production that lowers the survival probability of their offspring. The extensive use of foster-nurse females permitted resolution of this problem.

A number of different outcross-sire strains were employed, but the large majority of second generation offspring were produced from CBA or C3H sires. A total of 1,354 second generation progeny were classified; BLo/+ : 372, MoBR/+ : 324, BLo/Y : 314, MoBR/Y : 344. No +/- or +/- recombinants were observed, and all suspicious animals were progeny tested to ascertain the presence of either BLo or MoBR. It was assumed that any MoBR : BLo/+ recombinant would be phenotypically similar to MoBR/+; therefore, only half of the recombinants might be detectable. The data set a 0.5% upper limit (P = 0.05) to the recombination figure.

A collaborative research activity is also being conducted with the National Institute of Dental Research, Biochemistry Branch, in Bethesda, Maryland. Clinical Associates Dr. David W. Rowe and Dr. George R. Martin have been carrying out biochemical studies of the skin to identify the inherited collagen enzyme defect. There is a deficiency of elastin cross-linking in the affected animals though no differences in amino acid composition between normal and affected is noted. The tentative conclusion is that the conversion of certain lysines to aldehydes is impaired in both skin collagen and aortic collagen and elastin. Since cross-linking of both elastin and collagen involves enzymatic oxidation of lysine residues, a deficient enzyme activity could explain the defects.

REFERENCES
SUMMARY

William P. Norris, Group Leader

During the past year we have continued to emphasize and extend our studies of the effects of continuous (22 hr/day), whole-body γ irradiation in the pure bred beagle dog. Our first major experiment in this area, in which dogs are being exposed continuously until death at exposure rates ranging from 5 to 35 R/day, is still in progress after 1700 days (~4.6 yr) of irradiation. With the exception of one animal still surviving at 10 R/day, the experiment has narrowed to the dogs given 5 R/day. This study has already established several important relationships between radiation dose rate, total dose, clinical responses, and ultimate causes of death that previously have not been recognized. A large, long-lived subject such as the dog is absolutely essential in establishing these relationships. The uniform, and now predictable, response of the beagle to these exposure rates is of special significance. For example, myelogenous leukemia and related bone marrow disorders have been the primary cause of death in more than half of the dogs that have died at 5 and 10 R/day.

As dogs have died in this first experiment, the available γ-field space has been utilized primarily to generate groups of dogs given predetermined total exposures ranging from 1400 to 4000 R at the same exposure rates used in the first study. Data from these animals are beginning to accumulate, and it now appears that there will be important differences in the causes of death when these first two experiments are compared.

Pregnant beagles have been exposed continuously from conception to parturition at rates ranging from 5 to 17 R/day and have delivered normal litters. This study also emphasizes the importance of dose rate and demonstrates that the radiosensitivity of organogenesis in the fetus is about equal to that of hematopoiesis in the adult. The pups irradiated in utero are being followed to determine whether signs of radiation damage will become evident as they mature. So far, in spite of the substantial doses of radiation they received during fetal life, they appear normal and capable of sexual reproduction.

These studies suggest further experiments designed to elucidate mechanisms of radiation-induced injury. Light and electron microscopic studies of bone marrow reveal disorders in the maturation process of elements of leukemic cells. Because of the high incidence of myelogenous leukemia demonstrated in suitably irradiated beagles, these studies may now be extended to illustrate the development of this abnormal process in serial biopsy specimens of bone marrow taken from a single animal.
Beagles exposed to either 17 or 35 R/day often develop septicemias, but do not at either 5 or 10 R/day, even though they become severely leukopenic. These findings suggest that immunologic mechanisms remain functional at these lower exposure rates. Preliminary studies to quantitate the immunologic capabilities of normal, unirradiated beagles have already been done in collaboration with members of other groups in this laboratory (1).

The stress imposed by continuous irradiation is one to which the adrenal cortex may be responsive, and the known interactions of the adrenal hormones with hematopoiesis make this a potentially fruitful area for investigation. The results of preliminary studies are included in the following reports.

The data becoming available in dogs allow for important interspecies comparisons, which lead to extrapolations to effects in man, using similar data already available from earlier studies with mice in this laboratory. First comparisons indicate the need for additional data in dogs exposed at rates below 5 R/day. Plans for the construction of the irradiation facility needed to produce such data are well advanced and we hope to begin collecting this information in approximately one year.

REFERENCE


RADIATION TOXICITY IN DOGS

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MYELOGENOUS LEUKEMIA AND RELATED MYELOPROLIFERATIVE DISORDERS IN BEAGLES CONTINUOUSLY EXPOSED TO $^{60}$Co GAMMA-RADIATION*

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

Young adult beagles of both sexes are being exposed continuously to whole-body γ radiation from a $^{60}$Co source at rates varying from 5 to 300 R/22-hr exposure day. Thus far, dogs at the lowest exposure have been irradiated for up to 1,200 days. All deaths have resulted from damage to the hematopoietic system, manifested as three distinct syndromes: septicemia, anemia, and myeloproliferative disease (MPD), usually in the form of myeloid leukemia.

The causes of death are related to the exposure rate and total accumulated dose which are reflected in the time of survival. At exposure rates of 35 R/day and above, all deaths result from septicemia, but septicemia does not occur at 10 R/day or less. At 17 R/day all three terminal syndromes are seen with MPD occurring at significantly longer times of exposure.

When exposed till death at 17 R/day, 2 of 13 decedents had MPD after a mean 1,038 days of irradiation, while at 10 R/day 6 of 13 decedents had MPD after a mean of 719 days of irradiation. Only 6 of 24 dogs being irradiated at 5 R/day have died; 4 had MPD at a mean of 1,063 days.

These results demonstrate that the response of bone marrow varies with both dose rate and total dose. The data show that hematopoiesis in beagles, judged by peripheral blood values, tends to equilibrate with continuous irradiation, particularly at rates of 5 to 10 R/day. Myeloid leukemia and related MPD were observed only in those dogs whose blood values indicated such an attempt at equilibration.

*Abstract of paper to be published in Bibl. Haematologica.*

THE RESPONSE OF BEAGLE DOGS TO PROTRACTED EXPOSURE TO $^{60}$Co GAMMA RAYS AT 5 TO 35 R/DAY.

I. SURVIVAL AND CLINICAL OBSERVATIONS

**William P. Norris, Thomas E. Fritz, and Calvin M. Poole**

**PURPOSE AND METHODS**

Our four previous annual reports (1-4) have described the initiation and progress of a study in which young adult beagle dogs of both sexes are
placed in a $^{60}$Co γ-ray field and kept there until they die. Daily exposure rates are either 35, 17, 10, or 5 R/22-hr exposure day.

The objectives are to relate survival times, clinical signs of radiation-induced injury, and causes of death to exposure rate and to total accumulated radiation dose. The data will provide a basis for interspecies extrapolations aimed toward predicting effects in man.

Each dog is examined regularly for clinical abnormalities, with special attention to the eyes (for signs of retinal pathology and lens opacities) and body temperature (for indications of septicemia) (1). A complete hematologic examination on each animal, including a differential white cell count, enumeration of platelets, and measurement of certain biochemical parameters, is done at regular intervals. Bacteriologic examinations are performed on blood specimens from living dogs, as well as on post-mortem specimens, to define the significance of bacteremia in the overall response. All decedents are necropsied, and their tissues are collected for microscopic study.

PROGRESS REPORT

On October 31, 1972, the experiment had been in progress for 1710 days. By this time, all dogs that had been exposed continuously to either 35 or 17 R/day were dead, but 1 of the original 16 dogs given 10 R/day was still alive, as were 14 of the 24 dogs given 5 R/day.

The survival data, to date, for these groups of dogs are summarized in Table VIII-1. An exposure rate of 35 R/day is acutely lethal to beagles, but the lower exposure rates allow continued hematopoietic function and survival, depending on exposure rate, for up to 3 years, or more. Mean survival time in beagles exposed continuously to 5 R/day is currently projected to be in excess of 2000 days.

