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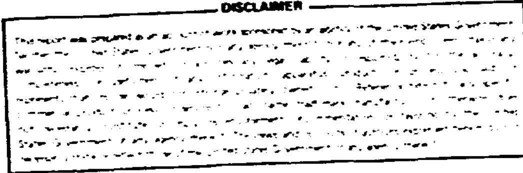

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BIOLOGY DIVISION PROGRESS REPORT

For Period of June 1, 1980 - July 31, 1982

Date Published: December 1982

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F. C. Hartman, Molecular and Cellular Sciences
L. B. Russell, Mammalian Genetics and Teratology
H. R. Witschi, Toxicology

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Oak Ridge, Tennessee 37830
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ORNL-2913	Period Ending February 15, 1960
ORNL-2997	Period Ending August 15, 1960
ORNL-3095	Period Ending February 15, 1961
ORNL-3201	Period Ending August 15, 1961
ORNL-3267	Period Ending February 15, 1962
ORNL-3352	Period Ending August 15, 1962
ORNL-3427	Period Ending February 15, 1963
ORNL-3498	Period Ending August 15, 1963
ORNL-3601	Period Ending February 15, 1964
ORNL-3700	Period Ending August 15, 1964
ORNL-3768	Period Ending February 15, 1965
ORNL-3853	Period Ending July 31, 1965
ORNL-3922	Period Ending January 31, 1966
ORNL-3999	Period Ending July 31, 1966
ORNL-4100	Period Ending January 31, 1967
ORNL-4240	Period Ending December 31, 1967
ORNL-4412	Period Ending December 31, 1968
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Introduction and Division Overview

R. A. GRIESEMER

The Biology Division is the component of the Oak Ridge National Laboratory that investigates the potential adverse health effects of energy-related substances. Almost all the work of the Division is experimental and utilizes mammalian and sub-mammalian systems to obtain data for predicting and understanding hazards to human health. Work directly with the human species is limited to studies of individuals naturally or accidentally exposed to environmental agents and to investigations utilizing fluids and cells that can be obtained from humans without harm.

The energy-related substances of interest are both physical (various forms of radiation) and chemical. Special emphasis is placed on problems associated with the emerging energy technologies, notably synthetic fuels of fossil origin. Since the energy-related substances to which people may be exposed tend to be complex mixtures of chemicals, the Division's activities concentrate on the adverse health effects from chemical or chemical-physical interactions and on methods to evaluate the effects of complex mixtures.

The Division's scientists are organized into multi-disciplinary teams that investigate the major disease endpoints: mutagenicity, reproductive disorders, carcinogenicity, and acute and chronic toxicity. A necessary part of such studies is parallel investigations of the normal structure and function of the body and the ways the body responds to injury. The studies range from molecular and cellular to the use of whole animals. Of the various organ systems, particular attention is paid to the skin and the respiratory tract, two major interfaces between the body and environmental agents.

The resources available for the Division's activities during the report period included a staff of approximately 90 at the doctorate level, 235 support personnel, 40 doctoral students, 45 post-doctoral students, and an average of 60 other visiting professors, students, and scientists. The Division occupies 500,000 sq. ft. of laboratory space with specialized facilities for 250,000 animals, a collection of 1,000 mutant stocks of mice, barrier facilities for the safe handling of hazardous substances, laboratories for recombinant DNA research, radiation sources, a facility for the production of large volumes of cells or microorganisms, and a library. The Division also makes extensive use of resources in other Divisions of the Oak Ridge National Laboratory including the Information Centers for mutagenesis and toxicology and the Analytical Chemistry Division where collaboration in research has been especially fruitful.

Among the significant changes in organization during this report period was the creation of a Toxicology Section under the direction of

Dr. Hanspeter Witschi to emphasize and expand this effort in keeping with the Division's major new initiative in toxicology. Another change was the formation of a laboratory for flow cytometry under the direction of Dr. Paul Mullaney. The Division has found the techniques of flow cytometry and the emerging science of cytometrics to have many applications in the biomedical sciences.

Technical progress during this report period is described in the following sections but a few highlights deserve special mention. Of the Division's several hundred publications each year, 18% are co-authored by predoctoral students, 23% by postdoctoral students, and 27% by collaborating scientists at other institutions. Special recognition came to Dr. Paul Selby who received the E. O. Lawrence Award in 1982 for his work on mutations in the skeletal system. A major Division-wide accomplishment was participation in a series of workshops on genetic toxicology (Gene-Tox Program) for the U.S. Environmental Protection Agency. J. L. Epler, W. M. Generoso, A. W. Hsie, E. Huberman, R. J. Preston, L. B. Russell, P. B. Selby, and W. Winton made major contributions to the workshops and acted as scientific advisers to the program. This activity and the resultant publications (listed with the reports of the Sections) emphasize the national lead role of the Biology Division at ORNL in research on genetic toxicology. The talents of Dr. Peter Mazur in cryobiology and of Dr. Liane Russell and her associates in mouse genetics were combined to enable the Division to successfully freeze and store mouse embryos. The Biology Division is one of two U. S. laboratories using this technique to preserve valuable genetic materials for future use at relatively low cost. Dr. Howard Adler developed a biological method, using enzymes in bacterial cell walls, to produce anaerobic environments. This technical breakthrough has already been applied to clinical aspects of the culture and identification of fastidious anaerobic microorganisms and holds promise for the production of hydrogen as well.

The body of this report provides summaries of the aims, scope and progress of the research of groups of investigators in the Division during the period of June 1, 1980, through July 31, 1982. At the end of each summary is a list of publications covering the same period (published or accepted for publication). For convenience, the summaries are assembled under Sections in accordance with the current organizational structure of the Biology Division; each Section begins with an overview. It will be apparent, however, that currents run throughout the Division and that various programs support and interact with each other.

In addition, this report includes an outline of educational activities, a listing of the members of the 1982 Advisory Committee, seminar programs in the Division, research conferences sponsored by the Division, extramural activities of staff members, abstracts for technical meetings by staff, and a table that summarizes funding and personnel levels for fiscal year 1982.

Division Staff – July 31, 1982

MOLECULAR AND CELLULAR SCIENCES SECTION

Section Head – F. C. Hartman
Secretary – S. P. Sands

Bio-organic Chemistry and Enzymic Mechanisms

F. C. Hartman
- M. I. Donnelly[1]
- I. L. Norton
- C. D. Stringer
- J. R. Totter[2]

Biosynthesis of Nucleic Acids

R. K. Fujimura
- B. C. Roop

S. Mitra
- A. Bhattacharyya[1]
- C. E. Snyder

S. K. Niyogi
- D. P. Allison
- R. P. Feldman
- M. Rorvik[2]
- M. L. Yette

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- V. A. Bonnewell[1]
- R. F. Fowler[2]
- D. L. Mykles[2]
- C. Soumoff[1]
- M. S. Spann[2]

A. L. Stevens

Comparative Nucleic Acid Biochemistry of Abnormal Cells

W. E. Cohn[3]

M. P. Stulberg
- E. B. Wright

E. Volkin
- M. E. Boling
- M. H. Jones

Theoretical and Applied Cryobiology

P. Mazur
- W. J. Armitage[1]
- K. W. Cole
- U. Schneider[2]

Genetic Damage: Repair and Physiological Consequences

W. E. Masker
- N. B. Kuemmerle
- M. A. Maupin

R. O. Rahn
- H. G. Sellin

P. A. Swenson
- J. G. Joshi[2]
- L. Riester

Macromolecular Structure and Function

J. R. Einstein

J. W. Longworth

D. E. Olins[3]
- G. J. Bunick[2]
- A. P. Butler[2]
- A. L. Olins[2]
- E. A. Wilkinson[2]

C. H. Wei

Molecular Genetics and Regulatory Processes

W. E. Barnett
- E. T. Howley[2]

K. B. Jacobson
- E. H. Lee[1]
- R. K. Owenby
- E. K. Wilkerson

Environmental Cellular Interactions

W. A. Arnold[3]

S. F. Carson[3]

J. S. Cook
- P. W. Braden
- W. S. Riggsby[2]
- C. J. Shaffer

CELLULAR AND COMPARATIVE MUTAGENESIS SECTION

Section Head - J. L. Epler
Secretary - N. P. Hair

Comparative Mutagenesis

- J. L. Epler
  - K. B. Allen
  - J. T. Cox
  - M. B. Jones
  - L. Oggs
  - W. Winton
- F. W. Larimer
  - A. A. Hardigree
- C. E. Nix
  - N. L. Forbes
  - L. M. Kyte
  - R. D. Wilkerson
- L. C. Waters
  - S. I. Simms

Genetics of Microorganisms and Drosophila

- H. I. Adler*
  - W. D. Crow
  - J. S. Gill
- D. Billen[3]
  - C. T. Hadden[2]
  - R. Y. Morris
  - A. E. Ralston
- E. H. Grell
  - R. D. Wilkerson
- R. F. Grell
  - E. E. Generoso
- R. F. Kimball[3]

Mammalian Cellular and Molecular Studies

- A. W. Hsie
  - P. A. Brimer
  - D. M. DeMarini[1]
  - R. Machanoff
  - S. W. Perdue
  - J. R. San Sebastian[2]
  - R. L. Schenley
- P. A. Lalley
  - J. Diaz
- R. A. Popp
  - E. G. Bailiff
  - D. M. Popp
- R. J. Preston
  - W. Au
  - M. L. Chandler
  - P. C. Gooch
  - T. Ho
  - H. E. Luippold
  - H. S. Payne
- J. D. Regan
  - W. L. Carrier
  - W. C. Dunn, Jr.
  - A. A. Francis
  - I. F. Fukushi[1]
  - I. Kusano[1]
  - W. H. Lee
  - B. G. Stanford

Development and Reproductive Physiology

- J. N. Dumont
  - T. W. Schultz
  - R. G. Epler

---

*Manager, Biotechnology Program

MAMMALIAN GENETICS AND TERATOLOGY SECTION

Section Head - L. B. Russell
Secretary - A. R. Smiddy

Mammalian Genetics and Development

- R. B. Cumming
  - L. W. Lewis
- W. M. Generoso
  - A. J. Bandy
  - K. T. Cain
- E. F. Oakberg
  - C. D. Crosthwait
- L. B. Russell
  - J. W. Bangham
  - D. A. Carpenter
  - C. V. Cornett
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  - S.-y. Hitotsumachi[2]
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  - C. S. Montgomery
  - E. L. Phipps
  - G. D. Raymer
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  - G. A. McBee
  - C. S. Richeson
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  - R. D. Thomas
  - P. S. White
  - E. L. Wilkerson

Mammalian Embryology and Teratogenesis

- R. W. Filler
  - C. Y. Horton

TOXICOLOGY SECTION

Section Head - H. R. Witschi
Secretary - J. M. Shover

Systemic Toxicology

N. W. Revis[2]
H. R. Witschi
- L. B. Brattsten[2]
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- C. Morse[1]
- C. W. Owenby
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- Y. B. Shah[2]
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- L. H. Smith

Skin Toxicology

J. M. Holland
- B. H. Chastain
- L. C. Gipson
- J. W. Hall
- J. Y. Kao
- L. R. Shugart
- T. J. Stephens
- M. J. Whitaker
- L. H. Woods

Respiratory Toxicology

W. E. Dalbey
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A. J. P. Klein-Szanto
- C. M. Fearick[2]
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- R. Morris[1]
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- S. F. Huang
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M. Terzaghi-Howe

Immunotoxicology

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- P. L. Glover

CARCINOGENESIS SECTION

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Radiation Carcinogenesis

R. J. M. Fry
- R. D. Ley
- L. E. Roberson

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- L. M. Adams
- N. D. Bowles
- K. C. Dearstone
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- L. C. Satterfield

Chemical Carcinogenesis and Tumor Promotion

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- K. A. Davidson
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- S. M. Fischer
- M. J. Gudzinowicz[1]
- L. G. Hardin
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- L. P. Yotti[1]

Chemical Carcinogen Metabolism

J. K. Selkirk
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- B. K. Mansfield
- P. A. Noghrei-Nikbakht
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Molecular Carcinogenesis

F. T. Kenney
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- K.-L. Lee
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Molecular Genetics of Carcinogenesis

W. K. Yang
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- J. C. Holloway
- J. O. Kiggans, Jr.
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- J. A. Otten
- R. W. Tennant[2]
- R.-W. Wong[2]
- D. M. Yang

CARCINOGENESIS SECTION - Continued

Molecular Immunology

S. J. Kennel
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- L. J. Foote
- E. T. Friedman[2]
- D. R. Hoffman[1]
- A. H. L. Huang[1]
- P. K. Lankford
- G. L. McNabb

Human Cancer Biochemistry

G. D. Novelli
- A. N. Best
- M. V. Long*
- E. F. Phares
- A. Solomon[3]

Cytometrics

G. R. Braslawsky**
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*Affirmative Action Representative

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Research Activities

Molecular and Cellular Sciences Section

SECTION OVERVIEW - F. C. HARTMAN

As part of the Department of Energy's life sciences program, our mission is to investigate basic aspects of adverse health effects of energy production. Since nuclear reactors and fossil fuels are our nation's major energy sources, interactions of radiation and chemicals derived from fossil fuels with biological systems are of primary concern. The more profound clinical manifestations of human exposure to these agents may include cancer, genetic damage, birth defects, and acute toxic poisoning, all of which represent perturbations of normal cellular processes. Thorough understanding of these disorders requires more basic knowledge about the structure, biochemistry, and physiology of cells and about defense mechanisms by which cells can cope with environmental agents. Thus, the Section has evolved a comprehensive, diversified program for probing the multifaceted aspects of health problems associated with energy production and utilization.

Cancer, mutations, and birth defects share common origins that entail modification of gene structure or alterations in nucleic acid enzymology. Major emphasis is therefore placed on gene structure and function. This central theme includes studies of the structure of DNA and chromatin, the interaction of nucleic acids with environmental agents, replication and transcription of DNA as well as their regulation, enzymology of repair of DNA damaged by chemicals or radiation, and molecular genetics. Within these general areas of molecular biology, some recent achievements are purification and characterization of key enzymes involved in the processing of precursor messenger RNA, direct documentation of the mutagenic potential of the alkylated base $\underline{O}^6$-methylguanine, development of an assay that can detect the binding of one molecule of benzo[a]pyrene per 10^7 bases in DNA and devising a strategy which should reveal the mechanism of a suppressor mutant in Drosophila.

Membrane biology is also considered of prime relevance, as membranes can be considered interfaces between the intracellular metabolic machinery and the external environment. In many cases, membranes are effective barriers to noxious chemicals and as such preclude the necessity of repair pathways; in other cases, membrane components are rendered inactive by combination with exogenous agents and repair mechanisms must then be invoked. Current studies emphasize membrane repair and turnover in response to drugs such as digitalis and growth stimuli such as tumor promoters. Another aspect of cellular-environmental interactions and membrane integrity which receives emphasis is cryobiology. Recently, the long-held view that injury to cells subjected to slow freezing is due to osmotic dehydration has been challenged. New data suggest that survival is

primarily dependent on the fraction of intracellular water remaining unfrozen rather than on the salt concentration therein.

Given the absolute dependence of life processes on catalysis and the adverse consequences of altering catalytic events, our long-standing interests in enzyme mechanisms continues. A major activity is the design of affinity labels for the characterization of catalytic sites. These studies also relate to toxicology in that acute toxic poisoning frequently reflects the specific interaction of a chemical with a key metabolic enzyme; e.g. the inactivation of acetylcholine esterase by nerve gases.

Although all of our studies are at least partially funded by DOE, supplemental support through grants from NIH, NSF, and USDA permits a somewhat broadened scope with enhanced scientific productivity. Declines in DOE budgets have virtually necessitated the securement of funds from other agencies to provide postdoctoral positions and other supportive personnel so essential to maintaining competitiveness.

Nucleic Acids: Structure, Function and Interaction with Carcinogens/Mutagens

STRUCTURAL ASPECTS OF DNA IN ITS REPLICATION AND REPAIR

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Many toxic by-products of energy production and utilization are mutagenic and carcinogenic and interact with DNA. The repair of lesions produced in DNA plays a critical role in its replication as well as in the ultimate expression of mutagenesis and carcinogenesis. In view of this, the research in this laboratory involves DNA structure, replication and repair and has two broad aims, namely (a) elucidation of the mechanism of genome replication and its control in mammals and (b) role of DNA repair in mutagenesis and carcinogenesis induced by simple alkylating carcinogens. In pursuit of the first goal, structure and replication of teratogenic mammalian parvovirus genomes are being studied because these linear single-stranded DNAs appear to be dependent on the host-specific synthetic and regulatory factors. For achieving the second aim, repair and mutagenic potential of base lesions produced by simple nitrosamines are being investigated.

<u>Structure and Replication of Single-stranded DNA Genomes of Parvoviruses.</u> We have extensively studied the comparative structure of DNAs of several nondefective mammalian parvoviruses and defective human adeno-associated viruses (AAV). These viral DNAs have duplex hairpin structures at both termini. We have established an evolutionary relation among the rodent viruses based on electron microscopic heteroduplex analysis. We

have found that about half of the DNA sequences from the 3' end are completely conserved and may code for some nonstructural proteins of similar nature. The other halves of the DNAs code for capsid proteins and are unique for each virus. We have also shown that Lu III, isolated from and specific for human cells, is indeed derived from or is an ancestor of a rodent virus. Nonetheless, this virus behaves like human AAV, and unlike rodent viruses, it encapsidates both (+) and (-) strands of DNA equally.

We have developed (in collaboration with R. Schmoyer of Computer Sciences Division) an empirical relation, superior to previous ones, to calibrate agarose gels for size determination of DNA and have accurately determined the size of parvoviral DNAs.

We have probed the 5' terminal structure of Kilham rat virus DNA by various techniques, determined the size of the hairpin and have shown that the 5' terminus is blocked by a group that allows ligation with the replicated 3' end in the form of a giant hairpin, but this terminus is not accessible to phosphatase/polynucleotide kinase. We are now looking for the putative site-specific endonuclease involved in replication of the hairpin and attempting to clone the intact viral genome into a plasmid.

Repair and Miscoding Properties of Alkylated Bases in DNA. O^6-alkylguanine, produced in DNA by simple alkylating carcinogens, is believed to be a critical premutagenic and precarcinogenic lesion. We have synthesized $[8\text{-}^3H]m^6dGTP$ of high specific activity and incorporated O^6-methylguanine (m^6G) as the sole modified base in synthetic DNAs. Using these substrates, we have shown that O^6-methylguanine is repaired by *in situ* demethylation of DNA in both bacterial and mammalian cells and have been able to quantitate the number of enzyme (methyltransferase) molecules that appear to act stoichiometrically. In collaboration with C. Hadden (this Division), and R. Day (National Cancer Institute), we have shown that, as in *E. coli*, *B. subtilis* contains an inducible methyltransferase and that human mer^+ and mer^- cell lines have wide differences in the enzyme level. It thus appears that the enzyme is tightly regulated in bacteria as well as in mammals.

Because of the current belief that m^6G is premutagenic as a consequence of its base-pairing ability with T, we have designed synthetic templates to study the base-pairing properties of m^6G and have shown that m^6G in the template has a large preference for T over C during DNA synthesis. Similarly, m^6dGTP base pairs with T in the template. Based on the above rationale, a collaborative effort of our laboratory and that of W. Masker (this Division) resulted in the first direct evidence for the *in vivo* mutagenic potential of m^6G. Finally, on the basis of kinetic studies, we conclude that m^6G in DNA rather than m^6dGTP in the nucleotide pool is the primary premutagenic lesion.

At present, we are studying the properties of homogeneous methyltransferase of *E. coli* and attempting to purify the enzyme and to clone its gene from human cells.

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THE MECHANISMS OF MUTAGENESIS AND DNA REPAIR

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Energy technologies create potentially deleterious effects on human health. Assessment of these health risks must include a consideration of the mutagenic and carcinogenic potential of environmental insults. Although mutagenesis screening systems can provide information on specific substances, accurate extrapolations and meaningful judgments with regard to the health hazards connected with energy production require an understanding of the mechanisms which cause mutation and the DNA repair pathways used by the cell to protect itself against genetic alteration. Our research effort is devoted to a study of DNA repair and the molecular mechanism of mutagenesis.

A major aspect of our work involves the use of in vitro DNA metabolic systems able to replicate, repair, and recombine DNA from bacteriophage T7 under relatively well defined biochemical conditions. A second in vitro system can encapsulate the product DNA recovered from the DNA synthesis system and produce fully infective T7 phage in vitro. Thus, DNA packaging provides a way of using biological activity as an endpoint in characterizing the physical integrity and genetic make-up of DNA that has been damaged by chemicals or radiation and then subjected to prior in vitro manipulations before being encapsulated into phage heads. We had previously used this sytem to demonstrate in vitro repair of ultraviolet radiation damaged DNA (6,7). Recently, we successfully demonstrated that DNA damaged by the mutagens ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) can be repaired with our system (4). Moreover, when DNA damaged by EMS is encapsulated to form viable T7 phage, these phage show reduced survival on repair deficient hosts (2) and accumulate mutations (to be published).

Application of the system described above to studies of in vitro mutagenesis was possible because of improvements to the DNA packaging system. By isolating capsids that are precursors to T7 phage and identifying components such as ATP and osmotic stabilizing factors that are important to DNA packaging, we have achieved efficiencies as high as 8% phage production per genome equivalent of DNA (5, to be published). These studies provide information regarding bacteriophage assembly and gave efficiencies high enough to allow us to examine the fidelity of DNA synthesis using the product recovered from the in vitro T7 DNA replication system. By replicating T7 DNA under normal conditions as well as potentially mutagenic conditions and then packaging that DNA, it was possible to examine the resulting phage for two types of mutations (amber and temperature-sensitive). Under standard conditions the frequency of mutation in vitro was virtually identical to what we found in vivo. In collaboration with S. Mitra and R. Foote, we included O^6-methylguanine triphosphate in our system and found a 10- to 70-fold increase in mutagenesis in the phage synthesized in vitro. This was the first direct demonstration that O^6-methylguanine, a suspected carcinogen, is a premutagenic lesion. Development of this in vitro mutagenesis system provides us with a sensitive experimental tool for examining the mechanisms of mutation induction.

Part of our program has focused on excision repair mechanisms in bacteria. In collaboration with R. Ley we used two distinct techniques to measure the extent to which "repair patches" incorporated as DNA damage are removed from the genome during excision repair. This demonstrated that very long repair patches are actually ligated to the contiguous parental DNA strand and therefore provide potential for both improved survival and mutation induction. Our data show long patches at about 5% of the incision sites (3,4). A large part of our effort on excision repair has concentrated on the uvrD gene of Escherichia coli. Although the precise role of the uvrD gene product is not known, it has been established that this genetic locus affects DNA repair, recombination, mismatch repair, and mutagenesis. In view of the complex repair response in human cells, it is not unreasonable that a function analogous to the uvrD protein may function in

higher cells. In addition to examining the effects of several alleles of the uvrD gene on various parameters of the excision repair of DNA damage in vivo, we have worked out a complementation assay that allows us to detect the uvrD gene product in vitro. We have used this assay to partially purify the uvrD gene product and are beginning to characterize this protein. It is hoped that these investigations will provide insight into the mechanism used both by bacteria and higher cells to cope with DNA damage and to help determine why mutations sometimes arise from DNA damage.

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mRNA PROCESSING IN YEAST

Audrey Stevens

Analyses of the health hazards of the procedures and materials involved in energy production can be studied best at the level of enzyme reactions carried out by living cells. Very important for these health studies is a knowledge of the most basic reactions of cells, which certainly include those involving RNA and DNA. Investigations in this laboratory continue to center on basic enzymatic reactions of RNA.

Still undefined are reactions involved in the conversion of precursors of mRNA (pre-mRNA) to mRNA in eukaryotes. The pre-mRNA is called heterogeneous nuclear RNA and is 2-6 times larger than mRNA. The conversion, called splicing, involves a removal of internal sequences called introns by endoribonuclease action followed by a rejoining of the 3'- and 5'-end

fragments, called exons, by ligating activity. It has not been possible yet to study the enzymes involved in vitro. Also undefined are reactions involved in the turnover or discarding of certain of the pre-mRNA molecules.

Yeast is a simple eukaryote and may be expected to have the same, but perhaps simpler, processing reactions as the higher eukaryotes. Two enzymes involved in the processing of pre-mRNA and mRNA in yeast are under investigation. Both enzymes have been partially purified from ribonucleoprotein particles of yeast. The first is a unique decapping enzyme which cleaves $[^3H]m^7Gppp$ $[^{14}C]$RNA-poly (A) of yeast, yielding $[^3H]m^7GDP$ and $[^{14}C]$pRNA-poly(A). That the enzyme is not a non-specific pyrophosphatase is suggested by the finding that the diphosphate product, $m^7GpppA(G)$, and UDP-glucose are not hydrolyzed. The enzyme differs from pyrophosphatases from tobacco and potato which cleave capped mRNA, because the latter enzymes are not specific and yield m^7GMP as the decapping product. The second enzyme is an endoribonuclease which converts both the $[^3H]$ and $[^{14}C]$ labels of $[^3H]m^7Gppp[^{14}C]$RNA-poly(A) from an oligo(dT)-cellulose bound form to an unbound, acid-insoluble form. The enzyme hydrolyzes the high molecular weight $[^3H]m/Gppp[^{14}C]$ RNA-poly(A), a large share of which may be pre-mRNA, several-fold faster than other single-stranded polyribonucleotides. The enzyme is inhibited by ethidium bromide, but fully double-stranded polyribonucleotides are not hydrolyzed. The hydrolysis of $[^3H]m/Gppp$ $[^{14}C]$RNA-poly (A) is stimulated about 2.5-fold by the addition of small nuclear RNAs U1 and U2 of Novikoff hepatoma cells. Results show that the stimulation involves an interaction of the labeled RNA with the small nuclear RNA. The inhibition of the enzyme by ethidium bromide and its stimulation by small nuclear RNA suggest that it may be a processing ribonuclease, requiring specific double-stranded features in its substrate. The characterization of the unique decapping enzyme and endoribonuclease may help to understand reactions involved in the processing of pre-mRNA and mRNA in eukaryotes.

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CONTROL OF TRANSCRIPTION IN ANIMAL VIRAL SYSTEMS

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In order to study the deleterious effects of various pollutants arising out of efforts towards increased energy production, there is a need to develop well-defined biological systems in which critical biochemical processes can be analyzed. Our investigations are specifically directed

towards elucidating the molecular mechanisms of transcription in two animal viral systems. We have extensively studied the DNA tumor virus, simian virus (SV) 40, and more recently, adenovirus 2. For such systems, particularly with SV40, transcription in vivo is known to occur at the level of chromatin, that is, DNA associated with histones, acidic proteins, host RNA polymerase II, and other regulatory proteins. Therefore, we are also conducting studies of the structure of the transcriptionally active viral nucleoprotein in order to fully understand the role of various proteins in viral transcription.

Specific, accurate initiation of transcription of class II (hnRNA and mRNA) genes has recently been achieved with three types of crude cell-free extracts, using as templates promoter-containing restriction fragments of various DNAs, and analyzing run-off transcripts. However, cell-free extracts, although capable of specific initiation and capping, are deficient in splicing, proper termination and polyadenylation of the nascent RNA product.

Since different homologous and heterologous crude systems can direct accurate transcription on various DNA templates, it is likely that a mechanism common to all three extracts may be involved in the recognition process, possibly at the level of the template. Although all the investigations involved naked DNA as the template, the possible alteration of the template structure has not been reported. We have found that interaction of SV40 DNA with the three different HeLa cell extracts capable of directing correct initiation of transcription leads to the formation of ordered nucleoprotein complexes that have a number of structural similarities with SV40 minichromosomes and eukaryotic chromatin. These nucleoprotein complexes can be conveniently purified by band sedimentation or gel filtration, and their sedimentation and elution properties resemble those of SV40 minichromosomes. Electron microscopy of purified complexes shows beaded structures that are sensitive to proteases, resulting in recovery of naked largely undegraded DNA. Contour lengths and compaction ratios of these nucleoprotein complexes as well as their digestion patterns with micrococcal nuclease and pancreatic DNase I are similar to those of authentic SV40 minichromosomes. Such nucleosome-like structures can also be obtained with linear SV40 DNA. However, unlike nucleosomes, no histones could be detected in the purified complexes. A non-histone chromosomal protein fraction (high mol. wt. and free of high mobility group proteins) prepared from the HeLa cell extracts could also generate similar beaded structures. We conclude that ordered nucleoprotein structures with certain common characteristics can be formed by interaction of DNA with non-histone chromosomal proteins as well as with histones. Only the former structures are generated in currently used cell-free transcription systems. We also find that only those purified nucleoprotein complexes containing the promoter can be actively transcribed in the presence of additional cell-free extract, suggesting that such structures and their protein components may be important in transcription. Our results indicate that the SV40 DNA-protein complexes formed with the in vitro systems mimic, in physical properties, the SV40 minichromosomes but differ from the latter in their protein composition.

Using the bacterial plasmid pBR322 as the cloning vehicle, we have recently cloned (with the help of Dr. Larry Boone of this Division) adenoviral DNA fragments containing the major late viral promoter. Upon incubation with the cell-free transcription extracts, these DNAs are also converted to ordered nucleoprotein complexes, as described for SV40 DNA, and direct specific initiation of transcription.

Our current studies are aimed at developing a "complete" in vitro transcription system that would truly reflect the situation in vivo. Such a system should involve histones, HMG proteins and nonhistone proteins, and hopefully, will correctly initiate and also terminate transcription and process the RNA to obtain the final viral RNA products. Such a system could then be utilized to test the effects of various mutagens and carcinogens at the various steps of eukaryotic transcription and RNA processing.

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CORRELATION OF STRUCTURE AND FUNCTIONS OF PROTEINS INVOLVED IN PHAGE T5 DNA REPLICATION: AN APPROACH TO STUDY OF MUTAGENESIS

R. K. Fujimura B. C. Roop

DNA polymerase, the enzyme responsible for DNA replication, also plays a key role in maintaining fidelity during DNA replication and repair. It is known that mutations in the structural gene for DNA polymerase increase the mutation or error rate. Mutations of other proteins in the DNA replication complex may also affect the fidelity of replication. It is our intention to alter these proteins by site-specific mutagenesis in vitro using a variety of mutagens. By these means we are hoping first to alter one or other of the multiple functions of the DNA polymerase, and then those of other proteins of the complex.

Many environmental agents are mutagens that directly alter DNA, and others may be modifying agents of proteins that alter functions of important enzymes. By direct studies of the consequences of changes in key proteins, we may be able to predict the effects of these agents and to suggest remedies.

Phage T5 DNA is a linear duplex with MW of about 7.7×10^7. In vivo analysis suggests that it is capable of initiating DNA replication from several sites with a primary site around the midregion of T5 DNA. The T5

replication system is intermediate in complexity between those of small circular DNA and bacterial DNA. It is our objective to isolate and characterize all the proteins required for every process involved in T5 DNA replication, particularly initiation, translocation, unwinding, and fidelity.

So far, two gene products coded by phage T5 DNA are known to be absolutely essential for DNA replication. These are the DNA polymerase coded by gene D7-D9 and the DNA binding protein coded by gene D5 (gpD5). There are several other phage gene products known to affect the onset, rate, and duration of DNA replication. Our studies suggest that some host proteins are also involved.

T5 DNA polymerase is a monomeric, multifunctional enzyme (6). Its most preferred primer-template is single-stranded DNA with a primer. It preferentially binds to such DNA at a 3'-OH primer end rather than at an end of a template strand or a single-stranded region. However, it does bind to a nick of duplex DNA and utilizes the 3'-OH end of a nick as a primer and elongates it from such an end, displacing the strand ahead of it. With a nicked circular duplex DNA, replication may continue until the copied segment is longer than the circumference of the initial circle. It is a highly processive enzyme. It is capable of translocating along a template incorporating at least 150 nucleotides. By our technique, it was processive to the end of poly(dA) used as a template. It has a 3'→5' exonuclease associated with it which also acts processively. The substrate is hydrolyzed until it is about 5 nucleotides long (1). We have shown that a single enzyme bound at a primer end can function as an exonuclease or as a polymerase depending on nucleoside triphosphates. It may reverse the direction in the midst of hydrolysis and start polymerization (2). Thus, the same polymerase is capable of acting as an editing enzyme correcting mistakes as it copies a template.

Gene product D5 has a dual role in DNA replication and control of transcription. It is essential for the shutoff of some early transcription and for the expression of late genes. It is an asymmetric protein of molecular weight 28,000. There are about 500,000 copies per cell, making it the most abundant DNA-binding protein synthesized in T5 phage-infected cells. It binds to both double- and single-stranded DNA, but binds preferentially and cooperatively to double-stranded DNA. Thus, it differs from bacteriophage T4 gp32 and *Escherichia coli* single-strand-binding protein, which are known as helix-destabilizing proteins; these bind preferentially and cooperatively to single-stranded DNA. The role of gpD5 in DNA replication is not clear. When nicked DNA is complexed to saturation with gpD5, DNA synthesis with all the DNA polymerases tested was inhibited.

Interaction of gpD5 with Duplex DNA and Its Effect on DNA Polymerase Functions. Interaction of duplex DNA with gpD5 was studied by velocity sedimentation of the complex to apparent equilibrium in a metrizamide gradient (7). The studies showed that duplex DNA saturated with gpD5 has a buoyant density of 1.17 g/cm^3. The binding of gpD5 to duplex DNA is cooperative in a buffer of low ionic strength, but apparently non-cooperative in the presence of 10 mM $MgCl_2$. Calculations based on these data show that

at saturation 40 base pairs are covered per gpD5 molecule. When nicked DNA is complexed with gpD5 to saturation, all DNA polymerases tested were inhibited. However, their associated 3'→5' exonucleases were not inhibited. E. coli exo III was inhibited. These findings suggest that enzymes that require properly H-bonded 3' OH ends of DNA are inhibited by gpD5 complexed to duplex region, but enzyme that preferentially acts on single-stranded DNA and distorted duplex DNA are not inhibited. Further kinetic studies of the polymerase inhibition suggest that the translocation process of DNA polymerase along a template is inhibited. This cooperative nature of binding to duplex DNA is being studied in detail.

Correlation of Structure and Function of T5 DNA Polymerase. By genetic analysis, the structural gene for T5 DNA polymerase is located at loci D7-D9 of the "D" segment of T5 DNA. (On the basis of recombination frequencies among various conditional lethal mutants of phage T5, T5 DNA is made up of four "segments"; D is the largest and occupies from the middle to the right part of T5 DNA consisting of genes that are transcribed from early to late period of infection.) However, by transfection of E. coli with restriction enzyme digests that were fractionated according to size and by use of such transfected cells as hosts for "marker rescue" experiments with amber mutants of the T5 DNA polymerase gene, we were able to identify the DNA fragment with the T5 DNA polymerase gene. Such DNA is used to clone the T5 DNA polymerase gene. We have several clones of the putative T5 DNA polymerase gene and they are being characterized. These clones will be mutagenized at several specific sites with the hope of isolating a variety of mutants of T5 DNA polymerase. We hope these mutants will consist of T5 DNA polymerase with altered properties of fidelity of replication, processiveness of translocation along the template and strand displacement. Essential structural properties of the polymerase for these processes will be studied.

Reconstitution of DNA Replication Complex. Preliminary studies suggest that T5 DNA is in a complex with gpD5 to varying extents depending on time after infection. When the DNA is saturated with gpD5, such DNA is not active in transcription or replication. DNA replication is initiated from multiple but specific sites. Presumably, such sites are not covered with gpD5 because of a weak affinity for gpD5. On the other hand, the DNA replication complex presumably has a high affinity for such sites. The key component of such a complex is DNA polymerase. By identifying other proteins that improve the affinity of the polymerase to DNA-gpD5 complex, and that affect the various functions of the DNA polymerase, we hope to reconstitute the DNA replication complex and to study its interaction with gpD5-coated DNA.

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NUCLEIC ACID BIOCHEMISTRY OF CELLS

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The present work of our group is concerned mostly with the relationship of the nucleotide precursor pool in eukaryotic systems to the synthesis and degradation of the nucleic acids. In particular, we are investigating the effect of a variety of metabolic agents, especially chemical mutagens and carcinogens, on the metabolic pathways involved in the biosynthesis of these precursors. Cellular functional compartmentation of the nucleotide pool plays an important role in these systems, and we are pursuing this aspect as well.

Nucleotide biosynthesis in Novikoff hepatoma cells is markedly altered by a variety of chemical mutagens, whether the mechanism of mutagenesis is by base substitution, covalent binding (adduct formation), intercalation, or cross-linking of DNA. The compounds investigated (N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroquinoline 2-oxide, 9-aminoacridine, and mitomycin C), at concentrations that cause some inhibition of RNA and DNA synthesis, bring about a large increase in the pool levels of all four nucleoside triphosphates. At the same time, reactions leading to the synthesis of CTP from exogenous uridine and GTP and ATP from exogenous hypoxanthine are severely inhibited. The formation of UTP from uridine and ATP from adenosine, by more direct phosphorylation reactions, appears relatively unaffected.

The increase in nucleotide pool size cannot be accounted for by a corresponding increase in de novo purine and pyrimidine nucleotide synthesis, as experiments with labeled formate and aspartate show similar inhibitions by the mutagens. With the salvage precursors, [^{3}H]uridine and [^{3}H]hypoxanthine, the mutagens can produce widely divergent reduction in the labeling of RNA-CMP versus RNA-UMP and of RNA-GMP versus RNA-AMP,

mostly a result of these agents causing large differences in the specific activities of the respective triphosphate precursors. These observations suggest that, in addition to the reactions with DNA, nucleotide biosynthesis could be another important biochemical target of chemical mutagens.

We have carried out double labeling experiments to determine whether a single, total pool serves as direct precursor for total RNA synthesis. One can estimate an apparent synthesis of an RNA nucleotide from the specific activities of the pool triphosphate's nucleoside (^3H cpm/nmol) and α-phosphate (^{32}P cpm/nmol) and from the total ^3H and ^{32}P incorporated into an RNA 5'-mononucleotide. When [^3H]Urd and $^{32}PO_4$ or [^3H]Hyp and $^{32}PO_4$ are used as precursors, the estimated synthesis is much higher when calculated from the pool [^3H]UTP and [^3H]CTP or [^3H]ATP and [^3H]GTP than from the corresponding α-^{32}P specific activities, with both types of cells and regardless of the times chosen. These results are independent of the specific activities of the [^3H]nucleoside or $^{32}PO_4$ used as precursors and hold true for poly(A)-containing RNA as well as total RNA. Our data are consistent with the hypothesis that the nucleotide pool is compartmentalized and that nucleosides are somehow rapidly channeled into a smaller functional pool used for RNA synthesis.

A severe imbalance in pool nucleotides has been invoked as a major cause of mutations. It will be important to determine whether or not chemical mutagens have a more selective inhibitory effect on the synthesis of the small, functional nucleotide pool than on the total pool.

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CHROMOSOME CHEMISTRY

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The major goal of this laboratory is to analyze and understand the macromolecular structure of eukaryotic chromosomes. This macromolecular structure is intimately involved in the three major functions of chromosomes: DNA packaging; transcription; and replication. Any of these functions can be damaged by exposure to abnormal physical or chemical agents. Our laboratory employs a wide range of biophysical, biochemical and ultrastructural techniques to work towards detailed macromolecular models. During the past two years we have concentrated our efforts in four major directions: 1) X-ray crystallography of avian erythrocyte mononucleosomes; 2) biophysical studies of nucleosome-nonhistone (i.e., HMG 14 and 17) complexes; 3) histone and chromatin structure in hypotrichous ciliated protozoa; and 4) 3-D ultrastructure of chromosomes in important physiologic states as determined by stereo-EM and by EM tomography. Our major accomplishments in each of these areas are summarized below:

1) X-ray crystallography of nucleosomes - Starting with very homogenous avian erythrocyte core mononucleosomes (i.e., 146 bp DNA plus inner histone octamer), we have discovered two new and useful crystal forms. One is in space group P_2, and has 2 nucleosomes per asymmetric unit. The other is in a very closely related space group C_2, with 1/2 nucleosome per asymmetric unit. Both forms give good diffraction with spots at better than 6 Å resolution. The C_2 form is of particular interest because it yields the first definitive evidence that the core nucleosome has an internal dyad axis.

2) Nucleosome - HMG 14 (or 17) complexes - The complex of nucleosomes with nonhistones HMG 14 (or 17) is believed to constitute a necessary first step in preparing chromatin for transcription. We have previously demonstrated that mononucleosomes have two binding sites for HMG 14 (or 17), symmetrically arranged with regard to the nucleosome diad axis. It further appears that in appropriate solvent conditions HMG binding to the nucleosome is cooperative, i.e., resembles an allosteric interaction. By examining HMG binding to formaldehyde - cross linked nucleosomes, we have shown that massive internal conformational changes are not a prerequisite for cooperativity. HMG interactions with nucleosomal DNA are an important part of the binding phenomenon. This has been demonstrated by changes in the nucleosomal DNA thermal stability, circular dichroic spectra, and radius of gyration (as determined by neutron scattering). In all probability, this key physiologic interaction involves only subtle changes in nucleosome conformation.

3) Histone and chromatin structure in hypotrichous ciliated protozoa - These simple eukaryotes accomplish miraculous feats with their chromatin.

The macronuclei of these cells (Oxytricha, Stylonychia and Euplotes) consist of a "bag" of highly polyploid linear chromatin fragments, believed to be individual genes. These nuclei are the major loci of cellular RNA synthesis. In addition, DNA replication in these macronuclei is easily observable in characteristic Replication Bands. We have developed methods to cultivate and harvest sufficient numbers of hypotrich cells to initiate biochemical studies of nuclear proteins, preparative fractionation of chromatin fragments, enrichment of individual genes as intact chromatin molecules, and enrichment of Replication Bands. In addition, we have developed a battery of pure gene probes to monitor chromatin fractionation, and begun to catalogue histone and nonhistone proteins by gel electrophoretic mobilities. The Replication Band has been extensively scrutinized by cytochemical and ultrastructural techniques. Future experiments will determine whether different genes are associated with different proteins, and which replication-related proteins are localized in the Replication Band.

4) 3-D Ultrastructure of Chromosomes - During the past two years, we have begun to develop 3-D ultrastructural models of chromosomes and nuclei in the various physiologic states: packaging, transcription and replication. Employing conventional sectioning and staining techniques, we have examined the following biological materials: avian erythrocyte and sea urchin sperm nuclei, and CHO metaphase chromosomes, as examples of nucleosome packing arrangements; puffs of polytene chromosomes (i.e., the Balbiani Rings of Chironomus salivary glands), as examples of localized transcription; and the Replication Bands of hypotrichous ciliated protozoa, as examples of localized DNA replication. Besides studying these materials by stereo-electron microscopy to develop semi-quantiative 3-D conceptions of the various chromosomal structures, we have developed a new and powerful technique, electron microscopic tomography. This method combines extensive data of tilted views of a specimen with computer reconstruction. The completely reconstructured object is subsequently visualized on a sophisticated image processing system to permit accurate 3-D model building. This technique has required the development of new programs and advancements at the frontiers of 3-D computer graphics.

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THE STRUCTURE AND ORGANIZATION OF THE EUKARYOTIC GENOME WITH SPECIAL EMPHASIS ON SATELLITE DNAs

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Our goal has been and continues to be the analysis in chemical, physical and molecular biological terms of satellite DNAs. We use Crustacea as model systems because of their cyclic growth phases and the wide spectrum of complexity of their satellites. In order to carry out this research, we work in two areas which are closely related and mutually supportive. The two areas are (1) the structure and function of satellite DNAs, and (2) the control and phenomenology of the crustacean molt cycle. Work in both areas has reached a point where their convergence and interrelatedness will be mutually most advantageous. For our studies on the function of satellite DNAs — some of which may be transcribed — we require RNA from animals at different growth stages. From our research on the crustacean molt cycle we can trigger precocious molts and synchronize the molt cycle of populations of animals. Our method permits their survival at ecdysis in contrast to earlier methods in which precocious ecdyses were initiated by the removal of a molt inhibitory hormone localized in the eyestalks. Thus, we can obtain macromolecules from animals after as well as before ecdysis.

Satellite DNAs occur at high and strictly regulated frequencies in many, if not all, eukaryotes. In some organisms, they account for as much as 67% of the DNA. Those satellites under current investigation in our lab are a G+C-rich (63%) satellite with a repeat unit (RU) of 2.07 ± 0.1 kilobases (kb), present in 1.6×10^4 copies and comprising three percent of the genome of the Bermuda land crab Gecarcinus lateralis, a pair of cryptic satellites with RU's of 156 and 80 bp that account for 30% of the DNA of a hermit crab Pagurus pollicaris, and an A+T-rich (74%) satellite with an RU of 1.3 kb that comprises 10 percent of the total DNA of the edible blue crab Callinectes sapidus. Preliminary experiments indicate that its RU (1.3 kb) is defined by Taq I sites, accordingly, we plan to insert this satellite into the Cla I site of pBR322.

Most of our research in the past two years has been concerned with the G+C-rich satellite of the land crab, the most complex satellite yet identified. Earlier experiments had indicated that the satellite did not

code for rDNA although the base composition of rRNA of the land crab is such that the DNA from which it is coded would have a density in CsCl gradients close to that of the satellite. In addition, the satellite is conserved in other crustaceans to an extent directly proportional to their evolutionary relatedness. Irreversible denaturation mapping of monomer (2.07 kb) and dimer (4.14 kb) RU's purified from Eco RI digests of satellite isolated from crab tissues identified domains rich in adjacent pyrimidines (TTs or CTs).

The satellite has been purified, restricted with Eco RI or Pst I and inserted into pBR322 at those sites. Sixteen Eco RI and 144 Pst I clones were recovered. Of the sixteen pGlE clones (Gl for G. lateralis, E for Eco RI), thirteen contain 2.07 ± 0.1 kb inserts, very close to the size of the RU of the satellite isolated from crab tissues. The range of the RU is 2.06-2.09 kb, and when these thirteen inserts are re-sized on a second gel, the same relative values are found as on the first, suggesting that the range of sizes is due to real microheterogeneity rather than statistical uncertainty.

Microheterogeneity is also seen in Hinf I maps constructed for three of the Eco RI clones and for six of the Pst I clones. The maps are all very similar, but despite family resemblances, to date no two clones with identical Hinf I restriction maps have been found, raising the question of whether each of the 1.6×10^4 copies in the parent satellite is different from all the others.

In addition to this microheterogeneity, two of the Eco RI clones show macroheterogeneity, containing significantly larger or smaller amounts of DNA than 2.07 kb. One is extended (EXT), with an RU of 2.7 kb; the other is truncated (TRU), with an RU of 1.69 kb. These variants have been stable through multiple rounds of replication in HB101 and therefore seem unlikely to have been derived from cloning accidents.

pGlE15 (RU = 2.09 kb) has been sequenced, as have large sections of the TRU and EXT variants. There are two distinct sets of domains in the satellite. Some domains are relatively simple in structure, such as a ~60 bp homocopolymer of $AG_{n = 1 \rightarrow 7}$, or fifteen tandem repeats of (C_2T), or several purine homopolymer tracts with several pyrimidine tracts some hundreds of nucleotides downstream on the same strand of DNA. Of functional importance, major deletions and/or insertions or amplifications occur at or near the simple repeated domains, leading to the EXT and TRU variants. Other domains are complex and of sufficient size to code for small proteins such as the cecropins (which convey immunity to some insects) or metal-binding metallothioneins, or repeating sequences such as the "anti-freeze" proteins. Some open reading frames in the satellite could code for proteins as long as 160 amino acids.

The EXT variant differs in several respects from the RU. The major and most striking difference is the 700 bp stretch of extra DNA consisting of 5 extra copies of a 142 bp fragment which has 83% homology with a 127 and a 130 bp sequence present as a single copy in the RU and TRU, respectively. In the acquisition of extra sequences by amplification, the

satellite differs from other satellites into which extra DNA is inserted. The major truncation that leads to the TRU variant is an extra Eco RI site caused by a C→G transversion approximately 370 bp upstream from the Eco RI site that usually delimits the 2.07 kb RU.

The satellite is of further interest in its high content of CG pairs which are markedly reduced in most eukaryotic DNAs. Computer analyses (ZFIND; R. J. Roberts, personal communication) show that the satellite has naturally occurring potential Z-form DNA in the form of two sets of $(CG)_3$, twenty sets of $(CG)_2$, four of them followed by a C, or preceded by a G or other alternating purines or pyrimidines (pu/py) several $(GC)_2$ and several tracts of nine to 25 other base pairs of other pu/py in their immediate surrounds. Overall, the potential Z-form DNA is ~2.3% alternating pu/py distributed in pairs, triplets or more. Some domains are much richer than others. Since the satellite is arranged in tandem repeats, the overall arrangement of the potential Z-form DNA is as follows: one domain (from bp 1527 to 2069) contains ~26% potential Z-form DNA; it is surrounded by several domains which contain much less or no potential Z-form DNA.

Recent research on the phenomenology of the crustacean molt follows up our earlier observation that the claw closer muscles of G. lateralis undergo a sequential atrophy and restoration during each molt cycle. Data suggest that one or more sarcoplasmic Ca^{2+}-dependent neutral proteinases (CDP) which degrade the major myofibrillar proteins are present. The greater CDP activity in atrophic muscles points to an important role for the proteinases in myofibrillar protein turnover and in the 40% reduction in muscle protein that occurs during proecdysis.

In vivo and in vitro studies continue on the determination of the pattern of change in ecdysteroid content of several Crustacean species and on the enzymology of the conversion of ecdysone to physiologically active forms.

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ENZYME AND GENE REGULATION IN DROSOPHILA

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An economy that depends on fossil fuel and nuclear energy will inevitably be forced to deal with the by-products of its energy sources. The biological consequences of exposure of humans to substances that normally are encountered in trace amounts must be understood. Examples are polyaromatic hydrocarbons, metal ions, and radioactive isotopes. Each of these becomes concentrated by energy production facilities at levels that are hundreds of times greater than normal. The biological investigation of the consequences of living with these substances is a necessary component of the development of an energy-based technology yet is an activity that generates little or no profit to a utility industry. For the public to be aware of and to deal with the dangers of these pollutants, information about their effects is needed. The role of determining these effects therefore falls on a government supported research program. Rather than scatter all of these biological investigations to other laboratories, it is beneficial to have expertise in the same organization that is developing energy strategies so that engineers and biologists can interact directly and appreciate each other's view points. A national laboratory is an appropriate place for the engineers and biologists to interact. In my research program two strategies are followed: (1) determine how the effects of certain mutations are neutralized by a natural mechanism; this is termed suppression and (2) seek the earliest events that occur when an animal is exposed to toxic metal ions and relate those to the physical-chemical properties of the metal ion.

Suppression. When the suppressor mutant su(s)[2] of Drosophila melanogaster is present, the effects of four mutations (vermilion, purple, speck, and sable) are alleviated. This suppressor has been known for decades but the mechanism by which the four mutations are reversed is poorly understood. The goal of our study is to determine the molecular mechanism by which the purple mutant is restored to normal by su(s)[2]. A closely related secondary goal is to determine the defects caused by purple that must be alleviated.

Our studies have revealed (1) the step in pteridine biosynthesis for which an enzyme is defective in purple, (2) the structure of one of the intermediates in this pathway (6-acetylpyrimidodiazepine), (3) a means to

synthesize the primary intermediate which is the product of the enzyme that is defective in the purple mutant and which is the immediate precursor of the 6-acetylpyrimidodiazepine, (4) a localization of the enzyme in question within the eye pigment granule where the pteridine pigments are located. These all are related to the secondary goal.