<table>
<thead>
<tr>
<th>Exposure Rate (R/day)</th>
<th>No. Dogs</th>
<th>Decedents Survival Range (days) Mean</th>
<th>Survivors No. Dogs</th>
<th>Survival to Date (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>8</td>
<td>45-68</td>
<td>57</td>
<td>None</td>
</tr>
<tr>
<td>17</td>
<td>13</td>
<td>85-1061</td>
<td>317</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>209-1622</td>
<td>608</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>390-1611</td>
<td>1127</td>
<td>14</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>303</td>
<td>7</td>
<td>1711</td>
</tr>
</tbody>
</table>
Fig. VIII-1.—Mortality in beagle dogs exposed continuously to either 5, 10, 17, or 35 R/day in a $^{60}$Co γ field. The arrows indicate the total exposure reached at the time of this report.

In the 5- to 17-R/day groups, the first deaths occurred when the accumulated exposure had reached about 2000 R (Fig. VIII-1), but (at 10 and 17 R/day) the accumulated exposure at 100% mortality was about 18,000 R.

Causes of death in all these animals have been directly related to hematopoietic failure or damage. At 35 R/day, the direct cause of death was always septicemia, usually from microorganisms carried in the throat and upper respiratory tract. At 17 R/day septicemia occurred in some dogs, while the others died of either anemia or myeloproliferative disease, generally taking the form of myelogenous leukemia. At either 10 or 5 R/day septicemias did not occur and causes of death, so far, have been due to either anemia or myeloproliferative disease. The consistency of these endpoints, and particularly the very high incidence of myeloproliferative disease, is an unexpected and highly important finding. More details of the pathologic findings are presented in another section of this report.

REFERENCES

THE RESPONSE OF THE BEAGLE DOG TO PROTRACTED EXPOSURE TO $^{60}$Co GAMMA RAYS AT 5 TO 35 R/DAY. II. EFFECTS OF TERMINATED EXPOSURES

Thomas E. Fritz and William P. Norris

PURPOSE AND METHODS

Groups of ♯13-month-old beagles of both sexes are being given pre-selected amounts of whole-body γ radiation at exposure rates of either 35, 17, 10, or 5 R/day (1,2). As the exposures are completed, the dogs are removed from the γ-ray field and maintained under observation in our standard kennel regimen for the remainder of their lives.

The intent is to create groups of dogs that have received either 4000, 2000, or 1400 R delivered at either 10 or 17 R/day, as well as groups receiving 1400 R at either 5 or 35 R/day. It is not possible to deliver higher total exposures at 35 R/day because 1400 R is the 50% lethal dose.

PROGRESS REPORT

The data collected at the present time are summarized in Table VIII-2. The summary includes the progress of two groups of dogs exposed for 100 days at either 17 or 24 R/day in an earlier study.

The three causes of death, septicemia, anemia, and myeloproliferative disorders (MPD), previously identified in dogs exposed until death at 5 to 35 R/day, are prominent in dogs given terminated exposures. Septicemia or anemia has occurred in all dogs that died within 69 days after irradiation was terminated and must be regarded as the direct, early result of the radiation treatment. Four cases of MPD have occurred between 250 and 405 days after terminated exposure to either 1700 R (17 R/day x 100 days) or 4000 R (10 R/day x 400 days). No cases of MPD have yet appeared at later times in these groups or in the several other exposure groups. Deaths from other causes are too few at this time to allow any interpretation; endometritis and fatal convulsive seizures are seen in untreated dogs in this colony.

CONCLUSIONS

The causes of death in dogs receiving terminated exposures to $^{60}$Co γ radiation are not different from those seen in dogs irradiated continuously until death at similar exposure rates. Thus far, the minimum total exposure that produces MPD is 1700 R delivered at 17 R/day and the induction period is about 400 days. Exposure rates higher than 17 R/day have never been related to induction of MPD.
TABLE VIII-2. Interim Data on Effects of Terminated Exposures to Protracted, Whole-Body \( \gamma \) Irradiation in Beagles

<table>
<thead>
<tr>
<th>Exposure Rate (R/day)</th>
<th>Total Exposure (R)</th>
<th>Survivors/No. Exposed</th>
<th>Time Post-Irradiation (Range in days)</th>
<th>Post-Irradiation Decedents</th>
<th>Mean Time to Death (Days)</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>1400</td>
<td>35/47</td>
<td>965-1528 (11/1/72)</td>
<td>15</td>
<td>13</td>
<td>Septicemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1276</td>
<td>Endometritis</td>
</tr>
<tr>
<td>24</td>
<td>2400</td>
<td>6/8</td>
<td>2498</td>
<td>1</td>
<td>69</td>
<td>Anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2024</td>
<td>Convulsive Seizure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2255</td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2291</td>
<td>Myositis(^a)</td>
</tr>
<tr>
<td>17</td>
<td>4000</td>
<td>11/19</td>
<td>192-770 (11/1/72)</td>
<td>1</td>
<td>12</td>
<td>Anemia</td>
</tr>
<tr>
<td>17</td>
<td>1700</td>
<td>8/8</td>
<td>2497</td>
<td>1</td>
<td>40</td>
<td>Septicemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>47</td>
<td>Anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>353</td>
<td>MPD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1825</td>
<td>Neurofibrosarcoma</td>
</tr>
<tr>
<td>10</td>
<td>4000</td>
<td>16/18</td>
<td>500-849 (11/1/72)</td>
<td>1</td>
<td>48</td>
<td>Anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>327</td>
<td>MPD</td>
</tr>
<tr>
<td>10</td>
<td>2000</td>
<td>6/6</td>
<td>259</td>
<td>1</td>
<td>30</td>
<td>Anemia</td>
</tr>
</tbody>
</table>

\(^a\) Necropsy diagnosis; histologic examination is incomplete.

REFERENCES

THE RESPONSE OF BEAGLE DOGS TO PROTRACTED EXPOSURE TO 
60Co GAMMA RAYS AT 5 TO 35 R/DAY. 
III. EFFECT OF CONTINUOUS IRRADIATION DURING PREGNANCY

William P. Norris and Calvin M. Poole

PURPOSE AND METHODS

It is important to compare the radiosensitivity of the developing fetus to that of the adult under conditions of continuous exposure to ionizing radiation delivered at reasonably low daily exposure rates. The developing fetus is regarded as being one of the most radiosensitive of all biological objects, but it has not been studied with due regard to the relative importance of dose rate and total dose.

Twenty-seven adult, virgin female beagles were bred to proven sires on two successive days. Immediately after the second breeding, 21 of these were placed in a γ-ray field where they were exposed to 60Co γ radiation at exposure rates of either 5, 10, or 17 R/22-hr exposure day for the full term of their pregnancies, or until palpation of the abdomen (after about 30 days) indicated that they were not pregnant. The latter were examined surgically and their uteri and ovaries were removed and fixed for microscopic examination.

The remaining six bred bitches were placed in the γ field, at an exposure rate of 17 R/day, at 20 days post-conception, a time at which the ova had implanted in the uterine horns. These were irradiated for the rest of their pregnancies. The entire gestation period of the beagle requires ~60 days, and organogenesis occurs during the 15 to 20 days immediately post-implantation.

Where possible, pups were delivered by cesarean section to eliminate complications associated with parturition. All surviving pups are being held for evaluation of their rates of growth, reproductive capabilities, and ultimate causes of death. Stillborn pups and other decedents were examined at necropsy and tissues were fixed for microscopic examination.

PROGRESS REPORT

The data from this experiment are summarized in Table VIII-3. Of four bitches exposed continuously to 5 R/day from conception until parturition, all delivered normal litters. At 10 R/day, 4/9 similarly exposed bitches whelped normal litters, while at 17 R/day only 2/8 bitches produced litters. All the females that were judged not to be pregnant at ~30 days after breeding were examined surgically, and none of their uteri showed signs of pregnancy.
TABLE VIII-3. Summary of Data on Reproduction in Beagle Bitches Exposed Continuously to \( \gamma \) Radiation during Pregnancy

<table>
<thead>
<tr>
<th>R/day</th>
<th>No. Bred Bitches Irradiated</th>
<th>Gestation Days Irradiated</th>
<th>Total Exposure (R)</th>
<th>Litters Delivered (%)</th>
<th>Total No. Pups</th>
<th>Pups Surviving on 10/31/72</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>0-Term</td>
<td>300</td>
<td>100 (4)</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>0-Term</td>
<td>600</td>
<td>44 (4)</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>0-Term</td>
<td>1020</td>
<td>25 (2)</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>20-Term</td>
<td>680</td>
<td>83 (5)</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>Totals</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>54</td>
</tr>
</tbody>
</table>

In view of the fact that the conception rate in this beagle colony is \( \approx 80\% \), the above data suggest that either 10 or 17 R/day may interrupt pregnancy prior to implantation of the ova. This possibility is supported by the finding that 5/6 bitches whose exposure to 17 R/day began after implantation was completed (20 days post-conception) delivered normal litters.

Of the 75 pups produced, so far, in this experiment at all the exposure rates, 54 (72\%) are surviving. None has shown signs of radiation-induced abnormalities. Similarly, there have been no indications of radiation-induced changes when factors such as litter size, birth weight, rate of growth, clinical appearance, hematologic values, and necropsy findings were reviewed.

The 16 pups exposed \textit{in utero} to 5 R/day are now about 18 months old. The males produce viable sperm in normal concentrations and 4/5 of the females have been observed in estrus. One of these has now been mated to a similarly irradiated male.

**CONCLUSION**

A continuous exposure rate throughout pregnancy of 17 R/day, already shown to be near the limit that permits continuing hematopoiesis in the adult, allows for normal fetal development in the beagle. No abnormalities have as yet been identified in pups exposed slowly to as much as 1000 R (\( \approx 750 \) rads) during pregnancy. There are, however, indications that either 10 or 17 R/day may prevent implantation of fertilized ova. So far, there are indications that dogs exposed continuously \textit{in utero} to 5 R/day (\( \approx 225 \) rad) are capable of reproduction, and breeding trials are in progress to verify this.
THE RESPONSE OF THE BEAGLE DOG TO PROTRACTED EXPOSURE TO $^{60}$Co GAMMA RAYS AT 5 TO 35 R/DAY.

IV. EFFECTS ON SPERMATOGENESIS

David V. Tolle, Robert F. Ladove,* and William P. Norris

PURPOSE AND METHODS

Spermatogenesis is highly radiosensitive and deserves study at even lower exposure rates than are presently available in our γ field. The purpose of this study was to establish the effect of continuous irradiation at 5 R/day (the lowest available exposure rate) on sperm production, and to correlate the changes observed with both dose rate and total dose. Four young adult male beagles, 12 to 15 months old, received 5 R/22-hr exposure day, while a fifth served as a control.

Semen collection was made at 2- to 3-week intervals using an estrus bitch to arouse the male. Semen samples were evaluated for the following: volume, color, opacity, viscosity, pH, motility, total sperm cell count, live-dead ratio, and percent of primary abnormalities. Samples were also prepared for viewing using the scanning electron microscope.

All dogs were physically normal with regard to testicular size. Two pre-exposure semen samples were normal for all parameters measured.

RESULTS

All four irradiated dogs were aspermic by 164 days (870 R) after the beginning of irradiation and have remained so on two subsequent collections. There were no significant changes in the total volume of ejaculate, but the semen became increasingly clear and thin with time in the radiation field. There were no significant changes in pH. Decreased sperm motility was evident after an accumulated dose of 600 R.

A plot of sperm concentration vs. accumulated dose (Fig. VIII-2) shows that numbers of sperm from irradiated dogs remained fairly constant for 60 days (300 R), after which there was a rapid decrease in numbers of sperm/mm$^3$. This decrease covered two log cycles in a period of approximately 80 days.

Increased numbers of dead sperm were observed in samples from three of the four irradiated dogs by 85 days (425 R). The mean percent live sperm in the control was 67.0 (13 samples), whereas the overall mean from the 4 irradiated dogs was 44.1 (34 samples). Sperm from all irradiated dogs showed an increase in numbers of primary abnormalities beyond

*Graduate Student, Northeastern Illinois University Participant in Summer Graduate Student Program in Biology, Supported by DNET-USAEC and NSF.
Fig. VIII-2.--Sperm cell concentration/mm³ of ejaculate and percent primary abnormalities in beagle dogs exposed continuously to 5 R/day ⁶⁰Co γ rays.

approximately 425 R (Fig. VIII-2). Corresponding values from the control dog remained below 10%. The major abnormalities observed were either beaded middle pieces or tightly coiled tails. The latter renders the sperm nonmotile.

CONCLUSIONS

Approximately 60 days of irradiation at 5 R/day (300 R) are required in the beagle before an exponential decrease in sperm cell concentration occurs. If the primary effect of irradiation is on the least differentiated spermatogonia, this period should correspond to the time required for spermatogonia to develop into mature sperm cells. It is interesting to note that the length of spermatogenesis in man is estimated to be 64 days (1).

REFERENCE

PURPOSE AND METHODS

Myeloproliferative disease (MPD) (1) in the beagle dog is a frequent and reproducible terminal consequence of $^{60}$Co irradiation at exposure rates of 5 to 17 R/day (2-4). The majority of these cases of MPD histologically and hematologically resemble myelogenous leukemia in man.

During the past year we have continued our studies on the fine structure of bone marrow cells from dogs with MPD. Our efforts have concentrated on more definitive identification of cell types, with particular emphasis on cells of the granulocytic series, and the ultrastructural characteristics of cytoplasmic organelles in leukemic granulocytes.

Methods of fixation and preparation for viewing have been previously described (5).

PROGRESS REPORT

The bone marrow tissue described in this report came from two dogs (1394 and 1381), which were irradiated at a dose rate of 17 R/day for 1015 and 1061 days, respectively. Both animals were killed when moribund. Preliminary studies of bone marrow, lymph node, liver, and spleen imprints, using conventional light microscopy, showed marked myeloid hyperplasia of the marrow, with leukemic infiltration and proliferation in the other tissues.

At first glance, electron micrographs of the granulocytic cells resemble those of normal bone marrow (5), but there are distinct differences. One important difference was an asynchrony of nuclear-cytoplasmic maturation. The nucleus appears to have continued to mature, while the cytoplasm has remained immature, basophilic, and sparsely granular or agranular. A few cells do have distinct cytoplasmic granules, yet have a large round nucleus with uncondensed chromatin and a distinct complex nucleolus, which is indistinguishible from the blast cell nucleus. This observation of asynchronous maturation has been reported previously, particularly in chronic myelogenous leukemia in man (6-8).

The cytoplasmic granules in early normal neutrophils are distinctly different in appearance from those of eosinophils. However, in the leukemic marrow of our dogs, both types of granules appear in nearly equal proportions within the same cell. Not only is the granule population mixed, but granules in all stages of maturation may be observed in the same cell. Auer bodies, frequently found in leukemic blast cells in man, have not been observed, but some cells have cytoplasmic granules concentrated near the cell limiting membrane that appear to be coalescent (9).
In both the electron micrographs and light microscope studies, cells of the erythroid, lymphoid, and megakaryocytic series appear morphologically identical to cells from normal canine bone marrow (5). The only difference noted was in the number of cells observed. The dogs with MPD have an increased myeloid-erythroid ratio and, thus, few erythroid precursors. Megakaryocytes and lymphoid precursors are rarely observed in the marrow relating to a terminal thrombocytopenia and lymphopenia. Plasma cells and phagocytic macrocytes are numerous in leukemic marrow and phagocytic activity, as judged by amount of engulfed debris (lytic nuclei, hemosiderin, and myelin figures), is greatly increased. Plasma cell nuclei appear normal, but in the cytoplasm the endoplasmic reticulum was swollen and there were increased numbers of electron dense secretory granules.