The primary goal was partially realized from constructing mutants (E. H. Grell, Drosophila Genetics Group) with different doses (1-4) of $su(s)^+$ and showing that the eye color and the activity of the defective enzyme of purple are further reduced as the dose of $su(s)^+$ is increased. This is a direct demonstration that the molecular basis of suppression can be addressed. Consequently an assay procedure was devised to measure the amount of "$su(s)^+$ substance" in a given genotype of Drosophila. This will allow this substance to be purified and characterized. Thus, this primary goal seems to be attainable.

Toxic Mechanisms. The physical chemical properties of metal ions have been measured in many ways, but our primary goal is to determine which of these many parameters are most relevant to the biological damage the metal ion causes. This project is an outgrowth of a seed money project and has become an interdivisional activity with two physicists (J. E. Turner and M. W. Williams, Health and Safety Research Division).

With Drosophila melanogaster we have: (1) determined the toxicity of 14 metal ions and compared the results with their toxicity on mice, (2) examined a number of physical and chemical properties of metal ions and shown that the hard-soft classification was most useful in that a good correlation between softness and toxicity to Drosophila and mice could be demonstrated, (3) shown that other physical and chemical properties, such as affinity for oxygen and sulfur, hold promise as correlates of toxicity, (4) demonstrated that the resistance to toxic metal ions decreases with age, (5) found that resistance to cadmium is a genetic trait since some inbred strains are 10 times more sensitive than others, (6) shown that the genetic trait(s) for resistance is (are) located on the X-chromosome, (7) found that the normal metal ion content of Drosophila is inversely related to the toxic effect of the metal ion (interdivisional project with Analytical Chemistry), (8) shown that macromolecular changes occur in response to administration of cadmium and certain other metal ions, both proteins and transfer RNAs being altered.

To understand how metal ions can affect transfer RNAs a physical chemical study was performed using Zn^{2+}, Cd^{2+}, and Mg^{2+} and measuring their effects on the chromatographic behavior (RPC-5) of the tRNA. The effects of Cd^{2+} and Mg^{2+} were mostly the consequence of their ionic strength but effect of Zn^{2+} was most unexpected. This is being studied further by comparing these three metal ions for their effect on the properties of tRNA as measured by nuclear magnetic resonance spectra, fluorescence spectra, ultraviolet absorption spectra, and melting temperature. We plan to extend this to infrared and Raman spectra in the near future. From these techniques the nmr and RPC-5 chromatography both seem to show unique effects of Zn^{2+} on tRNA structure. The studies on tRNA are interesting in themselves

and may also be considered model studies that would find application in the effects of metal ions on the structure of other RNAs and of DNA.

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Molecular Toxicology

ENVIRONMENTAL INSULTS TO DNA

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S. S. Cheng

The purpose of this work is to investigate the mechanisms by which a variety of chemical and physical agents cause damage to DNA. In particular, the aim is to develop analytical methods for quantitating very low levels of DNA damage. Emphasis is placed on the use of nonradiometric assays to measure this damage. Such assays have particular importance in evaluating damage incurred by animals exposed to pollutants in the environmnet, i.e., under nonlaboratory conditions. In short, it is desired to use DNA damage as an indicator of exposure. The agents that have been studied include ultraviolet radiation, metal ions, and chemicals in coal tar mixtures such as benzo[a]pyrene. The mechanism by which the substitution of halogenated bases into DNA sensitizes cells to radiation has also been investigated. Major tools used in these investigations include fluorescence spectroscopy and HPLC. The fundamental approach used in these studies involves the application of basic knowledge of DNA physical chemistry to the solution of analytical problems facing the environmental biologists.

The interaction between deoxyguanosine (dG) and *cis*-Pt leads to the 2:1 and the 1:1 dG-Pt adducts. These adducts were separated on an Aminex A6 cation exchange column by use of 0.01 M K_2CO_3 (pH 11) as an eluent. The stoichiometry of the adducts was determined from the ^{195m}Pt radioactivity and from the absorbance of the guanine chromophore at 280 nm. Time-course studies show that dG reacts initially with *cis*-Pt to form the 1:1 adduct, which then interacts with a second molecule of dG to form the 2:1 adduct. Acid hydrolysis (100°C in 88% formic acid for 5-15 min) of the 1:1 and 2:1

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Molecular Toxicology

ENVIRONMENTAL INSULTS TO DNA

R. O. Rahn H. G. Sellin
S. S. Chang

The purpose of this work is to investigate the mechanisms by which a variety of chemical and physical agents cause damage to DNA. In particular, the aim is to develop analytical methods for quantitating very low levels of DNA damage. Emphasis is placed on the use of nonradiometric assays to measure this damage. Such assays have particular importance in evaluating damage incurred by animals exposed to pollutants in the environmnet, i.e., under nonlaboratory conditions. In short, it is desired to use DNA damage as an indicator of exposure. The agents that have been studied include ultraviolet radiation, metal ions, and chemicals in coal tar mixtures such as benzo[a]pyrene. The mechanism by which the substitution of halogenated bases into DNA sensitizes cells to radiation has also been investigated. Major tools used in these investigations include fluorescence spectroscopy and HPLC. The fundamental approach used in these studies involves the application of basic knowledge of DNA physical chemistry to the solution of analytical problems facing the environmental biologists.

The interaction between deoxyguanosine (dG) and cis-Pt leads to the 2:1 and the 1:1 dG-Pt adducts. These adducts were separated on an Aminex A6 cation-exchange column by use of 0.01 M K_2CO_3 (pH 11) as an eluent. The stoichiometry of the adducts was determined from the ^{195m}Pt radioactivity and from the absorbance of the guanine chromophore at 280 nm. Time-course studies show that dG reacts initially with cis-Pt to form the 1:1 adduct, which then interacts with a second molecule of dG to form the 2:1 adduct. Acid hydrolysis (100°C in 88% formic acid for 5-15 min) of the 1:1 and 2:1

adducts results in their conversion to two new products which elute differently from the column but which still contain Pt bound in the same stoichiometric ratio to dG as in the nonhydrolyzed adducts. The hydrolyzed adducts show a negative diphenylamine reaction indicative of cleavage of the glycosidic bond. It is concluded that mild acid hydrolysis converts the 1:1 and 2:1 dG-Pt adducts into the corresponding guanine-Pt adducts which are chromatographically distinguishable. This hydrolysis-HPLC procedure has application to the identification of the Pt-adducts formed in DNA.

The fluorescence associated with the acid liberated tetrol has been used to quantitate the amount of benzo[a]pyrene diol epoxide (BPDE) bound to the DNA of mice treated with B[a]P. Previously, we have utilized low temperature enhancement of the fluorescence to measure BPDE bound to DNA with a sensitivity of one part in 200,000. In order to extend the sensitivity down to a part in 10^6 or lower, DNA-BPDE was subjected to acid hydrolysis followed by HPLC. A comparison of the relative quantum yields of the B[a]P moiety measured with a standard spectrofluorimeter under various experimental conditions shows that room temperature measurements of the liberated tetrol give signal levels commensurate with low temperature measurements on intact DNA-BPDE provided the room temperature measurements are done under deoxygenated conditions. Currently, a reverse phase C-18 column is being used to examine DNA-BPDE following acid hydrolysis for 60 min in 0.1 M HCl at 85°C, a treatment sufficient to remove most of the BPDE in the form of the tetrol. The column is eluted with 50% methanol, and the tetrol peak is monitored by its fluorescence at λ >360 nm using 246 nm excitation. The amount of tetrol detected in this way is ~4 pg per 50 µl of sample. Therefore, it is expected that in 50 µg of DNA, as isolated from a single mouse, one can detect approximately one molecule of BPDE per 10^7 bases.

DNA isolated from *Escherichia coli* and substituted with various amounts of iododeoxyuridine (12-95%) was irradiated at either 265, 300, or 313 nm and the frequency of chain breakage measured by sedimentation in either neutral or alkaline sucrose gradients. The wavelength dependence of the photochemical cross section for chain breaks paralleled that for iodine loss, and the average frequency of chain breakage per halogen loss was 0.50 ± 0.07. Approximately 80-90% of the breaks observed in alkali are alkali-labile bonds and are not observed under neutral conditions. The presence of ethanol during irradiation reduced the frequency of chain breakage by more than an order of magnitude. These results are interpreted in terms of a model in which photo-induced deiodination leads to the formation of a uracilyl radical which then abstracts a hydrogen atom from either a nearby sugar moiety or from another hydrogen donor such as ethanol. The resulting modified sugar can then rearrange to form either a clean chain break or an alkali-labile bond.

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MOLECULAR MECHANISMS OF TOXICITY, MUTAGENESIS AND CARCINOGENESIS BY METAL POLLUTANTS

S. K. Niyogi R. P. Feldman

Certain metals have been identified as toxic agents, potential environmental carcinogens and mutagens through occupational exposure as well as in the laboratory. Increasing quantitites and varieties of toxic metal compounds are being released as pollutants from mining, coal combustion, asbestos production, welding industries, etc. It is essential to understand the molecular mechanisms leading to metal toxicity in target tissues, in addition to screening for metal pollutants.

Since one of the prime targets of mutagens and carcinogens is the DNA genome, we initiated a study on the effects of a variety of metals on DNA-directed RNA transcription. Using bacterial RNA polymerase, metal chlorides were tested for their effects on misincorporation of nucleotides with poly(dA-dT) template and on the rates of overall transcription and chain initiation with phage T4 DNA template.

The results of misincorporation can be summarized as follows: Zn^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Na^{+}, K^{+}, and Li^{+} — all nonmutagenic and noncarcinogenic — do not increase misincorporation. Mutagenic and carcinogenic metal ions tested, for example, Mn^{2+}, Cu^{2+} and Cd^{2+}, increase misincorporation, Ni^{2+} slightly increases it, and Co^{2+} does not increase it at all. These results suggest the need for caution in proposing simple enzymatic assays for screening potential mutagens and carcinogens. Both the hard-soft character

and the carcinogenic potential of a metal ion are important in its effect on misincorporation.

Among the divalent metal ions tested, the concentration-dependent order of inhibition of overall transcription with T4 DNA is $Pb^{2+} > Zn^{2+} > Cu^{2+} > Be^{2+} > Cd^{2+} > Ni^{2+} > Ca^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Sr^{2+}$. Ca^{2+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Li^{+}, Na^{+}, and K^{+} — nonmutagenic and noncarcinogenic metals — decrease chain initiation at concentrations that inhibit overall transcription. Pb^{2+}, Cd^{2+}, Co^{2+}, Be^{2+}, and Mn^{2+} — all mutagenic or carcinogenic — stimulate chain initiation over a wide range of concentrations that inhibit overall transcription. Cu^{2+} and Ni^{2+} — both carcinogenic — stimulate initiation at very low concentrations that inhibit overall transcription. The effect of a metal ion on transcription initiation appears to be related both to its mutagenic/carcinogenic potential and its hard-soft character.

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Environmental-Cellular Interactions

RESPIRATION SHUTOFF IN ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM AFTER FAR-UV IRRADIATION

P. A. Swenson L. Riester

An important consequence of far ultraviolet (254 nm) irradiation (UV) damage to the DNA of *Escherichia coli* is cessation of respiration. The shutoff, which occurs about 60 min after exposure to UV, is dependent upon protein synthesis and upon the *recA*$^{+}$ and *lexA*$^{+}$ gene products. Other so-called rec/lex responses caused by damaged DNA include error-prone repair (as indicated by mutagenesis) and induction of lambda prophage. Our objective is to understand the genetic and biochemical bases of the shutoff process and its control.

We have found that much of the inner membrane disappears in irradiated cells that have their respiration shut off but that there is greatly increased synthesis of an inner membrane protein with a molecular weight of 51,000. In addition, two NAD^{+} binding proteins in the inner membrane of irradiated cells show decreased ability to bind to an NAD^{+} affinity column compared to those from unirradiated cells. We suspect that these alterations in the biochemical composition of the inner membrane are in part responsible for the complete and selective loss of pyridine nucleotides from irradiated cells during the course of respiration shutoff. This work was done in collaboration with Dr. J. G. Joshi, University of Tennessee, Knoxville.

It is well known that when Salmonella typhimurium cells harbor plasmid pKM101 they show increased resistance to UV and enhanced UV mutagenesis. The plasmid gene responsible for these effects has been localized and designated muc (mutagenesis-ultraviolet, chemical). We have shown that pKM101 prevents UV induced respiration shutoff and that the muc$^+$ gene is responsible. A Tn5 translocatable element within the 9000 base muc gene causes the irradiated S. typhimurium cells to behave as though no plasmid is present, but Tn5 insertions just beyond each limit of the muc gene have no effect.

In S. typhimurium supX mutants are known to be radiation sensitive. We have found one supX strain that is not protected by pKM101. The supX locus is identical with the top locus which specifies topoisomerase I. This enzyme removes negative superhelical turns from DNA; in its absence transcription of DNA is increased. We propose that the exaggerated shutoff of respiration and increased killing of supX cells occurs because of greater ease of transcription of an operon involved in UV-induced respiration shutoff.

It is important to localize the gene for UV-induced respiration shutoff. We have used an operon fusion technique for inserting the lacZ gene (which codes for β-galactosidase) next to promoter regulator regions of S. typhimurium operons that are induced by UV damage. Such mutants when irradiated make β-galactosidase instead of the normal gene product. We have isolated over 2000 of these mutants and have tested their abilities to shut off respiration after UV. We have not found a mutant with normal UV sensitivity that fails to shut off its respiration after receiving a UV fluence sufficient to cause shutoff in a wild type strain. However, two that do not shut off at such a fluence, but which are very UV sensitive, have been isolated. The genes responsible for the UV sensitivities have not been mapped.

We have recently found that three S. typhimurium strains, each deficient in a specific peptidase, are sensitive to UV with respect to killing and respiration shutoff. Until now these mutants have had no distinguishing phenotypes other than inability to hydrolyze a specific peptide bond. Peptidases play regulatory roles in cells and studies with these mutants may aid in the study of the processes of respiration shutoff and cell killing.

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REGULATION OF TRANSPORT SYSTEMS IN CULTURED MAMMALIAN CELLS

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Cells and whole organisms interact with their environment through their membranes, plasma membranes for cells and cellular epithelial membranes for the organism. As a protective measure, the lipid bilayer of the surface membrane serves as an effective barrier to most of the polar compounds that may be dissolved in the aqueous environment in which cells live. As a corollary, the dissolved substrates required by cells or organisms either for maintenance or for growth are commonly taken up by specific transport systems. The latter, in turn, are well regulated in terms of numbers of transporters, the affinities of the transporters, or their degree of coupling to metabolic systems. Our group has been studying various aspects of transport regulation in mammalian cells in relation to the metabolic requirements of the cells or, in model epithelial systems, the requirements of the organism. Special emphasis is given to changes in these systems in response to growth stimuli, including the effects of tumor promoters. Our principal findings are:

Differentiation of LLC-PK_1 Cells in Culture. LLC-PK_1 cells, derived by Hull (Indianapolis) from pig kidney, differentiate in culture into a transporting epithelium. We assay this transport as the Na^+-dependent uptake of α-methylglucoside (AMG), a characteristic property of differentiated renal proximal tubules. Differentiation occurs spontaneously in postconfluent cultures, but can be accelerated by inducers like hexamethylene bisacetamide (HMBA) or phosphodiesterase inhibitors like dibutyryl cyclic AMP, theophylline, or methyl isobutylxanthine (MIX); conversely, differentiation is inhibited by the tumor promoter TPA.

In our and other laboratories the concentrative uptake of AMG has been measured on populations of cells, and the cellular basis of the process is not known. We have conceived of six potential mechanisms that could contribute to the increased uptake and have been systematically testing them:

1) Since uptake is measured as a function of cell protein, the cell water space may enlarge postconfluence. However, measurements of water/protein utilizing the distribution volume of the nonmetabolizable 3-O-methylglucose have shown this factor to be constant.

2) A few transporting cells may become increasingly extensively coupled with nontransporting cells via gap junctions, and nontransporting cells may become loaded via the coupling junctions. However, in collaboration with R. Azarnia and W. Loewenstein (Miami) we have found by both electrical measurements and observation of injected fluorescent dyes that these cells are not coupled at any growth stage.

3) The driving force for Na^+-linked uptake, especially the membrane potential, may increase postconfluence. This postulate cannot account for

the simultaneous decrease in Na^+-linked neutral amino acid uptake. Estimates of membrane potential from the distribution ratio of the lipophilic cation tetraphenylphosphonium (TPP^+) suggest that the p.d. in growing cells is ~105 mV (inside negative) and falls to ~65 mV at confluence and remains constant. This mechanism accounts for the amino acid results, but is in the wrong direction to explain the increasing capacity for hexose accumulation.

4) The increased uptake capacity may be due to a decreased leak efflux over parallel pathways in postconfluent cells. Although AMG is taken up at the apical surface, there is a Na^+-independent transporter at the basolateral surface which may be down-regulated at confluence. However, we have shown that AMG is not a substrate for this transporter, and that the principal route for efflux appears to be simple diffusion. Although accumulation capacity is a resultant of uptake and efflux, the rate constant for efflux remains invariant throughout growth and postconfluent stages, ruling out a changing-efflux mechanism.

5) In the postconfluent state, the cells develop (over a period of a week or more) an increasing number of transporters per cell. We have estimated the number of transporters in cultures by the Na^+-dependent binding of the specific ligand [^{3}H]phlorizin and have found that the AMG-accumulating capacity is linearly related to the phlorizin binding. This then is a possible mechanism, although the phlorizin-binding results are equally compatible with mechanism 6 (below). The transporters-per-cell mechanism appears unlikely on the basis of uptake kinetics, but cannot yet be ruled out with certainty.

6) In the postconfluent state, individual cells independently and rapidly develop their full capacity for concentrative transport. The increasing accumulating capacity of the population, as well as the increasing degree of [^{3}H]phlorizin binding, reflects an increasing number of fully differentiated cells in the population. This postulate is more compatible than mechanism 5 with the uptake kinetic data, and is the postulate we favor. H. R. Kaback (Hoffman-LaRoche) is supplying us with dansylglucoside and dansylgalactoside with which we plan to test the hypothesis by direct quantitative observation under fluorescence microscopy.

Recycling of Surface Sialoglycoconjugates. Surface sialoglycoconjugates of HeLa and HTC cells were labeled with $NaB[^3H]_4$ after oxidation by $NaIO_4$. The labeling procedure cleaves the sialic acids to a neuraminidase-sensitive 7-carbon derivative, 5-acetamido-3,5-dideoxy-L-arabino-heptulosonic acid, termed $AcNeu^7$ (Van Lenten, L., Ashwell, G., J. Biol. Chem. 246: 1889-1894, 1971). After labeling, the radioactivity is lost from both cell types with biphasic kinetics. The half-time for the fast phase is about 4-5 hr; the slow phase has a half-time of 100-200 hr. About 30 hr after labeling and at later times, approximately 30% of the cell-associated radioactivity is susceptible to removal by external neuraminidase, suggesting an exchange with an internal pool that is twice the size of the surface pool. An internal pool of relatively high specific activity compared to the surface was generated by labeling as above, followed by a period of time to allow internalization and enzymatic removal of external

neuraminidase-sensitive radioactivity. During subsequent reincubation in growth medium, the surface became relabeled from the internal pool, again reaching a 30% neuraminidase-sensitive plateau. The relabeling of the surface was confirmed by radioactivity measurements on isolated plasma membranes. [^{3}H]AcNeu^7 cannot be reutilized by these cells in the de novo membrane biosynthetic pathway. The argument is made that the labeled sialoglycoconjugates are recycling intact through the internal pool.

When cells are allowed to internalize a significant fraction of surface label and the remaining surface label is removed with neuraminidase, the electrophoretic pattern of internalized labeled protein is similar to that of proteins on the surface, suggesting that internalization is relatively nonselective. On the other hand, when the surface is relabeled by recycling of internalized protein, a new surface pattern is observed suggesting that to some extent recycling is heterogeneous.

Following the partial separation of galactosyl-transferase-rich microsomes (Golgi marker) from β-hexosaminidase-rich particulates (lysosomal marker) in cell homogenates, we have preliminary evidence that a substantial fraction of internalized surface-labeled glycoproteins first move into the Golgi compartment and from there move into the lysosomal compartment. These pathways have not been completely worked out.

Regulation of Membrane Transport Proteins by Turnover. Many cases are now known in which, when eukaryotic cells are starved for substrates that are taken up by specific transporters, the cells respond by an adaptive increase in transport activity at the surface. This is almost invariably seen as an increase in transport V_{max} and is taken to represent an increase in the number of specific functional transporters. Such responses have been observed in hexose, nucleoside, neutral amino acid, alkali cation, and insulin deprivation. In principle, a change in the steady-state concentration of transporters could result either from a corresponding change in the rate of synthesis or an inverse change in the rate of turnover and degradation of the translocase.

We have previously shown that HeLa cells starved for K^+ in the medium respond with a doubling in the surface density of the Na,K-ATPase as titrated with the specific ligand [^{3}H]ouabain. The increase, and its reversal on restoration of normal K^+ levels to the medium, are due entirely to changes in turnover rate with no alteration in synthesis rate. A similar mode of regulation has been described by Lane and his associates (Johns Hopkins University) for the up- and down-modulation of insulin receptors in fat cells deprived of or surfeited with insulin.

In collaboration with T. L. Hayden (University of Kentucky) we have derived a computer model of this regulation. The formulation is comprised of simultaneous differential equations describing: (1) the rate of transport of substrate S as a function of the concentration [S] in the medium; (2a) the cellular concentration of product P as it depends on and is proportional to the transport rate of S as well as (2b) the metabolic loss of P; (3a) the proportionality between [P] and the rate of turnover of the transporter or, alternatively, (3b) the proportionality between [P] and

the rate of synthesis of the transporter; (4) the concentration of transporter as a function of synthesis and turnover, with allowance for (5) a transit time between onset of synthesis and the insertion of functional transporter in the cell surface.

Using known values for the Na,K-ATPase, excepting an assumed value for the metabolic flux of P, we can satisfactorily describe earlier data for the up- and down-regulation of the enzyme on the regulation-by-turnover model. Because of the transit time, with the same data it can be shown that regulation-by-synthesis leads to delayed responses and undamaged oscillations, i.e., no control at all. We suggest that regulation-by-turnover is physiologically advantageous for membrane proteins with significant transit times and may ultimately be found to be a common mechanism for other transporters as well.

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THEORETICAL AND APPLIED CRYOBIOLOGY

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The research of the Theoretical and Applied Cryobiology Group is devoted to low temperature biology, to the permeability and osmotic responses of cells, and to the effects of temperature and the state of the aqueous environment on the cell surface. The successful freezing of complex biological systems requires an understanding of the fundamentals of low temperature injury. However, an attractive feature of cryobiology is that a given experiment often yields information of both basic interest and practical utility. With this in mind, we are pursuing fundamental cryobiological studies, using for the most part cells that are themselves of practical interest in biology and medicine, namely, mouse and bovine embryos, mammalian erythrocytes, human granulocytes, and mouse skin.

Cells that can be successfully frozen to liquid nitrogen temperatures (-196°C) and thawed can be maintained for decades at -196°C with almost no loss in viability and essentially no physiological or genetic alterations. This ability of low temperature storage to stop biological time makes it useful in assessments of the health effects of the production and use of energy. For example, mutant strains of mice can be stored as early embryos at -196°C until needed. Multiple samples of skin from a given individual can be preserved at low temperatures, thus greatly reducing the varability introduced when skin from various individuals is used with athymic nude mice to assess various toxic compounds, mutagens, and carcinogens. Both these examples are currently under study in the Division, with consultation from our group.

There are other energy-related aspects of cryobiology. It would be desirable to minimize the considerable energy required to produce food, as for example by developing more efficient foraging livestock. In this regard, embryo freezing is playing a growing role in accelerating the breeding of select cattle. The use of fossil energy may also alter climate - hence, it would be desirable to preserve a wide variety of germ plasm to meet altered requirements, and it would be desirable to determine the basis of draught and freezing resistance in crop plants.

Current Research

Mechanisms of Slow Freezing Injury. As aqueous solutions freeze, the electrolytes in the external solution progressively concentrate, and, if cooling is slow, the cells undergo progressive osmotic dehydration. It has been generally thought that slow freezing injury is the result of either excessive salt concentration or excessive cell shrinkage. But, it is also conceivable that cell survival is affected by the size of the unfrozen channels in the solution or, more precisely, by the fraction of solution that remains unfrozen at any temperature. The phase rule dictates that solute concentrations and fraction unfrozen are reciprocally related and ordinarily inseparable. One can separate them, however, by suspending cells in ternary solutions of nonelectrolyte, electrolyte, and water which

differ in total solute concentration but maintain a fixed ratio of nonelectrolyte to electrolyte. We have performed these experiments with human red cells in solutions of glycerol-NaCl-water. Samples were frozen to various subzero temperatures which were chosen to produce various molalities of NaCl (0.24 to 3.30) while holding the fraction unfrozen constant, or conversely to produce various fractions unfrozen (0.03 to 0.5) while holding the molality of salt constant. (Not all combinations of these values were possible.) To our surprise, survival was far more dependent on the fraction in the solution remaining unfrozen than it was on the concentration of NaCl in that unfrozen fraction. Damage became extensive when more than 90% of the solution became frozen regardless of whether the salt concentrations were as low as 1 molal or as high as 2.4 molal. The results were essentially the same for cooling rates of 0.6 or 1.8°C/min, warming rates of 20 or 300°C/min, and cell suspension hematocrits ranging from 0.2% to 60%. These findings will require major modifications in how cryobiologists view slow-freezing injury and its prevention. They may be especially pertinent to the freezing of the whole organs.

Human Granulocytes. The transfusion of human granulocytes could be useful clinically if the storage stability (>48 hr at 4°C) of the cells could be improved. Freezing would lengthen the storage time indefinitely, but unfortunately claims of the successful freezing of these cells have not proved reproducible. We have found that the inability of human granulocytes to retain high functional viability after freezing appears not to be due to improper cooling and warming rates. Few cells survive contact with 2 M glycerol and subsequent dilution even in the absence of freezing. This detrimental effect of glycerol is seen only if cells are incubated at 37°C for about 60 min prior to the survival assay (chemotaxis or fluoroscein diacetate FDA). In contrast, nearly 100% of cells in phosphate buffered saline or Hanks' solution survive the 37°C incubation - provided that 0.1 to 0.6% autologous plasma is present. Interposing the 37°C incubation between the experimental treatment and the fluorescence measurements thus makes the FDA procedure a highly sensitive measure of granulocyte injury.

The damage from glycerol appears to be a true toxicity and not a consequence of osmotic shock during the removal of the glycerol. Determinations of the permeability coefficient of the cell to glycerol permitted us to design protocols for the stepwise addition and removal of glycerol which minimized cell shrinkage and swelling, respectively. Even with these precautions, exposure to 0.5 to 2.0 M glycerol was still damaging. Damage was lessened only when the total exposure to glycerol was held to 3 min at 0°C.

Studies with hyperosomotic solutions of the non-permeating solutes, sucrose and NaCl, showed that the cells are also damaged by osmotic shrinkage. The maximum osmolality tolerated was about 0.5. However, the tolerated osmolality of sucrose or NaCl solution increased as the degree of dilution was decreased. Thus, nearly all of the cells survived exposure to 1.4 osmol/kg solution when the subsequent dilution was to 0.7 osmol/kg. Volume data from directly observed cells showed that after dilution the cells returned to volumes predicted by a Boyle-van't Hoff plot. Thus, the damage observed in the hyperosmotic media does not appear to be explicable in terms of permeation of sucrose or NaCl into shrunken cells.

Permeability of Bovine Embryos to Dimethyl Sulphoxide (DMSO). Knowledge of the permeability coefficients of cells to protective additives provides a useful tool for minimizng the osmotic stresses involved in freezing cells. We have determined the permeability coefficients of bovine morulae and blastocysts to DMSO by observing the shrinkage and reswelling of the embryos when they are placed in hyperosmotic solutions of DMSO. The embryos were photographed at frequent intervals during the exposure. The cell perimeters were then digitized from their photographic images, and cell volumes calculated. Computerized numerical solutions to the differential equations describing permeation permitted us to select the value of the permeability coefficient (P_s) that provided the best fit to the data. The values of P_s range from 3×10^{-5} cm min^{-1} at 0°C to 2×10^{-3} at 35°C.

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Protein Structure and Enzyme Mechanisms

X-RAY DIFFRACTION

J. R. Einstein C. H. Wei

We have been interested in structural elucidation by X-ray diffraction of compounds of biological interest. Understanding exactly how atoms are arranged in three-dimensional arrays as molecules can help explain the relationship between structure and functions. The species investigated may vary in size and shape; our recent studies included such diverse substances as antischistosomal drugs, a complex of cadmium with nucleic acid base, nitrate salts of adenine, and proteins.

The behavior of hycanthone and its analogous antischistosomal drugs in biological systems has previously been ascribed to intercalation of these drugs with DNA. It has been considered essential for the thioxanthene ring system to be planar so as to provide a strong hydrophobic interaction with adjacent DNA base pairs. Furthermore, the terminal nitrogen atom of the side chain, diethylaminoethyl group, has been thought to interact with phosphate residues on the periphery of the DNA helix, thereby stabilizing the drug-DNA complex. Results of our structural investigations of three hycanthone analogs have indeed revealed that in each case the heterocyclic ring system of the molecule forms a planar configuration, with the long side chain taking up different conformations according to packing rules. The nitrogen atom of the side chain was found to invariably form various hydrogen bonds with neighboring ligands, thus indicating a strong possibility that this atom may be involved in interaction with phosphate groups of DNA. Although known to possess similar antischistosomal functions, two recently discovered drugs, amoscanate and oltipraz, have different molecular formulas from those of hycanthone analogs, and their molecular architectures were also elucidated unambiguously.

It is well recognized that interactions of certain metal ions with nucleic acids are essential to a variety of biological processes in animals and plants. In particular, cadmium, a known environmental pollutant, has been observed to be one of the most potent in diminishing the fidelity of DNA synthesis _in vitro_. The binding sites of cadmium to bases together with the hydrogen bonding scheme involved would be a first step in contributing to the knowledge of its molecular mechanism. When cadmium nitrate was reacted with 5'-AMP, three distinctly different crystal specimens were obtained. Only one of them contained cadmium, and was subsequently characterized by X-ray analysis to be the first known crystal structure of purine base coordinated to cadmium. Each of the remaining two specimens (with no cadmium) was later individually identified as a nitrate salt of adenine, only differing from each other in molecular composition and resulting hydrogen bonding network.

In the past, we have isolated and purified from soybean seeds five different trypsin inhibitors of which two species have been crystallized and reported. Our continued efforts have now resulted in successful

crystallization for the third species, which appears to be soybean trypsin inhibitor E-I (a member of the Bowman-Birk class of inhibitors; sequence known). In this case, a pronounced polymorphism in the crystals has been observed. The simultaneous presence of two cubic crystalline forms in the same sample of mother liquor is not unprecedented, as has been reported by other investigators for macromolecular samples.

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PROTEIN LUMINESCENCE: THE END OF THE BEGINNING

J. W. Longworth

My last 23 years have been devoted to the study of luminescence of proteins and include long periods of investigation of luminescence of nucleic acids. The conclusion from these years of work upon protein luminescence is the great diversity of behavior that is found in proteins, and this arises as a result of sidechain interactions created by the folding of the polypeptide chain. It would be of interest if an example of specific photophysical effect could be detected which arose as a consequence of a specific interaction, and as potential hydrogen bonds are rarely left unsatisfied in protein structures, interactions engaging the phenolic hydroxyl exist widely. The entire armory of my contributions to this topic of spectroscopy can be found in this study of tyrosine fluorescence of human serum albumin. This protein as yet has no established molecular structure but amino acid and gene sequences show that it is a result of a triplication of a double duplication block, where the fourth sequence block is extensively deleted and is a relic: that is, there are nine pronounced

homologous sequence blocks. One can expect the structure to be composed of three homologous folding domains and each domain to possess a three-fold repeat. Within this protein lie 18 tyrosines and 1 tryptophan.

Tryptophan absorbs ultraviolet radiation at longer wavelengths than does tyrosine, and thus, there is a spectral interval on the long wavelength edge of protein absorption where tryptophan alone can be selectively excited. Tryptophan interacts strongly with polar groups in its environment, and as a result it fluoresces to much longer wavelengths than tyrosine. Tyrosine also interacts with polar groups, which shift its absorption and fluorescence, such that there is a compensation and the fluorescence appears at a constant location. When both tyrosine and tryptophan are excited, the long wavelength spectral region of the fluorescence emission is overwhelmingly from tryptophan. These features, although complex, offer a simple means to isolate tyrosine fluorescence in the presence of tryptophan.

Fluorescence emission spectra are collected with a single photon counting spectrometer which sequentially steps through an emission spectrum. This act creates a file within a minicomputer, which can then be numerically manipulated. First a spectrum is collected where tryptophan is selectively excited, and a second where both tryptophan and tyrosine are excited. Emission intensities beyond a value where tyrosine contributes less than 1% are used to equalize the two spectra, and the resultant difference comprises the tyrosine emission.

Emission from tyrosyl residues of HSA is noticeably different than from free tyrosine. Suitable removal of the tyrosine fluorescence by comparing the short wavelength regions of the two emission spectra leave a spectrum with a peak at longer wavelength. The excitation spectrum of the long wavelength tyrosine component is identical with the excitation spectrum of the short wavelength component. This long wavelength component resembles the emission of ionized tyrosine, tyrosinate. Ultraviolet absorption studies 30 years ago disclosed that one third of HSA tyrosines absorb at longer wavelength than the majority and were believed to be involved in a complex with carboxylate groups.

The excited singlet state of tyrosine is a much stronger acid than the ground state, and when complexed with carboxylate in model complexes will emit as tyrosinate.

By dissolving HSA in an equivolume mixture of ethylene glycol and water, polar glasses form upon cooling to 77K. In such a glass oxygen is unable to diffuse and triplet states are now no longer quenched. Both fluorescence and phosphorescence can now be measured, and the phosphorescence separated due to its long lifetime with mechanical choppers. The tryptophan contribution can be removed through comparing spectra excited at the two excitation wavelengths and the luminescence of tyrosine from HSA is disclosed. Again, there is indication of tyrosinate fluorescence at 77K — is this a consequence of atom tunnelling? The phosphorescence is entirely from tyrosine. The triplet state of tyrosine is only a slightly stronger acid than the ground state and thus insufficiently acidic to protonate

carboxylates: upon intersystem crossing, the proton is recaptured by tyrosinate to create tyrosine! The singlet-to-triplet ratio is vastly different for HSA compared to tyrosine, where the protein has a much reduced relative triplet yield. Similar reduction in triplet yield is found for tyrosine interacting with disulphide linkages of HSA, and tyrosine is known to preferentially interact with disulphide links in proteins with known molecular structures.

The folding pattern of a polypeptide chain creates interactions which profoundly affect the excited states of tyrosine. The absorption of ultraviolet electromagnetic radiation creates excited states where the singlet ejects a proton, which is recaptured upon formation of a triplet and can then eject an electron. These chemical processes provide mechanisms to dissipate the electronic excitation energy, but form transient chemical species which ultimately reform the parent molecule. Thus, from spectroscopic measurements a view into the unexpected and extensive chemistry which accompanies absorption of radiation is provided.

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PROTEIN CHEMISTRY AND ENZYME MECHANISMS

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Claude D. Stringer

Since virtually all biological processes are enzyme mediated, studies of enzyme structure and function are clearly central to a broad-based program concerning health effects of environmental agents. Our efforts are focused on active-site characterization which bears directly on the elucidation of the principles underlying two key properties of enzymes: their stringent specificity and their enormous catalytic efficiency. The most versatile approach for the selective introduction of a chemical label into the active site, thereby providing structure/function correlations and identifications of active-site residues, is affinity labeling. In its traditional form, affinity labeling entails the use of reactive analogs of natural substrates to label substrate binding sites. Conceptually, it can

be viewed as combining features of competitive inhibitors and general protein reagents into a single molecule. The substrate-like features of the reagent direct it to the active site in a fashion completely analogous to the binding of competitive inhibitors. This binding step results in a highly localized concentration of reagent within the substrate binding site and thus increases the likelihood of modification of a residue within this site as compared with other positions of the protein molecule.

Many of our recent investigations have centered on ribulosebisphosphate carboxylase, the plant enzyme essential for the photosynthetic assimilation of CO_2. This enzyme also possesses inherent oxygenase activity which accounts for photorespiration, a nonessential, energy-wasteful process that reduces net CO_2 fixation. Thus, an understanding of the *in vivo* modulation of the carboxylase/oxygenase ratio and a determination of whether this ratio can be systematically manipulated by external means are of major significance to agriculture and production of biomass.

We have designed several affinity labels for the carboxylase all of which bear a structural relationship to ribulosebisphosphate. These reagents have permitted the identification of Lys-175 and Lys-334 as residues within the binding domain for substrate. Concurrent comparative sequence analyses of the carboxylases from spinach and *Rhodospirillum rubrum* revealed high degrees of homologies around both lysyl residues. These findings of species invariance in primary structures suggest that the lysyl residues subject to affinity labeling are functional in catalysis.

Another major effort involves the design of reactive analogs of α-ketoglutarate as potential active-site probes for the numerous enzymes that process this key metabolite. One such compound, (RS)-3-bromo-2-ketoglutarate, is a substrate, inactivator, and ligand for affinity chromatography of $NADP^+$-dependent isocitrate dehydrogenase. In collaboration with Dr. Roberta Colman (University of Delaware), we have shown that bromoketoglutarate is also an affinity label for NAD-dependent isocitrate dehydrogenase. Inactivation appears to correlate with the selective alkylation of an active-site sulfhydryl group.

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Cellular and Comparative Mutagenesis Section

SECTION OVERVIEW - J. L. EPLER

The Cellular and Comparative Mutagenesis Section contains a diversity of research but has the common goal of providing information relevant to the estimation of the genetic hazards of radiation and chemicals to man, especially as these biohazards are encountered in energy-related processes. Our strategy is to expand our understanding of the mechanisms of mutagenesis and to develop and validate a variety of test systems for assay of potentially hazardous agents.

The Section is divided into four research units, which have considerable overlap as well as interactions with other groups within the Biology Division and with other divisions of the Oak Ridge National Laboratory. These research units are labeled Comparative Mutagenesis, Mammalian Cellular and Molecular Studies, Genetics of Microorganisms and Drosophila, and Development and Reproductive Physiology. In each of these units there are research projects designed to provide a better understanding of the mechanisms of mutagenesis and also the utility of assays developed here or elsewhere. If these studies are to provide information to aid in the protection of the occupationally and generally exposed public from genetic hazards and to make possible the prediction of (or the reversal of) such damage, more must be learned about the way in which genes act and are expressed. Thus, continuing and parallel research yielding knowledge of genetics and reproduction in a variety of organisms, including man, must accompany or precede the development of test systems.

There are also inter- and intradivisional programs and interactions that involve facets of the Cellular and Comparative Mutagenesis Section, as examples, the recently instigated Biotechnology Program and the health effects research project within the Life Sciences Program in synthetic fuels. Furthermore, the Section contributes to graduate training in genetics within the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences.

In the area of genetic toxicology the main effort is the development and validation of assays for mutagenicity and teratogenicity. When coupled to the efforts in the other Sections, the array of bioassays represents a rather complete coverage of testing procedures in genetic toxicology.

The assays encompass bacteria (DNA repair and mutagenesis in Salmonella, *E. coli*, and Haemophilus), fungal systems (mutagenesis in yeast and Neurospora), Drosophila (sex-linked recessive lethal tests, nondisjunction, chromosome damage, and sister chromatid exchanges), amphibia (teratogenesis and embryo toxicity), mammalian cells *in vitro* (mutagenesis, chromosome aberrations, DNA repair, and sister chromatid exchanges), and mammalian cells *in vivo* (chromosome aberrations, mutagenesis, and sister

chromatid exchanges). The individual research summaries provide details of these various research programs. The point to be emphasized here is that information from short-term and longer-term assays using a wide variety of different systems provides a basis for extrapolation to potential effects in man.

Several aspects of the program are involved rather directly with extrapolations to man. The most direct approaches are naturally those involving studies on mammalian systems, such as, in vivo/in vitro cytogenetics or mutations assayed in mammalian hemoglobins. Other less direct approaches involve comparisons between Drosophila and mouse including those obtained from inhalation studies and from the mutagenic effects of diesel fuels. Since it is possible that mutagenicity is predictive of carcinogenicity (a correlation being studied by a number of investigators within the Section and in other institutions), it is hoped that mutagenicity assays can be used to predict the potential carcinogenicity of a compound to man. Finally, the Development and Reproductive Physiology Unit is involved in a variety of studies concerned with the teratogenicity and embryo toxicity of pollutants in amphibian systems. Validation of these end points as compared to mammalian systems could lead to a useful test system for the prediction of similar effects in man.

In order to better interpret the data from these various assays and to extrapolate to effects in man, it is essential that the underlying mechanisms of mutagenesis, carcinogenesis, teratogenesis, and clastogenesis be determined. It is clear that DNA repair is involved in the process of mutation induction, and a comprehensive study of the enzymology of repair is being carried out. Under this category is included the collaborative intersectional efforts in the area of photobiology. Detailed studies of recombination and gene expression in Drosophila provide basic information towards understanding mutation induction.

Other mechanistic approaches involve the study of metabolic activation, whereby nonmutagenic compounds are metabolized into mutagenic forms, and the study of the relationship between the structure and mutagenic potential of related compounds, e.g., nitrosamines, polycyclic aromatic hydrocarbons, and aromatic amines.

An end-product of mutagenesis research is information from which regulatory guidelines can be established. Several projects directly impinge upon regulatory guidelines, including an interdivisional project concerning the applicability of short-term tests and chemistry to the Resource Conservation and Recovery Act. In addition to contributing to the data base useful to the Environmental Protection Agency under the Toxic Substances Control Act, the Section has coordinated the "GENE-TOX" activity for the Office of Toxic Substances. The Section has also cooperated with EPA in research on potential genetic effects of diesel fuel emissions. Much of the developmental work on the use of short-term assays in the evaluation of health effects of coal conversion and shale oil technologies has been carried out within the Division under Ingeragency Agreements between the EPA and DOE. These contributions stand not only as examples of how our long experience in fundamental research can be applied to the

problems of modern technology but also as examples of cooperation between teams of investigators in various divisions of ORNL.

The following summaries will indicate how individual research projects fit into the above statement of purpose and how the information obtained might be used to estimate genetic effects on man from environmental agents.

Comparative Mutagenesis

COMPARATIVE MUTAGENESIS

J. L. Epler
F. W. Larimer
C. E. Nix
T. K. Rao
L. C. Waters
K. B. Allen
J. T. Cox
L. Dry
N. L. Forbes
A. A. Hardigree
L. Oggs
S. I. Simms
R. D. Wilkerson
W. Winton

The Use of Short-term Tests in the Isolation and Identification of Chemical Mutagens in Complex Mixtures. The feasibility of using short-term mutagenicity assays to isolate and identify the potential biohazard(s) of complex materials is being examined by use of various coupled chemical and biological approaches. Such research has usually involved a preliminary chemical characterization and preparation for bioassay, followed by testing for bioactivity (generally the mutagenicity test for Salmonella histidine reversion described by Ames). Subsequent fractionation procedures to further characterize the mutagens present are carried out, with the bioassay being used as a tool to follow the activity and guide the separations. The mutagenicity tests are intended to function as (i) predictors of profound long-range health effects such as mutagenesis and/or carcinogenesis; (ii) a mechanism to rapidly isolate and identify a hazardous biological agent in a complex mixture; and (iii) a measure of biological activity, correlating baseline data with changes in experimental (or environmental) conditions and, in the case of actual industrial effluents or streams, with changes in process conditions. With this combined chemical fractionation and short-term assay approach, information is being accumulated on the actual compounds responsible for the biological effect. Thus, the mutagenicity tests will also function as (iv) an aid in identifying the specific hazardous compounds involved and in establishing priorities for more definitive chemical analysis and monitoring along with further validative testing in comparative systems, including whole-animal testing, for mutagenesis and carcinogenesis.

Although our work has emphasized evaluation of test materials from the developing synthetic fuel technologies, the procedures are generally applicable to a wide variety of industrial and natural products, environmental effluents, and body fluids.

Short-term tests with bacterial and fungal mutagenicity assays appear to detect effectively the mutagenic potential of complex environmental or

industrial effluents; however, chemical fractionation is necessary to reduce toxicity and concentrate hazardous materials. Extension of the results to higher organisms, i.e., mammalian cells, Drosophila, and the mouse, appears to be valid but needs more research. Each individual system has its own strengths and weaknesses. Knowledge of basic mechanisms and understanding of the systems will improve the practical application of the assays.

Yeast Mutagenesis. In the yeast program research is conducted in three general categories: (a) a base program aimed at a fundamental understanding of the molecular nature of mutagenesis, necessary for interpretation, extrapolation, and development of the health effects program; (b) the Coal Liquids program, aimed at development of a general understanding of the nature of the mutagenic potential of synfuel materials; (c) support of health effects programs in specific synfuel processes under development.

a. Base program. Recent work has centered around the use of several well-defined frameshift mutations in studies of mutagenic mechanisms and the involvement of DNA repair processes in mutagenesis. Frameshifts in regions of GC base pair runs have been characterized as to their reversion behavior when exposed to a variety of intercalating agents or ultraviolet irradiation. Indirect evidence indicates that the agents examined to date predominantly induce -1 deletions. Chemically induced frameshift events are dependent upon the REV3 repair pathway; potential lesions appear to be repaired both with and without fidelity by this repair pathway.

The direct-acting nitrosamides methylnitrosourea (MNU) and ethylnitrosourea (ENU) were chosen as archetypes for small alkyl-lesion mutagenesis mechanism studies. Although ENU and MNU mutagenesis require RAD6/REV3 repair functions, allele-specificity studies indicate that a direct untargeted mechanism is not involved. Preliminary evidence suggests that yeast does not exhibit the "adaptive response" to small alkyl lesions. Work is in progress to characterize the repair of alkylated bases.

Two repair genes from yeast have been isolated by recombinant DNA techniques and have been expressed in bacteria. The RAD2 excision repair gene and the REV3 error-prone (mutagenic) repair gene were isolated by complementation of appropriate yeast mutant hosts transformed with hybrid plasmids bearing wild-type yeast DNA inserts. The RAD2 gene is expressed in E. coli maxi-cells and the gene product has been identified as a protein of ca. 30,000 dalton molecular weight. Characterization of the enzymatic function of the RAD2 protein is in progress. REV3 has been localized to a coding sequence of greater than 4.6 kilobasepairs. Transformants carrying REV3 display full mutability; dosage studies are underway, as are efforts to identify the REV3 gene product.

Three yeast genes used as target loci in mutagenesis assays, ADE2, ARO1, and HIS4, have been isolated, and new mutant alleles are being constructed in vitro. These new alleles will provide alternative defined test sequences for those markers now in use, and permit rapid recovery and sequencing of induced mutations.

b. Coal liquids. Fractionation studies of a variety of coal liquids have indicated that the ether-soluble base fractions, notably the primary amine-PAHs, exhibit great mutagenic activity. These materials are direct-acting mutagens, in contrast to their neutral fraction PAH analogs. The neutral PAHs are effective mutagens, but require metabolic activation. Examination of coal liquids that have been upgraded by hydrotreatment indicates that the mutagenic activity of both the base and neutral fractions is reduced. The bulk of the mutagenic activity in the base fractions is mitigated by relatively low levels of hydrogenation.

c. Specific process assessment. The major efforts in support of health effects research in specific process development have been directed principally, although not exclusively, to three processes: the University of Minnesota-Duluth low-BTU coal gasification project; the Paraho-SOHIO Shale Oil project; the H-Coal Direct Liquification PDU evaluation and current H-Coal pilot plant process samples. Results obtained from these three processes support the same conclusions: tars and other high-boiling materials enriched for PAHs and N-PAHs possess significant mutagenic activity; the mutagenic activity seen is notably greater than that observed for natural crude oils. These results corroborate similar findings using the Salmonella/microsome assay (reported elsewhere).

Chemical Mutagenesis in Drosophila melanogaster. It is clear that the toxic, carcinogenic, and mutagenic properties of chemicals present in the environment can have a profound biological impact. Evaluation of such biological hazards and risks is presently imprecise and based on a relatively small, though expanding, data base. It is also evident that populations, including man, exposed to potential environmental hazards are heterogeneous. Thus it becomes important to better understand the genetic and biochemical diversity involved in mutagenic processes.

Our goal is to better understand the mechanisms of mutagenesis in Drosophila. This serves at least three purposes: (1) the improvement of Drosophila as a tool in mutagenicity testing, (2) the addition to our knowledge of how mutations are produced in higher eucaryotes and (3) the improvement of our ability to predict potential risk for other organisms.

We have approached this problem by using a combination of genetic and biochemical techniques. A number of simple model compounds have been tested for mutagenic response in several wild-type and DNA repair deficient strains. Enzyme assays have been developed for several of these compounds and we have investigated the correlation between mutagenic and biochemical activity. In addition we have begun experiments to try to understand the genetic and biochemical basis for strain differences in the response to simple nitrosamines.

Five wild-type strains (Hikone-R, Oregon-R, Canton-S, Berlin-K, and Samarkand) have been extensively tested for the induction of X-linked recessive lethals by five simple alkylating agents (dimethylnitrosamine, DMN; diethylnitrosamine, DEN; methylethylnitrosamine, MEN, methylnitrosurea, MNU; and ethylnitrosurea, ENU).

Compounds that require no metabolic activation, MNU and ENU, were equally mutagenic in all strains over at least a 20-fold concentration range. On the other hand, strain as well as concentration dependent effects were detected when compounds requiring metabolic activation (DMN, DEN, and MEN) were used. In these experiments only 1- to 2-day-old males were treated with the mutagen. Since other workers have reported strain differences in mutagenic response when larvae are treated with 7,12-dimethylbenzanthracene, we plan to extend these studies to larvae. This approach is further encouraged by biochemical differences in DMN demethylase activity observed between larval and adult stages, e.g., qualitative and quantitative differences in cytochrome P-450 and mixed-function oxidases.

Information on the effects of DNA repair deficiencies on mutagenicity in Drosophila is sparse. We have attempted to address this problem by utilizing several repair deficient mutants in the X-linked recessive lethal assay. Three types of mutants were used: (1) those with a defect in some step in excision repair, mei-9; (2) those defective in postreplication repair, mei-41; and (3) various combinations of double mutants. Each was tested with seven simple alkylating agents (DMN, DEN, MEN, MNU, ENU, EMS, MMS) over at least a 10-fold concentration range. Thus far, two interesting results have been observed. Males containing the mutant, mei-9 and treated with the methylating agents DMN, MNV, and MMS produce 2-3× less mutations than wild-type males. Since two of the alkylating agents, MNU and MMS, require no metabolic activation, the observed effect is probably related to the repair defect. With the corresponding ethylating agents, DEN, ENU, and EMS, there was no significant difference between genotypes. When males were mutant at the mei-41 locus there was no observed difference with any of the alkylating agents, but double mutants, mei-9 mei-41, behaved like mei-9 alone. Several alleles at the mei-9 locus were tested and, though there were differences, all were less mutagenic than wild-type with methylating agents.

Another interesting observation, although not surprising, was the completely different mutagenic response when wild-type treated males were mated to repair deficient females (mei-9). Though these experiments are not complete, it appears that the methylating agents MMS and DMN are more mutagenic when mei-9 females are used. The effect is most pronounced in mei-9^{L1} females. Only one ethylating agent, EMS, has thus far been tested, but in both mei-9^{a} and mei-9^{L1} there is no significant increase over the spontaneous level.