CONCLUSIONS

The major ultrastructural differences in leukemic marrow from irradiated beagles are: (a) asynchrony of nuclear-cytoplasmic maturation, (b) a mixed population of cytoplasmic granule types within the same cell, (c) asynchrony of granule maturation and differentiation, (d) coalescence of cytoplasmic granules, and (e) increased proliferation of plasma cells.

REFERENCES


ROLE OF ADRENAL GLUCOCORTICOIDS IN RESPONSES OF THE BEAGLE TO CONTINUOUS WHOLE-BODY $^{60}$Co GAMMA-IRRADIATION

J. Patrick Stone

PURPOSE AND METHODS

Plasma glucocorticoid levels in the dog (1,2) increase in response to the stress of acute whole-body irradiation, but such an adrenal response has not been reported in animals exposed to continuous whole-body irradiation. Because physiologic changes suggestive of a stress condition have been reported in chronically irradiated beagles (3), a study was initiated to investigate how adrenal glucocorticoids modify and influence the physiologic responses of the beagle to continuous whole-body irradiation. This study was divided into three parts: (a) the effects of complete lack of glucocorticoids (adrenalectomy with mineralocorticoid replacement therapy) during irradiation, (b) the measurement of plasma glucocorticoid levels during irradiation, and (c) the correlation of glucocorticoid levels and physiologic changes produced by radiation, especially those involving the hematopoietic system.

Young adult male beagles (13 to 16 months old), bilaterally adrenalectomized or sham-adrenalectomized, were allowed to recover for 2 to 3 weeks; then they were irradiated at 35 R/day to death. Plasma electrolytes were monitored and maintained at normal levels by deoxycorticosterone (mineralocorticoid) injections and by sodium chloride given orally. In this study of the direct physiologic effects of irradiation, antibiotic treatments prevented bacterial complications; penicillin and streptomycin were injected three times weekly and either neomycin or sulfaguanidine was given orally twice a week. Jugular blood samples were taken from irradiated and control dogs at least twice a week for complete hematologic analysis (RBC, WBC, and platelet counts, plus differential WBC counts), for measurement of selected biochemical parameters and plasma electrolytes, and for adrenal corticoid assays. All decedents were necropsied, and their tissues collected for microscopic study.

Routine clinical methods for plasma glucocorticoid determination (4,5) were unsatisfactory for this study because substances which interfere with fluorometric glucocorticoid assays were found in beagle plasma. These plasma components were tentatively identified, using thin-layer chromatography, as cholesterol and cholesterol esters. Plasma glucocorticoid isolation methods (6) were modified to remove these interfering substances and to increase the efficiency of glucocorticoid extraction. Although the resulting procedure is complex, excellent sensitivity (2.5 μg/100 ml plasma) and specificity for cortisol and corticosterone can be obtained using only a 1.5-ml plasma sample.
PROGRESS REPORT

To date five adrenalectomized and five sham-adrenalectomized beagles have been exposed at 35 R/day to death; all died of anemia with mean survival times of 51 days (1773 R) and 72 days (2543 R), respectively. In previous studies, dogs irradiated at 35 R/day died of septicemia after 57 days (1995 R) (7). In the present study, a vigorous antibiotic treatment prevented septicemia and allowed exposure to continue to a terminal anemia.

Adrenalectomy per se produced a constant and significant (p< 0.01) decrease in circulating erythrocytes (=80% of sham-adrenalectomy controls) indicating that the glucocorticoids affect either the rate of erythropoiesis or the rate of erythrocyte loss from circulation. Other hematologic and biochemical parameters were not significantly affected by adrenalectomy.

Irradiation caused similar thrombocytopenias and leukopenias in both adrenalectomized and sham-adrenalectomized animals. However, the erythrocyte responses for the two groups were quite different, (Fig. VIII-3). The terminal erythropenia began after 10 to 20 days of irradiation in adrenalectomized beagles and after 20 to 30 days of irradiation in the sham-adrenalectomized beagles. The constant rate of RBC loss (per unit time), as irradiation continued, indicates that the bone marrow may have been completely suppressed. The rate of RBC loss (slope of the line) in the adrenalectomized dogs was considerably greater than that observed in the sham-adrenalectomized dogs, but both slopes (-1.9 and -1.1) were greater than that (-0.9) calculated from the normal beagle erythrocyte life span (8). This suggests that the loss of glucocorticoids (adrenalectomy) followed by irradiation may have: (a) reduced the life span of the erythrocytes, (b) caused an increased rate of erythrocyte loss to phagocytosis,

![Graph](Fig. VIII-3.--Erythrocyte responses in beagles exposed to continuous whole-body γ irradiation at 35 R/day.)
(c) increased radiation damage to capillary endothelial cells allowing erythrocytes to escape into interstitial tissues and lymph vessels (9), or (d) increased the radiosensitivity of the erythrocytes, causing their premature removal from circulation.

Fluorometric analyses of plasma samples are not complete; however, glucocorticoid levels six times pre-irradiation values are present prior to anemic death. These data suggest a correlation exists between increasing glucocorticoid levels and hematopoietic system damage. Completion of these analyses will permit a more positive interpretation.

CONCLUSIONS

With high levels of continuous whole-body irradiation (35 R/day), a vigorous antibiotic treatment permitted exposure to continue, without bacterial complications, to an anemic death.

A significant role for glucocorticoids in erythropoiesis seems evident because anemia was produced by adrenalectomy in the beagle. In addition, a lack of glucocorticoids during protracted whole-body irradiation caused the level of circulating erythrocytes to undergo an increased rate of decline. In normal beagles, radiation damage to the hematopoietic system seems to be correlated with increasing glucocorticoid levels.

REFERENCES

1. Lawrence, G. H. *Endocrinology* 45, 383 (1949).


CESIUM-137-LATE EFFECTS OF SINGLE INTRAVENOUS INJECTIONS IN BEAGLES

Thomas E. Fritz

PURPOSE AND METHODS

This study of the late effects of single intravenous injections of $^{137}$Cs in beagles began in 1961. A total of 73 dogs, in three age groups, were injected with doses near those found to be lethal within 30 days. Forty of these dogs survived more than 100 days and received integrated doses of radiation ranging from 1000 to 1400 rad during this time. Early mortality, pathology, dosimetry, tissue distribution, and the hematopoietic response have been previously reported (1-3).

PROGRESS REPORT

Since our report last year (3) an additional 11 dogs have died, the largest group to die in any one year since the study began. Eight of these died of neoplasms, two had endometritis, and one had advanced hepatic degeneration and anemia. Hepatic degeneration, varying in severity, was seen in all of the decedents during the past year.

Table VIII-4 lists the causes of death in the 32 dogs that are now dead. The additional tumors observed during the past year bring the total neoplasms regarded as the cause of death to 18, or an incidence of over 50%.

A review of the causes of death in colony controls has revealed only one tumor thus far in 24 decedents of a total of 122 control dogs. This finding supports our previous impression that $^{137}$Cs-injected dogs have a higher incidence of soft tissue tumors (3).

To evaluate further the effects of $^{137}$Cs, data from the 122 colony controls and the 40 $^{137}$Cs-injected dogs were used to construct life tables and compare the survival of the control and treated groups. Figure VIII-4 plots the survival of these two groups. It is obvious from this figure that the treated dogs are dying appreciably earlier than the controls. We expect the remaining $^{137}$Cs-treated dogs to die within the next year or two, and, as the controls continue to age and our data accumulate, the differences should become even more obvious.

CONCLUSIONS

In dogs, the late effects of injected $^{137}$Cs are hepatic degeneration and an increased incidence of soft tissue tumors. The survival time is also decreased as compared with untreated dogs maintained under identical conditions.
TABLE VIII-4. Causes of Death in Long-Term Survivors of Single Near-Lethal Injections of $^{137}$Cs

<table>
<thead>
<tr>
<th>Causes of Death</th>
<th>No. Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Neoplastic</td>
<td></td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>3 (1 tentative(^a))</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Lymphosarcoma</td>
<td>1</td>
</tr>
<tr>
<td>Vertebral reticulum cell tumor</td>
<td>1</td>
</tr>
<tr>
<td>Neurofibrosarcoma</td>
<td>5 (1 tentative(^a))</td>
</tr>
<tr>
<td>2 unclassified tumors(^a)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td>B. Non-Neoplastic</td>
<td></td>
</tr>
<tr>
<td>Endometritis</td>
<td>6</td>
</tr>
<tr>
<td>Hepatic degeneration</td>
<td>3</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>1</td>
</tr>
<tr>
<td>Splenic hematoma</td>
<td>1</td>
</tr>
<tr>
<td>Convulsive seizures</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Still alive</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

\(^a\) Necropsy diagnosis; histological examination incomplete.

Fig. VIII-4.—Survival of beagle dogs given near-lethal intravenous injections of $^{137}$Cs compared to untreated beagles. $^{137}$Cesium was injected at \(\approx\)150 or 400 days of age; these data exclude dogs dying of acute hematopoietic failure within 100 days of injection.
REFERENCES

IX. LABORATORY ANIMAL FACILITIES

SUMMARY

Robert J. Flynn, Assistant Director for Animal Facilities

The objectives of the Animal Facilities Group are twofold: (a) to provide sufficient and appropriate animals and animal management so that the Division's research programs involving such models have the greatest chance of successful conclusion; and (b) to study animal production, management, and use so that the Division's animal models and their management are being continually improved. Our current efforts directed toward providing sufficient and appropriate animal models are illustrated by the reports on the status of the colony and the continued development of inbred catalase mutant mice. Studies directed toward the refinement of animal management are illustrated by reports on murine pneumonia and Enterobacter cloacae infection of mice.

Animal facilities personnel also provide specialized professional support (pathology, microbiology, roentgenography, surgery) to other groups within the Division. This support can be identified in reports found elsewhere in this publication.

LABORATORY ANIMAL FACILITIES STAFF

REGULAR STAFF

Brennan, Patricia A. (Assistant Biologist)
Fritz, Thomas E. (Veterinary Pathologist)
Flynn, Robert J. (Senior Veterinarian)
Keenan, William G. (Scientific Assistant)
Poole, Calvin M. (Veterinarian)
Simkins, Richard C. (Scientific Assistant)
Tolle, David V. (Scientific Assistant)

THE SUPPLY AND MAINTENANCE OF DEFINED ANIMALS.
I. STATUS OF THE COLONY

Calvin M. Poole, Thomas E. Fritz, Patricia C. Brennan, and Robert J. Flynn

The June 30, 1972, animal inventory, including the number and kinds of animals produced or acquired and maintained in the Division's animal facilities during the past fiscal year is presented in Table IX-1.
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Inventory as of June 30, 1972</th>
<th>Total Acquired July 1, 1971 to June 30, 1972</th>
<th>Total Produced at Argonne July 1, 1971 to June 30, 1972</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Mus musculus</td>
<td>65</td>
<td>265</td>
<td>480</td>
</tr>
<tr>
<td>Germfree</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPP</td>
<td></td>
<td>43,073</td>
<td>0</td>
<td>64,731</td>
</tr>
<tr>
<td>Conventional</td>
<td></td>
<td>7,213</td>
<td>6,433</td>
<td>6,629</td>
</tr>
<tr>
<td>Wild</td>
<td></td>
<td>197</td>
<td>0</td>
<td>201</td>
</tr>
<tr>
<td>Whitefooted mouse</td>
<td>Peromyscus leucopus</td>
<td>367</td>
<td>0</td>
<td>285</td>
</tr>
<tr>
<td>Brush mouse</td>
<td>Peromyscus boylei</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>California mouse</td>
<td>Peromyscus californicus californicus</td>
<td>45</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>California mouse</td>
<td>Peromyscus californicus insignis</td>
<td>193</td>
<td>0</td>
<td>185</td>
</tr>
<tr>
<td>Canyon mouse</td>
<td>Peromyscus ornatus</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cactus mouse</td>
<td>Peromyscus eremicus</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Florida mouse</td>
<td>Peromyscus floridanus</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cotton mouse</td>
<td>Peromyscus goeppinus</td>
<td>7</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Harvest mouse</td>
<td>Reithrodontomys humulis</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Northern pygmy mouse</td>
<td>Baiomys taylori</td>
<td>94</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>Rat</td>
<td>Rattus norvegicus</td>
<td>244</td>
<td>2,669</td>
<td>196</td>
</tr>
<tr>
<td>Black rat</td>
<td>Rattus rattus</td>
<td>36</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>Multimammate mouse</td>
<td>Przemy (mabomya) natalensis</td>
<td>115</td>
<td>0</td>
<td>191</td>
</tr>
<tr>
<td>Pack rat</td>
<td>Neotoma albigula</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rice rat</td>
<td>Oryzomys palustris</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chinchilla</td>
<td>Chinchilla laniger</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chipmunk</td>
<td>Tamias striatus</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Oryctolagous curiculus</td>
<td>9</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Dog (beagle)</td>
<td>Canis familiaris</td>
<td>523</td>
<td>0</td>
<td>125</td>
</tr>
</tbody>
</table>

The studies involving these animals during this period may be identified by referring to other parts of this report. Animals produced within the Division's facilities are designated with the suffix "Anl" (for Argonne National Laboratory).

*Pseudomonas aeruginosa* infection has not been encountered in the mouse colonies for several months. Routine monitoring and control procedures have apparently been successful in eliminating this infection.

Attempts to control *Enterobacter cloacae* infection have been continued. The ground corn cob bedding, which was associated with the introduction of the organism into the colony, has been replaced with a pine chip bedding. The latter was extensively tested before it was put into use and it was found to be sterile, absorbent, and relatively free of dust.

Efforts to eliminate *E. cloacae* infection by rederviding the rodent colonies by cesarean section have been severely hampered by the appearance of the organism in animals shortly after they are removed from isolators. Further attempts will be made to start an infection-free breeding colony as soon as certain changes in sanitation practices have been instituted.
Murine pneumonia, the most important disease problem in every animal facility which houses animals for long-term studies, is still present in some of our stocks. Our recent work suggests that the primary inciting agent may be Sendai virus. Studies to confirm this and, ultimately, to eliminate the infection are in progress.

THE SUPPLY AND MAINTENANCE OF DEFINED ANIMALS.
II. THE DEVELOPMENT OF INBRED CATALASE MUTANT MICE

Calvin M. Poole, Robert J. Flynn, and Robert N. Feinstein

The program to introduce the acatalasemic locus (Cs\textsuperscript{b}) and the hypocatalasemic locus (Cs\textsuperscript{c}) into the C3Hf/Anl and C57BL/6 Anl inbred strains of mice was continued.

Development of these inbred mutant strains involves initial crosses between the mutants and the selected inbred strains with subsequent backcrosses between mutant carriers and stocks from the appropriate inbred strains. Mutant carriers are determined by tests of the blood catalase levels. Whenever possible only mutant carriers from large litters (8 or more weaned) with the physical appearance of the inbred strains are used for further breeding. Eight generations of backcrosses and eight subsequent sibling matings between mutant homozygotes are required to produce an inbred mutant that is genetically comparable to the catalase normal inbred strain.

Eight backcross generations and more than eight sibling mated generations in the C3Hf/Anl-Cs\textsuperscript{b} program have been completed. A small nucleus of this strain has been cesarean derived and is currently being bred to obtain sufficient numbers for use. The seventh backcross generation has been reached in the C57BL/6 Anl-Cs\textsuperscript{b} program and eighth backcross generation in the C3Hf/Anl-Cs\textsuperscript{c} and the C57BL/6 Anl-Cs\textsuperscript{c} programs. Ten to fifteen breeding pairs of each mutant inbred line are being maintained.

LABORATORY ANIMAL DISEASES.
I. STUDIES OF MURINE PNEUMONIA


PURPOSE AND METHODS

Our studies with experimental murine pneumonia during the last year have concentrated on defining the response of B6CF\textsubscript{1}/Anl[Anl 70] mice to respiratory challenge with \textit{Mycoplasma pulmonis}. Such data are lacking
for this mouse strain. The methods we used for these studies are the
same as those we have previously reported (1).