Our immediate plans are to continue the mutagenicity studies and, in addition, to initiate experiments concerning the possible specific biochemical defect in the mei-9 mutants.

Mechanisms of Mutagenesis: Metabolic Studies. We are continuing to exploit the Drosophila system in an effort to learn more about the basic biochemical mechanisms involved in chemical mutagenesis. Drosophila represents a useful alternative to mammalian systems for such studies. Some advantages of the Drosophila system include: (1) Drosophila is mutated by a wide variety of chemicals, (2) metabolic activation and mutation occur

within the same organism and within a time period sufficiently short to allow meaningful correlations between the two events to be made, (3) Drosophila cell-free extracts are as amenable to in vitro biochemical analysis as those from mammalian sources and (4) genetic variation in mutability exists in Drosophila which can be further manipulated and exploited for study.

A significant portion of the period covered by this report was used to develop qualitatively and quantitatively reproducible methods to extract the mixed-function oxidase (MFO) system from larvae and adult flies. On the basis of cytochrome P-450 content, we can now prepare microsomes from whole adults with fully one-half the specific activity observed in rat liver microsomes (e.g., 0.3-0.4 nmoles/mg microsomal protein). Activities of a number of MFO's, e.g., dimethylnitrosamine, benzphetamine p-nitroanisole demethylase; aniline and benzo[a]pyrene hydroxylases, are comparable to those in rat liver microsomes. Critical modifications of earlier procedures that resulted in improved extractions include: (1) the use of fresh material-freezing results in fragmented mitochondria that are difficult to resolve from microsomes by differential centrifugation, (2) the use of carefully sized teflon-glass tissue grinders to prevent excessive disruption of organelles and (3) the use of multiple centrifugations to remove the mitochondrial fragments that are produced.

From studies of mammalian systems, there is considerable controversy as to which metabolic pathway(s) is responsible for generating the ultimate mutagenic (or carcinogenic) form of dimethylnitrosamine (DMN). Because DMN is highly mutagenic to Drosophila, we are attempting to use this system to resolve this controversy. Toward this goal we have characterized a DMN-demethylase activity in whole-body extracts of both larval and adult forms of the Hikone-R strain of Drosophila. A microsomal enzyme, it has many properties of a cytochrome P-450-containing MFO. Current evidence from kinetic studies suggests that it is a single enzyme with a K_m for DMN of 10 mM. The content of this enzyme in a number of other Drosophila strains is less than 10% that observed in the Hikone-R strain. However mutability of most of these strains of DMN is equal to that observed in Hikone-R. Clearly a positive correlation between the level of this enzyme in whole-body extracts and DMN-induced mutagenesis among these strains does not exist. A number of possibilities to explain this lack of correlation were tested and ruled out. They include: (1) the presence of inhibitors of DMN-demethylase activity in extracts of the low-activity strains, (2) a sex bias in the Hikone-R strain in which the enzyme activity is confined to the females, (3) the possibility that DMN feeding induces DMN-demethylase activity in the low activity strains and (4) the possibility that Hikone-R has a more efficient DNA repair system than the other strains.

We recently completed a series of experiments designed to compare DMN metabolism in vivo to that observed in vitro. ^{14}C DMN was fed to males and metabolism was indicated by conversion of the radioactivity to an acid precipitable form. Uptake of DMN was proportional to the amount fed up to at least 1 mM and was nearly equal in Hikone-R and Oregon-R (a low DMN-demethylase activity strain). Metabolism was 1.5- to 2-fold higher in Hikone-R than in Oregon-R. Although the correlation of in vivo metabolism

of DMN with DMN-induced mutations (these are practically equal in Hikone-R and Oregon-R) is not precise, it clearly is better than the correlation with DMN-demethylase activity in which at least a 10-fold difference exists. These results suggest that the DMN-demethylase, characterized with a K_m for DMN of 10 mM, is only moderately, if at all, active in the major xenobiotic organs of Drosophila. Subcellular fractionation of extracts from flies labeled with DMN showed that the highest specific incorporation, i.e., radioactivity per mg protein, is in the microsomes, suggesting that the metabolism is via the MFO system. Pyrazole and phenobarbital, compounds known to affect DMN-demethylase levels in mammals, did not significantly alter the metabolism of DMN in vivo.

We are currently considering three possibilities to explain the data: (1) the α-hydroxylation pathway of DMN metabolism, catalyzed by DMN-demethylase, is not involved in DMN mutagenesis; (2) the DMN-demethylase activity that we have characterized is involved in DMN activation with the activity in the gonads of the various strains being nearly equal; (3) there is another DMN-demethylase activity in Drosophila, not readily detected at the level of sensitivity of our assay, with a K_m for DMN that is significantly lower than 10 mM, and the level of which is nearly the same among the various strains. Experiments designed to evaluate these possibilities are in progress.

The differential expression of DMN-demethylase activity among various strains of Drosophila provides an excellent system to study the genetic regulation of the MFO system. By systematically putting Hikone-R chromosomes into demethylase-deficient strains, we find the enzyme to be associated with chromosome II. The locus for DDT resistance, which Hikone-R has, is also on chromosome II (2-66.0). Whether the DMN-demethylase activity and resistance to DDT are closely linked is being investigated by screening other DDT-resistant strains. Whether expression of DMN-demethylase in Hikone-R is a result of a structural gene mutation or a regulatory gene mutation is being investigated. The fact that a number of MFO's are expressed to higher levels in Hikone-R than in other strains might indicate a mutation in a regulatory gene that controls a number of structural genes.

Overall we feel that during the period of this report we have demonstrated the utility of the Drosophila system for investigating the effects of genetic diversity on mutation induction and the biochemical mechanisms of mutagen metabolism.

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Mammalian Cellular and Molecular Studies

MAMMALIAN CELL GENETIC TOXICOLOGY

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Our goal is to study the mutagenic effects of energy-related chemicals and to estimate the risk to humans from exposure to these agents. Mammalian cells in vitro and in vivo have been used in this study, which has four main objectives.

1. The Chinese hamster ovary cell/hypoxanthine guanine phosphoribosyl transferase (CHO/HGPRT) assay has been used to determine cytotoxicity and mutagenicity of environmental agents, to validate the assay for predicting carcinogenicity, and to study mechanisms of chemical mutagenesis. Our studies showed that this assay permits quantification of gene mutation and cytotoxicity induced by various promutagens and direct-acting mutagens. The quantitative nature of the system provides a basis for the study of structure-mutagenicity relationships of over 80 chemicals including alkanesulfonates, alkylsulfonates, nitrosoguanidines, nitrosoureas, heterocyclic mustards (ICR compounds), platinum (II)chloroammines, quinolines, nitrosamines, haloethanes and polyaromatic hydrocarbons. Studies on the interrelationships of molecular lesions and mutation induction revealed that both the nature and the quantity of mutagen binding to DNA affect chemical mutagenicity. Since CHO cells do not possess biotransformation capabilities to convert promutagen to its mutagenic forms, a rat liver

metabolic activation system (S9) is coupled to the CHO/HGPRT system to determine mutagenic activity of promutagens such as dimethylnitrosamine (DMN). We found that addition of 10 mM $CaCl_2$ to the S9 and its phosphate-buffered salt mixture further enhanced both the mutagenic activity and cytotoxicity of DMN. Calcium phosphate, tricalcium phosphate, and alumina C_γ gels, but not DEAE cellulose, could substitute for $CaCl_2$ in the S9 mix. Increasing the time of contact between the S9 protein and the S9 salts increased the efficacy by which the S9 mix activated DMN, and is indicative of an adsorptive process by calcium phosphate gel. Further study showed that ethylene dibromide is a direct acting mutagen, and that inclusion of the S9 system further enhanced the cytotoxicity but not the mutagenicity of this compound.

2. The Multiplex CHO assay has been used to enumerate sister chromatid exchange (SCE) and chromosome aberrations, and to study the interrelationships of chemically-induced cytotoxicity, gene mutation, SCE and chromosome aberrations. We used three carcinogenic/non-carcinogenic pairs to study the interrelationship of these four distinct biological effects. These compounds include the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and its non-carcinogenic analogue N-methyl-N-nitroguanidine, and the procarcinogens benzo[a]pyrene (B[a]P) and DMN and their noncarcinogenic analogues pyrene and dimethylamine. We observed that all carcinogens tested, but not their noncarcinogenic counterparts, showed all four biological effects. Cytotoxicity, however, did not appear to correlate with any of the other end points. SCE induction appeared to be the most sensitive indicator of "genetic activity." MNNG appeared to be the most active, followed by B[a]P and DMN as ranked on a molar basis. This ranking is consistent in the expressed activity of these compounds with regard to both chromosomal aberration and gene mutation. These results indicated that the Multiplex CHO System is capable of discriminating divergent structural classes of carcinogenic and noncarcinogenic compounds. Both cytogenetic and gene mutation assays proved useful in quantifying the mutagenic effects of these carcinogens. A similar study has been conducted with a carcinogenic heterocyclic nitrogen mustard, ICR 170, and its noncarcinogenic congener ICR 170-OH. ICR 170 caused a concentration-dependent increase of cytotoxicity, gene mutation and SCE; it caused chromosome aberration only at a high concentration which also caused a high cellular lethality. On the other hand, ICR 170-OH exhibited only a cytotoxic effect.

3. We have used recombinant DNA technology to study the molecular basis of mutation of E. coli xanthine-guanine phosphoribosyl transferase (gpt) gene (equivalent to mammalian hgprt in CHO cells) which has been transfected into HGPRT deficient CHO cells using the calcium-phosphate precipitation technique. Some gpt^+ CHO clones are stable following passage in non-selective medium and E. coli XGPRT activity can be demonstrated electrophoretically in extracts from these cells. Genomic DNA derived from several of these stable gpt^+ CHO lines has been analyzed by Southern blot hybridization indicating the presence of one to several copies of pSV2gpt in the high molecular weight DNA. Treatment of one of these clones with ethylmethane sulfonate followed by selection in 6-thioguanine (TG) produced a dose-dependent increase of phenotypically gpt^- TG^r colonies. Several

spontaneous and EMS-induced TG^r clones were isolated and have been shown to carry sequences which hybridize to the pSV2*gpt* vector. These results indicated that the *gpt*$^-$ phenotype is not necessarily derived from the segregation and loss of *gpt* sequences. Recent analyses of spontaneous and X-irradiation-induced *gpt*$^-$ mutants indicated that *gpt* sequences are deleted in some mutants.

4. We have continued development of (a) a quantitative human cell mutation (at the hgprt locus) assay, (b) a chromosomal deletion assay in a CHO/human hybrid cell line, and (c) a Chinese hamster *in vivo* system to assay for mutation at the hgprt locus, chromosome aberration and SCE in spleen cells. Use of these multiphasic genetic toxicity systems at the molecular, cellular, and animal levels should provide reliable identification of suspected environmental mutagens and be valuable in studying mechanisms of mammalian mutagenesis.

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THE INDUCTION OF CHROMOSOME ABERRATIONS, SISTER CHROMATID EXCHANGES AND SPECIFIC LOCUS MUTATIONS BY RADIATION AND CHEMICALS, AND THE APPLICATION OF THE STUDIES TO POPULATION MONITORING AND RISK ESTIMATION

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The major portion of the research of the Mammalian Cytogenetics Group can be considered to be directed towards estimating the genetic risk, and potentially the carcinogenicity, of radiation and chemical exposures to man. The approach taken is to attempt to determine the mechanism of induction of chromosome aberrations, sister chromatid exchanges and specific locus mutations, and to apply the information obtained to the interpretation of data from currently used assay systems, or for the development of new, more sensitive, or more predictive, assays.

This report is divided into several sections, each one representing a separate series of experiments. There is a logical progression to the sections, and there is a clear relationship between them.

1. X-ray-induced Chromosome Aberrations and the Involvement of Repair of DNA Base Damage. It has been shown that cytosine arabinoside (ara-C) inhibits the resynthesis step of the excision repair of DNA damage induced by a wide variety of agents. As a result of this inhibition, single strand gaps are accumulated in the DNA. The inhibitory action of ara-C on DNA repair can be reversed by the addition of deoxycytidine (dC).

This first series of experiments was designed to determine if the specific damage, whose repair was inhibited by ara-C, could be converted into chromosome aberrations, and thereby provide information on the type(s) of DNA damage that are involved in the induction of aberrations and also on the mechanism of induction of chromosome aberrations.

The hypothesis was that if the strand gaps accumulated in DNA by ara-C inhibition could be converted into aberrations on reversing the inhibition with dC, then the frequency of aberrations would be increased when incubations in ara-C were given to treated cells. Also the frequency of aberrations should increase with increasing ara-C incubation times.

For a variety of reasons human lymphocytes were used in these studies. Non-stimulated G_0 lymphocytes were irradiated with 200 rad of 250 kVp X rays at 100 rad/min. Following irradiation, cultures were incubated for 0, 1, 2 or 3 hr in the presence of ara-C. At the end of the appropriate incubation time, ara-C was removed and dC was added, and the cultures were incubated until fixation (52 hr).

The frequencies of all aberration classes were increased in the X-ray plus ara-C cultures as compared to the cultures receiving X rays alone. It was also observed that the frequency increased with increasing ara-C

incubation time, up to 3 hr. This indicated that the lesions responsible for aberrations were continually being repaired over at least this 3 hr-period and that they were being repaired by an excision repair process, since ara-C requires the resynthesis of several bases in order to effectively inhibit repair. The results, therefore, showed that directly induced single and/or double strand breaks were not the initial lesions responsible for aberration production, but rather that the excision repair of X-ray-induced base damage was involved. This is contrary to what was usually considered to be the mechanism.

In order to show that the increased aberrations produced by the incubation in ara-C were not the consequence of a different mechanism than when aberrations were formed in the absence of ara-C incubation, phytohemagglutinin (Pha) stimulated lymphocytes were irradiated in the G2 stage of the cell cycle (100 rad). In these studies ara-C was added after irradiation and remained in the cultures until fixation. For cells irradiated only (i.e., no ara-C incubation), the frequency of chromatid deletions was about 2.0 per cell, and that of exchanges about 0.3 per cell. In cells incubated with ara-C after irradiation for 2 or 3 hr, the frequency of deletions was about 5 and 8 per cell respectively, but no exchanges were observed. These results show that the inhibition by ara-C of repair of X-ray damage that can be converted into aberrations prevents the formation of all exchange aberrations, and not just some fraction of them. Thus the aberrations produced when ara-C incubation is used appear to involve the same mechanism as those produced in the absence of ara-C.

In experiments performed in collaboration with Dr. Michael Bender of Brookhaven National Laboratory, aphidicolin, a specific inhibitor of DNA polymerase α, was used in place of ara-C in order to see if repair was similarly inhibited and if increased aberration frequencies could result. Essentially the same results were obtained with X rays plus aphidicolin as are reported above for X rays plus ara-C. These results lend further credence to the idea that the repair of X-ray-induced base damage is involved in the production of chromosome aberrations.

2. Hypothesis for the Mechanism of Induction of Chromosome Aberrations. These results allowed us to propose a novel mechanism for the induction of chromosome aberrations. In simple terms it is stated that chromosome aberrations are the result of the interaction of two repairing regions, either on the same chromosome or on different chromosomes, depending on the aberration type. The interaction will occur during excision repair of base damage, when gaps are present in the DNA during the resynthesis step, following excision. The most important feature is that, in order to be able to interact, the regions must be undergoing repair coincidentally, since the repair of any one region is rapid, and the time available for interaction short, despite the fact that the time to repair all the damage can take several hours. These repairing regions must also be close together spatially, in order that interaction can occur. The important consequence of this hypothesis is that the frequency of aberrations will be dependent not only on the amount of initial DNA damage, but also the probability that interaction between repairing regions can occur, i.e., upon the overall rate of repair of the DNA damage.

The following experiments (Sections 3, 4 and 5) were designed to test this hypothesis.

3. The Induction of Chromosome Aberrations in Lymphocytes from Down Syndrome Individuals. It was reported some years ago that the frequencies of X-ray-induced chromosome aberrations in G_0 lymphocytes of persons with Down syndrome were about 1.6 times those in lymphocytes from normal individuals. Our hypothesis would indicate that this increase could be due to an altered repair rate for that X-ray-induced DNA damage that was converted into chromosome aberrations. The amount of DNA damage induced by X-rays is generally proportional to DNA content. Since Down cells have only 1% more DNA (due to the extra chromosome 21), the DNA damage is expected to be the same in normal and Down lymphocytes. If the rate of repair of the damage that is converted into aberrations is faster in Down cells than normal cells, there is an increased probability of interaction between repairing regions that can result in an aberration, and the aberration frequency will be higher in Down cells.

The same protocol as described in (1) above was utilized, with lymphocytes being X-irradiated in G_0, and with post-irradiation in the presence of ara-C. As for normal cells, the frequency of aberrations in Down cells was greatly increased when cultures were incubated in ara-C after irradiation. However, the increase was much more rapid for Down cells than normal cells. For example, the frequency of abberations in Down cells following a 1 hr ara-C incubation was the same as that observed after a 2 hr ara-C incubation for normal cells. This result indicates a more rapid repair in Down cells of that damage that is converted into aberrations (also equivalent to that damage whose repair can be inhibited by ara-C). Thus the hypothesis suggested above allows for an explanation of the increase in X-ray-induced chromosome aberrations in Down cells as compared to normal cells.

4. The Induction of Chromosome Aberrations by Chemical Agents. Can the hypothesis developed above for X-ray-induced aberrations be expanded to include aberration induction by chemical agents?

In contrast to X rays, the vast majority of chemical agents do not produce chromosome type aberrations in G_0 and G_1 cells, but rather produce chromatid-type aberrations when the cell passes through the DNA synthesis or S-phase. The repair of the DNA damage produced by chemical agents is, in general, much slower than that for X-ray damage. Thus the probability of interaction between coincidentally repairing regions is low, and consequently the probability of producing aberrations is low. This probability of interaction will be greatly increased during or shortly after replication, as a result of gaps being left in the replicating strand opposite altered bases, thus explaining the increase in chromatid-type aberrations in chemically treated cells as a consequence of DNA replication.

What would happen if the probability of interaction is increased in chemically-treated cells prior to replication? This can be achieved by incubating cells with ara-C after treatment, thus accumulating gaps in the repairing DNA as a consequence of the inhibition of repair. Reversal of

this inhibition with deoxycytidine (as above) should result in an interaction between these accumulated gaps, and an increase in aberration frequency. Lymphocytes were treated in G_1 (Pha-stimulated cells) with 4-N-nitro-N-quinoline oxide (4NQO) and methyl methanesulfonate (MMS) and incubated with ara-C for 3 or 6 hr. There was an increase in aberrations, exclusively of the chromosome-type, i.e., the type induced by X rays in G_1-treated cells in the absence of any post-irradiation treatments. Thus by increasing the probability of interaction between repairing regions, by accumulating several hours of repairing regions by ara-C inhibition, the aberration frequencies induced by chemical agents are increased and the aberration types are those expected to be induced in G_1 cells. Thus the different spectrum of aberration types induced by radiation and chemical agents is the consequence of the different rates of repair of the DNA damage induced, that in turn gives different probabilities of having coincidentally repairing regions able to interact to give aberrations.

5. Interactive Effects of Radiation and Chemical Agents. A further test of the hypothesis is to carry out experiments to determine if the damage produced by different chemical agents can interact with that produced by X rays. For these studies two chemical agents were chosen: one, 4NQO, induces DNA damage that is repaired slowly, particularly in contrast to X ray damage, such that interaction would be unlikely; the other, bleomycin, is radiomimetic since the damage it induces is repaired rapidly, and the aberration spectrum it induces is very similar to X rays.

If treatment of lymphocytes with 4NQO is followed immediately by X-irradiation, the aberration yields observed are identical to the additive effect obtained following the individual treatments. However, if following the 4NQO treatment the cells are incubated with ara-C for 6 hr, and then X-irradiated, the aberration frequency is about twice as high as that for the additive effect of the individual treatments. Thus, by increasing the probability of having coincidentally repairing regions, 4NQO repairing regions can interact with those resulting from X-ray damage.

In contrast, if cells are treated with bleomycin and then with X rays, the aberration frequencies are much higher than the additive effects of the individual treatments in the absence of any ara-C incubation. Since the repair of the damage induced by both these agents is rapid, the probability of coincidentally repairing regions is high without any need to inhibit repair with ara-C.

These studies provide very strong supportive evidence for the hypothesis developed for the mechanism of induction of chromosome aberrations.

6. Risk Estimation and Population Monitoring. Without pursuing any lengthy discussion, it can be seen that the hypothesis discussed for the mechanism of aberration induction can add significantly to the ability to interpret aberration data obtained in different species and in different cell types, i.e., for data that are the basis for providing estimates of the genetic risk to man from radiation and chemical agents. The differences in sensitivities to aberration induction in different species and in different cell types within a species can be considered in terms of the

relative rates of repair of the damage that is converted into chromosome aberrations. Of course, the relative lengths of the cell cycle and the duration of the specific cell cycle stages will also be influential. However, these are readily measurable, and their influence can be estimated and already has been in some cases. We are currently pursuing this approach, and have some preliminary evidence to indicate that the different sensitivities for aberration induction by X rays in lymphocytes of different species is a consequence of the different rates of repair of ara-C inhibitible damage (i.e., that damage that is converted into aberrations).

We also have two rather large cytogenetic monitoring studies in progress, in collaboration with Dr. Michael Bender at Brookhaven National Laboratory. If we identify a variation in "spontaneous" or induced aberration frequencies, it will be important to determine if such differences could be accounted for by differences in ability to repair specific types of DNA damage, specifically the rate of repair of such damage.

This section tends to lose some of its impact as a result of brevity, but we feel it represents one of the most important future extensions of our current research.

7. The Mechanism of Induction of Sister Chromatid Exchanges and Specific Locus Mutations. The analysis of sister chromatid exchanges (SCE) provides a very sensitive assay of induced DNA damage observable at the chromosome level. However, the consequences of an increase in SCE are unknown and almost certainly require an understanding of the mechanisms of their induction before such consequences can even begin to be understood. Furthermore, the interpretation of SCE data would be greatly enhanced by understanding the mechanism of their induction.

In the following series of experiments we feel we have come a long way towards understanding this problem. In a parallel series of experiments we have also attempted to compare and contrast the mechanism of induction of specific locus mutations at the hgprt locus in Chinese hamster cells.

A requirement for observing SCE is that cells be grown in a thymidine analogue (usually 5-bromodeoxyuridine, BrdU) for two rounds of replication. BrdU by itself, when incorporated into the DNA, can result in SCE, and the frequency is proportional to the BrdU concentration over quite a wide range. We observed that another thymidine analogue 5-chlorodeoxyuridine (CldU) could also be used, but in this case approximately 7 times as many SCE were induced over a wide concentration range. This difference was not due to a greater incorporation of CldU compared to BrdU, because we demonstrated, using HPLC, that the amount of incorporation was identical for the same extra-cellular concentrations, over a wide range of concentrations, and thus percent incorporations. Merely changing the incorporated base causes a very large change in SCE frequency. The obvious hypothesis to test was, is it the incorporation or the replication of the particular base analogue that causes the increase in SCE?

By utilizing "double treatments" this question can be answered. A "double treatment" is one in which the first replication is in the presence

of CldU and the second with BrdU, or vice versa. The SCE frequencies observed are compared with those obtained when only BrdU or CldU is present during both replications. In summary, the frequency of SCE was always dependent upon the base analogue present during the first replication, i.e., high SCE frequencies were observed with CldU during the first replication, and much lower frequencies with BrdU present during the first replication. The conclusion is that the SCE are a consequence of errors of replication of the incorporated base analogue, and not of errors of incorporation itself. In the experiments designed to study mutations to thioguanine resistance ($hgprt^+ \rightarrow hgprt^-$) from growth in BrdU and CldU, it was shown that these mutations could also result from the replication of BrdU- or CldU-substituted DNA. However, it also appeared that such mutations could also result from incorporation of the analogue into the DNA, probably in place of deoxycytidine.

The mechanism of induction of SCE and specific locus mutations both appear to involve errors of replication but, in addition, mutations also appear to be a consequence of errors of incorporation.

8. Studies with a Transplantable Mouse Myeloid Leukemia - an Animal Model. These initial studies with a transplantable mouse myeloid leukemia are intended to determine whether or not there are differences in sensitivity to a variety of end points between normal and leukemic cells, and whether the factors contributing to any differences can be ascertained. Furthermore, some aspects of this investigation are intended to aid in further studies that are currently underway to determine if specific induced chromosome alterations are the cause of myelogenous leukemia, and also whether analysis of specific induced chromosome alterations can be used to predict the frequency of leukemias.

The studies to date make use of the transplantable leukemia cell lines originally developed by Dr. Arthur Upton at the Biology Division. These cells lines have been karyotyped and considered in terms of their suitability for further study. The most suitable line contained a single, clearly observed, metacentric chromosome that has been identified as an isochromosome 8 by chromosome banding. This marker chromosome makes the leukemic cell clearly distinguishable from the co-existing normal bone marrow or spleen cells when the leukemic cells are transplanted into the host animal. This model system will, therefore, permit the study of differential effects of normal and leukemic cells in the same animal.

Using this model system, SCE were analyzed in the mouse bone marrow and transplanted leukemic cells during the course of development of the leukemia over a 20-day period. There was no difference in SCE frequency in leukemic cells during the development of leukemia and no difference between the frequencies in normal and leukemic cells. It will clearly be of interest to determine if there are differences in induced frequency following treatment with a variety of agents, including chemotherapeutic drugs.

9. Summary. The intention of these studies is to improve our ability to extrapolate from data obtained with laboratory animals to the likely

outcome in man, in order to provide estimates of the genetic, and potentially the carcinogenic, risk to man from exposures to radiation and chemical agents. There are several studies that have been recently initiated but are not reported here because of limited results so far. These particularly involve the development or improvement of assay systems to provide a greater predictive value or greater sensitivity.

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DNA REPAIR IN HUMAN CELLS

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Our primary objective is to elucidate the molecular events in human cells when cellular macromolecules such as DNA are damaged by radiation or chemical agents. We study and characterize (i) the sequence of DNA repair events, (ii) the various modalities of repair, (iii) the genetic inhibition of repair due to mutation, (iv) the physiological inhibition of repair due to mutation, (v) the physiological inhibition of repair due to biochemical inhibitors, and (vi) the genetic basis of repair. Our ultimate goals are to (i) isolate and analyze the repair component of the mutagenic and/or carcinogenic event in human cells, and (ii) elucidate the magnitude and significance of this repair component as it impinges on the practical problems of human irradiation or exposure to actual or potential chemical mutagens and carcinogens.

The significance of these studies lies in (i) the ubiquitousness of repair (most organisms, including man, have several complex repair systems), (ii) the belief that mutagenic and carcinogenic events may arise only from residual (nonrepaired) lesions or that error-prone repair systems may be the major induction mechanisms of the mutagenic or carcinogenic event, and (iii) the clear association of repair defects and highly carcinogenic disease states in man [xeroderma pigmentosum (XP)].

Ultraviolet light induces dimer formation between adjacent pyrimidines in DNA, giving rise to thymine-thymine, thymine-cytosine, and cytosine-cytosine (T<>T, T<>C, C<>C) dimers. About 90% of the damage induced in DNA by ultraviolet light occurs with the formation of these types of dimers. Labeling of both thymidine and cytosine in human cells using [^{14}C]uridine as a precursor allows quantitation of all three dimers by two-dimensional paper chromatography. After 50 Jm^{-2} of 254 nm light, the ratio of C<>C, C<>T, T<>T in human cellular DNA is 10:24:66. Due to the light absorption characteristics of cytosine, not only are fewer C<>C dimers produced, but in addition their incidence levels off more at lower doses than does that

of the other forms. For example, while thymine-containing dimers may be induced to a level of about 10% of the total pyrimidines, the C◇C dimer will make up only 0.05% of the dimers when cells are irradiated with low biologically relevant doses of the wavelengths contained in sunlight. A low dose of filtered FS40 light (sunlamp) produces dimers at a ratio of 20:40:40 (C◇C, C◇T, T◇T) in human cells. The number and kinds of pyrimidine dimers induced in DNA by ultraviolet light are related to the DNA base ratio, the wavelength of UV light and the total dose. In human cells after low (biological) doses of 254 nm light, the thymine-containing dimers comprise about 75% of the total dimers - after "sunlight" the number is reduced to 60%. Whether or not this difference in the kinds of dimers seen at different wavelengths is biologically significant is at present unclear.

If cells are exposed to longer ultraviolet wavelengths, much higher doses are needed to create the same number of dimers in the DNA. Increasing the wavelength from 254 to 313 nm requires a 2000-fold increase in dose to produce one million dimers per cell. Human cells in culture exposed to 100 min of sunlight results in the production of about the same number of dimers as 10 Jm^{-2} of 254 light. However, it is estimated that only about 10% of the wavelengths around 300 nm would penetrate the skin to the sensitive basal layers so that one could conclude that a biologically significant dose of 254 nm radiation might be in the neighborhood of 1-5 Jm^{-2}.

Human cells in culture are able to excise about one million dimers over a 24-hr period. The capacity derived from initial rates appears to indicate that these cells are able to excise about 10^5 dimers per hr. We have performed experiments relating the number of dimers excised with the amount of dimers introduced into the DNA by ultraviolet light of 254 nm. At doses of UV-light 20-60 Jm^{-2} the number of dimers excised reaches a maximum of around one million per cell. Doses higher than 60 Jm^2 seem to inhibit dimer removal. We analyzed for dimer content by two dimensional paper chromatography of acid hydrolyzates of the DNA and by determining the number of enzyme-sensitive sites (ESS) present in the DNA. Both methods measure the amount of pyrimidine dimer in the DNA of UV-irradiated cells. At this time, we do not know the reason for the inhibition of dimer excision at doses greater than 60 Jm^{-2}. It is apparent from several lines of evidence that excision repair is a well coordinated multienzymatic process involving a number of steps leading to the replacement of damaged nucleotides. We observe that breaks occur in the DNA at high doses, and this may well reflect the uncoupling of the steps of excision repair.

We have developed a technique whereby 3-hr pulses of arabinofuranosyl cytosine (ara-C) and hydroxyurea (HU) are used to analyze the kinetics of repair with time after ultraviolet irradiation in human fibroblasts. We demonstrated that this technique offers a significant improvement over existing repair assays in its ability to visualize between 57 and 100% of all sites undergoing repair in a given period of time. In addition, kinetic analyses of repair are more easily made and yield more information than techniques such as repair replication or unscheduled DNA synthesis. We have also examined the nature of the inhibition event by ara-C and have determined that repair breaks accumulate in the presence of ara-C and HU only up to a certain time beyond which no further breaks appear. The time

needed to reach this saturation point depends on the number of sites undergoing repair during the treatment time.

We have continued our studies on DNA repair in human cells induced by chemical agents. The dBrU photolysis assay was used primarily to measure repair since it enables us to estimate both the number and the relative sizes of the repaired regions in DNA. We can, thusly, classify chemical compounds according to the type of DNA repair they induce. We have completed a study testing 32 chemical agents and UV- and γ-irradiation for their comparative ability to induce short-patch or long-patch repair. Those compounds which induce long-patch (30-100 nucleotides) repair have all been shown to be the most active direct acting carcinogens within the study. The lack of repair of such lesions in XP cells known to be deficient in the long-patch repair mode has been used to confirm the data obtained with the dBrU photolysis assay on normal cells. Typical patch sizes measured by this assay ranged from about 30 to 70 nucleotides for various chemical carcinogens in contrast to about 90 nucleotides for UV repair. Alkylating agents previously shown to induce short-patch DNA repair activity induced by chemical agents was generally higher with the more electrophilic agents.

In recent years, much effort has been directed toward the elucidation of DNA repair processes in both procaryotic and eucaryotic cells following chemical or radiation insult. Much of this interest has evolved from the suggestion that DNA repair might be important in the aging process, mutagenesis and/or carcinogenesis. While a vast literature exists regarding the types of lesions induced by ultraviolet radiation and the excision repair of the predominant UV-lesion, the pyrimidine dimer, relatively little is known about repair following alkylation damage. This is due largely to the fact that alkylating agents induce a wide variety of DNA base damages for which no simple assay technique analogous to those for pyrimidine dimers has been developed. Nevertheless, many studies have been carried out demonstrating quite clearly that repair processes do act following treatment of mammalian cells with monofunctional alkylating agents.

We were interested in studying three basic questions concerning alkylating repair: (1) What is the nature of this repair, i.e., does repair proceed via a long-patch or short-patch process? (2) Are xeroderma pigmentosum (XP) cells defective in any facet of alkylation repair? (3) What is the time course of alkylation repair?

It has generally been accepted that the alkane sulfonates induce a typically "short-patch" repair proceeding via the insertion of something less than five bases into the repairing site, although some evidence now exists suggesting that there may be a long-patch component also. It has been a matter of speculation as to which of the many alkylated bases in the DNA are repaired by classical repair processes and which are either not repaired at all or are repaired by minor repair processes. We have demonstrated that at least a portion of the alkylation damage incurred in the DNA following treatment with alkane sulfonates is repaired via a long-patch repair mechanism.

It might be predicted that if indeed there was a long-patch component involved in alkylation repair, XP cells might show a deficiency in this process owing to their deficiency in long-patch repair following treatment with UV and UV-mimetic chemicals. Xeroderma fibroblasts have been reported as being as proficient as normal human cells in repair replication following MMS or EMS treatment but showing a delayed repair following ethyl nitrosourea treatment.

We have used the technique of ara-C inhibition to establish the temporal pattern of alkylation repair in normal human and XP cells and have confirmed the existence of two distinct phases of repair, one complete in the first 3-5 hr after insult and the second extending from 9-35 hr post-insult. We showed that both phases of repair have a long-patch (UV-like) component, thus establishing for the first time the existence of this mode of repair in response to alkylation damage. While xeroderma cells display somewhat fewer alkaline labile sites in their DNA following alkylation treatment than do their normal counterparts (consistent with earlier observations suggesting a deficiency in a glycosylase or apurinic endonuclease), we are unable to demonstrate a deficiency of these cells in either of the two phases of repair.

Clearly, cells from normal individuals possess the ability to repair damage to DNA. Numerous studies indicate that individuals deficient in DNA repair are prone to cancer. Although not all cancers arise from defects in DNA repair, evidence is abundant that events leading to malfunctions in DNA replication and repair create a high potential for malignant transformation.

The biological relevance of induction and repair of DNA damage should properly emerge from a correlation of such damage and repair with (a) mutation, (b) cell killing, and (c) environmental carcinogenesis. It is encouraging that some correlations have been made and are consistent with the idea that more DNA and/or less repair lead to more mutation and cell killing. With regard to correlation of DNA damage and repair, and environmental carcinogenesis, the problem is more complex in that experimental carcinogenesis is properly studied in the whole animal while DNA repair experiments are most conveniently done in cells and culture.

One invaluable tool necessary to the understanding of the repair process in human cells would be either the development of specific mutant cell lines defective in the different steps of repair, or alternately, a clear characterization of the biochemical significance of the seven complementation groups found in xeroderma pigmentosum. The meaning of the multiple complementation groups of XP is unclear. Do seven genes control incision, the first step of excision repair? Or do these groups represent mutations in genes controlling different enzymatic steps in the repair pathway?

It is hoped that continued studies on the control of cellular processes involved in DNA damage and its repair, detailed kinetic analysis and correlations between biochemical events and changes in cell growth will lead to understanding and subsequent control of carcinogenesis.

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GENETIC ANALYSIS OF DNA REPAIR IN MAN WITH CELL HYBRIDS

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The long-range goal of this project is to investigate the expression and individual genetic components involved in the repair of DNA damage and to localize the genes involved in this complex system to specific human chromosomes. Such an elucidation of the genetic basis for the repair of DNA damage caused by physical or chemical agents is fundamental if we are to understand its pivotal role in carcinogenesis and mutagenesis. We have employed man × mouse somatic cell hybrids as our experimental system and bromodeoxyuridine photolysis, radiochromatography and molecular weight analysis as our assays for DNA repair to pursue this project. The use of somatic cell hybrids allows for the mapping of genes to specific human chromosomes and the genetic dissection of this polygenic system due to the isolation of its component parts as a result of the preferential segregation of human chromosomes in proliferating human × mouse somatic cell hybrids.

Five different human cell lines derived from unrelated individuals normal for the ability to repair UV-induced DNA damage were utilized in this study. The human cells included three fibroblast lines and two lymphoblastoid lines. The parental mouse cell lines consisted of the RAG line, which is deficient for hypoxanthine phosphoribosyl-transferase ($HPRT^-$) and B82, a line which lacks thymidine kinase (TK^-). Excision repair of UV-induced DNA damage was measured using the dBrU photolysis assay. Using this assay, it was shown that mouse cells have 5-10% of the magnitude of excision repair seen in human cells. This result demonstrates

that it is possible to distinguish the human DNA repair components from mouse DNA repair components in human × mouse somatic cell hybrids. Utilizing these parental cells, five independent sets of hybrids were formed. Sixty-five human × mouse primary hybrid clones were isolated from these five separate fusion experiments. The ability of human and mouse cell lines to repair UV-induced DNA damage was differentiated quantitatively with the mouse cell repairing DNA damage at 5-10% of the magnitude of human cells and individual hybrid clones having one of three categories of repair: (1) those having human-like repair capacity, (2) those having mouse-like repair, and (3) hybrids intermediate between the two.

The man × mouse somatic cell hybrids were analyzed for the presence or absence of each human chromosome by testing these hybrids for the expression of 35 enzyme markers. Genes coding for these enzymes have been assigned to each of the human chromosomes except the Y so that these enzyme markers provide a way of determining which human chromosomes are present in the hybrid clones. When the segregation of the ability to repair UV-induced DNA damage was compared to the expression of the 35 enzyme markers in three sets of hybrids made between human fibroblasts and mouse cells, there was a strong correlation between the presence of human chromosome 3 and the ability to repair UV-induced DNA damage and the loss of chromosome 3 and the loss of excision repair capacity. These data suggest that a gene or genes required for DNA repair are located on chromosome 3. In these same clones, there was a correlation between chromosome 14 and excision repair capacity. However, there were two clones which were positive for chromosome 14, but negative for repair. These clones are being checked to be sure they do not contain a fragment chromosome 14.

When we analyzed the data from two sets of hybrids made with human lymphocytes and lymphoblastoid cells, the correlation was not so strong. There were two clones which were intermediate in their repair capacity, but were negative for chromosome 3 markers. This may be due to chromosome breakage or to differences in the relative sensitivities of the enzyme assay for <u>ACY</u>, a gene located on chromosome 3 and the DNA repair assay. There was <u>only</u> one exception to a correlation between chromosome 14 and repair capacity in these hybrids. This hybrid was negative for chromosome 14 but positive for the ability to repair DNA damage. A possible explanation for the discrepancy of results in hybrids derived from human-fibroblasts vs. hybrids derived from lymphocytes or lymphoblastoid cells may be that there is a tissue specificity in the expression of the repair system as is seen for several other enzyme systems. This possibility is being actively investigated.

It is interesting to note in this regard that aberrations of chromosomes 3 and 14 have been associated with specific human malignancies, suggesting a possible association between disturbances of the repair system and carcinogenesis.

GENETIC DISSECTION OF DNA REPAIR IN XERODERMA PIGMENTOSUM CELLS

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The human skin disease, xeroderma pigmentosum (XP), demonstrates direct linkage between defective repair of damaged DNA, induction of mutations and induction of cancers. Studies utilizing the fusion of XP cells from unrelated patients have indicated that excision defective cells can be classified into at least seven different complementation groups designated A through G. This genetic heterogeneity in XP is perplexing since the biochemical lesion is thought to be in the UV endonuclease which initiates excision of UV-induced pyrimidine dimers in DNA. An elucidation of the basis for the genetic heterogeneity of XP is necessary if the functional relationships between DNA repair, mutagenesis, carcinogenesis, and risk assessment are to be fully understood and if xeroderma pigmentosum is to serve as a model system for such studies. Therefore, the specific objectives of this project are to (a) determine the genetic basis of the heterogeneity in XP complementation groups; (b) determine the number of different genes which may be involved in the expression of the xeroderma pigmentosum phenotype; (c) determine the chromosomal location of the gene(s) responsible for XP; and (d) determine the specific genetic contribution made by mouse cells to the XP cells which results in genetic complementation of their DNA repair capability. These studies were pursued using our ability to generate and genetically analyze man × mouse somatic cell hybrids for the presence of each human and mouse chromosome and the ability to repair DNA damage. The assays for DNA repair include (a) bromodeoxyuridine photolysis, (b) radiochromatography, and (c) molecular weight analysis. The use of somatic cell hybrids makes it possible to determine gene-chromosome assignments, to dissect this complex polygenic system by isolating its component parts and to investigate the genetic basis for complementation of defective repair in XP cells. To carry out these studies, several sets of hybrids were generated employing human parental cells derived from each of five XP complementation groups, namely Groups A-E. These human × mouse cell hybrids were formed such that they either segregated human chromosomes and retained mouse chromosomes or segregated mouse chromosomes and retained human chromosomes. Seven sets of hybrids were isolated from these fusion experiments.

The initial results of these studies demonstrated that mouse cells will complement the defective repair in four XP complementation groups, namely Groups A, B, C, and D, but not in a fifth group, Group E. In hybrids formed with complementation Groups A-D, human × mouse cell hybrids were isolated which had a repair capacity greater than the mouse and defective XP parental cell. This was never seen in hybrids between XP-E and mouse. These data suggest that the genetic defect in Group E is different from that found in the other groups.

When XP × mouse somatic cell hybrids segregated certain human chromosomes, they lost the ability to be complemented by the mouse cell. Presumably, these hybrids have lost the gene(s) coding for the repair step

that is rate limiting in the mouse. On the other hand, the mouse supplies the repair step that is defective in the XP cell. Hybrids between XP-A and mouse and XP-B and mouse have been analyzed for their human chromosome content. In XP-A × mouse hybrids, there is a strong correlation between the presence of chromosome 14 and the ability to be complemented and the loss of complementation and the loss of chromosome 14. No such correlation was seen with chromosome 3. In XP-B × mouse hybrids there is a correlation between the presence of chromosome 3 and complementation and absence of chromosome 3 and the loss of complementation. Chromosome 14 may also be associated with complementation since only one clone in the XP-B × mouse hybrids was discordant for complementation and chromosome 14. We have not yet finished our genetic analysis of the hybrids formed with cells from the other complementation groups.

Hybrids have also been generated between XP cells and mouse spleen cells under conditions which allow for the segregation of the mouse chromosomes and retention of the human chromosomes. These experiments were carried out to determine which mouse chromosomes carry genes involved in the complementation of defective repair in XP. Data derived from XP-A × mouse spleen cell hybrids suggest that mouse chromosome 4 carries a repair gene(s). However, our data cannot rule out other mouse chromosomes as possible sites for repair genes.

Preliminary experiments were carried out to determine whether this complementation of defective repair in XP cells by mouse cells is biologically significant. This was done by testing hybrid clones for resistance to UV irradiation. XP cells are highly sensitive to UV irradiation. Using this approach, viable XP × mouse hybrid clones were isolated following UV irradiation. The defective DNA repair capacity of the XP cells had been complemented by the mouse cells both enzymatically and biologically.

GENETIC BASIS OF MUTAGENESIS AND CARCINOGENESIS

Peter A. Lalley Judith A. Diaz

The primary objectives of this project are (1) to investigate the genes important for susceptibility to mutagens and carcinogens whether they be physical, chemical or biological agents; (2) to identify, characterize and determine the chromosomal assignment of these genes; and (3) to investigate the linkage relationships of homologous genes in man, mouse and other species. The data derived from these studies are important since an elucidation of the genetic basis of these systems is essential for the ultimate understanding of how genetic and environmental factors interact in the process of mutagenesis and carcinogenesis. Comparative mapping data is important for the extrapolation of biological and biomedical data from laboratory animals to man. These studies are being pursued utilizing somatic cell hybrids as our experimental system, which allows for the genetic dissection of complex polygenic traits by isolating their component

parts, and the determination of gene-chromosomal assignments due to preferential chromosome segregation.

We have been investigating the genetics of the induction of the aryl hydrocarbon hydroxylase system (AHH) in the mouse, a system of major importance in chemical carcinogenesis. Recently, we have shown that the gene controlling induction of this complex enzyme system in response to benzo[a]pyrene is located on chromosome 17, a chromosome which carries several genes (e.g., H-2 complex) which control the host response to foreign substances. In addition, employing restriction enzyme analysis with a cDNA probe specific for the P_{450} structural gene(s) we have shown that the structural gene is located on chromosome 2. These data have given us some insights into the complex genetic structure of an enzyme system directly associated with chemical carcinogenesis since it is involved in the conversion of polycyclic hydrocarbons to their carcinogenic forms.

We have utilized restriction enzyme analysis with cDNA probes specific for retrovirus genes to examine the chromosomal location of genes involved in murine viral carcinogenesis. The data from these studies demonstrate that the endogenous sequences for the Abelsom murine leukemia virus (A-MULV) and the endogenous sequences of viral sarcoma gene are located on mouse chromosome 2. These data, important for understanding the arrangement of the endogenous retrovirus genes, will aid in understanding the mechanisms of viral carcinogenesis. In collaboration with Drs. W. K. Yang and R. W. Tennant of the Biology Division, we have investigated the genetics of the RF virus, an inducible murine leukemia virus from the RFM mouse. We have determined that the gene controlling induction of virus and the endogenous viral sequences are located on mouse chromosome 5. These data have given some insights into the mechanisms of induction of a known leukemia virus.

The other genes investigated and mapped can be grouped into genes which code for enzymes, hormones, androgen-inducible proteins and globin genes.

Using Chinese hamster × mouse somatic cell hybrids segregating mouse chromosomes, we characterized and mapped the genes coding for inosine triphosphatase (ITP), an enzyme required for nucleic acid metabolism, and acid Phosphatase (ACP), an enzyme involved in hydrolysis of mucopolysaccharides, to mouse chromosome 2, and phosphoserine phosphatase (PSP), an enzyme required for serine metabolism and phosphoglycollate phosphatase (PGP) to mouse chromosome 5. The genes coding for two lysosomal enzymes, namely, α-fucosidase (FUCA) and acid lipase (LIPA), were mapped to mouse chromosomes 4 and 19, respectively. Deficiency of LIPA in man is associated with Wolman's disease and I-cell disease, while deficiency of FUCA is the cause of fucosidosis. All three disorders are fatal lysosomal storage diseases. Since deficiencies ACP and PSP in man have also been associated with specific human diseases, knowledge of the genetics of these enzymes is important for establishing animal model systems for studying these disorders. In addition, establishment of the clustering of phosphatase genes on two chromosomes suggests that these genes may have arisen by gene duplication and subsequent mutations. We have also mapped

the genes coding for aminocylase (ACY) and argininosuccinate lyase (ASL), two enzymes involved in amino acid metabolism to mouse chromosomes 9 and 5, respectively.

We have combined the use of somatic cell hybrids with recombinant DNA techniques to map genes coding for hormones which are not expressed in tissue culture. By using hybrids segregating mouse chromosomes together with restriction enzyme analysis with cDNA probes specific for the genes of interest, we have mapped the genes coding for insulin to chromosome 7, the gene coding for the α subunit of thyroid stimulating hormone (TSH) to chromosome 4 and the gene coding for somatostatin to chromosome 16. These data are important for understanding the organization and regulation of genes required for hormone production. We have also used this approach in studying genes whose expression is inducible by androgens, namely the major urinary proteins (MUP). There are approximately 25 of these proteins, all of which are inducible by testosterone. We demonstrated that the genes coding for these proteins are clustered on mouse chromosome 4 closely linked to the gene controlling induction, thus suggesting a mechanism of gene regulation in mammalian cells.

In collaboration with Dr. R. A. Popp of the Biology Division, we have demonstrated that in the mouse the α-globin gene pseudogenes are not located within the α-globin gene cluster as they are in other species studied, including man. Instead, they are located on two different chromosomes, namely chromosomes 15 (α-3) and 17 (α-4). These data demonstrate that evolution of this important genetic system has proceeded differently in rodents and man. This has implications for the extrapolation of data from laboratory animals to man when dealing with systems that have genetic components.

We have been working on procedures for carrying out gene transfer experiments in mammalian cells. Recently, we have been able to transfer ouabain resistance from mouse cells to human cells utilizing microcell mediated gene transfer. We have also transferred the long arm of the human X chromosome into mouse cells using isolated chromosomes and lipid vesicles as carriers.

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MAMMALIAN BIOCHEMICAL GENETICS

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The principal aim is to evaluate hazards to humans of exposure to nuclear and chemical by-products of energy production. Mice are used as experimental animals, and the data obtained must be extrapolated to man. The studies can be divided into the four general areas of research discussed below.

Comparative Mechanisms of Mutations Induced in Germinal Cells by Irradiation and chemicals. Alterations in the expression of the hemoglobin genes are being studied because mouse hemoglobins have been well characterized by us. The two tightly-linked adult α-globin genes in natural populations of mice encode seven unique α-globin polypeptides that appear in fourteen different combinations; the two tightly linked adult β-globin genes encode three β-globin polypeptides that appear in only two combinations. We have determined the basis for several variations in the expression of the hemoglobin genes induced by X rays, namely (a) three independent mutations were deletions of the compound α-globin locus in chromosome 11, (b) one mutation was a tandem duplication of a portion of chromosome 7 that included the compound β-globin locus, (c) one mutant resulted from double nondisjunction of chromosome 7, and (d) one mutant was a somatic cell mosaic for chromosome 7. Each of these mutations resulted from chromosomal aberrations.

Chemicals have also induced several hemoglobin mutations: (a) triethylenemelamine induced one deletion of the compound α-globin locus; (b) ethylnitrosourea induced an A → T transversion at the second letter of the natural codon for histidine at residue 89 in the α-globin gene, which then codes for leucine; and (c) ethylnitrosourea has also caused a change in the electrophoretic mobility of the β-minor chain of hemoglobin, which also may be a base substitution mutation.

Genetic Regulation of Gene Expression and the Organization of Genetic Information. The foregoing studies show that a fundamental understanding of the structure, function, organization, and regulation of genes and their

products is essential to interpret the mutagenic effects of irradiation and chemicals in germinal and somatic cells. Although deletion of the compound α-globin locus reduces the number of α-globin genes by one-half in mice heterozygous for this kind of mutation, we found that the quantity of α-globin produced is approximately 0.75 of normal. Analysis of the mRNA levels in reticulocytes of these mice established that the quantity of α-globin mRNA present is also approximately 0.75 of normal. Thus, the α-globin to β-globin mRNA sequences exactly reflect the α-globin to β-globin chain ratios; α-globin gene transcription appears to be elevated to approximately 0.75 of normal and compensates partially for the loss of one-half of the α-globin genes.

The Southern blots which showed that X rays and triethylenemelamine had deleted the adult α-globin genes in α-thalassemic mice also indicated that the α-globin-like pseudogenes had not been affected by any of the three independent deletion-type mutations at the α-globin locus. This result was quite unexpected because a close physical association had been found for the α-globin-like pseudogenes and the functional α-globin genes in several species of birds and mammals. Techniques of somatic cell hybridization and recombinant DNA were combined to show that pseudogenes α-3 and α-4 are located on chromosomes 15 and 17, respectively, rather than on chromosome 11 where the embryonic pre-α-globin and the two adult α-globin genes are located. These data explain why α-3 and α-4 were not affected by the three independently induced deletion-type mutations that cause α-thalassemia in mice.

The eye lens crystallins represent three homologous families (α, β, γ) of proteins that demonstrate age-specific changes in synthesis and assembly. The lens is particularly sensitive to radiation and chemically-induced damage. Two electrophoretic variants of mouse lens γ-crystallins have been described, and one locus has been mapped to chromosome 1 between Idh-1 and Pep-3. The second locus remains unmapped, but it is not linked to Len-1. To learn more about the nature of these lens protein variations and their possible association with cataract formation, the relevant γ-crystallins were purified and analyzed by tryptic peptide fingerprinting and amino acid analysis. The results of the chemical data support the genetic interpretation that two nonlinked structural loci encode for the two γ-crystallins and exclude an alternate possibility that one of the crystallins is a posttranscriptional modification of the other. These studies are part of an effort to understand the mechanisms of spontaneous and mutagen-induced lens dysfunction.

Pathophysiological Effects of Induced Mutations. The induced deletions of the embryonic and adult α-globin [illegible] clinical symptoms indistinguishable from those of α-thalassemia [illegible] in humans. In addition to the hematological indices reported [illegible] α-globin to β-globin synthesis ratio discussed [illegible] that α-thalassemic mice have an increased iron load [illegible] spleen, liver and kidneys. Humans with thalassemia, [illegible] and hemochromatosis also have iron overloads in these [illegible] in macrophages and reticuloendothelial cells), [illegible] recurrent infections. It has not been established [illegible]

infections in these patients are due to a suppressed immune recognition or the growth-promoting effects of available iron. To learn more about the cause of recurrent infections in humans with thalassemia, we are beginning a study on the effects of iron overloading on the immune competence of thalassemic mice.

Development of Tests to Compare Toxicity and Mutation Induction In Vivo. The hemopoietic system is a most sensitive indicator of insults from environmental agents. Aplastic anemia is the most severe form of hemopoietic failure in man; there is depletion of all hemopoietic stem cells of the bone marrow without signs of malignancy. Approximately one-half of the human cases of aplastic anemia result from exposure to drugs or toxins; the remainder are largely caused by hematological disorders, viral infections and autoimmune diseases. Aplastic anemia is different from the temporary marrow hypoplasia which appears following exposure to irradiation. Radiation-induced hypoplasia of bone marrow returns to normal quite rapidly following stem cell proliferation but aplastic anemia is not reversed because the individual does not have any stem cells. Radiation affects undifferentiated stem cells as well as more differentiated cell populations within the bone marrow of mice, but chemicals often preferentially affect cells at specific stages of the cell cycle or stages of differentiation. We have developed methods to detect and to express the relative cytotoxic effect of chemicals on different populations of cells in the bone marrow of mice treated with chemicals.

The number of stem cells in the bone marrow is determined by the spleen colony assay (CFU-s) which quantitates undifferentiated stem cells as well as stem cells committed to a particular pathway of differentiation. The toxic effect of a chemical is determined from its effects on bone marrow cellularity and the number of CFU-s in bone marrow after treatment. A sensitive cell index is calculated as the ratio of CFU-s (% of normal) to bone marrow cellularity (% of normal). If the index is 1.0, the undifferentiated stem cells and the more differentiated cell populations are equally affected by the chemical. The sensitive cell indices of 0.16 for 6-thioguanine, 0.94 for cyclophosphamide, and 1.66 for cortisone acetate indicate that 6-thioguanine is very toxic to dividing cells, cyclophosphamide affects differentiated and dividing cells equally, and cortisone acetate affects primarily the differentiated cells of the bone marrow.

A sensitive cell index of 0.16 and 0.05 was calculated for mice at 24 hr after they received 200 and 400 mg/kg, respectively, of ethylnitrosourea. An Ortho Cytofluorograph was used to show that the majority of the bone marrow cells ceased to synthesize DNA within 24 hr after exposure to ethylnitrosourea, but bone marrow cellularity, CFU-s and DNA synthesis usually returned to near normal values by 21 days after treatment.

A 6-thioguanine resistant stock of mice was developed by us to validate an in vivo system to detect mutations at the HGPRT locus in hemopoietic stem cells. CFU-s from the 6-thioguanine resistant stock of mice will develop into spleen colonies when transfused into lethally irradiated mice that are given 0.1 mM 6-thioguanine in their drinking water. CFU-s from normal mice fail to develop spleen colonies under similar conditions

but mutations that cause a deficiency of HGPRT enzyme activity in hemopoietic stem cells should permit those mutant cells to replicate and form spleen colonies. Using this assay, the spontaneous frequency of 6-thioguanine resistant cells in normal bone marrow was found to be 0.33×10^{-4} and the frequency was elevated to 1.5 to 7.0×10^{-4} in bone marrow of C57BL/6 mice treated four months previously with 250 mg/kg of ethylnitrosourea.

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Genetics of Microorganisms and Drosophila

DROSOPHILA CYTOLOGY AND GENETICS

R. F. Grell E. E. Generoso

The meiotic process constitutes the core of eukaryotic genetics. An understanding of the normal course of events during this period is basic to evaluating the ability of physical agents such as heat, and chemical agents including mutagens, to alter the process and lead to generally detrimental consequences. Despite the critical roles that they play, the mechanisms of the two principal early meiotic events, namely, synapsis and recombination, remain virtually unknown. We have been involved in studies of both events in the Drosophila pro-oocyte, the former through EM and EM autoradiographic approaches and the latter through genetic approaches, although a wide area of overlap has emerged. The aspect we chose to explore concerns the time of synapsis and recombination on the premise that a resolution of this parameter should contribute to a clarification of the mechanisms by eliminating from among the multitude of proposed models those inconsistent with our findings. The landmark to which both events are being related is premeiotic-S. Recognition of synapsis depends on visualization of the synaptonemal complex (SC), a structure revealed by EM, which lies between paired homologues.

During the current year we have completed a detailed study at the ultrastructural level of the development of the pro-oocyte nucleus. To provide an unambiguous temporal order, the first pro-oocyte in the developing pupal germarium has been followed at 6 hr intervals from its formation (~129 hr post-oviposition) until State 1 (168 hr) when its germarial phase is complete. This time interval encompasses the periods of synapsis and recombination. EM autoradiographs of sectioned germaria were first scanned at low magnification to locate the pro-oocyte in the first formed

(most posterior) 16-cell cyst and then photographed at higher magnification to look for the presence of label (= DNA replication) and SCs (= synapsis) in the same pro-oocyte nucleus. Label and SCs were found to co-exist at each 6 hr interval from 132 hr up to and including 162 hr. At 132 hr SCs were extensive with an average length of 50 μm/genome, providing unambiguous evidence that synapsis begins close to pro-oocyte formation and the initiation of premeiotic interphase. Since SCs are the visual expression of synapsis, it is evident that homologues are suitably positioned for recombination during premeiotic-S.

Complete serial sections of the pro-oocyte nucleus, taken at 6 hr intervals from 132 to 168 hr, provided reliable measurements, hitherto unavailable, of changes in SC length. Based on averages of a minimum of 6 nuclei/interval, we observe a sharp increase from 50 μm to 69 μm between 132 and 138 hr, a peak length of 75 μm at 144 hr, a decrease and subsequent plateau to 60 μm between 150 and 162 hr, and a further drop to 50 μm at 168 hr. Of great interest is the disclosure that maximum SC length at 144 hr coincides both with maximal recombination response to heat for the total genome and with midpremeiotic-S.

Our temporal study also provided reliable information concerning the time of appearance of a structure termed "recombination nodule" on the assumption that it is directly involved in mediating recombination. Its appearance has been assumed by some to mark the beginning of recombination and on the basis of calculations that 36 hr elapses between completion of the SC and formation of a nodule, it was concluded that recombination is well separated from snyapsis (Lindsley and Sandler, 1977). By contrast, we have detected a spherical nodule at 132 hr, coinciding closely with SC formation and by 138 hr their frequency has increased 8-fold. If their presence signifies recombination has begun, their appearance in the very early pro-oocyte would provide proof that recombination is initiated at early premeiotic interphase.

These studies establish beyond question that synapsis begins at early premeiotic interphase rather than at the zygotene stage of prophase; they provide additional evidence that recombination coincides with DNA replication at premeiotic interphase. Hence, a reappraisal of the current view, as incorporated in the Holliday and Whitehouse models, that replication, synapsis and recombination are sequential events, well separated in time, appears to be in order. Results of this study are now in press.

Two temperature-sensitive recombination mutants are known in Drosophila, both isolated in this laboratory. One, $\underline{rec\text{-}1^{26}}$, is an allele at a new locus, also represented by two temperature-insensitive alleles, $\underline{rec\text{-}1^{6}}$ and $\underline{rec\text{-}1^{16}}$. We have shown that $\underline{rec\text{-}1^{26}}$ responds to the restrictive temperature (31°) only during premeiotic-S; at the permissive temperature (25°) X-chromosome recombination is near normal and polarized with maximal reductions distally. Homozygotes of $\underline{rec\text{-}1^{6}}$ and $\underline{rec\text{-}1^{16}}$ as well as their heteroallelic combination at 25° show greatly reduced, virtually identical, recombination values and a reversed polarity pattern from $\underline{rec\text{-}1^{26}}$. Currently we are comparing the effects of one and two doses of each allele on recombination to determine if they act additively as expected for leaky

mutants. We find that two doses of rec-1[26] show a slight but significant increase over one dose and that the latter is associated with a polarity switch. In the cases of rec-1[6] and rec-1[16], recombination is unaltered by a reduction in dose. The similarity in their values and lack of dosage response for both alleles suggests that that these are null mutants and that the residual recombination we observe may represent a secondary pathway. This possibility will be explored.

The second temperature-sensitive mutant, c(3)G[28] localizes to a closely neighboring gene whose mutant alleles eliminate both meiotic recombination and the SC. Complementation between mutants at the two loci is complete. c(3)G[28] is a leaky mutant and at 25° permits a low level of recombination; if the temperature is reduced to 17°, the recombination frequency is increased ~3-fold. We wished to determine if the genetic leakiness is accompanied by a partial restoration of the SC. Studies thus far indicate this is the case. Moreover, measurements at the two temperatures show greater SC restoration at 17° correlating with increased recombination at this temperature.

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BIOCHEMICAL AND DEVELOPMENTAL GENETICS OF DROSOPHILA

E. H. Grell

Our broad objective is to determine how genes control the orderly growth and development of an organism. We employ a mutational analysis in the study. Mutant genes which cause a defect in a biosynthetic or developmental pathway have often been used to investigate the nature of the pathway. Mutants are selected on the basis that they affect the process under study and then a battery of genetic and other tests are used to learn as much as possible about the process. The advantage in using Drosophila is that it is possible to select gene mutations, deficiencies, duplications, reversions, enhancers, and other genetic alterations which are the raw materials for genetic analysis.

The homeotic mutants of Drosophila are extensively studied to reveal how genes control differentiation. The homeotic mutants cause an organ to develop in such a way that it resembles another organ. We have discovered a mutation which we believe is a homeotic mutant of a type not previously described. It causes a degree of confusion in dorsal-ventral differentiation. Normally the sternite plates on the ventral side of the abdomen are unpigmented and the dorsal tergites are partly or wholly melanized. The mutant has pigmented ventral sternites while the dorsal tergites are less pigmented than normal. The dorsal thorax also has differentiation of tissue which resembles some ventral tissue.

The mutation is a dominant "antimorph." Deletions of the wild-type allele of the locus do not cause the mutant phenotype. Duplications with a wild-type allele of the locus do not "cover" it. Two doses of the mutant are lethal in diploids and triploids. The mutation is easily mutilated by X-rays and EMS. The second-step mutations are partial and full reversions. This attribute should make it possible to clone the gene. A P-element induced reversion can be induced and the clones can be identified by their homology to P-element sequences.

MICROBIAL MUTAGENESIS AND CELL DIVISION

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Alicia Carrasco[2]
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W. D. Crow

Our group has been pursuing three related objectives. The first of these is a study of a mechanism by which the bacterium Escherichia coli repairs radiation-induced damage. In particular, we have observed that cells of certain strains of this bacterium, mutant at the lon locus, can be restored to viability after exposure to ionizing radiation if they are incubated in a nutrient medium to which a preparation of partially purified bacterial membranes has been added. These preparations stimulate division by producing chemical alterations in the nutrient medium and simultaneously creating a highly anaerobic environment. Our evidence indicates that the activity of the membrane fragments may be specifically directed at events required for cell septation rather than a more general repair of damage produced in DNA. In the absence of the membrane preparations, irradiated cells of a lon mutant grow into extremely long multinucleate filaments before dying. In the presence of the membrane preparation, the growing filaments form septa and divide into normal-sized viable cells (1, 3).

A second objective of the group was to make use of lon mutants for a rapid, sensitive, and inexpensive assay for chemical mutagens. Cells of lon mutants form long multinucleate filaments if exposed to a variety of agents that react with DNA. These filaments can readily be observed microscopically 2 to 3 hr after exposure to the suspect agent. We have defined conditions for performing this test and have surveyed a group of known mutagens and nonmutagens. The results are in good agreement with results obtained using the Salmonella histidine assay (4).

A third objective of our group has been to make use of the oxygen reducing properties of bacterial membrane preparations to stimulate the growth of anaerobic bacteria. Many anaerobic bacteria produce metabolic products that are of value as fuels, solvents, and intermediates in chemical synthesis. Our knowledge of these organisms is limited, at least in part, by the techniques available for growing and maintaining them. Some anaerobes are extremely oxygen sensitive and can be manipulated only by the use of techniques that seem cumbersome to one accustomed to working with organisms such as Escherichia coli. We have developed a method to prepare

membrane fractions from bacteria that contain a stable and highly active, cytochrome-based, electron transport system. This system will remove oxygen very rapidly and completely from liquid and solid microbiological media as long as a suitable hydrogen donor (lactate, succinate, etc.) is present. Neither the membrane preparation nor the reaction products are toxic to a wide range of bacterial species. We have been able to obtain rapid and luxuriant growth of eighteen species of anaerobic bacteria by using membranes as the oxygen reducing agent. No other precautions for excluding oxygen are required in most cases (2).

Our general goal is to develop basic microbiological techniques that will facilitate the application of genetic manipulation methods to important anaerobic species. To this end, we have developed a method, based on the use of membranes, that allows us to grow liquid cultures of *Clostridium acetobutylicum* from very small inocula to high titers without elaborate chemical or physical methods for excluding oxygen. We have also developed efficient methods for plating this bacterium that do not require the use of anaerobic incubators.

Clostridium acetobutylicum produces significant amounts of butanol, among other compounds, from various sugars. It is likely that growth of the organism is finally inhibited by the butanol produced. Our membrane based techniques now allow us to do quantitative mutagenesis studies with the organism and we are currently attempting to isolate butanol-resistant mutants. We hope that at least some of these will produce significantly greater quantities of butanol, ethanol, acetone, hydrogen, and other products of interest.

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GENETICS OF REPAIR OF RADIATION DAMAGE TO DNA IN BACTERIA

D. Billen C. T. Hadden

The goal of this project is to study the consequences to bacterial DNA of damage by radiation and chemical agents. By correlating the extent of

physical and biological damage to DNA, as expressed in various mutants defective in specific DNA repair pathways, we hope to determine mechanisms of biological inactivation of DNA and ways in which the damage can be repaired.

We have measured physical damage to DNA in Bacillus subtilis and Escherichia coli by use of alkaline sucrose gradient centrifugation, which indicates the distance between breaks or alkali-labile lesions in single strands of DNA. Biological damage is measured by loss of viability or by loss of transforming activity in treated DNA from B. subtilis, and by the production of sites for DNA repair synthesis by DNA polymerase I (Pol I) in toluene-treated E. coli. We have investigated effects of ultraviolet light (both far-UV and near-UV), ionizing radiation, and selected chemical agents, in the presence or absence of sensitizing or protective agents.

In studies of E. coli involving both alkylating agents and X-rays, we observed that the number of DNA single-strand breaks measured in alkaline sucrose gradients usually increased with dose along with repair synthesis. We measured the repair of X-ray induced breaks and found that only about one-half were repaired, even when ligase was maximally active. It appears that these non-repaired sites do not serve as primer ends for PolI-dependent repair synthesis in toluene-treated cells (1). Radioprotective agents that reduce the number of strand breaks also reduce the PolI-dependent repair synthesis. This radioprotection does not seem to be correlated directly with the OH radical scavenging ability of the protective agent. Therefore both the single-strand breaks and the resulting repair synthesis may result primarily from direct rather than indirect damage to the DNA.

Similarly, when toluene-treated E. coli whose DNA contained bromouracil in place of thymine were irradiated with ultraviolet light, both single-strand breaks and primer sites for PolI-directed repair synthesis were produced. However, the presence of cysteamine during irradiation reduced the number of strand breaks without significantly changing the amount of repair synthesis. This selective protection may represent a reduction in kinds of DNA damage that are not readily repaired in vivo.

A major goal was to characterize DNA repair processes in vivo in B. subtilis. A number of radiation-sensitive mutants were studied, with the result that we have learned a great many details about the repair of DNA in UV-irradiated cells:

(a) In experiments with E. coli, Ganesan showed that during post-replication repair of UV-irradiated cells pyrimidine dimers appear in the newly replicated DNA and are diluted by subsequent growth and recombination. We have shown that this kind of exchange occurs also in B. subtilis (3), and that in Rec$^-$ mutants it is correlated with ability to be transduced but not with transformability (2). In this system it is possible to follow the repair by excision and by recombinational exchange of about 50 pyrimidine dimers per genome, or about 10^{-5} lesions per base.

(b) Repair of UV-irradiated transforming DNA in several strains of B. subtilis was studied in order to determine the effects of excision repair and postreplication repair on transformation (4). Integrated DNA is apparently repaired by both mechanisms in wild-type cells and in uvr-1 recipients, although the latter excise only small numbers of pyrimidine dimers. In uvs-42 mutants, which are defective in incision at pyrimidine dimers, post-replication repair saves recipient cells from the lethal effects of integrated dimers, but the resulting recombination events greatly reduce the linkage between closely linked genetic markers. In a recG recipient, which does excision repair but not post-replication repair, linkage is altered only slightly, if at all, during repair of transforming DNA. Therefore the apparent reduction in size of integrated regions of UV-irradiated transforming DNA probably results mainly from post-replication repair of larger integrated regions.

(c) This system has been used to determine the relative contributions of post-replication repair and of the pyrimidine dimer excision system in repairing DNA damaged by some chemical agents. Of particular interest are compounds to which pyrimidine dimer excision mutants of E. coli are preferentially sensitive, e.g. 8-methoxypsoralen (8-MOP).

Upon irradiation with near-UV light (NUV), a mixture of 8-MOP and DNA will react so that both monoadducts and crosslinks are formed. We have found (5) that in B. subtilis the repair of 8-MOP monoadducts does not require the pyrimidine dimer excision system, although that system may be used if it is present. The repair of crosslinks does appear to require at least the first step in excision, which may be an endonucleolytic or a glycosylic attack on the affected base. There is no evidence that post-replication repair is involved in the repair of either monoadducts or crosslinks.

In E. coli pyrimidine dimer excision is a multi-step pathway in which the first step is an endonucleolytic cut at or near the pyrimidine dimer. In Micrococcus, however, the first step is a glycosylic cut that separates one of the damaged pyrimidines from its deoxyribose. Some properties of B. subtilis suggest that it may follow the Micrococcus pattern rather than that of E. coli. Both Bacillus and Micrococcus are Gram$^+$ whereas E. coli is Gram$^-$, and dimer excision is sensitive to caffeine in E. coli but not in Micrococcus or B. subtilis. We proposed to study the mechanism of this first step in B. subtilis by use of a monoadduct probe that is sensitive to the excision pathway. Our choice of 8-MOP as that probe was not highly successful because removal of 8-MOP adducts does not depend on the excision system. However, experiments are underway to characterize the 8-MOP excision product and to determine whether a specific product can be assigned to the dimer excision pathway.

(d) It has been shown that treatment of growing E. coli cells with low concentrations of alkylating agents results in the induction of an 0^6-methylguanine-DNA methyltransferase that removes methyl groups from the 0^6 position of guanine in DNA (Foote et al., Biochem. Biophys. Res. Comm. 97, 654, 1980). This transferase acts stoichiometrically and not catalytically (Robins and Cairns, Nature [London] 280, 74, 1979; Schendel and

Robins, Proc. Natl. Acad. Sci. USA 75, 6017, 1978). We have shown (Billen and Hellermann, J. Bacteriol. 137, 1439, 1979) that the same adaptive treatment increases the amount of Pol I-directed repair synthesis in toluene-treated cells.

We have now studied the induction of methyltransferase in _B. subtilis_ exposed to low concentrations of _N_-methyl-_N_'-nitro-_N_-nitrosoguanidine (MNNG). In collaboration with Sankar Mitra and R. S. Foote (Biology Division), we have shown that the basal level of methyltransferase in _B. subtilis_ is about ten-fold higher than in _E. coli_ and that there is about a ten-fold increase during adaptation. Along with the increase in methyltransferase activity there is a reduction of about ten-fold in mutagenesis by a high concentration of MNNG. Further experiments are in progress to determine whether induction of methyltransferase in competent populations has an effect on the biological activity of alkylated transforming DNA, and to study the specificity of reversion at nonsense and missense loci. These studies will provide fundamental data on the response of _B. subtilis_ to alkylation of DNA, a potential result of radiochemical reactions _in vivo_.

Our future studies will focus on the radioprotective effects of alcohols that act as OH radical scavengers but also react to irradiation by the formation of a radical on the carbon alpha to the hydroxyl. This alpha-hydroxy radical is hypothesized to react with irradiated DNA to reverse direct radiation damage to a base or sugar. These studies will help us assess the extent to which direct interaction of radiation with DNA rather than with other cell components is responsible for DNA damage _in vivo_.

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Toxicity Assessment

THE FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS (FETAX) - A NEWLY DEVELOPED SCREENING ASSAY FOR THE DETECTION OF TERATOGENS

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T. Wayne Schultz   Rhonda G. Epler

There is no question that a rapid and inexpensive screening tool is needed to assess potential teratogenicity. The now classical Ames or Salmonella test and others screen for potential mutagenicity and carcinogenicity. Although a number of relatively rapid bioassays, using test organisms ranging from the invertebrates through lower vertebrates to mammals, as well as cell, organ and embryo culture systems, have been used to examine teratogenicity, none has been adequately validated or widely accepted for routine use. Thus our laboratory has sought to develop and validate a model screening assay for teratogens. Our model, referred to as FETAX (Frog Embryo Teratogenesis Assay: Xenopus) has to date been validated with 25 known mammalian teratogens of varying potencies and applied to examine the relative teratogenic risk of a variety of chemicals and complex mixtures from coal-conversion and shale oil technologies. Their effects have been compared to those of similar materials derived from natural petroleums.

The FETAX Model. One reason the amphibian embryo is attractive as an assay system is that it has been and remains a classical model for experimental embryological studies. Unlike some of the other teratogenesis screens that use cell or organ cultures, the frog embryo is an intact developing system that undergoes events (e.g., cleavage, gastrulation, organogenesis) comparable to those of other vertebrates, including mammals.

Other attributes of the model which make it attractive are the following. (1) The assay is rapid, endpoints are routinely reached at 96 hr. (2) It is relatively inexpensive. (3) Methodologically, the protocol is simple. (4) Large numbers of embryos (3,000 to 5,000) are routinely available for testing from a single spawning. (5) The developmental stages are easily observed and scored. (6) Dose levels of teratogens are easily controlled. (7) Pulsed exposures to specific developmental stages are easy, as are prolonged exposures beginning at specific stages. (8) FETAX allows direct exposure of the embryo to either the teratogen or its metabolites and has the potential for in vitro metabolic activation by microsomal (S9) preparations. (9) The embryos are compatible with low concentrations of solubility vehicles including DMSO, propylene glycol, acetone, and alcohol. (10) The embryos are functional organisms. They are motile at 96 hr so that assessment of the functional state of the nervous and muscular systems can be made by simple observations of swimming ability. Prenatal development includes all stages prior to metamorphosis - stages when organ development and growth occur. Postnatal development can be considered to begin with metamorphosis and conclude with young adults in 2-3 months. (11) Finally, endpoints are easy to recognize and score. We routinely use the following: mortality,

anatomical deformities, growth, developmental stage attained, motility (behavior), and pigmentation. Despite concern that the jelly coat surrounding the embryo may act as a permeability barrier, our experience has been that it does not. In any case, the jelly can be easily removed by manual or chemical means.

A variety of endpoint data can be collected from the FETAX system. Those routinely obtained are outlined below.

1. The LC_{50} and LC_{100}, parameters which indicate embryolethality, and the EC_{50} and the EC_{100}, i.e., concentrations that induce terata in 50% (or 100%) of the surviving embryos are determined. Since the EC endpoints reflect abnormals among survivors, they represent a more realistic evaluation of teratogenesis.

2. The NOEC, i.e., no observable effects concentration, is also determined.

3. The developmental stage attained is determined against a standard reference table of development.

4. Growth (length) attained is determined by measuring the length of only normal-appearing embryos from various exposure concentrations. This method, which selects against observable terata, gives a more realistic estimate of normal growth.

5. Evaluations of motility (behavior) and pigmentation are made. These facets of development are related to neuronal control involving both the central and peripheral nervous systems. Thus, they provide clues to adverse effects on nervous and/or muscular system development and function.

6. Finally, gross anatomical observations are recorded. These observations allow evaluation of anatomical defects in major organ systems and are augmented by light and/or electron microscopy.

The endpoints enumerated provide a range of data that can be integrated into comparatively precise and detailed statements of relative potential teratogenic hazard. Other observations are possible including examinations for chromosomal damage as well as biochemical parameters such as RNA, DNA, and protein synthesis, and specific enzyme levels.

The above characteristics of FETAX make it attractive as a screen for teratogens. The usefulness of the system lies in its potential to establish relative teratogenic risks and thus set priorities for further testing in higher-level assays.

Teratogenic index. Teratogenic Index (TI) has been developed to permit comparisons of diverse compounds or mixtures with regard to their inherent teratogenic risks. The TI simply defines the relationship between the 96-hr LC_{50} and the 96-hr EC_{50}, i.e., between lethality and the number of surviving abnormal embryos. Thus, $TI = LC_{50}/EC_{50}$.

The Teratogenic Index makes it possible to relate teratogenic risk of highly toxic (lethal) materials to the risk of those which are relatively non-embryolethal. It allows for comparison between known and suspected teratogens. In this regard and for validation and standardization purposes, we have tested a series of 25 known mammalian teratogens with FETAX. Some of these materials, such as trypan blue, hydroxyurea, and retinoic acid are well established as 'blatent' mammalian teratogens. Others such as alcohol, saccharin, and aspirin are more subtle teratogens (or considered nonteratogenic). On the basis of TI's derived from these standardization compounds, we have established a TI value of 2.0 or greater as one which would indicate the need for further testing. TI values between 1.5 and 2.0 suggest materials that should be treated with suspicion and caution as potential teratogens and tested further in other assay systems. TI values below 1.5 reflect compounds which are more embryolethal, that is, are coeffective teratogens whose toxicity and teratogenicity are difficult to delineate and whose lethality may be more pertinent to risk assessment than teratogenicity.

Skeletal development. Older, premetamorphic embryos have been used to assess the effects of osteolath-genic teratogens (compounds that cause abnormal bone development). A series of eight of these compounds (thiosemicarbazide, semicarbazide, urea, thiourea, ethylene thiourea, ethylene urea, guanidine and aminoguanidine) have been used in an attempt to discover relationships between chemical structure and teratogenic activity. In this series of compounds the important molecular configuration appears to be the following association of atoms found in thiosemicarbazide, semicarbazide, ethylene urea and ethylene thiourea.

(S or O)

Thus the FETAX model, using older embryos, has the additional application of a short-term, inexpensive assay that responds to factors that affect skeletal development.

Application of the Model. The FETAX model provides a short-term, inexpensive assay that can be applied as a screen to a variety of materials ranging from pharmaceuticals to workplace hazards and industrial wastes. In addition, it is useful for monitoring aquatic environments.

Other characteristics of the model which make it applicable as a screening tool include: (1) It's relevant as an intact embryonic system including early cleavage stages through organogenesis. (2) Large numbers of embryos are available for testing. (3) The assay has the ability to discriminate between embryotoxic and teratogenic effects. (4) It displays quantitative dose-response relationships. (5) It has the potential for testing later premetamorphic events (i.e., skeletal development). (6) The model is adaptable to a variety of testing protocols.

To date, FETAX has been validated with 25 known mammalian teratogens. In each case the response, both qualitative and quantitative, has been distinct for each teratogen, i.e., different teratogens cause different abnormalities. We have used FETAX to assess a wide range of products and

by-products from coal conversion, shale oil retorting and natural petroleum refining processes. The data generated, especially the Teratogenic Index, are useful in ranking the relative teratogenic risks and in suggesting a priority for additional testing in higher level mammalian systems.

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CYTOTOXICITY OF FOSSIL FUEL RELATED PURE COMPOUNDS AND COMPLEX MIXTURES

T. Wayne Schultz James N. Dumont

The need for a time and cost effective means of screening or ranking the large number of industrial chemicals for their health effects has become the catalyst for recent renewed interest in quantitative structure activity relationships (QSAR) in ecotoxicology. QSAR permits the ranking of chemicals on the basis of meaningful correlations between their structure and their biological activity (e.g., toxicity). We have used a simple model system - the ciliate Tetrahymena pyriformis grown in axenic culture - to investigate QSAR among a variety of classes of pure chemicals known to be present in complex mixtures of materials obtained from several synfuel

industries. In addition, we have tested a number of the complex mixtures themselves. The endpoint of our assay reflects the ability of the test compound to impair growth (i.e., reproduction) of the culture. Using a large group of nitrogen heterocyclic compounds and a large group of napthalenes and their derivatives, the following relationships have been demonstrated. For dinitrogen compounds, increases in toxicity follow increases in methyl substitution, the number of aromatic rings, molecular weight, boiling point, number of carbon atoms and log of the partitioning coefficient (hydrophobicity). For the naphthalene compounds, their ability to accept or donate hydrogen and their hydropholic substituent constant have high correlations with toxicity. For complex mixtures, toxicity appears to be related to aromaticity and heteroatom substitution. The finding that hydrotreatment, which reduces both parameters, mitigates the toxic response supports this observation.

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LIFE SCIENCES SYNTHETIC FUELS PROGRAM

Coordinators	– J. L. Epler – R. J. M. Fry
Microbial Systems	– F. W. Larimer
Cytotoxicity and Embryo Toxicity	– J. N. Dumont – T. W. Schultz
Repair	– J. D. Regan
Mammalian Cells	– A. W. Hsie
Teratogenesis	– L. B. Russell – R. Filler
Mammalian Mutagenesis	– L. B. Russell – W. M. Generoso
Toxicology	– H. R. Witschi – L. H. Smith
Carcinogenesis	– J. M. Holland

A multi-divisional effort aimed at the integrated assessment of the health and environmental effects of various coal conversion and shale oil technologies is being carried out. The Biology Division's efforts are summarized below. Specific sites evaluated are the low Btu gasifier at the University of Minnesota, Duluth, the shale oil upgrading program, Paraho/SOHIO, and the coal liquefaction pilot plant (H-Coal) in Kentucky.

Statement of Problem. The principal objective of this project is to test the primary effluents and fugitive emissions from coal-conversion processes for their potential effects on man. The specific issues include (i) potential effects on man of process discharges to air or water, (ii) exploration of environmentally acceptable methods for disposal of solid waste produced, and (iii) determination of hazards associated with the handling and combustion of products.

Research Approach. Our approach to answering these questions, in an expeditious and cost-effective manner, involves a parallel, two-level program. Level one is cellular bioassays. These assays provide baseline data on typical effluents and emissions and ascertain how the relative toxicity of major effluents and fractions thereof vary as a function of changes in process conditions. In addition, biological effects studies using cellular assays will provide an essential data base for eventual determination of correlations with whole animal studies.

Level two consists of the mammalian toxicity and carcinogenicity bioassays. As data become available through analytical chemistry, area monitoring, and cellular bioassay programs, they can be used in guiding decisions concerning whether other materials should be tested and whether additional assays should be carried out in mammals.

With short-term mutagenicity assays, testing with only one microbial system has been shown to be faulty in a number of cases with pure compounds. We improve the reliability of information by using a battery of short-term genetic and/or DNA assays on the primary effluents and potential fugitive emissions. Thus, potential hazards from both the mutagenic and carcinogenic aspects will be more carefully "screened." Furthermore, some segments of the battery of assays are being used only on selected compounds determined by the coupled effort of chemical separation and analysis and initial biological screens. The actual components can then be characterized as either highly purified fractions or actual pure chemicals. Feedback to chemical screening then becomes a feasible monitoring method.

Following is the battery of tests used: (i) Salmonella histidine reversion (Ames test), (ii) mammalian cell and/or yeast gene mutation and recombination/conversion assays, (iii) DNA repair, and (iv) in vitro cell transformation. Only selected samples will be tested in the full battery of assays, depending largely on the preliminary results of the Ames screening and the chemical analyses. Cytotoxicity will be considered a normal component of the genetic/DNA screens; however, other assays that rapidly and specifically examine toxic effects on "cellular" systems will also be carried out with appropriate samples and selected active compounds.

The search for heritable genetic changes in mammalian germ cells is the most critical and definitive test for the assessment of genetic risk to human beings. Samples to be tested here must be carefully chosen and considerable progressive screening carried out before general application. The choice of samples to evaluate can be modified or number of samples increased in relation to the results of the cellular bioassays and/or chemical screening.

Five types of tests are available in the mouse system, two of which detect chromosomal damage and one the induction of point mutations. The fourth genetic test, the rapid "spot test," probably also detects point mutations but in somatic cells. The fifth mouse test is designed to be a sensitive indicator of germ cell killing by mutagens and of possible effects on reproductive physiology.

Initially it has been planned to include only dominant lethals and the spot test of the five mammalian tests in the comparative assessment of genetic risk from the low-Btu gasifier. The other assays (also including DNA repair systems) are currently being used in the generic approach with other fossil energy-related compounds and would interrelate with and reinforce the site-specific evaluations.

The preceding integrated program will provide the assessor with specific information on specific process materials. Information on the potential mutagenicity, carcinogenicity, and overall toxicity of the multiply tested materials can be compared with results from other gasifiers and other technologies. Such comparative information and published data on similar materials should permit an estimate of biohazard for each plant.

Mammalian Genetics and Teratology Section

SECTION OVERVIEW - L. B. RUSSELL

The work of this Section may be broadly classified into three areas: whole-mammal mutagenesis, basic genetics and cytogenetics, and the study of reproductive effects (including the gonads and developing embryos). There is a constant interplay between the basic and more applied aspects of the work, so that it is sometimes difficult to classify a given experiment. For example, the testing of specific chemicals can be carried out in such a manner as to contribute to method development or to our understanding of the detailed properties of the biological material; and the wealth of genetic variants that is generated in mutagenesis experiments provides models for human conditions, or can be used to increase our understanding of genetic expression and of the organization of the mammalian genome, which, in turn, can generate tools for studies in mutagenesis.

During this reporting period, method development and validation have been successful in areas as diverse as dominant-mutation screening, detection of chromosomal damage, prescreening for forward mutations, statistical formulations, direct measurements of DNA damage in reproductive cells, and finding indicators of teratogenicity.

- Dominant skeletal mutations, which are important for risk analysis because they provide a measure of genetic damage that is directly relatable to the human situation, can now be more rapidly screened for than before as a result of the successful development of three non-breeding-test methods, which are being validated in radiation and ethylnitrosourea experiments.

- Reciprocal translocations, which are the most prevalent and potentially most harmful of transmissible chromosomal rearrangements have been scored by two methods in parallel; one of these (test of heritability) was shown to be superior to the other (cytological analysis in the exposed male).

- For the detection of transmissible forward mutations, the specific-locus test (SLT) remains the most efficient method.

- Use of a multiple-decision statistical procedure led to the development of a grid of critical sample sizes that allows rapid evaluation of SLT results as positive, negative, or inconclusive.

- The mouse spot test, an *in vivo* somatic prescreen, has been shown, during this reporting period, to produce results related not only qualitatively but quantitatively to those of the SLT.

- Several of the whole-mammal mutagenicity tests have been evaluated for national and international committees, and protocols have been formulated to govern world-wide use.

- Considerable progress has been made in developing direct measures of DNA damage in the gonad. Thus, studies of unscheduled DNA synthesis have shown a good correlation between unscheduled DNA synthesis and SLT responses; and an alkaline-elution test for sperm DNA shows promise of measuring single-strand breakage in exposed mice and perhaps men.

- An in vitro prescreen for teratogenicity has been developed by demonstrating that induced restrictions in the developmental potential of embryonal carcinoma cells are well correlated with more direct measures of developmental damage. The importance of working with carefully defined critical periods (as in the homeotic-shift test) was illustrated by data analysis.

In the course of the method-development and validation studies as well as in direct experiments, a number of substances were tested for genetic or reproductive effects. Among these were ethylene oxide, Dichlorvos, Triclosan, benzo[a]pyrene and some coal-technology-related mixtures, ethylnitrosourea and the related compounds methylnitrosourea, ethylnitrosourethan, hydroxyethylnitrosourea, and several chemicals on the FDA GRAS (generally recognized as safe) list.

Several of the investigations are contributing information about the biological system and about the nature of its interactions with environmental agents. We have always considered an understanding of the kinetics of reproductive-cell formation and renewal to be essential to the interpretation of genetic data. This understanding is now being refined by the study of age effects and of circadian rhythms on cell-cycle properties, and by the analysis of delayed responses to chemically-induced germ-cell death. The nature and condition of the target cell strongly governs the type of genetic effect observed. Perhaps the most striking feature of the chemical mutagenesis results obtained with the mouse specific-locus method is that they have revealed the great complexity inherent in mammalian germ-cell responses. For example, the mutation frequencies in different germ-cell stages vary greatly, and the pattern of relative frequencies changes markedly with different chemicals. Furthermore, these drastic changes can be seen among closely related chemicals such as ethylnitrosourea and methylnitrosourea. Thus, many of the generalizations made in the past on the basis of results from simpler systems are shown to be untenable.

Another special feature of the mammalian germ-cell system (and, perhaps, specifically of spermatogonial stem cells) appears to be the capability to repair genetic damage from even a highly effective mutagen, such as ethylnitrosourea, if the dose is low. This conclusion was made possible by our coordinating genetic studies with molecular dosimetry experiments which showed that alkylations in the testis are proportional to

injected dose. Work in the latter area has now provided exciting indications that the ratio of O^6 to N^7 ethylations of guanine may be different at high and low doses of ENU.

In the area of chromosome aberrations, also, we have progressed in our understanding of the interactions of mutagens with the biological material. Several lines of evidence have led to the hypothesis that the kinds of chromosomal aberrations produced depend on the longevity of the critical alkylation product and the length of the interval between the formation of this product and sperm entry into the egg. Molecular dosimetry studies have shown that protamine-, rather than DNA-alkylation, may be the cause of chromosome losses induced by chemicals that react by an S_N2-type mechanism, the predominant alkylation site being the sulfur group of cysteine. The response to S_N1-type chemicals is different.

Finally, in the developing embryo, it was shown that an endogenous mixed-function oxidase system becomes operative as early as the blastocyst stage.

Many of the findings already mentioned, and some others, have a strong bearing on the area of genetic risk assessment. For example, the complexities of the responses of different germ-cell stages, the very different patterns obtained with closely related chemicals, and the capability of spermatogonial stem cells to repair genetic damage from some chemicals at low doses, all indicate that, for some time to come, each potentially hazardous chemical will have to be tested in the mouse, regardless of its similarity with other chemicals already tested, and regardless of its response in other mutagenesis systems. Our radiation data, too, have a strong bearing on risk assessment. In extensive experiments, we have now shown that at 0.8 R/min and below (to as low as 0.0007 R/min), mutation frequency is independent of dose rate. Qualitative analysis of mutations has indicated that the size of lesions produced, and (probably correlated with it) the proportion of lesions causing dominant effects, are larger for radiation than for ENU.

Among the findings in basic genetics and cytogenetics are several that increase our understanding of the meiotic process. Thus, the presence of the synaptonemal complex in preleptotene spermatocytes indicates that crossing over occurs by a copy-choice mechanism. Synaptonemal complex studies of several rearrangements (produced as by-products of mutagenesis experiments) show a good mapping correlation between meiotic and mitotic chromosomes and shed light on such problems as nonhomologous pairing in synaptic adjustment, and the length of the X-Y pairing segment. Experimental studies on genetic male sterilities resulting from certain translocations have eliminated several explanations of the cause of such sterilities. A series of valuable X-autosome translocations has been genetically and cytologically characterized and the results have shed further light on X-inactivation mechanisms as well as making these translocations increasingly attractive as tools for studies on gene action and genetic organization. Finally, detailed analysis of large numbers of mutations recovered in specific-locus experiments has identified vital and non-vital loci in certain chromosomal regions, has provided information on

genetic expression in mammals, has yielded tools for construction of extensive gene-dosage series, and is contributing answers to some of the pragmatic questions in germ-line mutagenesis. It has also indicated that spontaneous mutations have an admixture of types not present in populations of induced mutations and that this may pose problems with the use of doubling-dose calculations. Certain mutants, e.g., those affecting the skin or skeleton, are providing animal models for human conditions.

Mutagenesis

SPECIFIC-LOCUS MUTATION FREQUENCIES IN MOUSE STEM-CELL SPERMATOGONIA AT VERY LOW RADIATION DOSE RATES, AND THEIR USE IN THE ESTIMATION OF GENETIC HAZARDS OF RADIATION IN MAN

W. L. Russell   E. M. Kelly

Experiments were undertaken to augment the information on the lowest radiation dose rates feasible for scoring transmitted induced mutations detected by the specific-locus method in the mouse. This is the type of information most suitable for estimating genetic hazards of radiation in man. The results also aid in resolving conflicting possibilities about the relationship between mutation frequency and radiation dose at low dose rates.

There was no statistically significant difference between mutation frequencies obtained in spermatogonia with 300 R of γ radiation at two different dose rates, 0.005 and 0.0007 R/min, or between either of these frequencies and data obtained earlier at dose rates of 0.8 R/min and below. This refutes the possibility (Nature 238: 101-104, 1972) raised by Lyon et al. that as dose rate decreases below about 0.03 R/min, there may be an increase in mutation frequency. Even at the lower dose rate, the mutation frequency is significantly higher than in both the contemporary and historical controls. Thus, the data do not support the suggestion raised by both Newcombe and Abrahamson (Health Phys. 25: 105-107, 1973) that there might be a beneficial effect of radiation at low dose rates. The results do support the view in an earlier publication by one of us (W. L. R.) that, at approximately 0.8 R/min and below, mutation freguency is independent of dose rate. Because this independence is now shown to extend over the more than 1000-fold range from 0.8 to 0.0007 R/min, it seems likely that it would hold at still lower dose rates, perhaps even to the much lower dose rates encountered in most human exposures.

The new data have permitted a fresh review of the information on mutation induction at all dose rates in spermatogonia. Simple mathematical models are used to compute (a) a maximum likelihood estimate of the induced mutation frequency at the low dose rates, and (b) a maximum likelihood estimate of the ratio of this to the mutation frequency at high dose rates in the range of 72 to 90 R/min. In the application of these results to the estimation of genetic hazards of radiation in man, the former value can be

used to calculate a doubling dose - i.e., the dose of radiation that induces a mutation frequency equal to the spontaneous frequency. The doubling dose based on the low-dose-rate data compiled here is 110 R. The ratio of the mutation frequency at low dose rate to that at high dose rate is useful when it becomes necessary to extrapolate from experimental determinations (e.g. from skeletal or cataract mutation frequencies), or from human data, at high dose rates to the expected risk at low dose rates. The ratio derived from the present analysis is 0.33.

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DOSE-RESPONSE CURVE FOR ETHYLNITROSOUREA-INDUCED SPECIFIC-LOCUS MUTATIONS IN MOUSE SPERMATOGONIA

W. L. Russell — M. H. Steele
P. R. Hunsicker — K. F. Stelzner
G. D. Raymer — H. M. Thompson

The results from ethylnitrosourea (ENU) have provided the most extensive dose-response curve yet obtained for induction of specific-locus mutations in mouse stem-cell spermatogonia by any chemical. Seven doses, ranging from 25 to 250 mg/kg of body weight were tested, and after allowing for clustering, at least 120 independent mutations were scored in a total of 59,483 offspring. In the lower portion of the curve, below a dose of 100 mg/kg, the data fall statistically significantly below a maximum-likelihood fit to a straight line. Independent evidence, described in the next section, indicates that, over this dose range, ENU reaches the testis in amounts directly proportional to the injected dose. It is concluded that the spermatogonia are capable of repairing at least a part of the mutational damage when the repair process is not swamped by a high dose.

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EFFECT OF DOSE FRACTIONATION ON THE ETHYLNITROSOUREA INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN MOUSE SPERMATOGONIA AND ITS BEARING ON RISK ESTIMATION

W. L. Russell
P. R. Hunsicker
D. A. Carpenter
C. V. Cornett
G. M. Guinn

In order to estimate what happens at doses lower than those used in the dose-response curve, it was necessary to resort to a fractionation experiment. The specific-locus mutation frequency from a single dose of 100 mg/kg of ENU was compared with that from a total dose of 100 mg/kg fractionated into doses of 10 mg/kg injected at weekly intervals. The induced mutation frequency with the fractionated dose was only 13% of that with the single exposure, and the difference was statistically highly significant ($P < 1 \times 10^{-9}$).

Carricarte and Sega of this Section have measured the amount of unscheduled DNA synthesis occurring in mouse spermatids after intraperitoneal injection of ENU. Over the range from 100 to at least 10 mg/kg, and possibly lower, unscheduled DNA synthesis is directly proportional to the amount of ENU injected. In an additional study, Sega has shown that DNA ethylation in the testis after intraperitoneal injection of tritium-labeled ENU is directly proportional to injected dose over the range of 10 to 100 mg/kg. These findings demonstrate that the decrease in mutation rate below linearity in this range of doses, as described in the previous section, and the reduced mutagenic effect when the 100 mg/kg dose is fractionated, are not due to failure of the chemical to reach the testis in proportionate amounts. The obvious conclusion is that, at a dose of 10 mg/kg, the spermatogonia have the capacity to repair a major part of the genetic damage induced by ENU.

The existence of a repair mechanism against the most effective mutagen known in the mouse suggests that the repair capacity of spermatogonia may have a more general action, perhaps against other mutagens. This view is supported by the fact that many chemicals that are potent mutagens in other biological systems have given low or zero mutation frequencies in mouse spermatogonia, even when it is known that they or their active metabolites reach the testis. This demonstration of an effective repair mechanism in spermatogonia suggests that the genetic risk to humans from some chemicals that have been proved to be mutagenic in lower organisms, or even in mammalian somatic cells, may be considerably less than previously predicted.

It remains to be tested whether repair of ENU-induced damage might be even more effective at still lower doses. The possibility of a threshold dose is not excluded, although it should be kept in mind that this apparently does not occur with radiation-induced mutations in spermatogonia.

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EFFECT OF SEX AND CELL STAGE ON INDUCTION OF MUTATIONS BY ETHYLNITROSOUREA IN THE MOUSE

W. L. Russell E. L. Phipps
P. R. Hunsicker

For the estimation of genetic risk it is important to know the relative mutational sensitivities of the various germ-cell stages. The data now available for ENU show that, at doses of 100 mg/kg and above, postspermatogonial stages and mature and maturing oocytes are giving mutation rates approximately one order of magnitude lower than those obtained from spermatogonia. It should be noted, however, that even with the dose as low as 100 mg/kg to males, no offspring were recovered from matings made during the sixth and seventh weeks post-injection. So, for cells that were treated as differentiating spermatogonia or early spermatocytes, the mutational sensitivity to ENU is still not known. From exposure of arrested oocytes, scored in conceptions occurring more than six weeks after injection, only one mutation has been observed in over 10,000 offspring, a figure not significantly different from the control. Thus, so far, it appears that the major genetic hazard from ENU would lie in exposure of the stem-cell spermatogonia.

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NATURE OF SPECIFIC-LOCUS MUTATIONS INDUCED BY ETHYLNITROSOUREA IN MOUSE SPERMATOGONIA

W. L. Russell J. W. Bangham
P. R. Hunsicker S. C. Maddux

The frequency distribution among the seven loci of the mutations obtained from ENU in spermatogonia is strikingly similar to that observed for X and gamma irradiation of spermatogonia, with one exception. With the chemical or with irradiation, the mutation rate is relatively low at the a and se loci, intermediate at the c locus and high at the b, d and p loci. With radiation, however, the frequency is highest of all at the s locus, whereas with ENU the frequency at s is low, not much higher than that at the a and se loci. In view of the evidence presented below that the ENU-induced mutations may be primarily gene mutations, the general correspondence of the distribution among the loci with that for irradiation of spermatogonia provides some support for the view that the radiation-induced mutations may also be primarily gene mutations. This argument is based on the observation that when deficiencies are known to constitute a larger proportion of the mutations scored, as is the case with X irradiation of postspermatogonial stages or neutron irradiation of spermatogonia, then the distribution among the loci is noticeably changed.

More than 25% of the mutations induced in spermatogonia by ENU have a phenotypic expression intermediate between that of the viable null allele and wild-type. This indicates that at least this proportion of the mutations are probably intragenic changes. That the proportion could be much higher is supported by the following two facts. First, there have been no occurrences of d-se deficiency, a type of event that is not uncommon when the conditions of a radiation experiment are known to result in deletions. Second, at five of the seven loci the proportion of mutations that are lethal in homozygous condition is very low, less than 5%. From the above results, it is clear that there is no validity to the earlier speculation by others that our specific-locus method might be detecting only deficiencies and not gene mutations.

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METHYLNITROSOUREA-INDUCED SPECIFIC-LOCUS MUTATIONS IN THE MOUSE

W. L. Russell D. A. Carpenter
P. R. Hunsicker

Methylnitrosourea (MNU) is much more toxic than ENU, so we have had to limit our injected amounts to 80 mg/kg or less. Because MNU is a powerful mutagen in other biological systems, including Drosophila, and in some cases is much more potent than ENU, it was surprising to find that it has little or no mutagenic effect in mouse spermatogonial stem cells. Following injections of 70, 75, or 80 mg/kg (weighted mean dose 74 mg/kg), only three mutations have been obtained, to date, in 21,079 offspring from cells treated in spermatogonial stem-cell stages. This is only slightly, and not significantly, above the control mutation frequency. A comparable dose of ENU would have given about 41 mutations in an equivalent number of offspring. From research in our section — dominant-lethal studies by Generoso, the results of Sega et al. on unscheduled DNA synthesis and on molecular dosimetry, and from the data presented in the next paragraph, it is abundantly clear that MNU reaches the germ cells in the testis in active form.

Testing MNU for its mutagenic effect in post-stem-cell stages produced another surprising result. With a dose of 75 mg/kg to the male parents, the offspring conceived within the first five weeks after injection showed only a low mutation rate. However, offspring conceived in the following week had an exceptionally high mutation frequency that was totally unexpected. In 2,827 offspring, 18 mutations have been scored. This, with a dose of only 75 mg/kg, is similar to the mutation frequency obtained with 250 mg/kg of ENU in spermatogonia. According to Oakberg's timing of spermatogenesis, offspring conceived in the sixth week after injection probably came from cells exposed as differentiating spermatogonia or preleptotene spermatocytes. As was pointed out earlier, the mutagenic effect of ENU on these particular stages is not yet known.