During the last year we also studied a pneumonia which occurred spon-
taneously in C3Hf/Anl[Anl 70] mice and in old irradiated B6CF1/Anl [Anl 66]
mice in the JANUS JM-2 late-effects experiment. The gross and microscopic
lesions were similar to those we have described (2). Lungs from affected
mice were cultured for bacterial pathogens and for Mycoplasma species.
Portions were also collected and pooled for use in the Mouse Antibody Pro-
duction (MAP) test (3). In addition, serum from both affected and conval-
escent mice was collected for virus antibody titration.

PROGRESS REPORT

Intranasal instillation of 21-day-old B6CF1/Anl[Anl 70] mice with
1 x 10⁷ colony forming units (CFU) of M. pulmonis produces pneumonic lesions
similar to, but less severe than, those we previously described in CD-1/Crl
and CF#1/Anl[Anl 63] mice (1). The most extensive gross lesions were
observed 4 days after infection. After that the pneumonia began to resolve
and was not detectable after 8 to 10 days. The severity of the microscopic
lesions paralleled that of the gross lesions and was similar to what we
reported previously (1). The bronchi remained patent and vascular cuffing
with lymphocytes was a constant finding. Fluorescent antibody stained
sections showed the mycoplasma concentrated on the bronchial epithelium,
with few in the alveolar spaces.

The effective dose for 50% of the mice (ED 50/4), challenged intra-
nasally with M. pulmonis is defined as the number of CFU's necessary to
produce gross pneumonia in half the animals in 4 days. This value was
determined for 21-, 100-, and 300-day-old B6CF1/Anl[Anl 70] mice. The
results are shown in Table IX-2. Weanling and 100-day-old mice do not dif-
fer significantly in susceptibility to respiratory challenge with M. pulmonis
but both age groups are more susceptible than 300-day-old mice. The cause
of this age-related difference in susceptibility is presently unknown but
may reflect a more mature immune system in older mice.

TABLE IX-2. ED 50/4 of Mycoplasma pulmonis in B6CF1/Anl Mice

<table>
<thead>
<tr>
<th>Age in Days</th>
<th>ED 50/4 (in colony forming units/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>1.7 x 10⁹ (2.2 x 10⁸ - 1.3 x 10¹⁰)ᵃ</td>
</tr>
<tr>
<td>100</td>
<td>2.9 x 10¹⁰ (4.2 x 10⁹ - 2.0 x 10¹¹)</td>
</tr>
<tr>
<td>300</td>
<td>6.5 x 10¹² (7.1 x 10¹¹ - 6.0 x 10¹³)</td>
</tr>
</tbody>
</table>

ᵃ95% confidence limits
Tests to define the cause of the spontaneous pneumonia are incomplete but a viral etiology, possibly Sendai virus, is suspected. All cultures for bacterial pathogens have been negative.

Sera from affected and convalescent animals have shown no rise in titer to pneumonia virus of mice (PVM) or Reovirus 3 over levels found in stock mice, and titers to K virus have been negative. However a significant number of sera tested (80%) have had titers of 1:320 or greater to Sendai virus. These data suggest recent infection or reinfection with Sendai virus. The results of one MAP test are consistent with this hypothesis. A suspension of pooled pneumonic lungs was inoculated intraperitoneally into weanling B6CF1/Anl[Anl 70] mice and sera were tested 30 days later. A fourfold rise in titer (considered indicative of infection in this test) to Sendai virus was observed, but no increase in titer to PVM, Reovirus 3, and K virus was noted. A similar test using CF#1/CF mice and the intranasal route of inoculation failed to demonstrate an increase in titer to Sendai virus; however, the presence of sufficient neutralizing antibody in the recipient mice or a low virus concentration in the inoculum or both could account for these results. Further tests are in progress.

CONCLUSIONS

B6CF1/Anl[Anl 70] mice are susceptible to experimental infection with M. pulmonis but less so than randombred mice. Old (300 days) mice are less susceptible than either weanling or young adult mice.

Present evidence suggests that the spontaneous pneumonia observed in C3Hf/Anl[Anl 70] and old B6CF1/Anl[Anl 66] mice is caused by Sendai virus.

REFERENCES


LABORATORY ANIMAL DISEASE.
II. STUDIES OF ENTEROBACTER CLOACAE

Patricia C. Brennan, Calvin M. Poole, Richard C. Simkins, Wayne T. Kickels, and Robert J. Flynn

PURPOSE AND METHODS

We previously reported that Enterobacter cloacae was introduced into mice in the Division's animal facility via infected corncob bedding and that treatment of mice with neomycin reduced, but did not eliminate, the organism from the colony (1).

A number of additional measures were instituted to control E. cloacae infection. The use of corncob bedding was discontinued and pine chip bedding was substituted. A screening program for E. cloacae in the breeding colony was initiated and all positive cages discarded. Three separate shipments of germfree mice were purchased to provide uninfected foster mothers for a new cesarean derived colony. One of these shipments was placed in an isolation building physically separated from Building 202. An intensive study of the distribution and epizootiology of E. cloacae was undertaken.

PROGRESS REPORT

Intensive test and slaughter of breeding mice reduced the incidence of E. cloacae from 25% in November 1971 to 2% to 8% by June 1972. However, we were unable to eliminate E. cloacae by this method. Furthermore, in a split-dose gamma radiation experiment in June of this year 23% of irradiated decedents had E. cloacae septicemia. Thus, although the incidence was reduced in the breeding colony, B6CF1/Anl[Anl 70] mice continued to harbor the organism. Test and slaughter procedures were abandoned and efforts to derive a new colony by cesarean section were intensified.

In March, a group of germfree mice was obtained. They were housed under filter tops in a regular animal room and given a "cocktail" of normal flora as previously described (1). Cage samples were collected one month later, and 77% were positive for E. cloacae. These mice were discarded and the room disinfected. A small group of 6 cages of germfree mice was purchased, treated as before, and placed in the same room. These mice remained free of E. cloacae for 3 consecutive months. The colony was then expanded and in November 1972 only 1 cage of 48 was positive for E. cloacae. In the meantime a separate building was obtained and equipped to house animals. In September 1972, 48 cages of germfree mice were placed in it. The mice were given autoclaved water and food, the bedding was autoclaved, and the cages were covered with filter tops. Rigid sanitation procedures were used throughout and the number of people allowed to enter the building for servicing the mice was restricted to two. The mice were
given a "cocktail" and cultured one month later. Sixty-three percent were found to harbor *E. cloacae*; *Klebsiella pneumoniae* and *Proteus mirabilis* were also isolated. Clearly the mice were contaminated in some unknown way and an epizootiological study was begun in an effort to determine the method of introduction of *E. cloacae* into these mice.

Numerous samples were collected from the isolation building, Building 202, and other buildings on site, and cultured for *E. cloacae*. We found that the cage wash area in Building 202 was heavily contaminated with *E. cloacae* as were fomites in the isolation building. *E. cloacae* was isolated from the floors throughout Building 202 and from mops and buckets used by the maintenance personnel. The air supply systems in Building 202 were not contaminated, with the exception of the intermediate gamma room air exhaust. This room was also contaminated with *E. cloacae*. Other buildings on the site were free of *E. cloacae* but were contaminated with *E. aerogenes*, a closely related organism, and with other coliforms.

A test of disinfectant efficiency against *E. cloacae* showed that phenol compounds were highly effective, but quaternary ammonium compounds were without effect.

CONCLUSIONS

*E. cloacae* was introduced into germfree mice in the isolation building via infected fomites or the air exhaust from the intermediate gamma room or both. Personnel traffic through the animal facility has resulted in contamination of the floors of the first floor of the entire building. *E. cloacae* appears to be unique to Building 202. The air supply systems are not responsible for the spread of the organism, thus rigid disinfection of the cage wash area and the floors should do much to control *E. cloacae*. Such procedures are being instituted.