Regardless of that outcome, it is apparent, from the low mutagenic response of stem-cell spermatogonia to MNU, that results with ENU were not predictive of what would happen with MNU.

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DEVELOPMENT OF METHODS AND CRITERIA FOR DECIDING WHETHER SPECIFIC-LOCUS MUTATION-RATE DATA INDICATE A POSITIVE, NEGATIVE, OR INCONCLUSIVE RESULT

Paul B. Selby W. H. Olson[3]

The binomial approximation of the UMPU (uniformly most powerful unbiased) test for the equality of 2 binomial proportions was shown to be an easily applied and extremely accurate method for testing the hypothesis that a given mouse specific-locus mutation frequency is no higher than the spontaneous mutation frequency. Critical sample sizes were calculated, using the Fisher exact test, to show at a glance whether $P < 0.05$ for comparisons with the spontaneous male mutation frequency of 43 mutations in 801,406 offspring. Agreement with the critical sample sizes calculated using the binomial approximation of the UMPU test was perfect in three of the five comparisons made [$f_{(1)}$ through $f_{(5)}$], and the critical sample size was only off by one for the others.

The first hypothesis that the mutation frequency (induced + spontaneous) of treated mice is not higher than the spontaneous mutation frequency was combined with the second hypothesis that the induced mutation frequency of treated mice is no less than 4 times the historical-control mutation frequency to produce a multiple decision procedure with 4 possible decisions: inconclusive result, negative result, positive result, and weak mutagen. Critical sample sizes for the second hypothesis, also with $P < 0.05$, were combined with those for the first hypothesis into a grid that permits rapid evaluation of data according to these criteria. The justification for using these criteria in reaching decisious is the practical necessity of rapidly determining which chemicals are potent mutagens. These criteria are, of course, only reasonable if a high level of exposure has been given.

The grid shows that positive results can become apparent in relatively small samples; however, at least 11,166 offspring are required to obtain a negative result. If samples of 18,000 are routinely collected in specific-locus experiments on males (unless positive results are found earlier), 75% of tests of chemicals that are non-mutagens will give a negative result. If the question under consideration is not whether a chemical induces specific-locus mutations in male germ cells but, rather, whether the exposure received by humans causes any important risk from gene mutations, a much smaller sample size may be acceptable under certain conditions.

The criteria and the grid described above were applied by the GENE-TOX Work Group on The Mouse Specific-Locus Test.

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THE MOUSE SPOT TEST: EVALUATION OF ITS PERFORMANCE AS A PRESCREEN

Liane B. Russell C. S. Montgomery
G. M. Guinn

The Mouse Spot Test (MST) is an *in vivo* mammalian assay that can detect a considerable array of genetic events affecting a set of marked loci. It thus provides indicators of genotoxicity germane to predictions of human health hazards that arise from either heritable or somatic mutations.

Although some common loci are used in the MST and the specific-locus test (SLT), the array of scorable genetic events is broader in the MST. To determine the degree of success with which the MST, a somatic test, predicts the induction of heritable damage, quantitative comparisons were made between the MST and the SLT by calculating "unit" mutation rates (rates per locus per mole of exposure). In 26 of 27 comparisons, the "unit" rate for the MST was higher than that for the SLT. When both tests had yielded clearly positive results, the MST/SLT ratio was of the order of 1 to 10 (usually 1 to 5). Future MST results can thus be used with some confidence to make broad quantitative predictions about upper limits for SLT frequencies. Priority for initiating SLTs should be low when the MST is clearly negative, and should be high when the MST "unit" mutation rate is high. There are several possible reasons (e.g., special repair capabilities in germ cells) why an SLT might occasionally be negative when the MST is positive. In these and other cases, the SLT provides the more pertinent data for assessment of risk from heritable point mutations, the MST being merely the prescreen.

The MST has ancillary capabilities for directly detecting embryotoxicity, teratogenicity, and cytotoxicity. The occurrence and magnitude of such effects shows no clear correlation with mutagenic potency. In limited comparisons between MST results and those from carcinogenicity bioassays, the MST sensitivity was 81.3%, but no negative carcinogens have as yet been tested in the MST.

Because of its capabilities, the MST, developed at Oak Ridge, is now receiving increasing use in other laboratories, especially in Europe and Japan. Since some of the several types of spots scorable in the test are not due to genetic changes at the marked loci, there has been some confusion in the reporting of results, and it has become important to provide detailed discussions of the method and to prescribe protocols. This was done through (a) a GENE-TOX panel (chaired by one of us), (b) a small international workshop, and (c) a chapter in a handbook of

mutagenicity test procedures. The GENE-TOX panel also performed a thorough analysis of all MST results published to date.

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INVESTIGATION OF THE SUPERMUTAGEN ENU AND OTHER SUBSTANCES IN THE MOUSE SPOT TEST

Liane B. Russell C. S. Montgomery

Because ethylnitrosourea (ENU) was found by W. L. Russell to be the most efficient mutagen yet encountered in mouse spermatogonial stem cells, we investigated its ability to induce somatic mutations in the mouse spot test (MST). ENU was also compared with the chemically related compounds methylnitrosourea (MNU) and ethylnitrosourethane (NEC). We found that ENU readily induces recessive spots (RS) and, due to its low toxicity, can be applied at relatively high doses. This combination of properties makes it the most efficient spot-test mutagen, as shown in a comparison with 16 other chemicals, even though, on the basis of molarity, it is not the most potent one. The ENU mutation frequency in cells at risk, calculated per locus, per unit of applied dose, is roughly similar for melanocyte precursors (in the spot test) and spermatogonial stem cells (in the specific-locus test). MNU which, due to its high embryotoxicity, could be tested only at a low dose, is clearly mutagenic, and dose extrapolations indicate it to be more potent than ENU. (However, as yet incomplete results at an even lower dose of MNU indicate less mutagenicity per mole.) NEC, though it could be tested at higher molarities than ENU, is only weakly mutagenic.

The spot test, in addition to mutational data, also yields information on cytotoxicity (white midventral spots), embryotoxicity, and teratogenicity. The toxicity and teratogenicity findings parallel earlier results in

the rat. For all endpoints studied, ENU is more effective than NEC. Relative to MNU, ENU is less toxic, less teratogenic, and less mutagenic in the spot test; but it is much more carcinogenic (transplacentally) and more mutagenic in spermatogonial stem cells. We propose that MNU is more effective in inducing gross chromosomal damage than is ENU, while ENU induces relatively more gene mutations. The spot test scores both types of mutational damage, while mostly the latter type is recovered from spermatogonia.

The MST was also used to test Triclosan, a chlorophenoxyphenol present in several commercial products. Although the dose range tested overlapped the toxic, the frequency of presumed somatic mutations was not significantly greater in the experimental groups than in the methanol-injected controls; and the results rule out with 95% confidence a spot incidence 5 or more times greater than the control incidence.

During the reporting period, we also carried out or completed tests of various coal-technology-related substances by means of the MST, specifically Coal Liquid No. 2 (SRC process) and ESP Tar No. 83 (UMD low-BTU gasifier). Both were negative for mutational effects but probably positive for cytotoxicity and/or teratogenicity.

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FURTHER DEVELOPMENT OF TECHNIQUES FOR STUDYING INDUCTION OF DOMINANT SKELETAL MUTATIONS

P. B. Selby   T. W. McKinley, Jr.
G. D. Raymer

The breeding-test method for studying induction of dominant skeletal mutations was employed by us in an earlier experiment, the results of which have been applied by international and national committees in estimating genetic risk from radiation. The breeding-test method permits proof of transmissibility and the setting up of valuable mutant stocks, but it is slow and tedious and requires a high level of training for observers. As a result, emphasis has been put on developing a much more rapid method that could be used in a wider variety of applications. Three non-breeding-test methods have been developed to date, these being the sensitive-indicator method, the multiple-anomaly inferential method, and the mutational-index method. All three of these methods can be applied in studying a single sample of mouse skeletons. The total time required for all three methods

is less than one-tenth of that needed for the breeding-test method, and observers require much less training.

All three of the non-breeding-test methods are based upon information gained about normal variability and mutation-caused variability in our earlier large breeding-test experiment. Both the sensitive-indicator and multiple-anomaly inferential methods permit the identification of presumed dominant skeletal mutants. The first method identifies mutants by the presence of any one of 12 specific malformations (each termed a sensitive indicator) and the second does this by the presence of two or more rare (rare = less than 1 in 400 in control) major defects that do not seem to result from one accident of development. Alternatively, by the second method, a mouse can be concluded to be a presumed mutant if there is a single extremely rare major abnormality that is bilateral. These two methods together identify a large fraction of the serious dominant skeletal mutations that are induced, even though only parts of the alizarin-stained skeleton (anterior to the hips) are examined routinely. It seems certain that all but a small proportion of presumed mutants are actually mutants. To avoid bias, skeletons are coded until after classification.

The mutational-index method provides an almost entirely independent means of determining whether dominant skeletal mutations have been induced. Skeletons of control and experimental animals are examined for eight specific categories of anomalies termed index anomalies. The likelihood that a mouse with any one of the index anomalies is a mutant is not as high as the likelihood that a mouse with a "presumed mutation" is a mutant. However, the total frequency of index anomalies has been found to be useful for making comparisons between different samples. A statistically significant increase in the index of mutation over that in the control is not expected unless dominant mutations are being induced by a treatment.

The three methods described provide a much more rapid means than the breeding-test method of both assessing whether a given treatment induces dominant mutations and getting a measure of the amount of damage induced. Some of the results to date are described in the three reports that follow. One major advantage of these methods is that they can detect phenotypic damage caused by dominant mutations that have such low penetrance that it would be impossible to demonstrate their presence using the breeding-test method. If a treatment is found to induce a high frequency of dominant skeletal mutations, a follow-up experiment may be performed, using the breeding-test method, in order to collect interesting mutants and to determine what fraction of presumed mutations can be shown to be transmitted.

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STUDIES ON THE INDUCTION OF DOMINANT SKELETAL MUTATIONS BY RADIATION

P. B. Selby G. D. Raymer
T. W. McKinley, Jr. S. K. Lee

The non-breeding-test methods are being employed in analyzing the induction of dominant skeletal mutations by several radiation treatments. Our two-fold purpose is (a) to test the methods, and (b) to determine how well specific-locus mutation-rate results predict the amount of induced damage. Male mice of the inbred 101 strain were irradiated with either 300 R, 600 R, or 100 R + 500 R (24 hr between fractions) of acute (85-93 R/min) X radiation. Other male mice of this strain were irradiated with 600 R of gamma radiation delivered at 0.005 R/min (chronic). The control frequency of presumed mutations is 5/1599. In the experimental groups, only results on stem-cell spermatogonia are reported here. The presumed mutation frequencies in the 300-R acute, 600-R acute, 100 R + 500 R, and 600-R chronic groups were 0/388 (0%), 10/732 (1.4%), 7/448 (1.6%), and 2/178 (1.1%), respectively. In both the 600-R acute and 100 R + 500 R groups, the frequencies of induction are statistically significantly higher than the control frequency.

The indices of mutation (experimental minus control) in the control, 300-R acute, 600-R acute, 100 R + 500 R, and 600-R chronic experiments are 0%, 0.7%, 2.0%, 4.4%, and 2.7%, respectively. The indices at 600-R acute and 100 R + 500 R are both statistically significantly higher than the control, and the index at 100 R + 500 R is statistically significantly higher than that at 600-R acute. The incidence of index anomalies (from which the index of mutation is calculated by subtracting the control incidence) is much higher than the frequency of presumed mutations; for example, in the control there were 47 index anomalies, but only 5 presumed mutants, among 1599 offspring. The index in the chronic experiment drops to 1.1% when a correction is made for an apparently preexisting mutation that caused several occurrences of one index anomaly among the offspring of one male.

Although the samples are still small, it is interesting that so many significant differences have already been found. The results fit quite well with expectations from specific-locus results as to the relative responses for the different treatments. This strengthens the assumption made by committees estimating genetic risk that our estimate of dominant damage collected in a 100 R + 500 R breeding-test experiment could be extrapolated to expectations for low-level exposures by using multiplication factors determined in specific-locus studies. With regard to risk estimation, the most important dose group in our current experiments is the 600-R chronic exposure. High priority will be given to building up the size of that group in order to obtain a reliable estimate of damage.

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ETHYLNITROSOUREA IS ALSO A SUPERMUTAGEN FOR DOMINANT SKELETAL MUTATIONS

P. B. Selby G. D. Raymer
S. S. Lee T. W. McKinley, Jr.

The non-breeding-test methods have been used to study induction of dominant skeletal mutations by ethylnitrosourea, a chemical which was found by W. L. Russell and co-workers to be a supermutagen for inducing specific-locus mutations. Mutation induction was studied in spermatogonial stem cells for two exposure levels, 150 mg/kg and 250 mg/kg (i.p. injections). Because of a severe effect of ENU on fertility, only 8 offspring were collected at the higher exposure. The presumed mutation frequencies in the 150 mg/kg and 250 mg/kg experiments were 7/331 (2.1%) and 0/8, respectively. The first frequency is statistically significantly higher than the control frequency of 5/1599 (0.3%). Indices of mutation and the adjusted proportions of index anomalies (shown in parentheses) were 6.1% (30/331) and 35% (3/8). In both cases, the indices are statistically significantly above 0. In fact, the index at 250 mg/kg is statistically significantly higher than that obtained in any other experiment to date, and the index at 150 mg/kg is statistically significantly higher than that in any non-ENU experiment, with the sole exception of the 100 R + 500 R X-ray experiment.

These ENU experiments, together requiring less than one person-month of labor, provided the first demonstration that ENU induces serious dominant disorders. Results to date suggest that a smaller fraction (roughly one-half as many) of the mutations induced by ENU may be deleterious than of those induced by low-LET ionizing radiation. Larger experiments are planned to improve the accuracy of such estimates. This is important to pursue, because the commonly used doubling-dose approach for estimating genetic hazard assumes that an equal fraction of spontaneous and induced mutations is deleterious, regardless of the mutagen. If the fraction of induced mutations that is serious is found to be strongly dependent on the mutagen, methods for estimating genetic risk will have to rely on measures of dominant damage even more heavily than is thought at present.

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TEST OF DICHLORVOS TO SEE IF IT INDUCES DOMINANT SKELETAL MUTATIONS

P. B. Selby G. D. Raymer
T. W. McKinley, Jr.

Dichlorvos is a widely used chemical that has been shown to be a mutagen in a few short-term tests. It had not been tested for gene-mutation induction in mammalian germ cells. Our primary interest in this chemical stems from its usefulness in eradicating mites. Although some strains of mice do not seem to be affected much by mites, which are common parasites in mouse colonies, the C3Hf strain, which is used in skeletal experiments, is adversely affected. For this reason, in early 1980 we used dichlorvos to eradicate mites from the stocks of mice used in skeletal experiments. Before using dichlorvos routinely to exterminate mites from our colony, it was essential to determine whether this chemical induces enough mutations to interfere with our experiments.

In this application of the non-breeding test methods, both C3Hf-strain females and 101-strain males were exposed continuously for 80 days to one-third of a resin strip impregnated with dichlorvos (Johnson Wax BOLT brand) placed on top of each cage. The total exposure was at least 20 times higher than that which is sufficient to exterminate all mites. A concurrent control was also set up. Mice were distributed at random from our production stocks to the experimental and control groups.

Animals in the exposed group were pair mated immediately after treatment, with the result that offspring in the sample were derived from germ cells exposed chronically at many different stages of development in both sexes. About one-half of the data collected to date come from litters conceived within 7 weeks after the end of the treatment. The treatment had no effect on fertility.

Analysis of the presumed mutation frequencies was complicated by the finding of two preexisting mutations. However, examination of pedigrees and comparisons of phenotypes made it possible to correct for both of these. A modification has been made in the way our production stocks are maintained in order to greatly simplify the handling of this problem when it occurs in future experiments.

The corrected probable mutation frequency in the concurrent control is 1/505, and in the group with both parents treated it is 0/491. From these

experimental data, by using the binomial distribution, we can rule out an induced presumed mutation frequency of 0.33% at the 5% significance level. Because dichlorvos does not affect fertility (and there is, therefore, presumably no cell selection against mutant spermatogonia), we can assume that it is conservative to extrapolate linearly from the frequency ruled out to the maximum frequency expected for one-twentieth of the exposure (the amount needed to eradicate mites). Accordingly, the maximum induced presumed-mutation frequency from a treatment to eradicate mites is 0.016%, which is only a small fraction of the historical control mutation frequency of 0.29%. Such a low frequency would not be expected to interfere with skeletal experiments.

No correction was necessary in analyzing the results in the mutational-index method. The index of mutation for the dichlorvos group is -0.2% when comparison is made to the earlier control and -2.4% when comparison is made to the concurrent control. If a similar estimate to that in the paragraph above is made to see what index can be ruled out at the 5% significance level, it is found that the maximum index from a treatment to kill mites is 0.0008%. Obviously, no interference with skeletal experiments would be expected.

It should be possible to reconstruct exposure conditions in order to permit a determination of approximately what the exposure was in terms of ppm.hr. Once this is known, and this experiment is completed, our data could be applied in estimating human risk from this chemical for different conditions of human exposure.

MECHANISMS OF CHEMICAL INDUCTION OF CHROMOSOMAL ABERRATIONS IN MOUSE GERM CELLS

W. M. Generoso K. T. Cain
J. A. Bandy

Chromosome-breakage-related genetic anomalies contribute heavily to the total human genetic burden. The high efficiency of certain chemicals in inducing chromosome aberrations in germ cells of laboratory mammals makes this class of genetic damage a major concern when the potential hazards to humans of chemicals are evaluated. For example, we have shown recently that ethylene oxide, a widely used industrial chemical, effectively induces heritable translocations and dominant-lethal mutations in male mice. Because various types of aberrations have varying effects on the conceptuses, an understanding of the mechanism by which the different types of aberrations are induced in mammalian germ cells is essential for practical testing as well as for genetic risk assessment. Our laboratory's recent contributions to this understanding are as follows:

(1) We demonstrated that the formation of breaks and interchanges in chemically treated spermatocytes, spermatids, or early spermatozoa is

delayed, in marked contrast to the more immediate formation of X-ray-induced aberrations.

(2) We discovered that the fertilized egg of mice can repair certain premutational lesions present in the fertilizing sperm, and that the yield of dominant-lethal mutations and heritable translocations is affected by the strain of females used to mate with the treated males. The repair capability of fertilized eggs is genetically controlled and appears to be dominantly inherited. The practical significance of these results lies in their application to the use of the dominant-lethal and heritable-translocation tests in mutagenicity testing. The sensitivity of these tests can be affected greatly by the stock of females used to mate with treated males. It is noteworthy that for all the compounds we have studied so far, certain stocks of females consistently transmitted higher dominant-lethal frequencies than did all other stocks.

(3) We recently showed that the conversion of premutational lesions induced by ethyl methanesulfonate in spermatozoa and spermatids into reciprocal translocations occurs after the sperm has entered the egg.

(4) Data published earlier showed clearly that ionizing radiation and chemicals, which are effective in inducing dominant-lethal mutations in male postmeiotic germ cells, are also effective in inducing heritable translocations in these germ-cell stages. Consequently, the close association between the production of heritable translocations and the production of dominant-lethal mutations (both being end points of chromosome-breakage events) had become a widely accepted generalization. Our recent data with isopropyl methanesulfonate and benzo[a]pyrene clearly show that this generalization is not correct. These two compounds induced no, or only very few, heritable translocations, even though the doses used induced high levels of dominant-lethal mutations. This observation is of importance in elucidating the pathways by which the initial alkylation product becomes either a chromosomal lesion that leads to loss of part of or the whole chromosome, or, alternatively, a lesion that leads to a reciprocal translocation.

(5) Another key finding was that the length of the interval between treatment of postmeiotic male germ cells and fertilization had a marked influence on the relative rates at which chemically induced dominant-lethal mutations and heritable translocations were produced. Triethylene melamine induced considerably fewer heritable translocations, relative to dominant-lethal mutations, in males that were mated within 2.5 hr after treatment (fully mature sperm were treated) than in males that were mated 11 to 15 days after treatment (corresponding to middle spermatids). This observation indicates that the alkylation product has to be transformed into an intermediate lesion in order for chromosome exchange to occur. Without this transformation, the alkylation product results in deletions and chromosome loss and eventually in embryonic lethality.

The above findings provide the basis for our interpretation that the kinds of chromosomal aberrations produced from chemical treatment of postmeiotic male germ cells depend upon the longevity of the critical

alkylation product and upon the length of the interval between the formation of this product and sperm entry. The production of chemically induced heritable translocation (balanced interchanges) requires a transformation of the initial alkylation product into a suitable intermediate lesion before sperm enters the egg, whereupon, it is converted into chromosome exchange before pronuclear DNA synthesis. Conversely, when the alkylation product remains unchanged and persists up to the time of pronuclear DNA synthesis, the main types of aberrations produced are those that lead to dominant-lethality (i.e., deletions and asymmetrical exchanges).

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RADIATION INDUCTION OF HERITABLE TRANSLOCATIONS IN MICE

W. M. Generoso K. T. Cain
J. A. Bandy C. V. Cornett

Although heritable translocations are an important endpoint for the assessment of genetic risk from radiation, there has been a serious information gap with regard to their induction in spermatogonial stem cells, the most important cell stage for risk considerations. This led to uncertainty in estimating the magnitude of risk per unit exposure. Further, the relationship between the frequency of reciprocal exchanges scored by cytological analysis of the exposed male's meiocytes and the frequency of those transmitted to first-generation offspring could not be established.

In order to fill in these gaps, we conducted two radiation studies on spermatogonial stem cells in which heritable and cytologically detected translocations were scored in parallel.

(1) Dose-effect study. Doses of 150, 300, 600, or 1200 R of acute X rays were used. The frequencies of heritable translocations observed were 0.62% (19/3078), 1.38% (46/3342), 1.86% (20/1075) and 0.58% (6/1038), respectively. The control frequency was 0.018% (1/5433). The corresponding cytological values observed (translocations per cell) were 0.025, 0.078, 0.195 and 0.045. The control cytological incidence was 0 in 600 cells scored. In both heritable and cytologically scored translocations, the incidences at all doses are significantly higher than those for the corresponding controls, and both dose-effect curves are clearly "humped." However, the shapes of the rising portion of the curves appear to differ: the one for heritable translocations closely fits linearity, while the one for cytologically-scored translocations significantly deviates from linearity. There is no consistent relationship between the transmitted and the cytologically scored frequencies. The ratios of the observed frequencies to those expected on the basis of the cytological frequencies ranged from 0.44 to 0.98.

(2) Dose-fractionation study. Two exposure conditions were used: (a) 4 × 500R with 4-week intervals, or (b) 2 × 600R, with an 8-week interval. In the 4 × 500R series, we found 84 translocation carriers among 1135 male progeny tested (7.40%). In the 2 × 600R series, 64 translocation carriers were observed among 1198 progeny tested (5.34%). Corresponding cytological values were 0.468 and 0.298 translocations per cell. The ratio of the observed frequency of heritable translocations to that expected on the basis of the cytological data was 0.71 in the 4 × 500R series and 0.74 in the 2 × 600R series. The heritable-translocation frequencies for the two fractionation regimes did not differ significantly from those expected on the basis of additivity but the corresponding cytological frequencies were significantly lower than those expected from additivity.

The present data data are by far the most extensive ones available for heritable translocation induction in spermatogonia stem cells. They show a lack of consistency between cytologically and genetically scored translocations indicating the inadequacy of using cytological data in estimating the transmissible frequency. With respect to heritable translocations, the linearity of the rising portion of the dose-effect curve and the additivity of effects observed in the fractionation experiment allow us to estimate directly the number of transmissible translocations expected per unit exposure.

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USE OF THE MOUSE HERITABLE TRANSLOCATION TEST IN TESTING CHEMICALS

W. M. Generoso J. A. Bandy
K. T. Cain C. V. Cornett

Certain spermatogenic cell stages of the mammal are known for their high sensitivity to chromosome breakage effects of certain chemical mutagens. Consequently, this type of genetic damage is of serious concern in the evaluation of genetic hazards posed by chemicals in the environment. From this standpoint, the heritable translocation test (HTT) in mice is generally regarded as an important method both for detecting chromosome breakage effects and for assessing genetic risk to humans. We are currently working on the improvement of the HTT for use in practical testing. At the same time we apply this method in testing specific compounds that are suspected of being genetically hazardous to exposed humans. In the past two years, we have completed a study of three GRAS compounds — ammoniated glycerrhizin, butylated hydroxytoluene, and gum arabic. None of these compounds appears to be effective in inducing heritable translocations in male mice.

We found earlier that the compound ethylene oxide, when injected into male mice, is highly efficient in inducing dominant-lethal mutations and heritable translocations. Ethylene oxide is the first industrially important compound that has been shown to induce heritable translocations in mice. Because of this, and because of the fact that production and use of this compound involve wide-scale human exposure, ethylene oxide is a good model compound for studying the usefulness of the heritable translocation test in genetic risk assessment. Accordingly, we are currently performing a dose-response study with inhaled ethylene oxide, including the current U.S. maximum allowable workplace concentration of 50 ppm.

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THE ROLE OF WHOLE-MAMMAL MUTAGENICITY TESTS IN PREDICTING GENETIC RISK

Liane B. Russell   P. B. Selby

There is presently much offical concern, both in this country and others, about the extent to which mutagenicity tests in non-mammalian, or in mammalian in vitro systems can predict the transmission of heritable damage in a mammal. Several national and international bodies are at work in attempts to define the signals that should lead one to perform genetic tests in mammals, to sort out the type of information that can be derived only from whole-mammal tests (e.g., differential germ-cell stage responses), and to examine the sensitivities and special capabilities of the various mammalian test systems. We have been involved in the efforts of the following deliberating bodies: International Commission for Protection against Environmental Mutagens and Carcinogens, GENE-TOX (test panels and coordinating committee), and the International Agency for Research on Cancer. This has resulted in our producing a number of papers (listed below), as well as playing a part in formulation of committee documents that do not list authorship.

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CHEMICAL DOSIMETRY STUDIES IN MAMMALIAN GERM CELLS

Gary A. Sega   James G. Owens

Chemical dosimetry experiments performed at this laboratory are designed to increase our understanding of why the mutational response varies in different mammalian germ-cell stages and why different chemicals produce different spectra of genetic effects in the germ cells.

By the use of isotopically-labeled test mutagens, it is possible to determine the amount of chemical binding to the germ cells and to selected targets within these germ cells. Studies on developing mouse sperm have shown that both DNA and protamines are important molecular targets for

chemical attack that may lead to chromosome aberrations. (Mammalian protamines are small proteins, rich in arginine and cysteine, intimately associated with DNA and found in late spermatids and spermatozoa.) Our recent studies have shown that the sulfur group in cysteine is the predominant site of alkylation in the mouse protamines. Another important observation we have made is that not all chemical mutagens have a strong affinity for mammalian protamines. Chemicals that react by an S_N2-type mechanism interact much more strongly with mammalian protamines than do chemicals reacting by an S_N1-type mechanism. There are also differences in the patterns of genetic response of the developing germ cells to these two classes of chemical agents.

Recently, W. L. Russell showed that a single 100 mg/kg exposure to ethylnitrosourea (ENU) produced an ~6 × higher mutation frequency in spermatogonial stem cells of mice than did 10 weekly exposures of 10 mg/kg. Using tritium-labeled ENU and subsequently analyzing DNA recovered from mouse testis, we have obtained preliminary evidence that the ratio of O^6-ethylguanine (O^6-EG) to N^7-ethylguanine (N^7-EG) is greater after a single 100 mg/kg exposure to ENU than after a 10 mg/kg exposure. Also, the O^6-EG appears to persist longer in testis DNA after the high ENU exposure than after the low exposure. Ethylation of the O^6-position of guanine has been implicated as a potentially mutagenic (and carcinogenic) event. It is thus possible that the higher mutation frequency found after the single 100 mg/kg ENU exposure resulted from the production of relatively more O^6-EG and the persistence of this adduct in the germ-cell DNA over a longer time period. Such data have important implications for genetic testing in the mammal, where high exposure levels of a chemical agent may be altering the proportions of DNA alkylation products in the germ cells and possibly inhibiting the repair systems.

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DNA REPAIR STUDIES IN MAMMALIAN GERM CELLS

Gary A. Sega Valentina C. Carricarte[2]
James G. Owens

We have established that unscheduled DNA synthesis (UDS), assumed to be DNA repair, occurs in meiotic and in some postmeiotic germ-cell stages of male mice exposed to a number of mutagenic agents. To measure this UDS, males are treated with a test chemical and are also given testicular injections of [^{3}H]dThd. Sperm moving through the reproductive tract are recovered at various times after treatment and assayed for the unscheduled presence of [^{3}H]dThd.

A number of chemical agents have now been tested by us for their ability to induce a UDS response in mouse germ cells. We have observed an excellent correlation between a chemical's ability to induce a UDS response in the germ cells and its ability to produce mutations in the Russell specific-locus test. Of the chemicals producing a positive UDS response in the germ cells, only hycanthone methanesulfonate (weakly positive for inducing UDS) was negative in the specific-locus test. All chemicals giving a negative UDS response were also negative in the specific-locus test.

From the data currently available, it appears that a chemical's ability to induce a UDS response in mouse germ cells can serve as a good predictor of the agent's potential for being mutagenic. Since a test of the UDS response of mouse germ cells to a chemical agent is relatively fast and inexpensive to perform, it may be a useful pre-screen before large amounts of time and money are invested on genetic testing.

Recently, we have studied the ability of ethylnitrosourea (ENU) to induce a UDS response in mouse germ cells. This chemical has been found by W. L. Russell to be the most effective mutagen ever observed in mammalian germ cells. ENU did induce a UDS response in the germ cells of male mice, but the response was quite low compared to that produced by methylnitrosourea (MNU), the methyl homolog of ENU. Our chemical dosimetry experiments indicate that part of the difference in UDS response may be a reflection of the DNA alkylation levels produced by each chemical. For equimolar exposures to MNU and ENU, MNU alkylates testicular DNA 12 × more than does ENU.

While ENU is much less efficient at alkylating testicular DNA than is MNU, ENU is a much more powerful mutagen in the spermatogonial stem cells (though not necessarily in all germ-cell stages). The difference may lie in the sites of alkylation within the DNA or in the efficiencies with which these various sites are repaired in different types of germ cells. Continued studies of the DNA repair response of mouse germ cells to different chemical agents will be important for a better understanding of the molecular mechanisms giving rise to mutations in mammals.

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DNA DAMAGE IN MAMMALIAN SPERM ASSAYED BY ALKALINE ELUTION

Gary A. Sega James G. Owens

One of the important uses of the alkaline elution technique is to assay for single-strand breaks in DNA. A modified alkaline elution technique has been devised by us for monitoring the presence of DNA single-strand breaks in sperm recovered from mutagen-treated mice. In the procedure, sperm are recovered at various times after mutagen exposure and lysed on a filter and single stranded DNA is eluted through the filter with a high pH buffer. Small fragments of DNA resulting from single-strand breaks pass more readily through the filter than do the high-molecular-weight DNAs in which no breaks have been produced. The amount of sperm DNA passing through the filter is then used as a measure of the amount of single-strand breakage induced.

Using methyl methanesulfonate (MMS) as a test mutagen we have been able to demonstrate that DNA strand breaks are produced in the developing sperm after a single exposure to this chemical. The strand breakage was found to be greatest in sperm treated as mid- to late-spermatids, which are also the stages showing the highest frequencies of dominant lethals and translocations after MMS exposure. When sperm representing earlier germ-cell stages at the time of treatment were studied (longer exposure-to-sampling intervals), the amount of DNA strand breakage decreased. By 21 days after MMS exposure, DNA breakage was barely detectable. We know from our studies of unscheduled DNA synthesis (UDS) in mouse germ cells that these earlier stages undergo a DNA repair response to MMS. Presumably, many of the MMS-induced lesions in the DNA that would give rise to single-strand breaks are repaired before these early germ cell stages have reached the vas as mature spermatozoa.

Future work with other mutagens that act by different reaction mechanisms and produce different patterns of genetic effects in the developing mouse sperm cells will give needed additional data on the correlation between DNA strand breakage and the occurrence of genetic damage such as dominant lethals and translocations. Since we are now able to monitor DNA strand breakage in developing mouse sperm, we have an attractive model for validating the use of alkaline elution in screening for DNA damage in human sperm. Such a screen would be an important new approach in human genetic monitoring.

In these experiments, we have looked at alkylation of hemoglobin, testicular DNA, liver DNA, lung DNA, spleen DNA, kidney DNA, sperm cells from the vasa defferentia and the caudal epididymides, DNA from these sperm cells, and protamine from these sperm cells. We have examined these tissues from animals sacrificed from 10 min to 15 days after the end of exposure.

In addition to providing numbers for the alkylation efficiency of ethylene oxide in certain specific molecular targets, these experiments have given some unexpected results. The initial alkylation density in the DNA's of different tissues is very different. Liver DNA is alkylated to the greatest extent of any tissue DNA we have examined. Testicular DNA has an initial alkylation density of about 5% of that found in liver DNA. 2-Hydroxyethyl groups are removed from liver DNA more rapidly than from other DNA's, suggesting a different spectrum of molecular targets or differential repair capacity among the tissues. In the 1-3 ppm.hr exposure range, testicular DNA has an initial alkylation density of 1×10^{-7} alk/dN/ppm.hr and liver DNA is alkylated to the extent of 2×10^{-6} alk/dN/ppm.hr.

Alkylation of sperm heads and sperm-cell DNA shows a pattern very similar to that seen previously for ethyl methanesulfonate and methyl methanesulfonate. The pattern is readily detectable in the 1-3 ppm.hr range.

The fact that alkylations per nucleotide in germ-cell DNA can be readily measured and appear to show an at least roughly linear relationship to exposure in the 1-3 ppm.hr range is an interesting finding. Human exposure limits are currently 400 ppm.hr per day.

A SEMIDYNAMIC INHALATION EXPOSURE SYSTEM

R. B. Cumming

In a major study of the Molecular Dosimetry of Ethylene Oxide in the Mouse (see the previous report), it became apparent that existing inhalation exposure systems were not suited for certain problems encountered in inhalation molecular dosimetry. Inhalation chambers have classically been of two types - static and dynamic. Neither has the properties necessary for precise exposure for prolonged periods to materials which must be conserved (such as isotopically labeled compounds).

Conditions in static chambers cannot be held constant. Temperature, O_2 and CO_2 concentrations, humidity, and the concentration of the test agent change with time. In reasonably sized chambers these changes can be fairly rapid. The exposure to the agent is calculated by integrating the area under the concentration time curve, but this calculation is usually made with considerable uncertainty. It is very difficult to study high concentrations or prolonged periods of exposure.

Dynamic chambers have their own set of problems for work of this sort. The test agent is added to the incurrent air stream and this consumes relatively large quantities, particularly for high concentrations or prolonged exposure periods. For isotopically labeled agents it is simply not possible to supply the amounts needed. A system must be provided which conserves the test agent while maintaining exposure conditions constant.

In an attempt to solve this problem we have developed a semi-dynamic inhalation system which allows precise control of the exposure conditions while using the test agent very economically. In this system, the atmosphere is pumped through a circular path, where the unwanted materials (e.g. CO_2, moisture) are removed and the concentration of the test agent is continuously monitored. Oxygen is supplied at the rate at which it is consumed, and since this rate can be measured, this procedure provides a rough measurement of the metabolic rate of the animals in the chamber. The test agent is supplied at the same rate at which it is consumed, thus keeping concentrations constant for whatever time period desired.

The system has been used for molecular dosimetry of ethylene oxide under exposure conditions very similar to those used in inhalation mutagenesis experiments with this same agent. The apparatus consists of a chamber, a pump to create the flow of the atmosphere through the system, a series of traps to remove moisture and CO_2 while leaving the test agent in the stream, a Wilks Miran I infrared spectrometer with a 20 m path-length cell, a monometer to measure chamber barometric pressure, an oxygen supply system, and a syringe pump to supply the test agent at the rate at which it is consumed.

We have used this system for exposures of up to 8 hr, and the prototype works very well for time periods from 30 min to 8 hr. We are able to control inhalation exposure conditions with greater precision than we have in the past.

DOSE RESPONSE MODELING FOR CHEMICAL MUTAGENS

Robert B. Cumming William H. Olson

Extrapolation of animal test data for the purpose of estimating human genetic risk requires that one compare relatively high exposures given to the animals with much lower exposures expected to occur in human populations. One way of providing information that is useful in these extrapolations is to use molecular dosimetry in a suitable intact mammal. Even with this approach, modeling is necessary, and this modeling must be supported with appropriate logical and mathematical arguments.

In attempting to develop such modeling, we have been particularly interested in defining conditions under which one would expect linear relationships, and others under which such linear relationships would be expected to break down. We have defined Haber's Rule as it can be applied

to the relationship between the integral of concentration and time for exposure to tissue (target) dose for chemical mutagens. Haber's Rule is defined in the context of compartmental models based on mammalian anatomy and physiology. It is then proved that Haber's rule holds for any system of linear differential equations with constant coefficients which is physically realizable. We have also studied non-linearities and the Blum-Drukrey model for time-to-occurrence of tumors. We expect on theoretical grounds that linear relationships will hold at low concentrations of toxic agents, where most human exposures will occur, and that non-linearities will dominate at higher exposures, where most animal experiments are conducted.

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RISK ANALYSIS

Robert B. Cumming

Genetic toxicology and other major areas of research in the Biology Division involve the development of data that are used to estimate the health risks from environmental chemicals or to analyze technological options. There have been problems of communication between the various disciplines involved in the measurement of health risks, and between these disciplines and the governmental policy makers who use and rely on health-risk information. To help solve this communications problem, a new international journal has been established under the auspices of the Society for Risk Analysis. Biology Division personnel have been involved in this development. Robert B. Cumming is Editor-in-Chief of the new journal, which is called Risk Analysis, and he heads a large and prestigious international editorial board.

Currently, Risk Analysis is in its second volume and it has been well received. There are over 1000 subscribers in 15 countries at the present time, and circulation is growing rapidly. The early success of Risk Analysis in the face of declining library budgets and general economic uncertainty shows that the journal fills a real need.

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MUTAGENICITY OF NONVOLATILE ORGANICS IN UNDISINFECTED AND DISINFECTED WASTEWATER EFFLUENTS

Robert B. Cumming   Lynda R. Lewis

For the past several years we have been investigating the potential for adverse human health effects caused by toxic chemical species formed and released to the environment in the process of disinfecting wastewaters. It has been known for at least eight years that the most widely used disinfection procedure, chlorination, in the presence of suitable precursors can produce modified organics which have biological activity. It is now clear that the most likely chemical treatment alternative to chlorine, ozone, can similarly produce such organics in aqueous solutions. Wastewaters are rich in precursors for these biologically active molecules. What is still not clear is the overall health impact of the disinfection technologies as they are now practiced or are likely to develop in the future.

We have investigated the mutagenicity of nonvolatile organics from nine wastewater treatment plants in five states. The effluents were tested without disinfection and following disinfection with chlorine, ozone or UV irradiation. Six of the treatment plants provided samples which had been disinfected by chlorination; six provided samples disinfected by ozonation; and three by UV irradiation. Eight of the nine plants used secondary treatment and one plant provided only primary treatment. Samples were collected at one or another of the treatment plants in every month of the year except November, and each plant was sampled several times over a two and one-half year-period. The treated wastewaters came from a wide variety of sources. Some were limited to residential wastes while others received their wastewater primarily from industrial sources. Most had some contribution from both types of sources. The samples were concentrated 500-3000 fold by lyophilization and subsequently analyzed using HPLC, GC and Mass Spectrometry. Both the concentrates and samples derived from HPLC fractions were tested for mutagenicity. The initial tests were done in a variety of test systems, but most of the testing after the preliminary phases of the study were done using reversion to histidine prototrophy in *Salmonella typhimurium*, strains TA1535 (a base-pair substitution detector) and TA1538 (a strain which detects frame-shift mutations). Even those concentrates which showed the greatest mutagenicity in the bacterial tester strains gave negative results in a short-term *in vivo* mammalian test (unscheduled DNA synthesis in spermatids of mice). Consequently mammalian tests were not used in later stages of the study. Mutagenicity in the Salmonella tester strains was widespread in wastewater effluents tested either as concentrates or as separated samples derived from HPLC fractions. Mutagenic effects were demonstrated in eight of the nine plants studied and it is probable that mutagenicity could be demonstrated in the remaining plant with further work. There was no discernible pattern in the results and this might be expected from these extremely complex and highly variable mixtures. In most cases, there was greater total mutagenicity in the separated samples than in the concentrates from which they were derived. This is probably due to the separation of toxic from mutagenic components

of the mixture. But there were cases where the reverse was true, suggesting very complex interactions among components of the mixture. Most frequently disinfection (either chlorination or ozonation) increased the mutagenicity of the sample, but again there were cases where the reverse was true. Samples collected from the same treatment facility on different dates frequently gave very different results. For example, in samples taken at the Moccasin Bend Wastewater Treatment plant in Chattanooga, Tennessee, in September 1978, chlorination increased the mutagenicity of HPLC samples in strain TA1535 but slightly decreased it in strain TA1538. In samples taken at the same plant the following July, the reverse was true: chlorination slightly decreased the mutagenicity in TA1535 but increased it in TA1538. All possible outcomes are found in this large set of data. The samples themselves are extremely complex and quite variable from time to time and place to place.

It appears that at the early stages of treatment of these wastewaters with oxidating disinfectants, such as chlorine or ozone, new compounds are formed, some of which may be mutagenic. As the contact times or concentration of oxidant increase, mutagens and other organic compounds are destroyed. Thus, we suggest that a dynamic situation exists during the disinfection process. The net results, which are measured by chemical analysis and toxicological tests, are dependent on the precise conditions at the time the sample is collected. These results are likely to change dramatically with differences in the raw effluent, weather, or the conditions of disinfection.

Some insights can be derived from these studies about the assessment of human genetic risk from complex mixtures, such as the organic molecules present in wastewaters and other surface waters. Bacterial test systems have been proposed for assessment of mutagenicity, because of their flexibility and relatively low cost. These studies illustrate that the bacterial tests, when facing very complex environmental mixtures, give only rough qualitative information. We learn that mutagens (for Salmonella) are very widespread in the environment and are a matter of some concern. This is useful information, but it gets us no closer to a quantitative evaluation of human health risks from a particular technology.

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Basic Genetics and Cytogenetics

QUALITATIVE ANALYSIS OF MOUSE SPECIFIC-LOCUS MUTATIONS

Liane B. Russell, W. L. Russell, E. G. Bernstine, C. S. Montgomery, M. S. Steele, H. M. Thompson

Specific-locus tests, which have for years been in use in our laboratory to measure the rate of induction of point mutations, yield a wealth of genetic material that can be carried on in stocks for subsequent study.

Analysis of specific-locus (SL) mutations at three loci has identified over 33 distinct complementation groups — most of which are probably overlapping deficiencies — and 13 to 14 new functional units. Perhaps due to ease of ascertainment, the complementation maps that have been generated for the d-se and c regions include numerous vital functions; however, some of the genes in these regions are non-vital, i.e., the mouse can tolerate their total absence as produced by overlapping deficiencies. At such loci, hypomorphic mutants (as distinguished from nulls) must represent intragenic alterations, and some viable nulls could conceivably be intragenic lesions also.

Analysis of SL mutations has provided information on genetic expression in mammals. Homozygous deficiencies can be completely viable or can kill at any one of a range of developmental stages. Heterozygous deficiencies of up to 6cM or more in genetic length have been recovered and propagated. The time of death of homozygous deficiencies and the degree of inviability of heterozygous deficiencies are probably related more to specific content of the missing segment than to its length. Combinations of deficiencies with X-autosome translocations that inactivate the homologous region in a mosaic fashion have shown that organismic lethals are not necessarily cell lethal.

The spectrum of mutations induced (e.g., the ratio of nulls to altered-activity mutants) depends on the nature of the mutagen and the type of germ cell exposed. Radiation of spermatogonia produces intragenic as

well as null mutations. Spontaneous mutations (several of which may arise in the zygote or in early cleavage) have an admixture of types not present in populations of mutations induced in germ cells, and this raises doubts concerning the accuracy of "doubling-dose" calculations in genetic risk estimation.

The analysis of SL mutations has yielded genetic tools for the construction of detailed gene-dosage series (from 0 to 3, in steps of 0.5), cis-trans comparisons, the mapping of known genes and identification of new genes, genetic rescue of various types, and the identification and isolation of DNA sequences.

Results from the analyses of mutations will contribute answers to some of the pragmatic questions in germ-line mutagenesis and risk assessment in the following areas: the relative roles of intracellular conditions (e.g., nature of chromatin, presence of repair enzymes) and secondary circumstances (e.g., selection) in determining the quantity and quality of transmitted mutations; the validity of quantitative extrapolations, such as projections to low doses and calculations of doubling dose; and the relation between measures of mutation rate and projections of phenotypic damage.

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ANALYSIS OF THE ALBINO-LOCUS REGION OF THE MOUSE: CHARACTERIZATION OF 34 DEFICIENCIES

Liane B. Russell   C. S. Montgomery
G. D. Raymer

In our continuing detailed analysis of the albino-locus region, we have investigated 34 independent nonviable c-locus mutations, derived from radiation experiments using the specific-locus method. These were tested for involvement of nearby markers tp, Mod-2, sh-1, and Hbb: 10, 22, and 2

involved, respectively, none of these markers, Mod-2 alone, and Mod-2 plus sh-1. When classified on this basis, as well as according to developmental stage at which homozygotes die, and by limited complementation results, the 34 independent mutations fell into 12 groups. From results of a full-scale complementation grid of all 435 possible crosses among 30 of the mutations, we were able to postulate an alignment of eight functional units by which the 12 groups fit a linear pattern. Abnormal phenotypes utilized in the complementation study were deaths at various stages of prenatal or postnatal development, body weight, and reduction or absence of various enzymes. Some of these phenotypes can be separated by complementation (e.g., there is no evidence that mitochondrial malic enzyme influences survival at any age); others cannot thus be separated (e.g., glucose-6-phosphatase deficiency and neonatal death).

We conclude that all of the nonviable albino mutations are deficiencies overlapping at c, and ranging in size from 2cM to 6-11 cM. Since many of the combinations of lethals produce viable albino animals that resemble the standard c/c type, we conclude (i) that the c locus contains no sites essential for survival, and (ii) that viable nonalbino c-locus mutations are the result of mutations within the c cistron. Viable albinos (the majority of radiation-induced c-locus mutations) may be intracistronic mutations or very small deficiencies.

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GENETIC STUDY ON MUTANTS THAT WILL PROVIDE MODEL SYSTEMS FOR SKIN CARCINOGENESIS

K. F. Stelzner

Four dominant autosomal mutations affecting development of the skin and hair arose during mutation-rate experiments employing the specific-locus method or in stocks derived from such experiments. Because it appeared probable from the work of Fry and associates at this laboratory that these mutants might be useful for skin carcinogenesis studies, a genetic analysis was undertaken to determine the number of loci involved, their relationship to each other, and their relationship to the hairless locus, hr. This analysis showed that three of the mutations probably constitute an allelic series. These three have been given the symbols Frl^{a}, Frl^{b}, and Frl^{c}. The Frl series shows no evidence of linkage with hr. The fourth mutation, Hr^{n}, is a dominant and homozygous viable allele at the hr locus. With the possible exception of Frl^{b}, all mutants were of spontaneous origin. Because of their unique characteristics, these new mutants are of potential value as mouse model systems in studies of skin carcinogenesis and related areas of research.

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THE CHARACTERIZATION OF X-AUTOSOME TRANSLOCATIONS, AND THEIR USE AS TOOLS TO INVESTIGATE GENE INACTIVATION

Liane B. Russell C. S. Montgomery
N. L. A. Cacheiro J. W. Bangham

Not long after the first mammalian X-autosome translocations [T(X;A)'s] were reported from our laboratory 23 years ago, investigations of these rearrangements provided the evidence on which the single-active-X-chromosome hypothesis was developed by us independently of Lyon's inactive X hypothesis. In the years since then, a number of additional T(X;A)'s have been recovered and studied, and we have recently summarized some of our voluminous data.

Of at least 17 T(X;A)'s observed, 15 (including 11 that arose at Oak Ridge) have now been studied genetically and/or cytologically. All but one were induced by mutagens in postmeiotic germ-cell stages. Eight have involved Chromosome (Ch) 7, four Ch 4, and one each Chs 2, 11, 12, 16, and 17. While part of this nonrandomness of distribution results from bias of detection [most T(X;A)'s having been found in specific-locus experiments], some may be due to selection against T(X;A)'s involving certain autosomal regions.

All of the Oak Ridge T(X;A)'s are reciprocal translocations, but one found elsewhere is an insertion of an inverted Ch-7 segment into the X. Genetic mapping indicates crossover suppression near breakpoints of some of the T(X;A)'s, affecting primarily the short arms. Breakpoint order is the same in the genetic, mitotic, and meiotic maps, but relative distances indicate more recombination in distal than central portions of the chromosomes. Eleven of the T(X;A)'s provide long marker chromosomes (>Ch1), and at least 5, a short marker. Unbalanced types do not survive postnatally in any of the Oak Ridge T(X;A)'s, but several of our translocations fairly frequently produce XO; 2A daughters.

All Oak Ridge T(X;A)'s lead to mosaicism for autosomal gene inactivation. All informative genes that have been investigated in the A and/or X portion of the long chromosome were found to be subject to inactivation. In one of the translocations in which several genes in the reciprocal chromosome were studied, no inactivation was found. The latter result and most cytological findings make it likely that there is a single site (or contiguous region) in the central part of the X that is responsible for primary differentiation of the X's.

In the chromosome differentiated as the nonactive one, inactivation does not proceed in an uninterrupted steady gradient. It is thought that the site which controls primary differentiation subsequently governs the action of multiple inactivation units. Alternatively, inactivation may proceed linearly (in both directions) from this site, but the varying intrinsic properties of the genetic material along the way allow this material to be "turned off" with varying degrees of ease.

The mosaic composition of the organism for a given autosomal gene is determined by (1) whether primary differentiation of the X's is random, (2) how the spreading effect is accomplished, and (3) whether selection against functionally unbalanced cells occurs. Depending on the autosome involved, selection is different in different tissues, because certain genes critical to the development of certain cells are functionally hemizygous in some rearrangements but not others.

T(X;A)'s have been used as tools in developmental studies to investigate cytogenetic mechanisms, and to analyze gene action (e.g., local vs organismic). They can be used to enrich for specific X or autosomal DNA by flow sorting or by the use of cell hybrids.

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INVESTIGATION OF THE CAUSES OF GENETIC MALE STERILITY

Liane B. Russell M. M. Larsen
N. L. A. Cacheiro C. S. Montgomery

Every one of 16 reciprocal X-autosome translocations [T(X;A)'s] reported to date produces sterility in heterozygous males. This sterility results from stoppage of spermatogenesis in pachytene, with occasional spermatocytes in some of the stocks reaching metaphase I, particularly in males just past puberty.

Our recent work has eliminated several of the hypotheses that have been proposed to explain the male sterility. Hypothesis: Interruption in the normal Y-pairing segment of the X may interfere with X-Y synapsis. Finding: In translocations in which this X segment is interrupted, X-Y pairing has not been prevented (see report by Ashley et al., below); further, translocations in which the segment is not interrupted also cause sterility. Hypotheses: Some necessary step in spermatogenesis, which could depend on the sequestering of X and Y in the sex vesicle during meiotic prophase, may be interfered with by inclusion of autosomal material in the vesicle; or, the disturbance in the sex vesicle's function could result from the inclusion of only one segment of the X instead of the whole

X. Findings: the sex vesicle probably contains both parts of the X whenever quadrivalent SC formations are observed, as we find to be invariably the case in some of the T(X;A)'s; further, two paired chromosomal segments, presumed to be autosomal portions of the translocation, protrude from sex vesicles in several of the male-sterile T(X;A) stocks.

It has also been suggested that heteropyknosis of the X, which normally occurs in spermatogenesis, could exert a position-effect inactivation on contiguous autosomal material, thus interfering with some of the male fertility genes that may be scattered throughout the genome. To test this hypothesis, we made experimental chimeras having a male translocation (R6) component. The chimeric mice produced no progeny from that component, but did produce progeny from the normal component, if this was XY. If the normal component was XX, the presence of R6 in the XY portion of the chimera did not interfere with the male phenotype that is usual for XX⟷XY chimeras. The results indicate that the sterilizing action of R6 takes place in the germ cells, rather than organismically, as would have been more likely if autosomal male fertility genes had been involved.

In another experiment to test this hypothesis, each of five different T(X;7)s was combined with our Ch-7 tandem duplication. Since this duplication contains an appreciable portion of Ch 7, there was a chance that it might "cover" inactivated critical autosomal portions in at least one of the translocations. All males were, however, completely sterile. These results support those from chimeras in indicating that the inactivation of autosomal fertility genes is an unlikely explanation for the male sterility.

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MEIOTIC STUDIES OF REARRANGEMENTS BY SYNAPTONEMAL COMPLEX ANALYSIS

T. Ashley[2] N. L. A. Cacheiro
Liane B. Russell F. L. Russell

A number of chromosomal rearrangements of the mouse are being used in an ongoing study of meiotic mechanism, particularly the initiation, progression and maintenance of synapsis and the circumstances of X-Y pairing.

Synaptonemal complexes of surface-spread spermatocytes of male mice heterozygous for rearrangements R2, R3, R5, or R6 have been examined by light and electron microscopy (LM and EM). All four of these rearrangements are reciprocal translocations between the X chromosome and Ch 7. Measurements of the lengths of all the chromosome axes involved in the translocation configurations, and of the extent of synapsis, were used to calculate the position of the break points of the four translocations. The

breaks in Ch 7 were at 62, 70, 21, and 30% of the length of the chromosome (as measured from the centromere); in the X, they were at 27, 22, 83, and 75%, respectively. The phenomenon of nonhomologous pairing, found to occur in R2, R5, and R6, complicates LM analysis, but is easily recognized and interpreted in the EM study.

In the case of R2 and R3, translocation quadrivalents are formed almost exclusively; but in R5 and R6, the frequency of quadrivalents was only 20 and 42%, respectively, with most of the rest of the associations being heteromorphic bivalents (7^X-Y and X^7-7). The latter condition is interpreted to result from premature desynapsis of 7^X from 7. In turn, this may be caused by the X portion of 7^X conferring its property of early desynapsis (from the Y) on that portion of the 7 to which it is attached. Support for this hypothesis comes from the finding that the frequency of quadrivalents is consistently higher in female than in male T(X;7) heterozygotes. Despite the fact that, in R5 and R6, the break in the X lies within the potential pairing region of the sex chromosomes, univalent Y's were observed with only low frequency: 8-14% and 5%, respectively. They were never observed in R2 and R3.

We cooperated with Drs. Poorman and Moses (Duke University Medical Center) in a synaptonemal-complex (SC) analysis of our tandem duplication in Ch 7. The SC's exhibit a characteristic loop, representing the unsynapsed portion of the duplication; but the loop disappears in later pachytene stages, as a result of synaptic adjustment. The length of the duplicated segment is 22% that of the normal Ch 7. Ends of the segment were mapped at 0.50 and 0.72 (from the centromere) in pachytene SC's, and at 0.48 and 0.71 in G-banded chromosomes, demonstrating strong parallelism between meiotic and mitotic chromosomes.

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TIME AT WHICH THE SYNAPTONEMAL COMPLEX IS FORMED IN MOUSE PRIMARY SPERMATOCYTES

R. F. Grell E. E. Generoso
E. F. Oakberg

There has been a debate over the time as well as the mechanism of crossing over in eukaryotes. The mouse provides an excellent organism for a study of this problem, since our long experience in identification of spermatocyte stages can be used to select specific cells for study prior to analysis by electron microscopy. Presence of the synaptonemal complex in preleptotene spermatocytes in DNA synthesis was clearly demonstrated. This strongly suggests that crossing over occurs by a copy-choice mechanism. These results have significant implications concerning mechanisms of induction of genetic damage by radiation and chemicals.

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DEVELOPMENT OF A PROGRAM TO FREEZE MOUSE MUTANTS

M. M. Larsen L. B. Russell

We have initiated a program for freezing embryos from mutant stocks of mice that have arisen over the past three decades in mammalian mutagenesis tests and that are continuing to arise at high rates in current experiments. As shown in other reports in this volume, some mouse mutants can be good models for human conditions; others have great value for studies in mammalian gene structure and function. The objective of the freezing project is to reduce the workload and the space required to maintain these stocks, and, at the same time, to preserve the invaluable genetic material for further research as the resources for such become available here, or as other laboratories perceive a need for specific mutants in the future.

Methods for freezing mammalian embryos were developed by P. Mazur and S. P. Leibo in our Division and have been successfully used elsewhere. With the advice of these two investigators, we have equipped an embryo-freezing laboratory in the mouse genetics facility and have further adapted their methods to our special needs. The expertise for removing, manipulating, and reimplanting mouse embryos was already available in the Mammalian Genetics and Teratology Section, where we had in the past successfully produced numerous aggregation chimeras.

To date, successful freezing has been determined by live births following thawing of a sample of embryos from each stock, culturing, and reimplantation into recipient pseudopregnant females. Currently, our success rate varies from 10 to 25% for different stocks. If our success

rate remains consistent, we may shorten the test procedure in the future except in the case of especially valuable mutants. We freeze 500 to 800 embryos per stock, depending on whether the mutant is homozygous viable, lethal, sex-linked, etc.

Reproductive Biology and Teratogenesis

EFFECTS OF AGE ON THE SPERMATOGONIAL CELL POPULATION OF THE MOUSE

E. F. Oakberg C. D. Crosthwait

A study of the stem-cell population in (101 × C3H)F_1 males, ranging in age from newborn to 23 months, indicates that some gonocytes present at birth are long cycling and presumably form the stem-cell population, whereas other gonocytes immediately enter the differentiation pathway. Cell-cycle properties characteristic of the adult are established by 21 days of age, and remain unaltered throughout life. Stem-cell numbers, however, are highest in young adult males, and decline with increasing age.

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A TEST FOR A CIRCADIAN RHYTHM IN MITOTIC ACTIVITY OF SPERMATOGONIAL STEM CELLS

E. F. Oakberg C. D. Crosthwait

In spite of numerous studies spanning many years, the effects of radiation on spermatogonial stem-cell kinetics are not well understood. Claims of shortened cell-cycle times during the repopulation phase are not well documented. Our data from long-term thymidine labeling suggest that cell-cycle kinetics of the long-cycling stem cells are unaffected. An understanding of stem-cell kinetics is needed for the interpretation of dose-fractionation effects on chromosome breakage and mutation induction.

No one, however, has considered the possibility that spermatogonial stem cells may have a circadian rhythm. We are determining the mitotic index of isolated A spermatogonia throughout the 24-hr day by use of whole mounts of seminiferous tubules. DNA synthetic activity of the long-cycling stem cells is being determined by H^3-thymidine labeling, followed 24 hr later by 300 R irradiation. Mice are killed 207 hr after labeling. When these studies are complete, we should be able to plan experiments that will

yield objective data on the effect of radiation on the cell-cycle properties of spermatogonial stem cells.

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EFFECT OF ETHYLNITROSOUREA, METHYLNITROSOUREA, AND HYDROXYETHYLNITROSOUREA ON THE TESTIS

E. F. Oakberg C. D. Crosthwait

Cell killing and cell-cycle kinetics of spermatogonial stem cells are being studied to provide the basis for interpretation of genetic and fertility effects of radiation and chemicals. A comparative study of the effects of ethylnitrosourea (ENU), methylnitrosourea (MNU) and hydroxyethylnitrosourea (HENU) revealed a similar pattern of spermatogonial killing for all three compounds. On the other hand, W. L. Russell has shown that these three compounds have very different mutagenic effectiveness in spermatogonial stem cells: ENU is considerably more mutagenic than the other chemicals.

Cells exposed in leptotene to 100 mg/kg and higher doses of ENU were found to degenerate in pachytene 5 days later. It is not known whether this delay in response results from a delayed expression of damage induced at the time of treatment, or from the persistence of ethylations which become effective at a critical stage of cell development. The lack of parallelism between cell-killing and mutational responses of spermatogonia to ENU, MNU, and HENU, together with the selective action of ENU on the leptotene stage of primary spermatocytes, demonstrates the high specificity of the cellular response to this chemical. Further, as noted, the cell death response to ENU exposure is delayed.

Labeling with tritiated thymidine prior to exposure to ENU indicates that there is no significant difference between cells in S and those in other stages of the cell cycle in sensitivity to cell killing. This suggests that, with ENU, one is unlikely to find cell synchronization, resulting from the killing of sensitive stages of the cell cycle, as one does with radiation. The effect of dose fractionation on mutation frequency should be less marked for ENU than it is for radiation.

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TERATOGENIC MECHANISMS AND PRESCREENING SYSTEM

R. S. Filler Y. Horton

Embryonic development is the most sensitive phase of the mammalian life span. Therefore, teratological considerations may provide the limiting factor in setting tolerance limits for environmental pollutants. Because compounds produced in energy-related technologies have as yet largely unknown teratogenic potential, our aims are to establish a sensitive and rapid prescreen for identifying putative teratogens and to elucidate operative mechanisms involved in teratogenesis.

Embryonal carcinoma (EC) cells, obtained from aryl hydrocarbon hydroxylase "responsive" and "nonresponsive" mice, have developmental capabilities analogous to early-stage embryos. EC cells can bioactivate proteratogens to various reactive intermediates via the 3-methylcholanthrene-type mixed-function oxidase system, but not by the phenobarbital-type pathway. A positive correlation has been established between the developmental potential of EC cells and (1) the extent of biotransformation of proteratogens, (2) chemically-induced cytotoxicity and (3) restriction in the differentiational capability of EC cells in a coupled in vitro/in vivo teratogenic prescreen. The mixed-function oxidase (MFO) system is responsive to induction by a wide variety of 3-MC-type inducers, the most potent of which is TCDD (>200-fold induction), with a $t_{1/2}$ of 16-17 hr. In addition to bioactivation capabilities, these embryo-like cells can also deactivate reactive intermediates by as yet unknown pathways not involving conjugate formation with glucuronic acid, sulfate or glutathione as is observed with more developmentally advanced tissues.

In parallel to the EC experiments, murine preimplantation embryos have been assayed to determine if an endogenous embryonic MFO system is operative. The extent of embryonic response may be potentiated by such an in situ system, and this may result in selective embryotoxic effects, modification of cellular division rates, or alteration in the differentiation potential of primordial cell types. C57BL/6N and DBA/2N preimplantation embryos were cultured from the 2-cell to blastocyst stage of development in the presence of $[^3H]$-B[a]P, as a chemical probe for detecting MFO activity. The developmental onset of biotransformation activity coincided with blastocyst formation (gestational day 3 1/2). In addition to demonstrating a basal MFO activity, blastocysts were also responsive to enzymatic induction with the 3-MC-type inducer TCDD but not with PB-type inducers. As had been the case with EC cells, deactivation pathways were also present but did not involve glucuronide or sulfate ester formation. Since we have defined the biochemical competency of preimplantation embryos for activating various classes of chemicals, experiments are in progress to expose embryos in vitro to teratogens and then transfer such treated embryos to pseudo-pregnant females. Subsequent developmental effects are being assayed.

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UTILIZATION OF CRITICAL PERIODS DURING DEVELOPMENT TO STUDY THE EFFECTS OF LOW LEVELS OF ENVIRONMENTAL AGENTS

Liane B. Russell

Our development of the homeotic-shift method as a sensitive and quantitative prescreen for teratogenesis has illustrated the advantages of using sensitive subpopulations. We have surveyed the teratology literature, particularly with regard to radiation, and find that the frequent lack of definition of subpopulations has led to a loss in sensitivity in the measurement of risk. An example is provided by the microcephaly data for Hiroshima. When we focused on the data for exposures during weeks 6-11 of pregnancy, the plot showed a steep dose-response curve (albeit with wide error bars due to the smallness of the samples). By contrast, the plot for exposure at any time during pregnancy (with different mixes of stages at each dose point) indicates a much shallower curve, of different shape at the lower end. Extrapolation to low doses could presumably be different from the two tabulations. This epidemiological study was a very large one, and the sample size and dose range are not likely to be duplicated for other agents. It is thus improbable that one will be able to deduce, from human data alone, critical periods for other endpoints. If one cannot do so -- and therefore has to work with an array of exposed stages -- the low-dose extrapolation will always be an underestimate as far as the real sensitive subpopulation is concerned.

For experimental studies, careful definition of critical periods in the development of selected characters can result in systems that may be highly useful in studying risk at low levels of exposure.

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Toxicology Section

SECTION OVERVIEW - H. R. WITSCHI

Toxicology is a predictive science. It examines the effects of chemicals in a great variety of experimental systems. The observations made are then used to predict potential risks to human health and well-being and also to assess safety.

Two ways are open to reach this goal. In one approach, suitable biological systems are exposed to defined chemical agents. The results are observed, measured and recorded. This is the time-honored approach of toxicity testing. The alternative is to focus on certain biological phenomena and to try to understand them in mechanistic terms. In this approach, chemicals are used as tools to probe biological processes. The first to advocate this was Claude Bernard.

The testing approach would be entirely satisfactory if it were the sole purpose of any toxicological study to predict acute toxicity only. There would be no need for mechanistic studies. However, it has become more and more evident that the real concern for a highly industrialized society is to predict chronic toxic effects following long-term low level exposure. Testing will allow us to make predictions for this purpose, too, although there is a general uncertainty and even fear that sometimes even the most thorough test scheme may fail to recognize potential hazards. As a result, test systems tend to become increasingly complex, larger and more expensive. There is a serious danger that the gap between the testing conditions called for in regulations and the physical and human resources available to do the work can no longer be bridged.

We also must realize that to rely on more and more complex test systems involving an ever increasing number of assays and animals is actually a gesture of defeat. In taking such an approach, toxicology tacitly acknowledges its failure to understand the biology underlying the untoward effects produced by chemicals. Only if we understand mechanisms can we expect to make true progress. This calls for more basic research in toxicology.

It must not be overlooked that there is presently an enormous imbalance between the amount of work and money spent on carcinogenesis and mutagenesis compared to other problems in toxicology. Cardiovascular disease, chronic disease of the respiratory tract, many degenerative processes in the liver, kidney, the skeletal and the nervous systems, blood dyscrasias and even behavioral abnormalities, to name just a few, may be, and probably are up to a certain point, caused by chemicals. Yet much less effort is made to evaluate the role of chemicals in these diseases.

The ongoing work in the Toxicology Section focuses at present mainly on two organ systems, the respiratory tract and the skin. To concentrate on these two systems is a rational choice: respiratory tract and skin are the two major areas of exposure to toxic agents present in the environment. Both skin and lung can also serve as routes of entry for toxic agents.

Mechanisms of toxicity are studied in both organ systems at several levels of biological organization. In whole animal work, care is being taken to expose animals under conditions which approximate potential human exposure. Such studies involve skin painting experiments in mice and inhalation studies in which rats are made to inhale carefully generated and accurately characterized aerosols of chemicals. Organ function studies, biochemical measurements, and histopathology are used to evaluate the effects of the toxic agents being examined. However, to study mechanisms associated with lung or skin toxicity in more depth it has become necessary to develop _in vitro_ sytems. To study carcinogenesis in the respiratory tract, the tracheal implant system has been developed to a stage where it is now possible to expose isolated tracheas repeatedly to airborne toxic agents without interfering with respiration. To examine penetration of foreign agents through skin and their metabolism in skin, a new technique was developed which provides both quantitative and qualitative information under carefully controlled conditions. Finally, the maintenance and study of epithelial cell lines derived from lung under _in vitro_ conditions remains a powerful research tool.

Two other organ systems besides skin and lung have and continue to receive some attention: the cardiovasclar system and the immune system. Additional information has become available on the role of trace elements in the pathogenesis of arteriosclerosis. Of particular importance is the observation that interactions between trace elements, particularly Ca and Pb, appear to play a determining role. The effects of chemicals on the immune system is another area of active research.

As an applied science, toxicology needs input from many fields of expertise. In several projects, collaboration with members of other sections within the Biology Division or from other Divisions of ORNL has been and continues to be initiated. The following summaries of research activities indicate how individual research projects fit into the general purpose of the Toxicology Section: to understand more about mechanisms of disease.

SYSTEMIC TOXICOLOGY

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W. M. Haschek L. B. Brattsten[2]
L. H. Smith C. L. Paton
C. C. Morse[1] C. S. Owenby
C. A. Sigler[1] M. J. Harden

The Systemic Toxicology Group continued to be active in three main areas of research: (a) pathogenesis of acute and chronic lung disease, (b) the mouse lung tumor system as a possible short-term *in vivo* assay for carcinogenesis, and (c) a formal toxicity testing program for various coal conversion products.

(a) We had shown before in mice how diffuse alveolar damage produced by the antioxidant butylated hydroxytoluene (BHT) can be aggravated by oxygen. The resulting lung lesion resembles strikingly a not uncommon human pathologic condition, adult respiratory distress syndrome (ARDS). People suffering from acute respiratory failure often require oxygen therapy. Unfortunately oxygen is toxic in itself and apparently the damaged lung is much more susceptible to oxygen toxicity than is an undamaged organ. In a series of experiments we then tried to establish limits of safety for oxygen in our animal model. This proved to be impossible; too many factors, such as percentage of oxygen in inspired air, onset of oxygen therapy in relation to acute lung injury, duration of oxygen therapy, and also the extent of the initial lung injury all proved to be determining variables.

We also attempted to prevent the development of fibrosis in ARDS by various therapuetic manipulations. Animals were treated with steroid hormones, anti-inflammatory drugs, or scavengers of free radicals such as superoxide dismutase or WR2721. None of these treatments had any influence upon the development of fibrosis. We were therefore unable to find an effective drug combination which would prevent the development of excessive collagen formation following acute alveolar injury aggravated by oxygen.

Of particular interest was an observation made in some long term studies. Whenever animals with diffusely damaged lungs are exposed to oxygen, the initial proliferative lesion resembles diffuse interstitial pulmonary fibrosis. With time the proliferative lesion subsides, although with biochemical techniques it is possible to demonstrate elevated levels of lung collagen for up to one year. However, during this time the collagen becomes rearranged as witnessed by a shift in type I to type III collagen ratio in the lungs. At the same time the histological signs of fibrosis disappear and a diffuse enlargement of the airspaces throughout the entire lung occurs. Although it is questionable whether this airspace enlargement represents truly emphysematous changes, it is nevertheless of interest to see an initial proliferative lesion being replaced with time by a chronic degenerative lesion.

Finally, we established that diffuse interstitial fibrosis not only results from an interaction between BHT and oxygen but that the same result can be obtained by using several different compounds. In mice it was possible to enhance lung damage with oxygen whether damage was caused by methylcyclopentadienyl manganese tricarbonyl (MMT), bleomycin, cyclophosphamide or inhaled cadmium chloride. Interestingly we found a species difference; in rats we could only potentiate lung damage produced by the two anticancer drugs, cyclophosphamide and bleomycin; cadmium chloride or MMT damage could not be potentiated with oxygen. The reason for this species difference needs to be explored further.

(b) Validation of the mouse lung adenoma assay was a second major research effort in the Systemic Toxicology Group. Several mouse strains, particularly A/J and Swiss Webster mice, develop multiple lung tumors following single or repeated intraperitoneal injections of carcinogens. The mouse lung tumor assay has been considered as a possible short term _in vivo_ bioassay for carcinogenesis. In order to validate this assay, we treated A/J mice with a total of 36 compounds, whose identity was unknown to us. Simultaneously the same compounds were tested independently in a different laboratory. The assay was completed in summer 1982. Although we do not know the exact identity of the compounds studied, we already know and tentatively conclude that, contrary to prior expectations, the lung tumor assay will not be a suitable _in vivo_ bioassay for carcinogenesis. Out of the more than 30 compounds tested, only 2 to 3 gave a questionable positive response. Since it can be surmised that at least one-third of the compounds given to us to test were proven animal carcinogens, it can be tentatively concluded at this time that the lung tumor assay is not sensitive enough to detect most carcinogens.

During the same time we did additional work on the promotion of lung tumors in mice by BHT. We have now established that BHT is also effective as a promoting agent if fed to mice in the diet. Several preliminary observations furthermore suggest that the promotion of lung tumors in mice may not necessarily require repeated cell proliferation of the target population in the lung. Whether cell proliferation can indeed be dissociated from tumor promotion in mouse lung is currently under active investigation.

(c) Toxicology Testing Program. Five tests are used routinely in our laboratory to evaluate the toxicity of synthetic fuels and the associated substances resulting from their production from coal or petroleum: acute oral and intraperitoneal toxicity in mice (LD_{50}); acute dermal toxicity in rats; skin and eye irritation in rabbits, and delayed-type allergic sensitivity in guinea pigs. We use test procedures that are adopted by many toxicology laboratories for estimating potential hazards of substances. We are also testing _in vivo_ selected substances for their effect on the _in vitro_ proliferative capacity of colony-forming cells of mouse bone marrow.

Five substances from a coal liquifaction plant (H-coal) and three product tars from a coal-gasification plant (UMD) were subjected to five toxicologic tests. Results with coal liquids showed that two were moderately toxic while three were essentially non-toxic according to oral

and intraperitoneal LD_{50} estimates. None manifested dermal toxicity or delayed-type hypersensitivity, but two were moderately but transiently irritating to the eyes and skin. Two of the three tars from the coal gasifier plant (UMD) were moderately toxic orally and were moderately irritating to the eyes but not to the skin.

Eleven substances obtained by treatment of liquid products from coal conversion processes were evaluated for toxicity by determining the oral LD_{50}. These substances ranged from slightly to moderately toxic, and it appeared that hydrotreatment of the starting material reduced its toxicity.

None of the 15 coal conversion products tested appeared to be more than slightly toxic to that compartment of mouse bone marrow which gives rise to formation of cell colonies <u>in vitro</u>. The test used reflects the <u>in vivo</u> effect of a single LD_{50} dose of a substance 24 hr after treatment on the granulocyte-macrophage compartment of the bone marrow.

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Respiratory Toxicology

INDUCTION AND PROGRESSION OF NEOPLASIA IN TRACHEAL EPITHELIUM

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The respiratory tract of man is continually accosted by hazardous agents in the environment that are potentially carcinogenic, cocarcinogenic or which may promote the development of lung cancer. Many of these hazardous agents are introduced into the environment as particulates, gases, aerosols, etc. during the refining and utilization of most, if not all, energy sources. There is a great need to develop mammalian model systems that will test for these potentially hazardous agents under experimental conditions that are well controlled in terms of target site exposed, as well as dose and duration of exposure to the agent. It is also important to define and use endpoints that are relevant to the human situation. Therefore, in this program, in vivo, in vivo-in vitro and in vitro models have been developed and are utilized to define and quantify cellular changes that identify particular stages in the evolution of neoplasia in the epithelium of the rat trachea, a tissue which is similar to the human bronchus.

In Vivo Exposure to the Test Agent. Several years ago, the rat tracheal implant model was developed in this laboratory to study the induction and pathogenesis of lung cancer. In this model, a well-defined target site is exposed to test agents released from cylindrical pellets molded to fit the tracheal lumen. This model has the disadvantages that a pellet can be introduced into a tracheal implant only a limited number of times, and release of the agent from the pellet is always initially abrupt and, most likely, highly toxic. To circumvent these problems, an open-ended, flow-through tracheal implant (FTTI) was developed which allows unlimited numbers of exposures to test agents of any physical form, e.g. solutions, gases, particles. The FTTI have the added advantage that the cells which exfoliate into the lumens can be periodically collected for diagnostic cytopathology. In this way the appearance and progression of lesions in the trachea can be followed, similar to that done with human sputum

samples, without sacrificing the animals. The early toxic effects of formaldehyde (HCHO), an ubiquitous environmental pollutant, have been studied with this model. Exposure for 1 hr twice a week to HCHO gas mixed with humidified air to doses of 5, 8 or 12 ppm HCHO induce basal cell hyperplasia and keratinizing squamous metaplasia within a few weeks. It is our interest to study the long-term effects of HCHO given alone and in various combinations with benzo[a]pyrene (B[a]P) to establish whether HCHO is a carcinogen, cocarcinogen or promoter of cancer in the respiratory epithelium. The development of the FTTI model opens up the possibility of testing a wide range of hazardous agents for their toxic and carcinogenic properties on respiratory epithelium in a quantitative fashion not possible before.

In Vivo-In Vitro Model. In vivo models are essentially limited to morphological endpoints. To overcome this limitation, a combined in vivo-in vitro model of carcinogenesis was developed which makes it possible to study a gamut of cellular and biochemical changes in cell populations derived from the tracheal tissue at different times after carcinogen exposure in vivo. The approach is to expose tracheal implants to known doses of carcinogen for pre-selected periods of time. The tracheas are then cut into explants and placed in organ culture for 24 hr. A cytopathological diagnosis is then made on the cells which exfoliated into the medium, and the explants are placed on the bottom of tissue culture dishes to initiate outgrowth of epithelial cells and establish primary cultures. Carcinogen-altered cells are selected out by placing the 14-day primary cultures in a medium deprived of pyruvate, a component we found to be necessary for the long-term growth of normal tracheal epithelial cells. Further alterations in the selected cell populations are identified by testing for ability of the cells to survive subculture, acquisition of anchorage-independent growth in agarose and tumorigenicity when inoculated back into a suitable host. We have found dose-dependent effects after exposure to dimethylbenz-[a]anthracene (DMBA) in terms of the number of explants which yield carcinogen-altered cell populations, decrease in time to anchorage-independent growth as well as to tumorigenicity. Since lesions on the explants are initially identified from the cytopathology of exfoliated cells, cellular and biochemical properties of cell populations derived from specific lesions can be studied and correlated directly to conventional morphological markers of the progression of neoplasia. We have found that the ability of cells to survive the pyruvate-deprived medium is a very early marker of carcinogen-induced alterations. Experiments are in progress to determine the metabolic differences between normal, the selected-for preneoplastic cell populations (PNCP), and tumor-derived cell populations in terms of glucose transport, glucose and pyruvate utilization in biosynthetic and energy pathways as a basis for understanding underlying mechanisms of neoplastic disease. Experiments are also planned and in progress to study the effects of the well-known promoter, tetradecanoyl phorbol acetate, on the frequency of induction of PNCP and the progression of neoplasia in these cell populations following initiation with DMBA. Similar studies are planned for FTTI exposed to B[a]P and HCHO. Quantitating the number of PNCP following exposure of tracheal implants to a test agent(s) appears to be a highly sensitive and relatively rapid method for determining the carcinogen potency of the agent in respiratory epithelium.

This assay should help to bridge the gap between long-term whole animal studies and short-term mammalian cell culture and mutagenesis assays in risk assessment.

In Vitro Models. In vitro models have been developed in which (a) organ cultures, (b) primary cultures, or (c) established cell lines are exposed to the carcinogen, and markers for the progression of neoplasia are subsequently followed. The main attraction of (a) is the possibility of exposing isolated but structurally intact tissue under controlled in vitro conditions, free from the confounding influences exerted by other systems. With this system we have demonstrated that a "preneoplastic" marker in primary cell cultures established from the organ cultures is morphologically altered foci of small, packed, highly proliferating cells. More recently we have shown that alterations in the requirement for pyruvate precedes the appearance of the foci. The presence of the foci is a hallmark for the acquisition of subculturability by the cells. Markers for the progression of neoplasia are then followed during repeated subculture of the cells. With (c), i.e., exposing established tracheal epithelial cell lines to carcinogen, only the late markers of neoplasia can be studied, but this model has the advantage that it is highly quantitative. In these systems additional markers for the progression of neoplasia are being investigated following exposure to the carcinogens, methyl-nitro-nitrosoguanidine, B[a]P or B[a]P metabolites. These markers include the change in requirements for Ca^{2+} and the formation of multinucleate cells in the presence of cytochalasin B.

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NORMAL AND NEOPLASTIC GROWTH IN EPITHELIAL CELLS

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<u>In Vitro Studies on the Dynamics of Neoplastic Development In Vivo</u>. The development of neoplastic disease *in vivo* is characterized by a long asymptomatic latency period. The length of this latency period is dependent on the dose, dose rate, and potency of carcinogenic insult. We have developed an *in vitro* technique referred to as the epithelial focus (EF)-assay whereby quantitative and qualitative changes taking place soon after carcinogen exposure can be monitored. Cells giving rise *in vitro* to EF which survive and proliferate when seeded into culture dishes were frequently observed immediately after carcinogen-exposure in rat trachea, esophagus, and lung. They were rarely observed in cultures established from nonexposed control tissues. Three types of EF could be distinguished experimentally: EF which could not be subcultured (EF_o); EF that could be subcultured (EF_s); and EF_s which did not grow in agarose ($EF_{s,ag}-$); and EF_s which did grow in soft agarose ($EF_{s,ag}+$). We previously investigated the effect of carcinogen-dose and time elapsing post exposure on the dynamics of neoplastic development in implanted rat tracheal epithelium as determined by changes occurring in the EF, EF_o, EF_s, $EF_{s,ag}+$ and $EF_{s,ag}-$ forming units (cell populations) *in vivo*. More recently, we have carried out similar experiments examining the effects of the promoter TPA on the dynamics of neoplastic development in tracheas exposed to low doses of carcinogen. Cells were harvested for the EF-assay 0-18 months after the end of carcinogen exposure or beginning of IPA exposure. Control tracheas contained <1 EF forming units/10^4 viable cells harvested. Carcinogen exposure resulted in a 6-20-fold increase in EF frequency between 3 and 12 months after exposure. TPA treatment of DMBA exposed tracheas did not further increase the EF forming unit frequency. With few exceptions, TPA did not appear to affect the growth rate in culture of DMBA-altered cell populations. There was, however, a marked effect of TPA on the maintenance of the size of the compartment of epithelial focus forming units with anchorage independent offspring ($EEFU_{s,ag}+$). By 3 months after exposure 15% EF_s were agarose-positive. No difference in frequency of $EEFU_{s,ag}+$ was noted at that time between DMBA and DMBA/TPA exposed tracheas. However, by 12 months following exposure to DMBA alone there was a significant

decrease in frequency of agarose-positive EF to 2%. This decline was prevented by treatment of such tracheas with TPA. In a parallel two-stage carcinogenesis study with tracheal transplants, a significant enhancement of the DMBA induced tumor response by TPA was observed. At 20 months after exposure 4% and 37%, respectively, of DMBA and DMBA/TPA exposed tracheas developed invasive carcinomas.

The data suggest that TPA either promotes progression from $EFFU_s$ to $EEFu_{s,ag}+$ or stimulates repliction of $EEFU_{s,ag}+$ at a time when without TPA exposure neither one is occurring at a high enough rate to maintain the $EEFU_{s,ag}+$ compartment in a steady state. An alternative explanation is that TPA prevents reversion of $EFFU_{s,ag}+$. The increased level of the $EEFU_{s,ag}+$ compartment seemingly increases the risk of frankly malignant cells to develop, thus resulting in a higher tumor incidence.

In Vivo Studies of Neoplastic Development in Cultured Normal and Preneoplastic Rat Tracheal Epithelial Cells. We have developed a technique referred to as tracheal repopulation whereby the growth of normal and preneoplastic epithelial cells previously isolated in vitro can be studied in vivo in de-epithelialized tracheal implants. This model attempts to incorporate the desirable features of both in vivo and in vitro systems for studying neoplastic progression. Tracheas denuded of their epithelium are inoculated with suspensions of normal or pre-neoplastic rat tracheal epithelium and then implanted subdermally into isogenic recipients. Within 2 weeks of implantation, a new epithelial lining is established. In recent experiments, reconstituted tracheas were exposed to carcinogen (MNNG), a promoter (TPA) or to vehicle alone (controls). In these experiments we hoped to be able to study the effect of previous exposure history on the oncogenic response in vivo of cultured epithelial cells. Unfortunately, little information could be gained from these experiments due to the high frequency of immunologic (macrophage) rejection observed particularly in those irradiated nude mice implanted with tracheas containing cells subsequently exposed to either TPA or MNNG. Too few tracheas were left in these groups to significantly compare neoplastic development with that observed in controls.

In Vivo Studies of Neoplastic Development in Cultured Normal Human Epithelium. Using the technique of tracheal repopulation, we have been able to "culture" in vivo normal human tracheal and esophageal epithelium previously maintained in outgrowth cultures in vitro. Normal non-exposed tracheal and esophageal epithelium, respectively, form a normal-appearing mucociliary or stratified squamous epithelium within 2-3 weeks of cell inoculation and tracheal implantation into irradiated nude mice. Exposure of tissues to carcinogen (as described above) was again associated with an increased frequency of immunologic rejection. To date, no tumors have been observed in carcinogen exposed human epithelia. Due to lack of funds these experiments had to be prematurely terminated.

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INHALATION TOXICOLOGY

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An unusual use of diesel fuel is in the production of a visual obscurant by the military. We have produced in the laboratory an aerosol by flash vaporization of diesel fuel, in the absence of oxygen, and subsequent recondensation, that resembles that produced by the military under field conditions. The aerosol is then used in rat studies to determine the potential health effects of this visual obscurant.

The first phase of this work was to establish a maximum tolerated concentration for single exposures of animals. Sprague-Dawley rats of both sexes were exposed to concentrations ranging from 2.7 mg/l to 16 mg/l for 2, 4, 6 hr. Deaths occurred during the first 48 hr following exposure and seemed to be associated with pulmonary hemmorhage and edema. Mortality was highly correlated with the product of particulate concentration and duration of exposure with 83% of the variation in mortality being explained by the concentration-time product.

We have also found that acute exposure to an aerosol of diesel fuel causes a concentration related increase in lavaged pulmonary free cells. During the first two days following exposure there was a large influx of neutrophils. Later, 4 days after exposure, an increased number of macrophages was found.

Startle responsiveness to an auditory stimulus was also affected by exposure with a significant decrement in performance remaining even 24 hr after exposure to concentrations greater than 2 mg/l; this indicates potential neurotoxicity.

During exposure breathing frequency is depressed, the degree of depression dependent on aerosol concentration.

Results from the above studies were used to design a protocol for a series of experiments in which animals were exposed a total of nine times once per week or three times per week. The purpose of these experiments was to determine the relative importance of concentration (1.2 to 6 mg/l), duration of exposure (2 hr vs. 6 hr) and frequency of exposure. A matrix design was used in order to reduce the impact of shipment and other block effects. Following exposure pulmonary function, startle responsiveness and the phagocytic activity of pulmonary free cells were studied. Tests were carried out either two days or two weeks after the end of exposure. The main effect appeared to be related to diesel fuel aerosol concentration; however, both duration and frequency of exposure did have some minor influence, particularly on pulmonary function.

In ongoing work we are looking at the longer term (3 month) effects of low concentrations (0.25 mg/l to 1.5 mg/l) on animals exposed for 4 hr per day, twice per week.

Our close association with the Analytical Chemistry Division and experience in the exposure and monitoring of complex hydrocarbon mixtures should present a natural opportunity for becoming involved in the acute inhalation toxicology of synfuel process stream samples as well as other hydrocarbon mixtures.

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Skin Toxicology

DERMAL PATHOLOGY PROGRAM

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The focus of this program has been to characterize the local and systemic toxicity of topically applied synthetic fossil liquids. Activity is organized into four overlapping but independent areas: (a) quantitative analysis of skin carcinogenesis; (b) *in vitro* evaluation of local cutaneous toxicity and chemical-skin interaction; (c) the dosimetry of PAH carcinogens in skin; and (d) extrapolation and risk estimation. Detailed discussion of each area accompanies this overview and summary. Substantial progress has been made in the first three areas, and we have every reason to believe that accumulating information will advance our ability to accurately predict potential health impacts associated with human percutaneous exposure to synthetic fossil liquids.

In summary, we can classify complex materials as to their degree of skin carcinogenicity by expressing potency relative to that of benzo[*a*]-pyrene (2, 3, 5). This information can then be used to evaluate several *in vitro* biochemical measures of general tissue injury (6), rate and extent of metabolism of marker compounds and extent of covalent macromolecular binding (7, 8), and the predictive validity of the Ames test in dermal carcinogenesis (4).

In the future, we anticipate that the emphasis of the program will shift from empirical dose response bioassays to short term biochemical measures applied to skin in organ culture. The establishment of a cryopreserved human skin tissue bank for comparative studies will pace the progress under area (d) above. With the possible exception of a chronic nephropathy (1), cutaneously administered fossil liquids do not appear to be systemically toxic. But much more work is required to determine both the relevance of nephrotoxicity as well as other possible forms of systemic toxicity to workers exposed to these materials.

(a) Quantitative Analysis of Skin Carcinogenesis. Empirical studies have been conducted to determine the optimum experimental design for establishing the potential skin carcinogenic potency of a given material or mixture. Among the factors that have been examined, those determined to have the greatest influence have been the level of exposure, interval between exposures, degree of irritancy, presence of hair follicles and genetic susceptibility.

To minimize the uncertainties which accompany comparisons of response to different materials tested at different times, a relative measure of carcinogenic potency has been developed. The expectation is that physiologic and environmental factors that influence the responsiveness to the reference carcinogen will have a similar influence on the unknown materials and thus bioassays, in terms of relative potency, can be more readily compared.

Statistical methods that are needed to quantify the potential carcinogenicity of coal, shale, and petroleum liquids have been developed. The response variable in these studies is T, the time to occurrence of the first skin tumor in C3H mice that are being exposed to a test material on a weekly basis. In certain situations the response is "censored" due to death of the animal or end of the study, and special methods are needed to deal with this problem. T is assumed to follow a three parameter Weibull distribution, and a dose-response curve that is used to describe the relation between the Weibull scale parameter and exposure has been developed. A known carcinogen, B[a]P, is used as a reference standard, and under comparable test conditions maximum likelihood estimates of the relative potency of the test material are obtained.

A research data management system that utilizes the computer resources of the Computer Sciences Division has been developed to facilitate the analysis of these data. Data entry and quality control procedures are implemented through DBMS 1022 on the DEC PDP-10, and statistical analyses are obtained using SAS on the IBM 3033. Limitations of the Weibull analysis are being evaluated and alternative methods are being developed.

(b) In Vitro Evaluation of Local Cutaneous Toxicity and Chemical-Skin Interaction. In order to better establish a quantitative basis for the extrapolation of cutaneous toxicity observations from animal to man, we have developed *in vitro* systems to study the toxicity, translocation and coupled biotransformation of topically applied materials by mammalian skin (9).

Two systems for monitoring metabolically viable and structurally intact mouse skin as short term organ cultures (24-48 hr) have been developed. An important property of the culture system is that material of interest is applied to the skin surface in a manner similar to exposure in vivo and that the material reaches the epidermal cells by diffusion through the various strata of the epidermis.

Studies with mouse skin maintained in the "static" system with tributyltin chloride, a known skin irritant, have demonstrated that in addition to the inhibition of in vitro incorporation of ^{3}H-thymidine and ^{14}C-leucine into epidermal DNA and protein respectively, there was leakage of intracellular enzymes into the culture medium in a dose and time related manner. Changes in the incorporation of radiolabeled precursors into cellular macromolecules and the release of intracellular enzymes provide sensitive general indicators of toxic tissue injuries. Our studies demonstrating that changes in these biochemical parameters occur in the in vitro skin organ culture system following chemical insults suggest that this in vitro approach may offer a simple, sensitive and early quantitative indicator to assess the cutaneous toxicity of chemicals and complex mixtures such as coal derived liquids. In initial studies with H-coal distillate and benzo[a]pyrene (B[a]P), a model PAH, changes in these selected biochemical parameters were observed and they appear to reflect the dermal toxicity of these mixtures following topical exposure in vivo.

Toxicokinetic studies on mouse skin with the "dynamic" organ culture system using ^{3}H-B[a]P have shown that at the doses investigated the rate of translocation of B[a]P across the intact mouse skin is related to the metabolic status and viability of the skin. Translocation of B[a]P across frozen-thawed skin was significantly impaired, while translocation across mouse skin which had previously been induced with ICDD in vivo was increased by several-fold. Preliminary analysis demonstrates the presence of essentially only polar metabolites of B[a]P in the effluent medium; they included the dihydrodiol and small amounts of glucuronides and sulfates together with an unknown major component, whose identity is currently being investigated. In vitro coupled biotransformation and translocation of B[a]P in mouse skin was dose dependent, and sex and strain differences in the overall translocation were also apparent. This observation that translocation of topically applied foreign chemicals through the skin is coupled to biotransformation and is influenced by factors which modulate the activities of drug metabolizing enzyme has important toxicological implications which need to be examined. Cutaneous metabolism may play an important role in the penetration and subsequent physiological disposition and toxicity of xenobiotics.

These in vitro culture systems developed for mouse skin are potentially adaptable for use with skin samples from other species. This offers a method whereby interspecies comparison may be made under defined conditions and thus provide a quantitative basis for extrapolation of dermal toxicity observations from laboratory animals to man. Indeed, a singular advantage of a totally in vitro approach is that viable human skin obtained from surgical specimens can be evaluated directly. However, before experiments with human skin can be conducted, it is essential to develop methods

for cryopreservation of viable pieces of intact human skin with retention of morphological and biochemical integrity. The availability of non-pathological human skin is simply too unpredictable to support a research program in this area without a skin banking capability. Preliminary studies to develop a protocol for the cryopreservation of skin have demonstrated that with either 1.5 M DMSO or 1.5 M glycerol as cryopreservatives, pieces of mouse skin in culture medium, cooled to -70°C at a rate of 2°C/min and stored in liquid nitrogen are viable after thawing. Criteria of viability included morphology, incorporation of radiolabeled precursor into tissue macromolecules, and successful transplantation to athymic nude mice with hair growth on the grafted tissue 31 days after surgery. The procedure proven to be successful with mouse skin will serve as a starting point for our efforts to develop a protocol for cryopreservation of pieces of human skin of sufficent area to allow for their use in *in vitro* dermatotoxicology investigations.

(c) The Dosimetry of PAH Carcinogens in Skin. Future development of the synthetic petroleum industry will result in potential worker and general population exposure to complex organic mixtures. Given that one of the most probable routes of exposure is dermal, we are interested in devising measures of cutaneous exposure to these materials that will quantitate the overall extent of protocarcinogenic tissue insult. From such a measure, we expect to obtain estimates of human cutaneous exposure risk.

Studies have shown that model PAH skin carcinogens undergo metabolic activation to proximate carcinogens which subsequently bind stereospecifically to target tissue chromatin and nucleic acids. It is our intent to measure the overall extent of such interaction under *in vitro* conditions which allow control of tissue exposure parameters as well as independent evaluation of cytotoxicity.

Previous reports from this laboratory have shown that room temperature fluorimetry, with its intrinsically high sensitivity and when coupled with HPLC, is an alternative method to radiometric detection of benzo[*a*]pyrene binding to DNA. This technique has been applied by us toward an analysis of the problem of chemical induction of carcinogenicity in mouse skin. We have devised a simple procedure for the isolation and purification of DNA from B[*a*]P-treated mouse skin and demonstrated that adduct formation can be detected and quantitated by acid-induced liberation of the pyrene moieties in the form of highly fluorescent tetrols.

Our analysis of DNA isolated from mouse skin in organ culture after topical application of B[*a*]P demonstrates that adduct formation occurred via the metabolic activation of the carcinogen to the diol-epoxide which is similar to results of others who used radiolabeled B[*a*]P. Furthermore, when considered with other data of ours, we believe that mouse skin in organ culture is a suitable diagnostic technique for studying the carcinogenic potential of chemicals and complements our research in the whole animal.

(d) Extrapolation and Risk Estimation. Our intention will be to use correlative biochemical measures developed using mouse skin to monitor

exposure of human skin in organ culture following exposure to model carcinogenic hydrocarbons. Through knowledge which relates the relative extent of metabolic activation and coupled macromolecular binding to the empirical potency of a given material or mixture as a skin carcinogen, we can infer the level of carcinogenic risk to human skin that is associated with a given level of metabolism and binding.

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Cardiovascular Toxicology

CARIDOVASCULAR TOXICOLOGY

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Two major studies were conducted during this period: (a) the effect of cadmium on the arterial system in various strains of rats, and (b) the

dose-response effect of calcium and iron on lead and cadmium-induced hypertension and atherosclerosis.

We have observed during this period that the effects of cadmium on blood pressure in rats are both strain and dose dependent. For example, blood pressure in the Wistar strain is significantly more sensitive to 1 ppm cadmium in the drinking water than to 5 ppm. In fact, following exposure for 6 months to 5 ppm cadmium, these strains had significantly decreased systolic pressure. The salt-insensitive strain did not show a significant change in blood pressure at 1 or 5 ppm cadmium but systolic pressure following exposure for 18 months to 5 ppm cadmium, was significantly elevated. The Sprague-Dawley and Fischer strains both showed a significant increase in blood pressure at both 1 and 5 ppm cadmium.

The factor(s) explaining these differences may be related to the intestinal absorption of cadmium. The concentration of renal artery cadmium in the salt-insensitive group did not show the marked changes observed in other strains. Furthermore, plasma noradrenaline in the salt-insensitive strain did not significantly change at 1 or 5 ppm cadmium during the 18-month experimental period.

The level of cadmium in the renal artery in the Wistar rats following exposure for 6 and 18 months was higher than in the other strains of rats. After exposure for 6 months to 5 ppm cadmium in the drinking water, Wistar rats had significantly reduced systolic pressure, but their renal artery cadmium was significantly higher than that of other strains. This suggests that cadmium absorption and/or tissue retention in the Wistar rats is significantly different from that in other strains. However, the levels of plasma noradrenaline in the various strains do not support this suggestion.

Plasma noradrenaline appeared to be related to renal artery cadmium rather than to changes in blood pressure. In previous studies, we have shown that the exposure to relatively high levels of cadmium (i.e., 50 ppm) reduces blood pressure, whereas exposure to low levels (5 ppm) results in a significant increase in blood pressure. If the arterial concentration of cadmium determines the direction of change in blood pressure (whether directly or indirectly), then these results may have the following explanations: (i) cadmium stimulates the release of noradrenaline from the nerve terminal, resulting in an increase in plasma noradrenaline; (ii) increased levels of this neurotransmitter stimulate or prolong vascular smooth muscle contraction, which leads to an increase in blood pressure; (iii) as the level of vascular cadmium continues to rise, the vascular effect of noradrenaline is antagonized by the toxic effect of cadmium on the contractile proteins of the smooth muscle cells, which results in either no change in blood pressure or a decrease. Previous results from this laboratory support this explanation. Thus the lack of a significant rise in blood pressure in the rats which show a significant elevation in plasma noradrenaline (i.e., Wistar rats exposed to 5 ppm cadmium for 6 months) may be related to the toxic effect of cadmium on muscle proteins and at the nerve terminals.

The mechanism responsible for the significant rise in plasma noradrenaline is presently being determined. We have measured the clearance of [^{3}H]noradrenaline in cadmium-treated rats and observed a significant decrease in rats exposed for 6 months to 5 ppm cadmium. However, significant differences were not observed in rats exposed to 1 ppm cadmium for 6, 12, or 18 months or in rats exposed to 5 ppm cadmium for 12 or 18 months. Thus the observed increase in plasma noradrenaline does not appear to be related to the effect of cadmium on the uptake of noradrenaline. The fact that COMT and MAO activities from the renal artery are reduced may in part explain why noradrenaline is elevated in cadmium-treated rats. Cadmium may also stimulate the release of this neurotransmitter. We have observed in both cats and rats that blood pressure increases by 12-30 mmHg following an intraveneous injection of cadmium. However, if animals are given reserpine (an agent which depletes the stores of noradrenaline at the nerve terminals) 36 hr prior to injection of cadmium, blood pressure does not increase. These results strongly suggest that cadmium stimulates the release of the neurotransmitter noradrenaline.

At present we are attempting to define more clearly these strain differences with respect to the effect of cadmium on noradrenaline metabolism and blood pressure. We are also studying the absorption rate of cadmium as defined by strain differences. These latter studies also involve determining the dose-response effect of calcium on cadmium absorption and tissue deposition.

During this period we have also been investigating the hypertension and atherosclerotic effect of cadmium and lead in the white Carneau pigeon. We have observed hypertension and aortic atherosclerosis in pigeons exposed to drinking water containing lead (0.8 ppm) or cadmium (0.6 ppm) for 6, 12, 18, 24, and 36 months. The cadmium effects were completely antagonized by calcium, whereas calcium and magnesium were both required to antagonize the lead effects. Magnesium alone was not effective in protecting the cardiovascular system against the effects of cadmium and lead. In drinking water containing lead and cadmium the severity of hypertension and atherosclerosis was greater than observed with these elements alone. These results suggest that (i) soft water (i.e., drinking water devoid of calcium) containing lead and/or cadmium induces cardiovascular disease and (ii) hard water (i.e., drinking water containing 100 ppm calcium and 30 ppm magnesium) protects the cardiovascular system against lead and cadmium-induced effects on the cardiovascular system. If these results could be extrapolated to humans, they would predict that the prevalence of cardiovascular disease would be lower in hard-water areas depending on the level of lead and magnesium.

In the studies described above we know that calcium antagonized the cardiovascular effects of cadmium. However, we don't know the dose-response effect of calcium on cadmium-induced changes in the cardiovascular system. For example, if drinking-water calcium were reduced to 50 ppm, would this element still protect the cardiovascular system against cadmium? Furthermore, can the lead effects be antagonized by calcium at concentrations greater than 100 ppm? Lead is known to decrease the gastrointestinal

uptake of iron. The question may be asked, does iron at appropriate concentrations decrease the uptake of lead? The concentration of iron is generally higher in hard water than in soft water. Thi the levels of calcium and/or iron may be factors affecting the absor n of lead in humans.

We are presently engaged in studies designed to determine (i) the dose-response effects of calcium and iron on the accumulation of lead in various tissues and on lead-induced hypertension and atherosclerosis, and (ii) the dose-response effects of calcium on the accumulation of cadmium in various tissues and on cadmium-induced hypertension and atherosclerosis.

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Immunotoxicology

CHEMICAL EFFECTS ON THE IMMUNE SYSTEM

E. H. Perkins P. L. Glover

In an effort to explore the effects of selected energy related and non-energy related chemicals on immunity and host defense mechanisms, two primary areas are under investigation. The first is mechanistic studies aimed primarily toward understanding the role of the immune system in chemical carcinogenesis. In contrast to spontaneously arising tumors, tumors arising following treatment with chemical carcinogens often bear specific non-crossreacting neoantigens. Under these circumstances altered immune competence could be of significance in initiation and progression of tumor growth. We have demonstrated a positive correlation between declining immune competence and early mortality associated with diethylnitrosamine (DEN) carcinogenesis in aging mice. Comparative studies using mice of three different immunologic constitutions demonstrated that decreased immune competence enhanced squamous forestomach carcinogenesis, had no effect on DEN-induced liver tumors and decreased the incidence of induced lung adenomas. The lack of correlation between tumor induction of different tumors in three different organs and altered immune competence demonstrates the importance of both immunologic (e.g., immunogenicity) and non-immunologic factors.