REFERENCE

X. EDUCATIONAL ACTIVITIES

POSTGRADUATE TRAINING

During 1972, 22 postdoctoral appointees, visiting scientists, and research associates contributed to the research programs of the Division. Three of these were Presidential Interns. The postdoctoral appointees during 1972, their schools, and their staff members with whom they were affiliated are as follows:

Neil S. Angerman
Gregory A. Antipa
Joy Archer
Metin Bara *
David W. Baxter
Anthony V. Carrano
James J. Colaianne +
Stanley R. Gawlik
Rowland L. Girling
David L. Hachey
Ray R. Hinchman
Norman S. Kondo **
Ronald D. Ley
Miran Menon +
Kenji D. Nakamura
Meyrick J. Peak
Karl V. Rinehart +
Richard H. Ross
John E. Sherwin
J. Patrick Stone
Julian E. Thomas
Rex M. Tyrrell

University of Alberta
University of Illinois
University of London
University of Istanbul
Indiana University
University of California, Berkeley
Purdue University
Marquette University
University of South Carolina
University of California, Santa Barbara
University of Chicago
University of California, Riverside
Oregon State University
University of Washington
Oregon State University
University of California, Riverside
Indiana University
Michigan State University
University of California, Santa Barbara
Wayne State University
Atlanta University
University of Bath

S. S. Danyluk
C. F. Ehret
W. K. Sinclair
S. A. Gordon
A. Lindenbaum
D. Grahn
D. Grahn
J. S. Miller
A. B. Edmundson
P. D. Klein
S. A. Gordon
S. S. Danyluk
R. E. Krisch
B. N. Jaroslow
F. Schlenk
R. B. Webb
H. E. Kubitschek
G. A. Sacher
S. A. Gordon
W. P. Norris
C. Peraino
H. E. Kubitschek

* Visiting Scientist
+ Presidential Intern
 ** Research Associate
In addition, there were 15 Faculty Research Participation (FRP) 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 teaching and research activities on their home campuses. The names of the faculty research participants, their schools, and the staff members with whom they were associated are as follows:

Winston A. Anderson  University of Chicago  G. A. Antipa
Robert J. Dinerstein  University of Chicago  P. D. Klein
Laszlo Hanzley  Northern Illinois University  T. N. Tahmisian
Sr. Petra Lenta  College of St. Scholastica  B. N. Jaroslow
Eugene W. McArdle  Northeastern Illinois University  C. F. Ehret
J. Emory Morris  SUCNY, Brockport  C. Peraino
Richard A. Morton  McMaster University  S. S. Danyluk
Daniel G. Oldfield  DePaul University  R. J. M. Fry
Paul Personne  University of Chicago  G. A. Antipa
Leonard Price  Xavier University  H. E. Kubitschek
Ronald M. Rogowski  Northwestern University  P. D. Klein
Lawrence Sweetman  University of California, San Diego  P. D. Klein
Thomas A. Victor  Northwestern University  S. S. Danyluk
Donald Van Ostenburg  DePaul University  S. S. Danyluk
John B. Watkins  Boston University  P. D. Klein

SUMMER GRADUATE STUDENT PROGRAM IN BIOLOGY

Twenty-one students from 15 different universities were enrolled in the 1972 program. The course, which was co-ordinated by Dr. E. John Ainsworth of this Division, ran for 12 weeks and consisted of 34 lectures in radiation biology given by 17 members of the Divisional staff over a 7-week period. Each student spent the remainder of his time working in the laboratory of a staff member. The students received financial support from a National Science Foundation grant, administered by CEA. Most of the students received academic credit for the course.

Four graduates of the 1971 course and three from this year's have subsequently returned to the Division as Thesis Parts or Laboratory-Graduate participants under other CEA programs. The success of the program in Biology has prompted the organization of similar programs in other divisions of the Laboratory.

The students, their schools, and their staff supervisors are as follows:

Adrienne F. Becker  University of Illinois at Chicago Circle  S. A. Gordon
William P. Craig  Atlanta University  R. J. Flynn
David E. Cunningham  University of Delaware  A. B. Edmundson
Carolyn Curry  Atlanta University  W. P. Norris
Harry Dalsey  Northeastern Illinois University  C. F. Ehret
Joseph M. Gergerich  
Maureen Groer  
Kenneth R. Groh  
Gary Horacek  
Robert F. Ladove  
Ronald G. Lindahl  
Calvin Meineke  
Raymond M. Miller  
John C. Prioleau  
Bobby Scott  
Stephen G. Sligar  
Bessie L. Smith  
Dana Strayer  
Terry F. Werner  
Donald J. Witt  
Charles Zeller  

University of Wisconsin, Milwaukee  
University of Illinois at Medical Center  
University of Illinois at Chicago Circle  
Kansas State University  
Northeastern Illinois University  
Wayne State University  
University of Illinois at Chicago Circle  
Illinois State University  
Atlanta University  
University of Illinois  
University of Illinois  
Tuskegee Institute  
SUONY, Brockport  
St. Louis University  
Ohio State University  
Northern Illinois University  

Joseph Firca  
Eleanor Blakely  
Raymond Spiewak  
David E. Cunningham

University of Cincinnati  
University of Illinois  
DePaul University  
University of Delaware

S. A. Tyler  
Y. E. Rahman  
W. K. Sinclair  
F. Schlenk  
W. P. Norris  
R. N. Feinstein  
W. E. Kisielewski  
J. S. Miller  
P. Brennan  
G. A. Sacher  
H. E. Kubitschek  
C. K. Yu  
C. Peraino  
D. Grahn  
R. E. Krisch  
R. J. M. Fry

OTHER GRADUATE PROGRAMS

One student, George H. Yoakum of the University of Missouri, is doing research for his Ph.D. thesis, under the supervision of Dr. Robert B. Webb, as a Laboratory Graduate Participant in a program administered by CEA. A related program, called Thesis Parts, allows graduate students to perform pertinent parts of their research at Argonne. Students in this program in 1972, their schools, and their supervisors are as follows:

Joseph Firca  
Eleanor Blakely  
Raymond Spiewak  
David E. Cunningham

University of Cincinnati  
University of Illinois  
DePaul University  
University of Delaware

A. B. Edmundson  
W. K. Sinclair  
S. S. Danylik  
A. B. Edmundson

UNDERGRADUATE TRAINING

During 1972, a total of 19 college undergraduates received training in the Division of Biological and Medical Research, in cooperation with CEA. Three programs were involved: the Associated Colleges of the Midwest (ACM), the Summer Honors Research Participation Program (SHRP), and the Spring and Fall Honors Program (formerly the Central States Universities Incorporated). Drs. Karl V. Rinehart (Lawrence University) and Benjamin F. Cooksey (Monmouth College) were the resident faculty advisers for the ACM students.

The students, their schools, and their staff supervisors are listed below:
Seven high school students from the Chicago area participated in this program, which is supported by the U. S. Department of Labor. The students and their supervisors are as follows:

Erma Green
Ruby Harris
Hugh Jenkins
Melinda Ross
Cordy Vivian
Myrtle Warfield
Rufus Williamson

A. H. Anderson
C. V. Fox
J. E. Thomas
C. F. Ehret
J. E. Thomas
C. V. Fox
R. B. Webb

During 1972, 17 staff members held a total of 29 faculty appointments at universities in the Chicago area. These appointments comprise limited
teaching activities, generally of a specialized nature, that involve reg-
eral contact with students, principally at the graduate level. They have
also led to cosponsorship of graduate students and to collaborative research
efforts with faculty members, some of which are described in this report.