In _in vivo_ studies, tumor lines were established by serial passage but by using the transplantation-excision method and the reportedly more sensitive Winn neutralization test we were unable to induce significant protection against most tumor lines. Sera of mice immunized by these procedures showed no antitumor antibody using antibody binding tests. Collectively, these results showed that DEN-induced tumor lines are non- or very weakly immunogenic at best, and that the more tumorigenic, metastasizing tumors were least immunogenic.

Simultaneously with the foregoing studies, epithelial cell lines were established _in vitro_. Transplantability was tested after primary culture and at every fifth passage thereafter up to the 30th-50th passage. The results of these experiments showed that with increasing _in vitro_ passages the number of cells required to produce tumor in the syngeneic host increased, that is, immunogenicity increases with increased _in vitro_ passage. Mice inoculated with a late _in vitro_ passage (passage 20 or above) were then challenged with the same tumor line used for immunization. It should be noted that although we were unable to induce significant protection using _in vivo_ passaged tumor cells, significant protection was observed with some of the _in vitro_ lines.

To consider a tumor cell line to be a single entity with similar immunological characteristics seems inappropriate because of the cellular heterogeneity observed among these forestomach carcinomas. Therefore, we isolated ~20 clones from each cell line using early _in vitro_ passages

(passage 2-5). The isolated clones were further propagated for 3-5 additional passages. Some of the isolated clones failed and some appeared microscopically identical. However, generally 5-6 different clones from each line grew when tested for their tumorigenicity in syngeneic hosts. However, as many of the isolated clones were not tumorigenic in immunocompetent hosts we attempted to determine whether these clones were (1) nontumorigenic for the host of origin, or (2) tumorigenic but rejected by immunocompetent hosts due to a higher degree of immunogenicity. Cells from these clones were therefore inoculated into immunosuppressed (400R) syngeneic mice. These experiments showed that in most cases tumors developed in the X-irradiated hosts, indicating that the clones are tumorigenic, but were rejected by the immunocompetent host due to increased immunogenicity. These experiments confirmed experimentally that a tumor line is heterogeneous in cellular make-up and can consist of cells with different immunogenic properties. More importantly, some highly immunogenic cells are masked or are too infrequent to be demonstrable in the primary tumor cell population but can be cloned out. An extremely important aspect of these studies will now be to determine if cross reactivity exists among immunogenic clones isolated from the same cell line as well as those isolated from different cell lines. We will raise monoclonal antibody for these studies. Future effort will also determine if we can prevent, or at least reduce, metastases by immunizing the host with highly immunogenic clones following isolation and/or serial transplant. Enhancement of tumor immunogenicity and an understanding of the basic mechanisms underlying this enhancement are paramount to bring such studies to a level of clinical applicability.

The second area is the establishment of the technology to evaluate the activity of suspect chemicals with regard to immunologic toxicity and to obtain fundamental information on the immunodestructive effects and reversibility or recovery from such insults, i.e., the acute and chronic effects of such injury at or near environmental levels, and further to determine an order of magnitude for significant immunosuppressive activity, successful recovery and/or subsequent late effects. In vitro antibody production has proved to be a sensitive and economical screening system to recognize potentially toxic substances. Efforts to measure antigen induced lymphoproliferative events, increased DNA, RNA and protein synthesis have been successful. Toxicity unrelated to cell killing can be readily distinguished and this provides an opportunity for sophisticated mechanistic studies, although the practical application of this system is one of screening for toxicity. Current toxicity testing of the immune system is conducted using different animal models. However, the ultimate objective is to establish acceptable levels and/or risk estimates for man. Furthermore, extrapolation to man of immunotoxicological effects from a single animal species can be misleading because the toxic effects of certain chemicals demonstrate species specificity. Therefore, in vitro comparative studies of several important immune parameters are being developed using human peripheral blood lymphocytes and their subpopulations. The selected parameters are: (1) natural killer cell activity, (2) antibody response, (3) T-cell mitogenesis, and (4) lymphocyte helper and suppressor functions. These studies will provide, for the first time, vital information on the relative sensitivity of important immune functions in man as compared to

one animal species, the mouse, and are a part of our continuing effort in this area.

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RADIATION IMMUNOLOGY

E. H. Perkins P. L. Glover

The purpose of this research is to define the role of immunodepression in radiation-induced carcinogenesis. Two models of leukemogenesis, thymic lymphoma and myeloid leukemia, appear particularly suited for these immunobiological investigations. Efforts focus on determining (1) if the inherent acute immunodepression is a critical obligatory component in the pathogenesis of these radiation induced leukemias, (2) the significance of autogenous immunity in the age related resistance to radiation leukemogenesis, (3) if tumor induction can be restricted by immunologic manipulation (immunoregulation), (4) the relationship between immunocompetence and target cell susceptibility, and (5) the elimination of susceptible target cells, thereby interrupting the pathogenic process. Thymic lymphoma and the myeloid leukemia induced in sublethally irradiated RFM mice are indistinguishable from that which occurs spontaneously. However, the incidence is increased many times above normal and the induction period is dramatically shortened following X-irradiation. Both leukemias are induced more effectively at an intermediate dose level than at lower or higher levels. In both, latency appears to vary inversely in relation to the dose. However, immunodepression increases and remains depressed longer in direct relationship to the radiation dose. Thymic lymphomas are dramatically inhibited and immunocompetence preserved by shielding hemopoietic tissue. Injection of bone marrow but not spleen cells, reduces lymphoma incidence, but does not enhance the restoration of immune competence, whereas injection of spleen cells enhances immune competence but fails to significantly reduce the incidence of lymphoma. In contrast, induction of

granulocytic leukemias is inhibited to a lesser extent, if at all, by partial body shielding. Age related susceptibility to thymic lymphoma is relatively high from birth to early adult life, after which it decreases sharply (after 6 weeks of age) apparently in association with the involution of the thymus, whereas granulocytic leukemia increases during maturation and remains relatively constant thereafter, at least until the sixth month of life. Radiation-induced thymic lymphomas are significantly higher in RFM females than in males; on the contrary, granulocytic leukemias are much more common in males than in females. Thymectomy inhibits the induction of thymic lymphomas, reimplantation restores susceptibility, but thymectomy has little effect on the induction of myeloid leukemia. Splenectomy inhibits the induction of myeloid leukemia but does not appreciably affect the induction of lymphoma. In RFM mice a number of radiomimetic alkylating agents are leukemogenic for thymic lymphoma but do not increase the incidence of myeloid leukemia. The viral etiology in the pathogenesis of both these malignancies emphasizes that autogencus immunity may still be a significant contributing factor in the observed age-related resistance. Because the above complex interrelationships can be independent or associated with immune competence, similarities or differences in the response of these two leukemias to immuno-manipulative and restorative procedures should contribute to our understanding of the importance of immune depression in the induction of these leukemias.

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Carcinogenesis Section

SECTION OVERVIEW - R. J. M. FRY

Since our last report the Toxicology Section has been formed, reducing the size of the Carcinogenesis Section but not the collaboration and interaction. However, we have had some major losses from our senior staff. Three previous members of the section have been appointed to major posts - R. W. Tennant, Chief, Cellular and Genetic Toxicology Branch of the National Toxicology Program; E. Huberman, Director of the Division of Biological and Medical Research at Argonne National Laboratory; and T. Slaga, Director-elect, University of Texas System Cancer Center, Science Park Research Division, Smithville, Texas. They will be missed since they played very important roles in our research activities but they leave a legacy on which we must build.

The Carcinogenesis Section is concerned with those areas of the biology of cells and tissues that are essential to the understanding of how chemicals, viruses, and radiation cause changes that can lead to cancer. Since understanding how neoplasia arise entails molecular, cellular, tissue and whole animal studies, research by the staff of the Section is at these different levels. In the case of some carcinogenic agents, especially radiation, dose-response relationships and the physical factors, such as dose rate and radiation quality and the biological factors that influence the responses are an important aspect of the studies.

The objective of the Molecular Carcinogenesis group is to elucidate the mechanisms of the control of gene expression. The normal control of gene expression drives differentiation and these controls are altered in malignantly transformed cells. Central to these studies has been the preparation of a cloned $cDNA^{TAT}$ because the main system used is the expression of the enzyme tyrosine aminotransferase (TAT). As a result of these efforts a $cDNA^{TAT}$ probe suitable for current studies has been prepared but since there are considerable advantages of a single-strand probe, work on developing such a probe will continue.

A plasmid has been identified that contains sequences that appear to be specific for tryptophan dioxygenase, which is another liver enzyme under strict control. The ability to investigate the control of this enzyme will be an important addition.

The studies on the hormonal effects of TAT expression are at an intriguing stage. While it seems clear that hydrocortisone acts at a transcriptional level, insulin and cAMP may increase the production of gene transcripts. The apparent differences in the mechanisms of control of the gene involved in TAT induction raise interesting questions. The cloned DNAs will also provide a method of investigating the repression of the genes involved in TAT and TO in cells of some liver tumor cell lines.

The research of the Molecular Genetics of Carcinogenesis Group is focused on the role of gene transposition in cancer induction and how different agents may bring about the changes. The induction of myelogenous leukemia in RFM/Un mice by radiation is the model system that has been chosen. These studies form an important part of a larger collaborative study of radiation-induced leukemia at the whole-animal, chromosomal and molecular levels and are based on many years of work at ORNL on this malignancy. Using an appropriate probe it has been shown that in the DNA of RFM/Un mouse cells there is a single locus of the retrovirus thought to be associated with the disease. In collaboration with Peter Lalley of the Division, gene mapping techniques have been used to demonstrate that the DNA related to the ecotropic virus is present on chromosome 5 of the mouse cells. While normal cells of these mice have a single copy of the ecotropic retroviral gene, the myeloid leukemia cells have three additional copies.

Other studies are directed at dissecting the relationships of endogenous retroviral genes to the expression of oncogenes. It is thought that retrovirus-specific long terminal repeat sequences may provide the promoter that governs the activity of an inherent oncogene.

The other side of the coin concerning retroviruses and cancer is the role of genetic control of the prevention of viral gene integration. This has long been an area of research for this group but now the mechanisms by which the presence of the so-called Fv-1 genes in certain strains of mice prevents viral gene integration are being studied successfully with recombinant DNA techniques.

The research of the Molecular Immunology group has taken advantage of the monoclonal antibody technology. This technology is being applied to fundamental questions about tumor cell surface proteins and to the practical and clinical question of drug targeting and tumor imaging. A lung tumor cell surface protein has proven useful for both these areas of research.

Specific antibodies to tumor antigens are expected to home to the tumor cells bearing the particular antigens. Therefore, it is possible, on the one hand, to radiolabel the antibody so that techniques of radio-imaging can be used for locating both primary tumors and any associated metasases. On the other hand, chemotherapeutic agents can also be delivered to specific cells by tagging with the appropriate specific antibody. In our recent work monoclonal antibodies have been attached to liposomes, an effective carrier of drugs, but up until now they have been insufficiently selective for targeting.

Monoclonal antibodies to lung proteins are being prepared with the idea of detecting lung damage with a greater sensitivity than is possible currently.

Antigens provide useful markers of neoplastic change and another area of research carried out by this group is studies of the antigenic changes

in relation to neoplastic development. The findings can thenbe applied to the devlopment of techniques for identifying preneoplastic cells.

The Cytometrics group suffered a great loss with the death of Paul Mullaney, but the facility continues to be used extensively by investigators. The group serves two purposes - first, to provide help to investigators to apply flow-cytometry to specific research problems and second, to develop new techniques, especially in the area of statistical analysis and modeling.

The Chemical Carcinogen Metabolism group continues to unravel the complexities of the metabolism and the action of metabolites of carcinogens. This program is a good example of the absolute necessity of very fundamental studies for providing the understanding of chemical carcinogenesis that is required for a rational basis for risk estimates and safety standards.

The riddle still exists of why benzo[a]pyrene should be a relatively potent carcinogen whereas benzo[e]pyrene, although similar in structure, is either not a carcinogen or is a weak carcinogen. In fact, this group has shown that the claims of the weak carcinogenicity of benzo[e]pyrene are probably due to contaminants. It is tantalizing to establish that movement of a benzo ring is sufficient to change a compound from a carcinogen to a non-carcinogen and not know why. Obviously the question is of great importance since the answer can help in the understanding of structure-function relationships.

Nitrogen-containing heterocyclics occur as the result of certain forms of combustion, such as occur with diesel engines. An understanding of the metabolism of this class of compounds is becoming important and is an active area of research for this group.

In the course of the various metabolism studies it has become clear that there is a difference in the metabolism between cell-free and intact cell systems. Thus, results for tests of mutagenicity that have been obtained using cell-free microsomal preparations for activation of the test compounds may not reflect what happens in the living animal.

Initiation and promotion have been the realm of activity for the Skin Carcinogenesis group. These studies are based on the premise that cancer develops through at least two stages that can be dissected and studied separately. The evidence produced by this group suggests that promotion may not be a single step but perhaps at least three separate stages.

A major task has been the development of an in vitro system that is suitable for obtaining information comparable to that obtained with whole animals. A mutagenicity assay has been developed using epidermal cells in culture for activation of the compounds under test and V-79 fibroblasts for detection of mutagen activity.

The role of prostaglandins in inflammation is becoming clearer and since it is a widely held view that inflammation is an important feature of

promotion, studies of prostaglandins have been designed to test these views.

The studies of Radiation Carcinogenesis involve both ionizing and ultraviolet radiation. There is an increasing interest in neutron radiation-induced cancer. One of the reasons for this interest is that many people believe that experimental animal studies will have to provide the data for protection standards for neutrons as they consider it unlikely that information from survivors at Hiroshima will be adequate. Until very recently there was a misplaced confidence in the belief that estimates of risk of cancer induced by low doses of neutrons could be obtained by linear extrapolation from high doses. It is now clear that dose-response curves for neutron-induced cancer in animals are saturation-type curves and that they bend over at doses below 20 rad. These observations have made it clear that we need a new model for high-LET radiation dose responses and that experiments will have to be carried out with very low doses. Such studies will not be trivial but as the current protection standards for high-LET radiation are being questioned, they are essential.

The development of _in vivo-in vitro_ systems is making it possible to study a number of factors involved in the mechanisms of radiation carcinogenesis. Furthermore, these techniques make the studies of interactions between different carcinogens more informative.

The studies in ultraviolet radiation (UVR) fall into two categories, the first being the development of new model systems and of information necessary for future experiments. For example, an action spectrum for the induction of pyrimidine dimers in the basal epidermal cells of hairless mice has been determined. This investigation is a prerequisite for the study of the action spectrum of UVR-induced skin cancer and the determination of the role of pyrimidine dimers in the cancer induction. Second, are the studies of the interactions of ionizing radiation and UVR and chemical carcinogens and UVR. Our results suggest that UVR can act as a promoter of cellsinitiated with other agents.

Since the last progress report there has been a considerable activity in the field of radiation carcinogenesis. We hosted one of two National Cancer Institute workshops that were held prior to the announcement of the new National Institutes of Health program in low-dose radiation effects. As part of this new program, three new interagency agreements were made between DOE and NCI for studies of radiation carcinogenesis.

Recently, an external radiation carcinogenesis interlaboratory group has been set up with J. B. Storer of the Biology Division as chairman. The group consists of representatives from Argonne National Laboratory, Brookhaven National Laboratory, Lawrence Berkeley Laboratory, The University of California-Davis, and The University of Wisconsin. This group will attempt to obtain standardization in dosimetry and histopathology classification and provide DOE information on research needs.

Students continue to keep us on our toes and hopefully we exercise their minds. There is a predoctoral- and a postdoctoral training program

in cancer research. A predoctoral training program in radiation studies with an emphasis on radiation carcinogenesis has been approved and hopefully will be funded in the coming year. Finally, our education in mechanisms of carcinogenesis was plesantly and effectively advanced by this year's Gatlinburg Symposium on Genetic Mechanisms of Carcinogenesis, organized by W. K. Yang. The subject was timely as the exciting story of the oncogene was ripe for the sort of discussion that a Gatlinburg symposium engenders.

Molecular Carcinogenesis

REGULATION OF GENE EXPRESSION

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The objectives of this research are to define in molecular terms the mechanisms controlling expression of specific genes in mammalian cells, how these effect the process of differentiation, how they are regulated by hormones and other specific effectors, and how they are altered in the dysfunction of gene expression in cells transformed to malignancy. Much of our work is focused on expression of the rat liver enzyme tyrosine amino transferase (TAT), a convenient model since expression is modulated in several important ways and the system is amenable to detailed biochemical analysis. Recent results have provided reagents that permit comparative analyses of other genes expressed in liver and exhibiting interesting regulatory properties. Significant progress has been made in a number of experimental approaches.

Preparation of Reagents: Molecular Cloning. Our principal objective here was the preparation of a cloned $cDNA^{TAT}$, a reagent crucial to further analysis of all aspects of TAT regulation. After hydrocortisone treatment, rat liver mRNAs were purified by size selection to yield a preparation enriched for the very low abundance $mRNA^{TAT}$ to at least 10% of total mRNA; by translation assay $mRNA^{TAT}$ was the principal mRNA species present. By conventional techniques the corresponding cDNAs were inserted into the Pst I site of pBR-322, grown in *E. coli* C-600, selected for antibiotic resistance properties and then for clones carrying inserts of interest. Three have been identified as carrying $cDNA^{TAT}$ inserts that can be removed intact by Pst I digestion. pTAT-1 contains 270 bp; sequence analysis reveals 40-50 GC pairs at each end and a similarly sized region of AT pairs at the 3' end, which make this small cDNA unsuitable for use as a hybridization probe. pTAT-2 and pTAT-3 appear to be identical, both containing *ca* 1000 bp and yielding identical restriction maps; either of these is a suitable probe for current needs.

Identification of these cloned cDNAs as cognate to $mRNA^{TAT}$ is based on the following. (1) In Northern blot analyses all hybridize primarily to a 21S (3000 nucleotide) mRNA that is induced ca 8-fold by hydrocortisone treatment, and secondarily to smaller species. (2) When fixed to DBM or nitrocellulose filters, then hybridized to liver poly(A) RNAs they selectively remove $mRNA^{TAT}$ as determined by translation assay of the non-hybridized RNA. (3) Elution of the hybridized mRNAs followed by oligo(dT) chromatography and translation assay yields a primary protein product that migrates with authentic TAT on SDS gels and is immunoprecipitable by antibody to TAT. Northern blot analysis of the eluted RNAs shows that some of the 21S $mRNA^{TAT}$ species is present but much of it has been degraded to smaller forms. An unequivocal identification will require the matching of amino acid and nucleotide sequences, but as yet sequence data are not available. Another research group is working toward protein sequence, and we plan to work out the DNA sequence. The cloned cDNAs now available (1000 bp) are unlikely to contain coding sequences; for this and other objectives we will require a full length (or nearly so) cDNA. Attempts to synthesize this reagent using a 600 bp fragment of pTAT-2 as primer for reverse transcription with partially purified $mRNA^{TAT}$ as template have not succeeded; the enzyme adds ca 175 nucleotides and then stops, under all conditions tested. This effort is continuing and will be complemented by a resurvey of the original clone bank to find larger $cDNA^{TAT}$.

We have explored a number of methods to prepare the cloned cDNA for use as a hybridization probe. The preferred method is digestion of the plasmid with restriction enzymes Hae III and Hha I. This degrades most of the plasmid to ca 200 bp fragments plus an easily separable fragment of 850 bp containing 700 bp of the 3' end of $cDNA^{TAT}$ and 150 bp of pBR322 sequences contiguous to the 3' end. This fragment has the required blunt configuration at both ends. Another method is partial digestion with the exonuclease III activity of T4 DNA polymerase, followed by resynthesis using the polymerase activity of this enzyme and incorporating [^{32}P]dCTP into the newly synthesized strands. Owing to the structure of this molecule the isotope is incorporated almost exclusively into the complementary strand of the cDNA, a predicted result that has been tentatively confirmed by strand separation on RPC-5 chromatography under alkaline conditions. The $cDNA^{TAT}$ prepared and labeled in this way is entirely suitable for current needs in hybridization analyses but future objectives will be better met with a single-strand probe. Chromatographic separation may prove to be satisfactory but has been erratic in the past. As an alternative we have recently succeeded in subcloning the cDNA insert from pTAT-2 into the M-13 single-stranded phage. This provides a source of both plus and minus strand versions of $cDNA^{TAT}$ that will be important in future work.

A number of other plasmids containing cDNAs cognate to rat liver mRNAs other than $mRNA^{TAT}$ have been partially characterized. The inserted cDNAs range from 2000 to 250 bp, and at least eight clones contain discrete sequences as indicated by restriction mapping, two of which are hydrocortisone-inducible. Of the latter, that termed p35 contains a 250 bp insert that appears to be specific for tryptophan dioxygenase (TO), another highly

regulated "liver-specific" enzyme with interesting developmental properties. This cloned cDNA hybridizes specifically to an 18S mRNA (Northern blot) that is increased 6- to 8-fold by hydrocortisone in adult liver and is very low in fetal liver, all attributes expected of $mRNA^{TO}$. After hybridization selection and elution the translation assay yields a principal protein product that migrates with authentic TO on SDS gels and is immune precipitable by antibody to TO, but this identification is not yet completed to our satisfaction. A large amount of p35 has been prepared and a labeled hybridization probe is currently being synthesized by the T4 exo III/polymerase method described above. Our restriction map of the 2000 bp insert of p69 is very similar to that published by another group for a cDNA specific for phenolpyruvate carboxykinase; we will further characterize p69 and other cloned cDNAs as resources permit.

Hormonal Control of TAT Expression. We have now completed a number of hybridization analyses of the inductions of TAT synthesis by hydrocortisone, insulin, or cAMP, using pTAT prepared and labeled as described and the "dot blot" method of relative transcript quantitation. Results with the steroid as inducer are as anticipated: hybridizable transcripts are increased in accord with the many earlier studies implicating a transcriptional control mechanism in this induction, as well as with studies elsewhere demonstrating transcriptional mechanisms in steroid-mediated control of gene expression.

The mechanisms by which either insulin or cAMP may induce the synthesis of liver enzymes have been the subject of much controversy among research workers in this area. We have been proponents of the view that both are likely to affect post-transcriptional steps in the readout of genetic information, based upon a number of indirect indicators from earlier work. But an unequivocal analysis, requiring the quantitation of gene transcripts, has only now become possible. Our experiments are not yet complete but nevertheless indicate in a straightforward fashion that both insulin and cAMP induce TAT largely via an increased production of gene transcripts. We can not yet exclude the participation of other mechanisms such as accelerated transcript processing or shifts in compartmentalization, but the available data suggest that these are unlikely to be very significant in either response.

These unexpected, but nonetheless exciting results, lead to a host of questions that will be the focus of future work, especially (1) how to reconcile previous results with current data suggesting transcriptional control in all three modes of induction of TAT, and (2) how gene or chromatin structure is altered by three quite different mechanisms to accelerate transcription from an active gene.

Enzymic Differentiation. Earlier work at the enzyme level has suggested that expression of TAT is repressed prior to birth, the repression being lifted almost immediately in the first hours after birth. We have completed an analysis of this differentiation in terms of functional gene products (levels of enzyme and of translatable $mRNA^{TAT}$) and aimed at defining the role(s) of inducing hormones. Results can be summarized as follows. (1) From 18 days postconception to term at 22 days (rat) both

functional gene products are present at levels 10- to 15-fold below adult levels. (2) This limited expression in fetal liver is completely refractory to modulation by glucocorticoids but can be temporarily increased to a limited extent by cAMP, an effect which does not confer glucocorticoid responsiveness on expression. Insulin is without effect throughout the fetal or perinatal period. (3) Immediately after birth both gene products are rapidly increased, rising to very high levels at 12 hr and dropping to the usual adult level by 24 hr postpartum. This shift in expression is accompanied by attainment of the capacity to respond to glucocorticoids. (4) The postnatal "overshoot" of expression between 9 and 18 hr is a consequence of induction by the high levels of endogenous glucocorticoids present at this time, with perhaps some input by cAMP-modulated induction as well. (5) We find no evidence for a necessary role of any of the inducing hormones in the postnatal shift from fetal to adult levels of expression.

We are now completing analysis of TAT differentiation in terms of hybridizable gene transcripts; again our results are puzzling but most intriguing. These data show clearly that TAT transcripts increase well before birth. Transcripts in the 21 day fetal liver are elevated 3- to 4-fold over the fetal level of 18-19 days, and at birth have increased 12-fold to the adult level, presumably owing to comparable changes in rate of gene transcription although this remains to be shown. That this increase in transcripts is not manifested as functional gene products until well after birth points definitively toward a strong element of post-transcriptional control operating during the perinatal period; yet our concurrent analyses of known effectors of control of TAT gene expression in the fully differentiated liver seem to exclude posttranscriptional mechanisms.

Changes in DNA methylation have been implicated in differentiation-associated activation of gene expression in a number of experimental systems, most involving cultured cells. We have found that direct treatment of fetuses in utero with the methylation inhibitor, 5-azacytidine, will precociously activate TAT expression, but the results further complicate the emerging puzzle. Eighteen hours after treatment of 20-day fetuses with an optimal dose of 5-azacytidine, hybridizable TAT transcripts are elevated to the highest level observed, comparable to that of the adult liver after glucocorticoid treatment. This is in accord with the known action of this inhibitor, and suggests that interference with DNA methylation activates transcription of the TAT gene. But under these circumstances the gene transcripts are fully functional; both translatable $mRNA^{TAT}$ and enzyme are also elevated to the high levels of the fully-induced adult liver. Our current understanding of the action of 5-azacytidine does not explain this capacity to overcome the apparent block in the production of functional gene products that is found in the untreated fetal liver late in gestation. Further analysis of this phenomenon is now under way; we see it as the most likely route to providing insight into the currently obscure picture of the mechanisms involved in hepatocyte differentiation.

Another approach to this question is being developed using the putative tryptophan dioxygenase-specific clone p35. We expect to confirm this identification soon; if this succeeds, we are prepared for a comparative analysis of the developmental activation of TO. Like TAT, expression of this gene in adult liver is regulated by glucocorticoids. But its activation during differentiation is quite different, expression being very low in fetal liver and for several days after birth and not reaching the adult level until the second week. How the mechanisms of differentiation-associated gene activation differ in this situation of otherwise similarly regulated genes is expected to be informative. Other cloned cDNAs can also be used in similar comparative analyses of mechanisms in differentiation.

Gene Expression and Malignancy. We have completed an initial study of the effects of the metallocarcinogen, beryllium, on gene expression using the TAT model system in cultured H-35 cells. At the low concentration of 1 μm this otherwise toxic metal did not influence cell growth, an indication that nucleic acid and protein synthesis per se were not affected. Basal levels of TAT, requiring continual rapid synthesis of both enzyme and mRNA, were likewise unaffected. Enzyme synthesis could be induced quite normally by either insulin or cAMP, but the induction by glucocorticoid was specifically inhibited by 50% or greater. Selenium, a toxic but non-carcinogenic metal, inhibited induction by insulin to the same extent as that by glucocorticoid, and other metals had no effect at similar concentrations. Thus it appears that the carcinogenic metal has a very subtle effect on regulation of gene expression, perhaps owing to its demonstrated capacity to bind to nonhistone nuclear proteins. This effect may be related to carcinogenicity and should prove useful as well in delineating the differences in mechanisms by which all three hormones increase production of gene transcripts.

Several rat liver-derived cultured hepatoma cell lines have been examined and found not to express either TAT or TO in terms of functional gene products. In the H-35 line TAT is expressed but TO is not. We are beginning analyses of expression of these genes in these hepatomas using the cloned cDNAs and aiming at determining mechanisms involved in what appears to be malignancy-associated repression of gene function. The relationship between these phenomena and the activation of expression occurring during differentiation should provide mechanistic insight on the concept of "dedifferentiation" in cancer cells.

Turnover of Gene Products. Expression of TAT, TO and similar genes programmed for regulation is characterized by rapid intracellular turnover of both mRNA and protein gene products. We have analyzed mechanisms involved in turnover of TAT in an apparently representative cell-free extract of H-35 cells and find that the soluble enzyme is rapidly aggregated into a very high Mr, inactive form in which the basic subunit structure remains intact. This appears to be the rate-limiting reaction responsible for rapid turnover; presumably the aggregated form enters lysosomes in the cell and is there degraded at a rate typical of other denatured proteins. We found no evidence for the participation of other macromolecules in the aggregation reaction.

Mechanisms of mRNA turnover are poorly understood; most evidence favors the participation of a 5'-exonuclease acting processively, a mechanism that would not yield discrete intermediates. On Northern blots of liver poly(A)-RNAs probed with pTAT we find well-defined species of $mRNA^{TAT}$ smaller than the functional 21S molecule, and the pattern is very consistent. At present some data suggest that this reflects breakdown occurring during manipulation; others indicate that intracellular turnover may be involved. We will continue to explore this phenomenon as well as the potential role of altered degradation of gene transcripts in hormonal inductions, during differentiation, etc., but we are otherwise temporarily phasing out direct studies of mechanisms involved in mRNA and protein turnover.

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MOLECULAR GENETICS OF CARCINOGENESIS

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The specific aims of our research are to understand the molecular mechanism of gene transposition in relation to cancer and to elucidate the effects of various environmental agents on this mechanism. Particular emphasis has been placed on murine leukemia-related retroviruses as an experimental model. Currently, three major research projects have been developed to investigate, respectively, the etiologic role of a particular retroviral genome in radiation-induced myelogenous leukemias, the novel forms of chromosomal genes with proviral DNA structure, and host gene control of retroviral gene integration and expression.

-induced Myelogenous Leukemias of RFM/Un Mice. Following
RFM/Un male mice develop a high incidence of myeloid leukemias
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i) Embryo cell cultures of RFM/Un mice are virus-free but can be
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DNA.

(ii) Dr. Peter Lalley, in collaboration with us, obtained various
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ecotropic virus-specific DNA fragment are concordant with the presence of
the specific genetic markers of mouse chromosome 5.

(iii) Normal RFM/Un cells contain a single ecotropic retroviral gene.
We have recently established that cells from a radiation-induced myeloid
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Our preliminary data indicate that addtional integration of the ecotropi
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Our current efforts are placed on further characterization
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Second, to follow up a previous observation that treatment of cells with cycloheximide in the first 4 hr of retrovirus infection leads to subsequent inhibition of supercoiled retroviral DNA formation in the cells, we examined the effect of other protein synthesis inhibitors and also studied the retroviral DNA integration. Anisomycin, NaF and puromycin gave an inhibitory effect similar to cycloheximide. By infectious center assay, as well as by direct DNA transfection assay, the retroviral linear DNA intermediate formed in the cycloheximide-treated cells was shown to be biologically intact. Also, we have devised a method to detect integration of ecotropic viral DNA in NIH3T3 cells, and with the method have shown a concomitant inhibition of the retroviral DNA integration and the supercoiled viral DNA formation in anisomycin- or cycloheximide-treated cells. These results indicate that a newly synthesized protein or proteins is essential for retroviral DNA circularization and integration.

Mechanism of Fv-1 Gene Restriction. The finding of Fv-1 genes, that affect the incidence of virus-induced leukemias, implies that retroviral genes are subject to host genetic control. Previous results from this laboratory and others have demonstrated that Fv-1 restriction acts to inhibit the formation of supercoiled closed circular DNA by the restricted virus and to prevent the gene integration. We have developed a working hypothesis, postulating that the synthesis and the function of LTRs are maintained by certain virion core proteins and that Fv-1 gene products will interact with these virion core proteins to disrupt the LTR synthesis and/or the LTR function. Thus, our studies have focused on testing the hypothesis and the results are summarized as follows: (1) We have

molecularly cloned the DNA of various virus strains, which include WN1802B, WN1802N, Gross N and RFV, determined the LTR nucleotide sequences of these viruses and observed interesting features of evolutional changes in this regulatory sequence. (ii) We have performed genetic analysis by crossing BALB/c mice ($Fv\text{-}1^{b/n}$) and RFM/Un mice ($Fv\text{-}1^{n/n}$), and observed an apparent segregation of the inhibitory action (on linear DNA synthesis by B-tropic viruses) with RFM-derived $Fv\text{-}1^{n}$ genotype in the progeny mice. (iii) In vitro recombinants were made by exchanging homologous fragments between N- and B-tropic viral DNA clones and were tested for changes in the host range properties by DNA transfection and examination of the progeny recombinant viruses. Preliminary results indicate that genetic determinants of N- and B-tropism are located in a specific area in the gag gene, corresponding to the middle portion of p30 protein.

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Normal and Malignant Cell Biology

TUMOR CELL SURFACE PROTEINS

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A. H. L. Huang[1]
P. K. Lankford

Tumor Antigen Characterization. Cell surface proteins mediate interaction between cells and their environment. Unique tumor cell surface proteins are being identified and quantified in several tumor systems to address the following questions: (i) How do tumor-specific proteins arise during cell transformation? (ii) Can these proteins be used as markers of tumor cell distribution *in vivo*? (iii) Can cytotoxic drugs be targeted specifically to tumor cells using antibody? (iv) Can solid state radioimmunoassay of these proteins provide a means to quantify transformation frequencies?

A tumor surface protein of 180,000 M_r (TSP-180) has been identified on cells of several lung carcinomas of BALB/c mice. TSP-180 was not detected on normal lung tissue, embryonic tissue, or other epithelial or sarcoma tumors, but it was found on lung carcinomas of other strains of mice. Considerable amino acid sequence homology exists among TSP-180's from several cell sources, indicating that TSP-180 synthesis is directed by normal cellular genes although it is not expressed in normal cells. The

Radiation-induced Myelogenous Leukemias of RFM/Un Mice. Following irradiation, RFM/Un male mice develop a high incidence of myeloid leukemias which have pathological features similar to radiation-induced chronic granulocytic leukemias in man. Transmission of the disease by cell-free extracts of the leukemic tissues was reported, but no conclusive evidence for the presence of an oncogenic retrovirus was given. Our work on the possible etiologic role played by an endogenous retroviral gene in the formation of this neoplastic disease is summarized in the following.

(i) Embryo cell cultures of RFM/Un mice are virus-free but can be induced to produce a single type of murine leukemia-related N-ecotropic retrovirus by iododeoxyuridine treatment. We have studied the virological and biological properties of this virus and also isolated recombinant DNA clones of the total genome as well as specific gene regions of this RFM/Un endogenous retrovirus. Using an eco-specific env gene probe, we have found that RFM/Un mice contain a single locus of this virus in the chromosomal DNA.

(ii) Dr. Peter Lalley, in collaboration with us, obtained various hybrid cell clones of Chinese hamster fibroblast, E36, and RFM/Un mouse spleen or bone marrow cells. We have examined these hybrid cell clones and demonstrated that both the virus induction and the presence of the ecotropic virus-specific DNA fragment are concordant with the presence of the specific genetic markers of mouse chromosome 5.

(iii) Normal RFM/Un cells contain a single ecotropic retroviral gene. We have recently established that cells from a radiation-induced myeloid leukemic cell line, established originally by Dr. A. C. Upton and coworkers, has at least three additional copies of this gene, as demonstrated by gel blotting analysis of EcoRI-digested chromosomal DNA. Individual cell clones isolated from this leukemia cell line gave the similar gel blot patterns, suggesting a monoclonal nature of this tumor. Our preliminary data indicate that addtional integration of the ecotropic retroviral genome is also present in primary myelogenous leukemic tissues of irradiated RFM/Un male mice.

Our current efforts are placed on further characterization and molecular cloning of the ecotropic retroviral genes and the adjacent cellular sequences from the individual primary myelogenous leukemias and examination for possible association with cellular protooncogenes.

Carcinogen-cell Genome Interaction. We believe that important genetic targets for environmental carcinogens, mutagens, and/or teratogens are gene elements that are capable of reaction to injuries, that play a regulatory role, that are inherited in the germ-line of the animal, and that are present in multitude in chromosomes. Endogenous retroviral genes associated with chromosomal DNA of the mouse appear to fit these criteria, since they can be induced by chemical or physical perturbation of cellular DNA metabolism, may transduce "oncogenes" and govern their expression by providing the promoter in the form of the retrovirus-specific long terminal repeats sequence, occupy specific loci in the chromosome and belong to an interdispersed long repeat gene family. We have studied the retroviral DNA

sequences in mouse chromosomal DNA by the recombinant DNA approach and made attempts to understand the molecular mechanism of retroviral gene integration.

First, with the consideration that the majority of murine leukemia virus-related sequences in mouse chromosomal DNA are not known to express as complete and infectious retroviruses, we made attempts to isolate these sequences from BALB/c mice as recombinant DNA clones with the Charon 9 lambda phage vector. Of the 14 clones isolated, all were found to contain provirus-like structure. One particular clone, AL-10, showed a restriction enzyme map similar but distinct from the ecotropic and xenotropic murine leukemia viruses of BALB/c mice. The integration of this AL-10 provirus was not found in NFS/N Swiss mice nor in a California Lake Casitas wild mouse. As revealed by nucleotide sequence analysis, the long terminal repeat (LTR) of the AL-10 provirus contains a 170 bp insertion-like element in the U3 region. This insertion-like element appears to be a novel interdispersed short repeat sequence of the mouse genome. The r and U5 region of the AL-10 LTR are similar in nucleotide sequences to those of known murine leukemia viruses. However, there are 5 base changes in the primer tRNA binding sites of AL-10 so that it does not bind proline tRNA or other eukaryote tRNA of known sequences. With the complete nucleotide sequence of gag gene of AL-10 clones obtained, we are able to place the specific oligonucleotide sequences of N- and B-tropic type C retroviruses to a specific location within the p30 protein gene, and also, observe a similarity between the AL-10 and the B-tropic virus in this location.

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Normal and Malignant Cell Biology

TUMOR CELL SURFACE PROTEINS

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Tumor Antigen Characterization. Cell surface proteins mediate interaction between cells and their environment. Unique tumor cell surface proteins are being identified and quantified in several tumor systems to address the following questions: (i) How do tumor-specific proteins arise during cell transformation? (ii) Can these proteins be used as markers of tumor cell distribution *in vivo*? (iii) Can cytotoxic drugs be targeted specifically to tumor cells using antibody? (iv) Can solid state radioimmunoassay of these proteins provide a means to quantify transformation frequencies?

A tumor surface protein of 180,000 M_r (TSP-180) has been identified on cells of several lung carcinomas of BALB/c mice. TSP-180 was not detected on normal lung tissue, embryonic tissue, or other epithelial or sarcoma tumors, but it was found on lung carcinomas of other strains of mice. Considerable amino acid sequence homology exists among TSP-180's from several cell sources, indicating that TSP-180 synthesis is directed by normal cellular genes although it is not expressed in normal cells. The

regulation of synthesis of TSP-180 and its relationship to normal cell surface proteins are being studied.

Monoclonal antibodies (MoAb) to TSP-180 have been developed. The antibodies have been used in immunoaffinity chromatography to isolate TSP-180 from tumor cell sources. This purified tumor antigen was used to immunize rats. Antibody produced by these animals reacted at different sites (epitopes) on the TSP-180 molecule than did the original MoAb. These sera and MoAb from these animals are being used to identify normal cell components related to the TSP-180 molecule.

Drug Targeting and Tumor Imaging. Antibody reagents reacting specifically with tumor antigens should "home" to target cells *in vivo*. Using radiolabeled antibody, tumor cells and their metastases can be located by radioimaging. Pure antibody reagents needed for these studies are now available in the form of MoAb. Studies using iodinated MoAb to TSP-180 indicate that radioiodinated antibodies are not well suited to *in vivo* imaging, and other labeling procedures are being explored. Furthermore, a detailed study of interaction of MoAb with cell surface antigens has been done in three different tumor systems. These results show that quantitatively, the interaction follows a simple mass action expression for bimolecular reactions. Thus for effective tumor imaging, the factors that are important are (i) the purity of the labeled antibody, (ii) the affinity constant of the antibody, and (iii) the antigen concentration of the tumor cells.

If antibodies can localize at tumor cell sites, they should be useful for delivery of cytotoxic chemicals to the tumor cells. Intermediate carriers, such as dextran and liposomes, should allow large numbers of drugs to be delivered by a limited number of antibody molecules. Monoclonal antibodies have been covalently derivitized with fatty acids and attached to liposomes. These liposomes bind specifically to target cells *in vitro* but show very little increase in efficiency of drug delivery relative to free drug. Methods to promote more efficient drug transfer from liposomes to tumor cells and specific liposome targeting *in vivo* are now being tried. This specific chemotherapy should be useful as adjuvant therapy to surgery or radiation for primary tumors.

Tumor Therapy with MoAb. Mice were treated with monoclonal antibody to TSP-180 in an attempt to prevent artificial lung metastasis formation from i.v. cell injection. This passive immunization was not effective. Several factors, including antibody class and subclass, antibody specificity, tumor antigenicity and amount of antibody administered, could be important factors.

Syngenic MoAb has been developed to an antigenic tumor (Moloney Sarcoma) and has been shown to be tumor specific with a high affinity constant. This antibody is therapeutic for the Moloney sarcoma tumor if administered before cell challenge or after tumors are well established. The molecular mechanism of this therapeutic effect is under active investigation.

Solid State Radioimmunoassay for Carcinogenesis. Tumor-specific proteins represent cell phenotypes characteristic of cell transformation. Quantitation and sensitive detection of these proteins should provide a method of early detection of transformation. Automated solid state radioimmunoassay techniques have been developed that can be used to screen thousands of cell clones for transformed phenotypes. Assays using monoclonal antibody coupled to solid supports should allow assay of more than 10^6 cells at a time. Details of solid state assays with MoAb have been worked out. Affinity constants of antibodies for antigen in fluid phase or coupled to a solid support do not vary significantly; however, antigen presentation on solid supports depends on the configuration of the support as well as the mode of attachment of the antibody. Monoclonal antibody to fragment D(FgD) of human fibrinogen has been used to develop a solid state assay for FgD which should be diagnostic of plasminogen activator activity. Although the affinity constant of the specific MoAb is relatively low (10^7 l/mole), the assay has been made sensitive at the ng/ml range by coupling the specific MoAb with non-specific antibodies of high affinity (6×10^9 l/mole). This assay should be useful in screening clones of cells for plasminogen activator activity and for analysis of serum FgD levels in cancer patients.

Monoclonal Antibody for Lung Toxicology. Analysis of toxic injury to lungs can be obtained chiefly through invasive procedures of biopsy or at autopsy. In contrast, assay of enzyme levels in serum has proven to be diagnostic of liver damage. The enzymes found in serum are those released from damaged or regenerating liver cells. If antibodies to lung specific proteins can be developed, they may be useful as reagents to detect lung proteins in serum as a function of toxic injury. Rat MoAb to mouse lung proteins have been isolated from rats immunized with two different lung protein immunogens. These antibodies are currently being analyzed for their binding specificity and for their suitability for assays of lung proteins released into the serum of toxin treated animals.

Study of Antigenic Markers in Developing Neoplasia. Most malignant tumors in man arise from epithelia via a multistep process, in which new cell populations having altered biological and morphological properties appear and progress. Recently there have been reports that describe this progressive nature of neoplastic disease *in vitro*. The purpose of this research project is to define and quantify cellular markers that can identify altered cell populations appearing in transformation of rat tracheal epithelial cells, and to delineate their role in the development of neoplastic cell populations. Identification of such markers would not only aid in understanding preneoplastic disease, but also offer new approaches for detection and quantitation of such alterations in cells exposed to putative oncogenic agents well before the appearance of the neoplastic phenotype. Our approach has been to define early phenotypic changes in short-term cultures of rat tracheal epithelial cells exposed to chemical carcinogens. Altered phenotypes have been identified on the basis of an increase in relative DNA content as compared to non-altered cells and expression of antigen that is not expressed on normal cells. These changes appear early occurring in precursor cells that eventually give rise to neoplastic populations and correlate with the initial breakdown of those

mechanisms which regulate normal growth and differentiation. Our goals are to be able to predict the probability of neoplastic cell types appearing in exposed cell populations well before the appearance of the malignant cell phenotype.

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CYTOMETRICS

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The major goal of this program is to develop new approaches using flow-cytometric measurement techniques that can identify and quantitate cellular changes that occur as a result of toxic or mutagenic agents. Major areas of investigation involve methods to improve data and image analysis and application of those methods to biological assay systems. The program also functions as a multi-user facility. Investigators last year included D. M. Popp, R. A. Popp, A. J. P. Klein-Szanto, A. Marchok and R. Klann.

The facility for flow cytometry and cell sorting consists of an Ortho Cytofluorograph Model 50H/H, which is connected to an Ortho Instruments 2150 Data Acquisition and Analysis System. The 2150 system consists of two Data General MP200 microNova processors, a 10 Mbyte disk drive (5 Mbyte removable cartridges), a VT200 terminal, a Grinnell image display system, and a Tektronix hardcopy. The software packages supplied by the manufacturer perform data acquisition and display, and provide some standard methods of off-line data analysis. In addition, original software can be developed in FORTRAN and PASCAL.

In the current configuration of the instrument there are two argon-ion lasers available for excitation and analysis of fluorescent compounds. One laser (Lexel 95) can be tuned to 488nm for excitation of DNA staining probes (i.e., propidium iodide) and fluorescein-isothiocyanate (FITC) labeled probes. The instrument was modified during the last year to include a Spectra Physics 165-8 laser that can be tuned to the near UV-region (353-360 nm). This increased the capabilities of the facility by allowing analysis using different fluorescent probes such as H033342 (a vital stain for DNA content) and carcinogenic polycyclic hydrocarbons (i.e., benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene). The polycyclic hydrocarbons can be excited directly using the Spectra Physics laser, allowing detailed study of the interaction of carcinogenic compounds with cellular receptors of living cells.

Statistical Analysis of Flow Cytometric Histograms. Flow-cytometry has been applied to estimations of nuclear DNA per cell, cell-cycle analysis and chemically-induced cellular differentiation. The extraction of information from DNA histograms has been a major research effort although most of the methods are not restricted to that particular application. In a variety of applications it is of interest to detect

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*Deceased

significant differences between two single or groups of histograms, in other words, to test the hypothesis that two measured histograms are samples from the same probability density function. For that purpose parametric tests, as well as non-parametric statistical tests, have been implemented on the 2150 and a new testing procedure devised. One other closely related problem is the classification of measured data patterns into two or more groups. Classifiers using data stored in LIST mode have been implemented. Using the results on the mixture identification problem, these classifiers can now be applied to HIST mode data.

Several algorithms which identify various components of histograms were implemented on the 2150 data analysis system (cell cycle analysis programs). Three new methods which use different deterministic and statistical approaches to identify the components of a mixture of normal or log-normal density functions were devised and their performance is being studied. The objective is to identify and quantitate individual components from mixed cell populations exposed to putative transforming or toxic agents. These methods are also applicable to measurements made of more than one parameter.

Slit-Scan Flow Cytometry: S. .rability Properties of Cell Features. A model was developed to compare the separability of cell populations described by features measured in low resolution slit-scanning flow systems with their separability when the features are extracted from high resolution digitized cell images. The results show that although the accuracy of the feature measurements deteriorates for increasing slit width, this is not necessarily true for the discriminatory power of the features. Depending on their original position in the high resolution feature space, the cell populations may even be located farther apart in the space of low resolution slit-scan features for reasonabl" small widths of the slit. Results obtained with images of cervical cells and simulated slit-scan measurements could be explained by the model.

Carcinogenesis in Respiratory Epithelium. Several investigators have correlated changes in DNA content with particular stages in the development of neoplasia of the respiratory tract epithelium. Changes in DNA content have been shown to be a reliable marker for identifying new populations appearing in carcinogen-exposed tracheal epithelial cells. Observations on the change in DNA content are consistent with multistep models of malignant transformation of cells in primary culture.

Tracheal implants exposed to dimethylbenz[a]anthracene develop numerous metaplastic and dysplastic lesions. In the preneoplastic lesions dark epithelial cells appear and increase in number as the lesions progress to neoplasia. The characteristic staining properties of these dark cells is thought to be due to an increased production of RNA and/or keratin. The percentage of dark cells present in the epithelial lesions was determined by staining the cells with pyronin-Y.

Toxicology of ENU in BALB/c Mice. Bone marrow from BALB/c mice removed at intervals (up to 56 days) following exposure to three non-lethal doses of ethyl nitrosourea (ENU) was examined by flow-cytometry for changes

in cell-cycle kinetics. The percentages of cells in G_1, S and G_2 +M phases of the cell cycle were determined using several cell cycle analysis programs. At all doses of ENU (2, 4, 6 mg/mouse) a dose-dependent reduction in the number of cells in S was observed at 24 hr. The early recovery of cells in S was dose dependent and long term recovery suggests a permanent displacement of cell cycle phase compartments. Although all compartments recover immediately (within 3 days) following the lower dose, the G_1 and G_2 + M compartments continue to decrease following the two higher doses, suggesting significant perturbation of stem cell proliferation and differentiation. These results show that flow cytometry can be used to analyze the toxicity of unknown substances on proliferating systems.

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Chemical Carcinogenesis

CARCINOGEN ACTIVITY IN HUMAN AND RODENT CELLS AND TISSUES

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Our laboratory has progressed in a number of areas in understanding the carcinogenic potential of both polyaromatic carcinogens and nitrogen-containing heterocycles, which are proving to be powerful mutagens and carcinogens and are major components of fossil fuel combustion. Earlier work in our laboratory suggested that the major reason for the lack of carcinogenic potential of benzo[e]pyrene was its inability to metabolize a reactive bay-region diol-epoxide. It appeared that the major pathway for metabolism by the monooxygenase system was at the opposite side of the

molecule in the K-region. We have now extended these observations to include early and later time points of metabolism of benzo[e]pyrene by hamster embryonic cells. Our earlier results dealt with a single 24 hr time point. We felt that it was possible that longer incubation times might allow formation and accumulation of bay-region metabolites. We followed the turnover of benzo[e]pyrene by hamster embryonic cells over several days during which a steady state metabolism was established. At no time point was the bay region benzo[e]pyrene-9,10-diol detectable. Our observations established an upper limit of 0.1% of total metabolites for this derivative. Furthermore, several treatments which were known to induce or increase bay region metabolism failed to yield greater amounts of benzo[e]pyrene-9,10-diol. This indicates to us that the weak carcinogenicity of benzo[e]pyrene that is occasionally reported in the literature is most likely due to contamination with benzo[a]pyrene in the sample. We are still faced with the enigma of why movement of a benzo-ring on benzo[a]pyrene to form benzo[e]pyrene completely destroys its carcinogenic capability.

We have continued our studies attempting to understand the stereochemical specificity of benzo[a]pyrene diol-epoxides with cellular macromolecules. Previous evidence had suggested that hamster embryonic cells metabolize benzo[a]pyrene-9,10-diol to a non-bay region diol epoxide-9,10-dihydroxy-7,8-oxy-7,8,9,10-tetrahydro-benzo[a]pyrene (reverse-BPDE) and that this metabolite bound covalently to nuclear protein but not to DNA when incubated with intact cells. In an effort to understand this binding specificity we have examined the physical and covalent interactions of reverse-BPDE with DNA *in vitro*. As a control, we have used 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydro-benzo[a]pyrene (DPDE), which is a major metabolite of benzo[a]pyrene that forms covalent adducts with DNA in intact cells. Both BPDE and reverse-BPDE formed non-covalent intercalation complexes with DNA with similar association constants. This intercalation can lead to either hydrolysis or covalent binding. For both diol epoxides, the ratio of the rate constants for these two processes is the same, so that a high DNA concentration BPDE and reverse-BPDE bind equally well to DNA. The larger differential in intact cell DNA-binding may be due to differences in the rates of repair of BPDE-DNA adducts compared to reverse-BPDE-DNA adducts. This stereochemical specificity surely plays a critical role in determining how benzo[a]pyrene causes malignant transformation in a given cell type.