The affiliations are as follows:

University of Chicago
Robert N. Feinstein
R. J. Michael Fry
Peter D. Klein
George A. Sacher
Fritz Schlenk
Warren K. Sinclair

University of Illinois at Chicago Circle
Douglas Grahn
Bernard N. Jaroslow
Herbert E. Kubitschek
Carl Peraino
Fritz Schlenk
Warren K. Sinclair
John F. Thomson

Loyola University
Bernard N. Jaroslow
Walter E. Kisielewski
Arthur Lindenbaum
Marcia W. Rosenthal

Northern Illinois University
James C. Copeland
R. J. Michael Fry
Douglas Grahn
Karl D. Hardman
Bernard N. Jaroslow
Herbert E. Kubitschek
Carl Peraino
Y. E. Rahman
Fritz Schlenk
Warren K. Sinclair
John F. Thomson

Northwestern University
Peter D. Klein

SEVENTH ANNUAL AUA-ANL BIOLOGY SYMPOSIUM

The 1972 symposium, "Photobiology," was held at Argonne National
Laboratory on October 16-18 under the joint sponsorship of the Division of
Biological and Medical Research and CEA. The chairman of the program com-
mittee was Solon A. Gordon; Robert B. Webb served as co-chairman.

The symposium was directed toward graduate students, primarily (but
not exclusively) from member institutions of the Argonne Universities
Association. Of the total attendance of nearly 300, there were 55 fully
supported graduate students, 23 partially supported students, and 138 non-
supported students and faculty. The remainder were speakers and Argonne
staff members.
A particularly successful feature of this symposium was the interaction between the students and the distinguished speakers. This included two informal evening sessions, one on repair mechanisms, led by Kendric C. Smith, and one on photosynthesis, led by Winslow R. Briggs, both of which were lively and well attended. An opportunity was also provided for students to visit the Division of Biological and Medical Research and to attend a demonstration of the biological spectrograph.
XI. PUBLICATIONS

OPEN LITERATURE


ABSTRACTS


Copeland, J. C. Regulation of chromosome replication in Bacillus subtilis. 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., April 23-28, 1972, p. 49.


XII. 1972 SEMINARS

The Division of Biological and Medical Research (BIM) and the Radiological and Environmental Research Division (RER) jointly support a seminar program that features speakers, principally from outside the Laboratory, who are selected by a committee, acting on recommendations of the staff. At the beginning of 1972, the committee consisted of Drs. T. E. Fritz (Chairman), E. J. Ainsworth, S. S. Danyluk, and Y. E. Rahman (all BIM); and D. J. Simmons (RER). In August, Drs. Fritz and Simmons were replaced by Drs. R. E. Krisch (BIM) and S. Spigarelli (RER), with Dr. Rahman succeeding Dr. Fritz as Chairperson.

The speakers were as follows:

Dr. Tatsuo Matsushita, Princeton University
Aspects of DNA Replication
January 13, 1972

Dr. Uldis Streips, University of Rochester
Studies on Competence for the Transfection and Transformation of Bacilli
January 27, 1972

Dr. F. Schlenk, Division of Biological and Medical Research, ANL
Whither Methyl?
February 3, 1972

Dr. John Autian, University of Tennessee Medical Units
Testing Materials for Toxicity and Biocompatibility
February 15, 1972

Dr. Bernard Roizman, University of Chicago
Structure and Function of Herpesvirus DNA
February 17, 1972

Dr. A. T. Ganesan, Stanford University
In vivo and In vitro Synthesis of DNA in Bacillus subtilis
February 24, 1972

Dr. J. F. Loutit, MRC Radiobiology Unit, Harwell, Didcot, England
Tumors in Bone and Bone Marrow Induced in CBA/H Mice by 90Sr and 226Ra
March 2, 1972

Dr. J. A. DiPaolo, National Cancer Institute
Quantitative Studies on Chemical Carcinogenesis In Vitro
March 9, 1972
Dr. P. R. J. Burch, Medical Research Council, Univ. of Leeds, England
*Carcinogenesis: A New Approach*
March 20, 1972

Dr. Richard C. Adelman, Fels Research Institute, Temple University
*A Biochemical Approach to the Comprehension of Mammalian Senescence*
April 6, 1972

Dr. Toyozo Terasima, National Institute of Radiology Sciences
*Bleomycin on Cultured Mammalian Cells*
April 10, 1972

Dr. R. T. Prehn, Institute for Cancer Research
*Immune Response in Carcinogenesis*
April 21, 1972

Dr. Harry V. Gelboin, National Cancer Institute
*Microsomal Hydroxylases: Induction and Relation to Polycyclic Hydrocarbon Action*
April 27, 1972

Dr. R. Dickerson, California Institute of Technology
*Cytochrome C: Oxidation States and Protein Conformation*
April 28, 1972

Dr. K. Biemann, Massachusetts Institute of Technology
*The Application of a Gas Chromatograph-Mass Spectrometer Computer System in Organic Chemistry and Biology*
May 9, 1972

Dr. D. Newton, A.E.R.E., Harwell, Didcot, England
*The Assessment of Plutonium-239 and Californium-252 in Man*
May 25, 1972

Dr. Charles Heidelberger, University of Wisconsin
*Cell Culture Studies of the Metabolic Activation of Polycyclic Hydrocarbons*
June 1, 1972

Dr. Ursula Heine, Dept. of Health Education and Welfare, National Institutes of Health
*Herpesviruses and Their Relation to the Infected Cell: An EM Study*
June 15, 1972

Dr. Gerald Wogan, Massachusetts Institute of Technology
*Naturally Occurring Carcinogens*
June 22, 1972

Dr. Donald G. Baker, Mt. Zion Hospital and Medical Center
*Tumor Incidence and Life Expectancy in Rats Exposed to a Low Environmental Temperature*
August 24, 1972
Prof. Leonard Hayflick, Stanford University
Human Cells and the Biology of Aging
September 8, 1972

Dr. Abraham W. Hsie, Oak Ridge National Laboratory
Control of Phenotypic Expression by Cyclic AMP and Hormones in Mammalian Cells
September 24, 1972

Dr. Bernice L. Neugarten, The University of Chicago
Social Psychological Patterns of Aging
September 28, 1972

Dr. M. M. Elkind, Hammersmith Hospital and BNL
DNA Damage and Radiation Cell Killing: Cause and Effect?
October 5, 1972

Prof. D. MacDonald Green, University of New Hampshire
The Process of DNA Infection of Competent Bacillus subtilis
October 12, 1972

Dr. Lawrence W. Kessler, Zenith Radio Corporation
Acoustic Microscopy of Biological Specimens
October 20, 1972

Dr. Allen B. Edmundson, Division of Biological and Medical Research, ANL
X-Ray Diffraction Studies of Immunoglobulins
October 26, 1972

Dr. A. L. Brooks, Lovelace Foundation
Frequency of Chromosomal Aberrations in the Liver Following Injection of Alpha and Beta Emitters
November 2, 1972

Dr. Sarah E. Stewart, Georgetown University
Activation of Viruses in Human Cell Lines
November 9, 1972

Dr. Paul V. Harper, Jr., ACRH
Explorations in Nuclear Medicine
November 16, 1972

Dr. W. L. McLaughlin, National Bureau of Standards
Aromatic Dye Precursors with Unusual Radiation Response Characteristics
November 21, 1972

Dr. Cornelius A. Tobias, University of California, Lawrence Berkeley Lab.
Biomedical Research with Accelerated Heavy Ion Beams
November 28, 1972

Dr. J. F. Fowler, Mount Vernon Hospital, London, England
Recent Progress on Work with Radiosensitisers of Hypoxic Cells
November 30, 1972
Dr. Vittorio Defendi, The Wistar Institute
Induction of Cell DNA Synthesis and Integration of SV40 Viruses
December 7, 1972

Dr. Penelope Allderdice, Columbia University
Chromosomal Fluorescence Studies in Hybrid Mammalian Cells
December 12, 1972

Dr. Martha L. Ludwig, University of Michigan
Crystal Structure of Clostridial Flavodoxin
December 13, 1972

Mr. W. J. Whitehouse, MRC Radiobiological Research Unit, Harwell, Didcot, Berks, England
Scanning Electron Microscope Studies of Trabecular Bone
December 21, 1972

In addition, several informal seminar series in specialized subjects—microbiology, mammalian cell biology, plant physiology, and biophysics—were held during the year. Most of the speakers were members of the Divisional staff; on occasion, scientists from the Chicago area also presented seminars.
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