We have begun a series of experiments to determine the parallelism between polycyclic hydrocarbon carcinogenesis as major products of fossil fuel combustion and nitrogen-containing heterocyclics which appear to form in high-pressure lower-temperature combustion systems as seen in diesel engines. The metabolism of two nitrogen-containing polycyclic aromatic hydrocarbons, 6-nitrobenzo[a]pyrene and 10-azabenzo[a]pyrene which are known environmental contaminants and mutagens, was studied in hamster embryonic cells and cell-free systems using microsomal homogenates prepared from liver. High pressure liquid chromatography (HPLC) was used to separate organic solvent-soluble metabolites. Metabolism of both compounds by hamster embryonic cells yielded both organic solvent-solvent and water-soluble (conjugated) metabolites, whereas with microsomes, only organic

solvent-soluble products were formed. Glucuronide conjugated products were released by treatment with beta-glucuronidase and could also be determined by HPLC using the same column and gradient system.

It was found with hamster embryonic cells that dihydro-diols were the main metabolites present in organic extracts of both 6-nitro and 10-azabenzo[a]pyrene. However, treatment of the aqueous phase with beta-glucuronidase released a substantial quantity of phenol. Microsomal metabolism appeared to form free phenols as the predominant metabolites, with much smaller amounts of dihydro-diols. Dihydro-diols are identified by using trichloropropane oxide as a specific inhibitor of epoxide hydrase. Phenols were detected by virtue of their characteristic red shift when their UV visible spectra were determined under alkaline conditions. The identification of the exact chemical structure of these metabolites is presently under way. Our data have shown a difference in metabolism between cell-free and intact cell systems, the former being routinely used for short term mutagenicity testing. This suggests that the use of microsomes as cell-free activators may not necessarily reflect the fate of the chemical in the intact animal or human.

Covalent binding of 6-nitrobenzo[a]pyrene and 10-azabenzo[a]pyrene to nuclear macromolecules was also investigated and compared to the binding profiles seen for benzo[a]pyrene. Benzo[a]pyrene binds to both DNA and RNA with a specific activity for nuclear proteins being particularly high. 6-Nitrobenzo[a]pyrene exhibited similar characteristics with an even higher affinity toward binding to nuclear protein. However, specific binding of 10-azabenzo[a]pyrene to RNA and DNA in nuclear proteins was several times lower than 6-nitrobenzo[a]pyrene and benzo[a]pyrene itself. Therefore, substitution of a nitrogen atom in the 10 position of benzo[a]pyrene may hinder events such as metabolic activation and macromolecular binding and may mediate against derivatives of this compound being strong carcinogenic agents.

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SKIN CARCINOGENESIS: TUMOR INITIATION AND PROMOTION

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The overall goal of this research program is to investigate and understand the mechanisms whereby the polycyclic aromatic hydrocarbon (PAH) carcinogens cause skin cancer. This problem is being approached by both in vivo as well as in vitro studies using cultured keratinocytes. Skin carcinogenesis can be divided into at least two distinct stages, referred to as initiation and promotion; the components of each stage can be defined and studied individually. More recent studies have shown that the tumor promotion stage can be further divided into two and possibly three stages. Use of different kinds or classes of tumor promoters have added significantly to understanding those events that can cause promotion. Inhibitors and stimulators of tumor initiation are being employed to better determine the critical events in PAH tumor initiation with particular regard to interaction with nuclear components. The critical events of tumor promotion are being determined through the use of modifiers, especially those specific for one or more of the promotion stages. Currently, the importance of the increase in "dark cells" that are thought to be primitive stem cells, the changes in states of differentiation and the role of prostaglandins in promotion are being pursued.

Keratinocyte Culture. Our proposed goal is to develop not only a reliable and quantitative in vitro transformation system using epidermal cells but also one in which two-stage transformation is operational using phorbol-ester tumor promoters. To this end it has been necessary to establish both optimal culture conditions and to understand and develop ways to measure the normal and tumor promoter-induced changes in cell differentiation processes. Recent improvements in culture conditions, which include use of low calcium media and collagen substratum, have made the growth of adult epidermal cells feasible. Additionally, a simple and reproducible means of separating populations of epidermal cells based on their buoyant density has been developed. Keratinocyte buoyant density decreases as the cells terminally differentiate. Using this procedure for the preparation of subpopulations of keratinocytes, it was found that topical treatment of SENCAR mice with complete, first and second stage promoters, but not hyperplastic reagents, accelerated the rate of terminal differentiation of cells with densities less than 1.074 g/cm^3, but had little effect on keratinocytes with a greater density. TPA treatment also converted dense basal cells to cells with a lower density, reduced plating efficiency and increased rate of differentiation suggesting that TPA induces a subpopulation of basal cells to commit to terminal differentiation and accelerates the rate of differentiation of committed cells.

Keratinocyte Cell-Mediated Mutagenesis Assay. The mouse skin two-stage carcinogenesis protocol (initiation-promotion) is widely used in the bioassaying of suspected carcinogenic chemicals and putative modulators of the initiation phase of chemical carcinogenesis. However, the expenses associated with caring for large numbers of animals and the length of time necessary for tumor development emphasize the need for in vitro short-term model systems which have similar capabilities. Although there are exceptions, most carcinogens are mutagens. Consequently, mutational assays are often used as preliminary screening systems for hazardous chemicals. We have developed and characterized a cell-mediated mutagenesis assay that uses living cultured newborn murine keratinocytes for the metabolic activation of promutagens and Chinese hamster lung V-79 fibroblasts for detection of resulting mutagens. Mutations at, or affecting the hypoxanthineguanine phosphoribosyl-transferase locus are scored by resistance to 6-thioguanine. The relative mutagenicities of several polycyclic aromatic hydrocarbons (PAHs) and the non-PAH promutagens, dimethylnitrosamine and sterigmatocystin, correlated with their in vivo skin tumorigenicity determined in a two-stage carcinogenesis protocol. Metabolic activation of the promutagens to ultimate mutagens was dependent upon the presence of the keratinocyte feeder layer. 7,8-Benzoflavone, a potent inhibitor of DMBA dependent initiation in mouse skin, inhibited DMBA dependent mutagenesis in the cell-mediated assay. Collectively, these results suggest that the keratinocyte cell-mediated mutagenesis assay is an excellent in vitro model for predicting promutagen activation in mouse skin.

Several stocks and strains of mice differ dramatically in their susceptibility to two-stage carcinogenesis. SENCAR mice are very sensitive to DMBA initiation and TPA promotion, whereas C57Bl/6, BALB/c and DBA/2 mice are less susceptible to DMBA initiation and TPA promotion. An important question is whether the differences in susceptibility to two-stage carcinogenesis are determined at the initiation or promotion stages. Using the keratinocyte cell-mediated assay as an in vitro model system for the initiation phase of two-stage carcinogenesis, we have determined that cultured keratinocytes from SENCAR, DBA/2, BALB/c and C57Bl/6 mice all activate DMBA to mutagenic derivatives, and that the mutant yields do not differ by a factor of 2 for any of the mice over a range of DMBA concentrations. These results indirectly suggest that differences in response to promotion with TPA are responsible for mouse strain-stock differences in susceptibility to two-stage carcinogenesis.

Role of Prostaglandins in Tumor Promotion. The tumor promotion stage in skin carcinogenesis is characterized in part by the presence of an inflammatory state. Since most studies have suggested that inflammation is essential for promotion, the effects of some of the mediators of inflammation are being studied, particularly those related to arachidonic acid metabolism. Earlier work showed that use of specific prostaglandins could have either an inhibitory or enhancing effect on promotion. Use of inhibitors specific for the various branches of the arachidonic acid pathway, including the thromboxanes, the hydroperoxy fatty acids and the prostaglandins, has provided valuable information on their involvement

in promotion. These studies strongly suggest that products of the lipoxygenase pathway are essential for promotion since inhibitors of this pathway inhibit promotion. The particular metabolite(s) responsible are being investigated. Further work is needed to determine how and in what manner tumor promoters disturb the normal balance of products from the arachidonic acid pathway and specifically how these agents alter tumor promotion.

Interactions of Polycyclic Aromatic Hydrocarbons with Epidermal Macromolecules. The mechanism of carcinogenic action of polycyclic hydrocarbons is being investigated through the use of tumorigenicity and covalent binding studies in the epidermis. Tumorigenicity studies with benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), the ultimate carcinogenic metabolite of B[a]P, have established a differential activity of the diastereomeric forms of BPDE in the two-stage skin tumorigenesis system. Racemic BPDE-anti diastereomer exhibited tumorigenic activity in SENCAR mice approaching that of the parent hydrocarbon, whereas racemic BPDE-syn showed no tumorigenic activity above control levels. The covalent interactions of these BPDE stereoisomers with epidermal macromolecules were examined in vivo. Our results suggested that the anti- and syn- diastereomers exhibited different time courses of covalent binding in whole epidermis, with the carcinogenic anti-diastereomer bound to DNA at levels twice that of the noncarcinogenic syn-diastereomer after 24 hr exposure time in vivo. Binding of the two diastereomers was also compared in the epidermal basal cell population following various exposure times in vivo. The results of this study demonstrated that nearly equivalent levels of both diastereomers were detected covalently bound to DNA, RNA and protein at all time points. Since the epidermal basal cell layer is the presumed target cell population for initiation, the absence of any observable difference between the amount of anti diastereomer (the carcinogenic form) and syn diastereomer (the noncarcinogenic form) bound to basal cell DNA would suggest a lack of correlation between extent of DNA binding and the previously reported relative carcinogenicities of the two diastereomers. Additional studies are underway to investigate the binding of the two optically pure enantiomeric forms of BPDE-anti in SENCAR epidermis. An examination of the time course of covalent binding to DNA in whole epidermis and in the basal cell layer will be correlated with the various DNA adduct profiles as detected by HPLC.

Studies on the differential interactions of BPDE stereoisomers with cell macromolecules have also been carried out in the avian erythrocyte nucleus, a system permitting the isolation of various populations of mononucleosomes enriched for high mobility group (HMG) proteins. Our results demonstrated a high degree of binding by the carcinogenic BPDE-anti diastereomer to HMG 14 in mononucleosomes isolated from nuclei exposed to the carcinogen in vitro. In contrast, the noncarcinogenic BPDE-syn diastereomer did not interact with HMG 14, although it associated at significant levels with the lysine-rich histones, H1 and H5. Further studies are underway to determine whether the specific interaction of the carcinogenic stereoisomer with HMG 14, a protein associated with actively transcribed chromatin, may constitute a biologically important interaction which reflects the tumorigenic ability of the BP-diol epoxide.

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Radiation Biology

RADIATION CARCINOGENESIS

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The research efforts of the ionizing radiation carcinogenesis program consist of studies on neutron carcinogenesis, time-dose relationships, the role of host factors in radiation carcinogenesis, and the carcinogenic interactions of radiation and chemicals. These problems are being pursued with in vivo studies as well as in vitro and in vivo/in vitro approaches. A common theme among all of these studies is the examination of mechanisms and the establishment of general principles which may allow a better understanding of the risks to humans from radiation exposure. Data from all of these studies are also being used to examine more direct methods of extrapolation of animal data to human risks.

The program in ultraviolet radiation carcinogenesis (UVR) is concerned with the following studies: (1) development of model systems, methods and

background information that is necessary for designing quantitative UVR carcinogenesis experiments; (ii) the role of interactions of UVR and chemicals; and (iii) interactions between ionizing and ultraviolet radiation in skin carcinogenesis.

Neutron Carcinogenesis. The largest research effort is directed toward the study of carcinogenesis after exposure to neutrons. The objective of these studies is to define dose response relationships, RBE-dose relationships and dose rate relationships for the induction of several murine tumors. In one study emphasis has been placed on the induction of lung and mammary adenocarcinomas in BALB/c mice, while in another study using neutrons we are examining the induction of myelogenous leukemia in RFM mice.

A major unresolved issue with respect to late effects (tumor induction and life shortening) from neutron exposures is the shape of the dose-effect curve at doses less than about 40 rads. Currently there are two principal schools of thought on the subject. One holds that the curve is convex (a function of the square root of the dose; the other holds that the most likely relationship is a simple linear function. It is of considerable theoretical as well as practical importance to establish which (if either) of these hypotheses is correct. If, as is now widely believed, the dose-effect relationship for low LET radiations (e.g., γ rays or X rays) can be approximated by a linear slope at low doses or dose rates, then it follows that the relative biological effectiveness (RBE) of neutrons will rise to very high values at very low doses if the true curve for neutrons is convex. If it is linear, then the RBE will have a constant value in low dose range.

In our studies to date on tumor incidence in mice, we have been unable to reject (on statistical grounds) either hypothesis. We recently analyzed survival time data from recent experiments for which the histopathology is not yet complete and plotted shortening of the mean life span as a function of dose. In one case, namely when the doses were delivered in two fractions separated by 30 days, we could reject the square root of dose function while a linear function gave an acceptable fit. We also tested goodness of fit of the alternative functions with data available in the limited literature on life shortening at low neutron doses. In most cases, either function gave an acceptable fit but in the three instances where one function or the other could be rejected, it was always the square root function that was rejected while linearity provided an acceptable fit.

The most promising approach to distinguishing between these alternatives is to conduct fractionation studies with doses per fraction of about 20 rad and long intervals (about 30 days) between fractions. Such studies are planned.

In addition to studies on the life shortening and carcinogenic effects of neutrons, studies are also underway to understand the mechanistic basis for the observed effects. One aspect of this work involves in vivo/ in vitro techniques which have been developed and which are being developed to study cell killing, transformation and progression to neoplasia using mouse mammary epithelial cells. A second aspect of the studies on

mechanisms involves the RFM mouse myelogenous leukemia model. In collaboration with Drs. Preston and Au (Biology Division), the possible role of specific chromosome aberrations in myeloid leukemogenesis is being examined. In another collaborative study Dr. Yang of this Division is examining the possible role of transposition of genetic elements in the development of myelogenous leukemia.

Estimation of Cancer Risk for Human Populations Exposed to Fission-Energy Neutrons. Recent reassessments of the dosimetry for the Japanese survivors of the atomic-bombings raise the possibility that the previous estimate that a significant component of dose in Hiroshima was due to neutrons may be in error and that, in fact, the neutron dose was trivial. If this reassessment proves to be correct, then it follows that estimates of risk from neutron exposures must necessarily be based on data from animals since there is no other human study group available for making such estimates.

We have attempted to make risk estimates for human populations based on the following considerations. We have shown elsewhere that, in mice, the relative risk of death from malignant neoplasms following gamma irradiation is remarkably similar to the estimates of the human lifetime risk of death from neoplasms developed by the BEIR committee or as calculated from the Japanese experience. Our first assumption, then, is that the relative risk for neutrons will also be essentially the same for mouse and man. It has been generally observed that the incidence or relative risk of various neoplasms in neutron-exposed mice begins to plateau at between 25 and 50 rad. For this reason, to obtain risk estimates in the relevant low dose domain, it is necessary to restrict analysis to mice exposed to doses of less than 50 rad. Data on tumor incidence (deaths) are limited. From one study we have performed, we estimate that the relative risk of death from a malignant neoplasm increases at a rate of 5%/rad in the low dose range. (In other words, the mortality from cancer doubles at 20 rad).

While there are limited detailed experimental data on neutron-induced cancers, there is a much larger amount of information on neutron-induced life shortening. We and others have shown that in the low-to-moderate dose range, life shortening in mice from gamma ray exposures is due almost entirely to the increased incidence and/or earlier onset of fatal malignancies. We have recently shown that this conclusion also holds for neutron exposures. The problem, then, is to go from estimates of life shortening to estimates of the relative risk of death (presumably the relative risk of death from fatal malignancies). We have been able to show that the relative risk of death can be estimated directly from life shortening by a rather simple equation. We have calculated relative risks of death from cancer in neutron-exposed mice from our data and from data in the literature on life-shortening. These estimates, by strain and sex, range from a 3.5 to 5% increase in relative risk per rad.

Estimates of the lifetime relative risk to human populations exposed to low doses or dose rates of gamma rays, developed by BEIR-III, ICRP, and UNSCEAR, indicate an increase in risk of from 0.05 to 0.14% per rad. If

the occupational dose limit for gamma rays is based on these risk estimates, then our indirect analysis would suggest that, in order to make risks from neutrons and gamma rays comparable, either the dose limits for gamma rays should be increased by a factor of 5 to 10 or the dose limits for neutrons should be reduced by a similar factor. Note that the current limits are 5 rad for gamma rays and 0.5 rad for neutrons. It would seem imperative that more data on cancer incidence in neutron-exposed animals be obtained as soon as possible.

Time-dose Relationships. Because of the many factors involved in tumorigenesis, interpretation of differences in the effects of different rates of exposure on tumorigenesis may be confounded by their effects on factors influencing tumor expression rather than the induction of initial events. To develop reliable estimates of radiation risk and to determine the general applicability of the principles derived from experimental studies, more information on time-dose relationships for the induction of solid tumors and the basis for these relationships is required. Further information on the underlying mechanisms for these time-dose relationships is essential. Over the last year we have initiated studies designed to examine time-dose relationships for radiation carcinogenesis. The objectives of this project are to (i) examine time-dose relationships for the induction of lung adenocarcinomas and mammary adenocarcinomas in BALB/c mice after gamma ray irradiation, including the influence of dose rate and fractionation; (ii) determine whether dose rate influences are a result of repair or recovery from initial carcinogenic events or due to effects on mechanisms related to tumor expression; and (iii) examine the persistence of latent carcinogenic effects. For the second and third objectives, experiments will examine early, radiation-induced alterations in growth potential (transformation) of mammary epithelial cells. Studies on each of these aspects are currently underway.

Host Factors in Radiation Carcinogenesis. In vitro studies by others have amply demonstrated that radiation causes transformations (presumably malignant) in cultured cells. There is good reason to believe, however, that host factors in intact animals may be of over-riding importance in determining whether transformed cells progress to a frank malignancy. For example, we have shown that relatively minor endocrine manipulations in intact mice markedly affect the incidence of certain radiation-induced tumors. Further, we have shown that many radiation-induced tumors are not independent and that radiation-induced alterations in host factors sometimes led to animals developing both tumors (positive association) or one tumor but not the other (negative association). These associations were seen especially in tumors of endocrine organs or in tumors believed to be endocrine related.

We are currently conducting studies in two strains of mice which differ significantly in the spontaneous incidence of various tumors to determine whether radiation-induction of tumors is related to the spontaneous incidence. If so, we will have further evidence of the major role played by host factors in radiation carcinogenesis. The experiment will also provide evidence for whether the relative risk or the absolute risk model is appropriate for evaluating carcinogenic risk.

Carcinogenic Interactions of Radiation and Chemicals. The potential risks that may result from interactions of low-dose and dose-rate exposures of ionizing radiation with chemical carcinogens cannot be assessed from presently available data. Although chemicals and radiation are additive in their carcinogenic effects at high doses, it is not known whether this is true at low doses and dose rates. In fact, some data suggest that an assumption of additivity is incorrect and may underestimate the potential risks. The objective of this project is to examine the nature of the interactions between radiation and chemicals at low doses and dose rates with particular emphasis on the potential tumor enhancing or cocarcinogenic activity of low-dose-rate radiation exposures. Specifically this research examines (i) whether the carcinogenic effectiveness of the radiation dose influences the nature of the interactions between radiation and a chemical carcinogen, (ii) the nature of the interaction of a chemical carcinogen and low-dose-rate radiation, and (iii) the influence of radiation quality on these interactions. In these studies, thus far, we have been examining the effects of radiation and 7,12-dimethylbenz[a]anthracene (DMBA) on the development of mammary tumors in BALB/c female mice. Data from these studies suggest that the nature of the interaction between DMBA and radiation can be influenced by: (i) the dose of the chemical carcinogen, (ii) the radiation dose, (iii) the dose rate, (iv) the quality of the radiation, and (v) the sequence.

Cocarcinogenesis: Ionizing and Ultraviolet Radiation; Chemicals and Ultraviolet Radiation. These experiments are designed to (i) determine the influence of ultraviolet radiation (UVR) with and without photosensitizers on the induction of skin cancer by ionizing radiation, (ii) determine the persistence of initiation events in the skin induced by ionizing radiation, and (iii) determine whether UVR acts as a promoter of systemically administered chemical carcinogens.

We had established that UVR acts as a promoter of psoralen-UVA-(PUVA) induced tumors and there is clinical evidence that PUVA treatments may act as a promoter of X-ray-initiated cells. When regimens of either PUVA or UVR treatment, that alone produced no skin cancers, were followed by treatment with the promoter TPA, high incidences of skin cancer were found. The skin provides an excellent model system for the study of interactions since it is possible to study separately the factors that influence initiation and expression and the mechanisms involved. In collaboration with E. Perkins (Biology Division) and G. Rowden (Loyola University, Chicago), we are studying the role of the changes in the immune system induced by UVR, PUVA and X-rays. PUVA and UVR treatments reduce the number of Langerhan cells, as identified by a fluorescent antibody technique, but with the low doses that we use in our multifraction treatments the decreases are small.

We have found that urethan administered intraperitoneally produces no skin tumors but subsequent treatment with either TPA or UVR does produce skin tumors. These results suggest that UVR may play a role in skin cancers due to chemical carcinogens that reach the epidermis by other routes than topically.

Development of Model System for UVR Skin Carcinogenesis Studies. These studies are collaborative efforts involving K. F. Stelzner, L. B. Russell, E. Perkins and J. M. Holland. Four autosomal dominant mutants producing a hairless phenotype were identified. These mutants have been tested for allelism with each other and with hairless hr, the mutation carried by the two stocks that we currently use in UVR experiments; SKH:hairless-1 and HRS/J/Anl. It appears that three of the mutations belong to an allelic series which have been designated Frl^a, Frl^b and Frl^c (Frl = fur loss). This Frl series is not allelic with hr nor does it show linkage with that locus. The fourth mutation has been designated Hr^n as it is a dominant and homozygous allele at hr locus. The mutations of the Frl series are being backcrossed to BALB/c, and the Hr^n to C57BL/6.

The Frl series is of interest because the epithelium of the skin is multilayered and considerably thicker than haired or other hairless mice. There is an absence of the numerous residual follicles characteristic of the hr mutation. A small number of nonproducing follicles that may be awl or monotrix are present.

The immunologic characteristics of the various backcrosses have been studied and now at the seventh backcross the members of the Frl series appear similar to each other and to BALB/c. It has been found that the number of Langerhans cells per unit area of epithelium of the skin is much higher than in any other strain or stocks of mice that we have studied. Since the Langerhans cell is now considered a peripheral sentinel of the immune system concerned with processing of antigens, this finding is of some interest.

Ultraviolet Radiation-induced Change in Optical Properties of Mouse Skin. Precise determinations of the amount of damage induced in target cells of the epidermis are essential for meaningful comparative studies in UVR carcinogenesis. Meaningful dose-response studies, wavelength dependency effects and interspecies comparisons will require more than surface fluence measurements. Dosimetry is difficult since most skin carcinogenesis experiments require multiple exposures to UVR. It cannot be assumed that equivalent levels of damage will be induced by each exposure. Initial exposures may result in altered epidermal thickness, which in turn will result in decreased penetration of the radiation in a wavelength dependent manner during subsequent exposures. The magnitude of the change in optical properties of the skin during the course of an experiment will undoubtedly depend upon the fluence per fraction, the fractionation interval, the wavelengths being studied, and the species of animal used. We have used the induction of DNA damage as a form of biophysical dosimetry to measure the effective dose of carcinogen to reach the basal layer in mouse epidermis. The induction of pyrimidine dimers in DNA may be used to measure the effective dose of UVR that reached basal cells under conditions used in tumor induction studies.

To measure changes in the optical properties of mouse skin, groups of SKH:hairless-1 mice were subjected to multiple exposures (3 times per week for 6 weeks) from an FS40T12 fluorescent sun lamp (280-400 nm) prior to the determination of the rates of induction of pyrimidine dimers in basal cell

DNA. Epidermal DNA in pre-exposed animals was labeled by multiple intraperitoneal injections of tritiated thymidine. The mice were then exposed to graded fluences from the FS40 sunlamp, and the presence of pyrimidine dimers in tritium-labeled DNA extracted from exposed skin was determined with dimer-specific nucleases from Micrococcus luteus. The presence of endonuclease-sensitive sites (pyrimidine dimers) in the DNA was determined from the reduction in molecular weight of the DNA as measured by sedimentation in alkaline sucrose gradients. Eighteen exposures to a fluence that is approximately one minimal erythema dose (MED) altered the thickness of the epidermis and resulted in a significant decrease (~25%) in the level of pyrimidine dimers induced per basal cell per exposure. Multiple exposures, 3 per week for 6 weeks, to a fluence equivalent to two MEDS (~3000 J/m^2) resulted in a two-fold decrease in the level of dimers induced per basal cell per exposure. The influence of this level of decrease on the final incidence of tumors is not known. Future quantitative UVR carcinogenesis studies should take into account whether the penetration of UVR to the basal layer is altered as a function of daily dose and number of fractions.

Induction of Pyrimidine Dimers in Epidermal DNA of Hairless Mice by UV-B: An Action Spectrum.* Determination of the ultraviolet action spectrum for a given biological effect is of value in the identification of primary chromophores involved in the photobiological actions. In the case of ultraviolet radiation (UVR)-induced skin carcinogenesis, it is generally accepted that wavelengths in the UV-B region (280-320 nm) are more effective than longer wavelengths; however, information on the relative efficiencies of the various wavelengths in photocarcinogenesis is sparse.

There are many problems associated with the determination of wavelength dependency for the induction of skin cancer in experimental animals. For instance, monochromatic light must be generated of sufficient intensity to allow the irradiation of a large number of animals in a reasonable period of time. In addition, the quality of the monochromator must be such that scattered light is not a complicating factor. Once these two criteria are satisfied, the problem of selection of appropriate fluences at the various wavelengths remains to be resolved. Because of the absorption of UVR in the upper layers of the epidermis, surface fluences are of little value in comparative studies of wavelength dependency for tumor induction.

We wish to test the hypothesis that the action spectrum for photocarcinogenesis is the same as that for the induction of DNA damage. In order to do this, skin surface fluences at each wavelength should be adjusted so as to induce the same level of DNA damage in basal cells (target cells for tumor induction) of the mouse epithelium. To make the proper surface fluence adjustments at various wavelengths in the UV-B

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*Carried out in collaboration with M. J. Peak at Argonne National Laboratory.

spectrum, we have determined an action spectrum for the induction of pyrimidine dimers in basal cell DNA at five wavelength regions that have peak emissions at 288, 293, 298, 302, and 307 nm with half bandwidths of 4 nm. Epidermal DNA in SKH:hairless-1 mice was labeled by multiple intraperitoneal injections of tritiated thymidine. The presence of pyrimidine dimers in tritium-labeled DNA extracted from exposed skin was determined with dimer-specific nucleases from Micrococcus luteus in conjunction with sedimentation of the treated DNA in alkaline sucrose gradients. The rates of induction of pyrimidine dimers were calculated to be 1.79×10^{-10}, 2.03×10^{-10}, 1.49×10^{-10}, 1.20×10^{-10}, and 0.32×10^{-10} per dalton per Jm^{-2} for 288, 293, 298, 302, and 307 nm, respectively.

These rates of induction of pyrimidine dimers provide a sound basis for the selection of exposures to induce the same level of DNA damage in basal cells regardless of UVR wavelength employed. If pyrimidine dimers are the lesions responsible for the initiation of neoplastic transformation, equalization of the number of dimers induced per basal cell regardless of wavelength should result in equivalent levels of skin tumors induced.

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27. Ullrich, R. L., Tumor induction in BALB/c female mice after fission neutron or gamma ray irradiation. Radiat. Res., in press.
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Educational Activities

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences

W. E. BARNETT - DIRECTOR

The University of Tennesee-Oak Ridge Graduate School of Biomedical Sciences is located within the Biology Division of Oak Ridge National Laboratory. The program is primarily designed for training leading to the Ph.D. degree although there are a few Master's degree candidates. Ph.D. students are supported by the University of Tennessee in the form of research assistantships or by federal training grants awarded to the School by the National Cancer Institute and by the National Institutes of Health. The School currently has 46 students working toward the Ph.D. degree and 3 in the Master's program. As of August 1982, 78 students have been awarded the Ph.D. degree.

The fall quarter of 1982 marks the beginning of the School's seventeenth year. W. Edgar Barnett is the Director. The School also has three full-time faculty members: Daniel Billen, Donald E. Olins, and Mark D. Mamrack. A major portion of the School's teaching and research training is provided by the staff of the Biology Division who serve as "shared" faculty.

The students form a very active group of investigators in training, and their names appear on a number of manuscripts each year. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division.

Postdoctoral Training Programs

Postdoctoral training is an important feature of Division activities, providing benefits both to the trainees and to the Division. Support for these training activities is derived from a variety of sources and is administered by the University of Tennessee through a subcontract with Union Carbide Corporation. As of July 31, 1982, there were 25 trainees enrolled in this activity.

After a two- or three-year period of research in the Biology Division, trainees have obtained positions in universities, industries, or other government laboratories.

Undergraduate Training Programs

The Biology Division participates in three undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Southern Colleges University Union Science Semester (SCUU), and (iii) Oak Ridge Associated Universities Summer Student Trainee (ORAU). Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors are offered opportunities for independent research in the life sciences. Ten to twenty students who possess the educational qualifications and the potential for a successful scientific career spend 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) doing research under the guidance of Biology Division staff members.

Although the principal purpose of the programs is to provide a training experience for the students, it often allows division staff members an opportunity to broaden their areas of research. Upon completion of their research activities in the laboratory, students prepare a formal scientific paper and present a talk on their work. The programs, in which over 500 students have participated during the past 20 years, have received the enthusiastic endorsement of the students and the members of the Biology Division.

Appendices

Advisory Committee - 1982

Dr. Boris Magasanik	Department of Biology, Massachusetts Institute of Technology
Dr. Dean R. Parker	DOE, Division of Biomedical and Environmental Research, Retired
Dr. Henry C. Pitot	McArdle Laboratory for Cancer Research, University of Wisconsin
Dr. Robert T. Schimke	Department of Biological Sciences, Stanford University
Dr. Arthur C. Upton	New York University Medical Center, Institute of Environmental Medicine
Dr. Gerald N. Wogan	Department of Nutrition and Food Sciences, Massachusetts Institute of Technology

Seminar Programs

INTERNAL SEMINARS AND JOURNAL CLUBS

Aging Research Journal Club	Semimonthly
Biochemistry Journal Club	Semiweekly
Cancer Research Seminar	Weekly
Genetics Seminar	Weekly
Histopathology Slide Seminar	Biweekly
Mutagenesis Journal Club	Biweekly

SEMINARS BY OUTSIDE SPEAKERS

The following seminars were given in the Biology Division by scientists from research organizations in the United States and abroad during the period June 1, 1980 through July 31, 1982.

Speaker	Affiliation	Subject
Alberts, B. M.[a]	University of California San Francisco, California	Mechanisms and enzymology of DNA replication in T4 bacteriophage system
Astrin, Sue[b]	Institute for Cancer Research Fox Chase, Philadelphia, Pennsylvania	Oncogenic expression in tumor cells
Avivi, Lydia	Tel-Aviv University and The Weizman Institute Tel-Aviv, Israel	Differential sensitivity to colchicine of the achromatic apparatus of different age groups of humans
Baglioni, Corrado[a]	State University of New York at Albany Albany, New York	Mechanisms of action of interferons
Bhatt, T.	The Wistar Institute Philadelphia, Pennsylvania	Chemistry, metabolism, and tumorigenicity in the cyclopenta[*a*]phenanthrene series
Bidy, Stephen P.	Middlesex Hospital Medical School London, England	*In vitro* bioassays for thyroid-stimulating autoantibodies: A comparative review of current techniques
Bowden, D. H.[b]	University of Manitoba Winnipeg, Canada	Cellular interactions in lung injury and repair

Bowden, F. R.	University of Arizona Medical School Tucson, Arizona	Tumor promoter induced alterations in EFG binding to cultured cells
Boyland, E.[c]	London School of Hygiene and Tropical Medicine London, England	The significance of promoters in dose-response and evaluation of carcinogen risk
Bryan, Philip N.	University of Zurich Zurich, Switzerland	Chromatin structure of tRNA genes in *Xenopus laevis*
Chu, Ernest H. Y.[d]	University of Michigan Medical Center Ann Arbor, Michigan	Regulation of CTP synthetase activity in mammalian cells
Cohen, G.[b]	University of Surrey Surrey, England	Hydrocarbon-deoxyribonucleoside adducts *in vivo* and *in vitro* and their biological significance
Coohill, T. P.[d]	Western Kentucky University Bowling Green, Kentucky	Action spectra in mammalian cells
Courtney, R. J.[b]	University of Tennessee Knoxville, Tennessee	Studies of herpes simplex virus glycoprotein
Devoret, R.	Frederick Cancer Research Center Frederick, Maryland and Laboratoire d'Enzymologie, CNRS Gif-sur-Yvette, France	Trans induction of *E. coli* K12 by UV-irradiated F-replicon
Donnelly, M.	University of Minnesota St. Paul, Minnesota	Biological formation of methanol from aromatic methoxyl groups

Drets, Maximo	Instituto de Investigaciones Biologicas Clemente Estable Montevideo, Uruguay	The clastogenic action of benzoquinones of animal origin on human and mouse chromosomes
Easley, James R.	College of Veterinary Medicine University of Tennessee Knoxville, Tennessee	The acute effect of lithium chloride on renal morphology and sodium-potassium-ATPase in dogs
Ehling, Udo H.[d]	Institüt für Genetik Neuherberg, Germany	Mutagenicity testing and risk estimation with mammals
Eicher, Eva[d]	Jackson Laboratory Bar Harbor, Maine	Interference with primary sex determination by a Y-linked mouse mutation
Elkind, M. M.[b]	Argonne National Laboratory Argonne, Illinois	"Single hit" kinetics and issues of public health
Epstein, Joshua[b]	Roswell Park Memorial Institute Buffalo, New York	Effects of cytosine arabinoside and anthracylin on DNA synthesis and clonogenicity of acute myeloblastic leukemia cells
Estensen, Richard[b]	University of Minnesota Minneapolis, Minnesota	Studies on the mechanism of action of phorbol ester tumor promoter induced-mitogenesis in human lymphocytes
Foard, D. E.[e]	Purdue University West Lafayette, Indiana	*In vitro* synthesis of soybean protease inhibitors and cloning of their genes
Friedman, Susan	University of Alberta Medical School Calgary, Alberta, Canada	Membrane-active drugs in cancer

Name	Institution	Research topic
Gaitan, Eduardo	University of Alabama Birmingham, Alabama	Chemical and biological characteristics of goitrogenic substances in drinking water
Galibert, Francis[b]	Hospital Saint Louis Paris, France	Comparison of nucleotide sequences of human and woodchuck hepatitis virus genomes
Grunberger, D.[b]	Columbia University New York, New York	Structural and conformational damage induced in DNA by chemical carcinogens
Hager, Lowell P.[a]	University of Illinois Urbana, Illinois	Biochemistry of SV40 large T antigen
Harrison, S. D., Jr.[b]	University of Kentucky Lexington, Kentucky	Phenothiazines, calmodulin, and nephrotoxins: Investigations of nephrotoxicity
Hastings, J. W.[c]	Harvard University Cambridge, Massachusetts	Drug induced singular behavior in the circadian rhythm of bioluminescence in *Gonyaulax polyedra*
Henderson, Rogene[b]	Inhalation Toxicology Research Institute Albuquerque, New Mexico	Pulmonary response of rats and mice to inhalation of diesel exhaust
Hill, M.	Institut de Cancérologie et d Immunogénétique Villejuif, France	Integration pattern suggesting transpositions of Rous sarcoma provirus in virus-transformed Chinese hamster cells
Hilliker, Arthur	Commonwealth Scientific and Industry Research Organization Canberra City, Australia	Gene organization in Drosophila

Hook, Gary[e]	National Institute of Environmental Health Sciences Research Triangle Park North Carolina	Tubular myelin formation in lungs of patients with pulmonary alveolar proteinosis
Horowitz, Marshall[b]	Albert Einstein College of Medicine New York, New York	Synthesis of adenovirus DNA *in vitro*
Huang, A.[b]	University of Tennessee Knoxville, Tennessee	Monoclonal antibody coated phospholipid vesicles vs. targeted carriers of chemotherapeutic agents
Johnson, N. P.[e]	Laboratory of Pharmacology and Toxicology Toulouse, France	DNA lesions, repair, and structure activity relations of platinum antitumor compounds
Kohn, H.[b]	Harvard University Cambridge, Massachusetts	The CONAS Report: Energy and risk
Kondo, Sohei[d]	Osaka University Osaka, Japan	Molecular biology of 4-nitroquinoline 1-oxide
Kootstra, A.	Swiss Institute of Experimental Cancer Research Lausanne, Switzerland	Interaction of benzo[*a*]pyrene with chromatin
Kraemer, K.[b]	National Cancer Institute Bethesda, Maryland	Xeroderma pigmentosum
Kubitschek, H.	Argonne National Laboratory Argonne, Illinois	Bilinear growth

Kuehn, Glenn	New Mexico State University Las Cruces, New Mexico	Phosphorylation of ornithine decarboxylase by a polyamine-dependent protein kinase
Kuroki, Toshio[b]	The Institute of Medical Science The University of Tokyo Tokyo, Japan	Epidermal keratinocytes: Their response to benzo[*a*]pyrene and cholera toxin
Lennarz, William J.[a]	Johns Hopkins University School of Medicine Baltimore, Maryland	Topological aspects of glycoprotein synthesis. Glycoproteins and embryonic development.
Little, John B.[b]	Harvard University Boston, Massachusetts	Factors affecting radiation transformation *in vitro*
Littlefield, Gayle[d]	Oak Ridge Associated Universities Oak Ridge, Tennessee	Cytogenetic studies on a patient with severe contamination with AN^{241}
Lucchesi, John C.[f]	The University of North Carolina at Chapel Hill Chapel Hill, North Carolina	Control of X-chromosome transcription in Drosophila
Luning, K. G.[d]	University of Stockholm Stockholm, Sweden	Genotype and cytoplasm - test with inbred lines of Drosophila
Manson, Jeanne M.	University of Cincinnati Cincinnati, Ohio	Cell death and somatic mutation in teratogenesis
McCarty, Kenneth S.	Duke University Medical Center Durham, North Carolina	Metallothionein induction and cadmium detoxification in mammalian cells

Medina, D.[b]	Baylor College of Medicine Texas Medical Center Houston, Texas	Selenium inhibition of mammary tumorigenesis: Possible mechanisms
Meyrick, Barbara	Vanderbilt University Nashville, Tennessee	Pathogenesis of hypoxia- and crotalaria-induced pulmonary hypertension
Middleton, Marshall[b]	Central Toxicology Laboratory Macclesfield, Cheshire, England	New approaches to dermato-toxicology
Murray, A. W.[b]	The Flinders University of South Australia Bedford Park, South Australia	Modification of cell surface properties by tumor promoters
Nakeff, Alexander	Mallinckrodt Institute of Radiology Washington University School of Medicine St. Louis, Missouri	Clonogenic cell assays: Their usefulness in predicting bone marrow toxicity
Natarajan, A. T.[d]	Sylvius Laboratories Leiden, The Netherlands	DNA primary lesions and their relationship to radiation-induced chromosomal aberrations and sister chromatid exchanges
Painter, R. B.[d]	University of California San Francisco, California	A new explanation for radiosensitivity in ataxia telangiectasia
Parker, J. C., Jr.[b]	University of Tennessee Knoxville, Tennessee	Metastatic brain tumors
Paweletz, N.	German Cancer Center Heidelberg, West Germany	Microtubules and membranes in the mitotic apparatus of mammalian cells

Peto, R.[b]	Radcliffe Infirmary Oxford, England	Statistical aspects of the design and conduct of animal carcinogenesis experiments. An epidemiological perspective on cancer.
Prescott, David[a]	University of Colorado Boulder, Colorado	Gene and chromosome structure in hypotrichous ciliates. Genetic studies on the mammalian cell cycle.
Qualls, Charles, Jr.	Louisiana State University Baton Rouge, Louisiana	Pyrrolizidine alkaloid toxicity in the horse
Rabin, R.	University College London, England	Role of degranulation of the endoplasmic reticulum in chemical carcinogenesis
Roberfroid, M.[b]	Catholic University Louvain, Belgium	Isolated hepatocytes in suspension as an *in vitro* model to study the biochemical mechanisms of chemical toxicity: Possible application as a screening test for toxicity
Roden, Geoffrey[b]	Loyola University Medical Center Maywood, Illinois	The Langerhans cell immune surveillance and the skin
Rohrschneider, L.[b]	Fred Hutchinson Cancer Research Center Seattle, Washington	Involvement of $pp60^{src}$ in the mechanism of transformation by Rous sarcoma virus
Roop, Dennis	National Cancer Institute Bethesda, Maryland	Structure and expression of a chicken gene coding for U1 RNA
Roos, I. A. G.	Cancer Institute Peter MacCallum Hospital Melbourne, Victoria, Australia	*Cis*-platinum DNA interactions

Salzman, Gary	Los Alamos Scientific Laboratory Los Alamos, New Mexico	Chromosome slit scan scanning with flow instruments
Schloss, John V.	University of Wisconsin Madison, Wisconsin	Potential transition-state analogs for aconitase and isocitrate lyase
Sirotkin, Karl[d]	University of Tennessee Knoxville, Tennessee	Developmentally regulated transcription of heat shock cluster in 67B in Drosophila
Sobels, F. H.	Sylvius Laboratories Leiden, The Netherlands	Modification of mutator- and X-ray induced mutability by repair-deficient mutants in Drosophila
Solt, Dennis[b]	Harvard University Cambridge, Massachusetts	Liver carcinogenesis: An experimental approach
Sorsa, V.[d]	University of Helsinki Helsinki, Finland	Electron microscopic revision of the salivary gland chromosome maps of *Drosophila melanogaster*
Stiles, C. D.[b]	Harvard Medical School and the Sidney Farber Cancer Institute Boston, Massachusetts	Molecular analysis of platelet-derived growth factor
Swenberg, J.	Chemical Industries Institute of Toxicology Research Triangle Park, North Carolina	DNA alkylation and replication during chronic carcinogen exposure
Taylor, Milton A.[d]	Indiana University Bloomington, Indiana	Mechanisms of mammalian cell mutation

Trosko, James E.[b]	Michigan State University East Lansing, Michigan	The role of inhibition of intracellular communication in the promotion phase of carcinogenesis
Tryka, A. Franci	Harvard University School of Public Health Cambridge, Massachusetts	Bleomycin and hyperoxia; a morphologic assessment of early and late effects
Turk, James[g]	Washington State University Pullman, Washington	Ultrastructural characteristics of bovine fibrosing alveolitis and evaluation of the 3-methylindole experimental model
Turk, Margaret[g]	Washington State University Pullman, Washington	Equine bronchiolitis induced by 3-methylindole
Veleminsky, Jiri[d]	Insitute of Experimental Botany Prague, Czechoslovakia	DNA repair in higher plants after treatment with alkylating agents
Verma, A. K.	McArdle Laboratory for Cancer Research University of Wisconsin Madison, Wisconsin	Modulation of mouse skin carcinogenesis by retinoids
Vore, Mary	University of Kentucky Lexington, Kentucky	D-ring steroid glucuronides: A new class of cholestatic agents
Waddell, W. J.	University of Louisville Louisville, Kentucky	Whole body autoradiography
Walker, Graham C.	Massachusetts Institute of Technology Cambridge, Massachusetts	Mutagenesis of cellular responses to DNA damages

Wigler, Michael[d]	Cold Spring Harbor Laboratory Cold Spring Harbor, New York	Somatic replication of DNA methylation
Wigley, Caroline B.[b]	Imperial Cancer Research Fund Laboratories London, England	Neoplastic transformation in mouse salivary gland epithelial cells *in vitro*
Williams, Jerry R.[b]	George Washington University Medical School Washington, D.C.	Evaluating mutagenic/carcinogenic hazards of chemicals: Problems and possible solutions
Wilson, Douglas[g]	University of Tennessee Memorial Hospital Knoxville, Tennessee	Overview of approaches to evaluation of human leukemias and lymphomas
Wood, Alexander W.[b]	Hoffmann-LaRoche, Inc. Nutley, New Jersey	Chemical and metabolic determinants of polycyclic aromatic hydrocarbon carcinogenicity
Wu, Cheng-Wen	State University of New York at Stony Brook Stony Brook, New York	Promoter recognition and search by RNA polymerase

[a]Molecular and Cellular Sciences Seminar

[b]Cancer Research Seminar

[c]Distinguished Lecturer Seminar

[d]Genetics Seminar

[e]Biochemistry Journal Club

[f]Genetics and Developmental Biology Seminar

[g]Histopathology Slide Seminar

Research Conferences

A symposium on Molecular and Cellular Mechanisms of Mutagenesis was organized by the Biology Division of Oak Ridge National Laboratory and held in Gatlinburg, Tennessee, April 6-9, 1981. Chairman of the organizing committee was Jeffrey F. Lemontt. The proceedings of the symposium were published in 1982 by Plenum Publishing Corporation, New York.

A symposium on Genetic Mechanisms of Carcinogenesis was organized by the Biology Division and held in Gatlinburg, Tennessee, April 11-15, 1982. W. K. Yang served as chairman of the organizing committee. The proceedings will be published in 1983 by Academic Press, New York.

Extramural Activities

1. Officer of Society

J. S. Cook	- President, Society of General Physiologists, 1979-1980
J. L. Epler	- Councilor, Environmental Mutagen Society, 1979-present
P. Mazur	- Board of Governors, Society for Cryobiology, 1981-1985
R. O. Rahn	- Councilor, American Society for Photobiology, 1980-1983
L. B. Russell	- Councilor, Environmental Mutagen Society, 1977-1980 Executive Committee, Environmental Mutagen Society, 1979-1980
G. A. Sega	- Councilor, Environmental Mutagen Society, 1978-1981

2. Society Committees

H. I. Adler	-	Education and Training Committee, Radiation Research Society, 1980-1983
J. S. Cook	-	USA National Committee for the International Union of Physiological Sciences (Vice-Chairman), 1977-1982 Publications Committee, Society of General Physiologists, 1982-1986 Publications Committee, Federation of American Societies for Experimental Biology Annual Symposium, 1983 (Co-organizer), Society of General Physiologists
J. L. Epler	-	Membership Committee (Chairman), Environmental Mutagen Society, 1982
F. C. Hartman	-	Nominating Committee, American Society of Biological Chemists, 1982 Nominating Committee, American Chemical Society, Division of Biological Chemistry, 1982
J. W. Longworth	-	IX International Congress on Photobiology, 1984 (Secretary-General), American Society for Photobiology
P. Mazur	-	Publications Committee (Chairman), Society for Cryobiology, 1974-present
A. L. Olins	-	Gordon Conference on Chromatin, 1984, 1986 (Co-chairman) International Congress of Cell Biology, Chromatin Symposium (Organizer), 1980
D. E. Olins	-	Gordon Conference on Chromatin, 1984, 1986 (Co-chairman)
R. J. Preston	-	Finance Subcommittee, Environmental Mutagen Society, 1982-present
L. B. Russell	-	Nominating Committee (Chairman), Environmental Mutagen Society, 1979-1980 Nominating Committee (Chairman), Genetics Society of America, 1979-1980 Publications Policy Committee, Environmental Mutagen Society, 1981-1982

D. M. Skinner - Nominating Committee, Biophysical Society, 1980
Representative to AAAS, Society of General Physiologists, 1980-present
Committee on Equal Opportunities for Women, American Society of Biological Chemists, 1980-1983
Public Policy Committee, American Society for Cell Biology, 1981-present
Member-at-Large, Section G, Biological Sciences, AAAS, 1982

3. Advisory Committees

J. S. Cook - Special Study Section, National Institutes of Health, 1981, 1982 (Chairman)
Member of the Corporation, Mount Desert Island Biological Laboratory

R. J. M. Fry - Scientific Committee 40, National Council on Radiation Protection and Measurements
Council Member, National Council on Radiation Protection and Measurements
Advisory Committee, Radiation Effects Research Foundation, National Academy of Sciences

W. M. Generoso - Committee of Gene Tox Program Assessment Panel, Environmental Protection Agency, 1981-present
Work Group on Dominant Lethal Test in Rodents, International Commission for the Protection Against Environmental Mutagens and Carcinogens (ICPEMC), 1981-present
Committee on Toxicology, Panel on Anticholinesterase Chemicals, National Research Council, 1981-1982

R. A. Griesemer - Subcommittee to Evaluate Effects of Short-Term Exposures to Drinking Water Contaminants, Committee on Toxicology, National Research Council, 1982
Pesticide Information Review and Evaluation Committee, National Research Council, 1981-1982

J. M. Holland - Joint Coordinating Committee, Comparative and Experimental Medicine Program, University of Tennessee, 1979-present

		Committee on Animal Models for Research on Aging, National Research Council, 1980 Technical Reports Review Subcommittee of the National Toxicology Program Board of Scientific Counselors, 1981-present Sudden Infant Death Pathology Working Group, Epidemiological Study of Sudden Infant Death Syndrome (SIDS) Risk Factors, National Institute of Child Health and Human Development Cooperative, 1980
A. W. Hsie	-	Special Study Section, Environmental Health Sciences, National Institutes of Health, 1980 Member of Scientific Directorate, Coordinating Council for Cancer Research, Villejuif, France, 1978-present
F. T. Kenney	-	Advisory Committee for Personnel in Research, American Cancer Society, 1978-present
R. J. Preston	-	Committee on *In Vivo/In Vitro* Cytogenetics of Gene-Tox Task (Chairman), Environmental Protection Agency, 1979-1981 Cytogenetic Adviser to Ethylene Oxide Council and to Health Industry Manufacturers Association, 1981-present Health Effects Advisor to Clinch River Breeder Reactor Project, 1981-present
L. B. Russell	-	Committee on the Biological Effects of Ionizing Radiation (BEIR III), National Academy of Sciences, 1977-1980 Committee I, International Commission for Protection Against Environmental Mutagens and Carcinogens, 1977-present International Committee on Standardized Genetic Nomenclature for Mice, 1977-present Committees (2) of Gene-Tox Task (Chairman), Environmental Protection Agency, 1979-1981 Coordinating Committee of Gene-Tox Task, Environmental Protection Agency, 1980-present Committee on Risk Assessment of Gene-Tox Task (Chairman), Environmental Protection Agency, 1980-present Science Advisory Panel, Litton Bionetics, 1980-present

		Technical Support Group, Workshop on Status of Risk Assessment for Reproductive and Teratogenic Effects, Environmental Protection Agency, 1981 Board on Toxicology and Environmental Health Hazards, National Academy of Sciences, 1981-1984
W. L. Russell	-	Scientific Adviser to U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation Committee on the Biological Effects of Ionizing Radiation (BEIR III), National Academy of Sciences, 1977-1980
P. B. Selby	-	Committee on Chemical Environmental Mutagens, National Academy of Sciences, 1980-present Work Groups (2) of Gene-Tox Task, Environmental Protection Agency, 1980
J. K. Selkirk	-	Breast Cancer Task Force, National Institutes of Health, 1979-1982 Committee on Pyrene and Analogs, 1981-1982
D. M. Skinner	-	Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-present
T. J. Slaga	-	Study Section on Chemical Pathology, National Institutes of Health, 1976-present; 1978-present (Chairman) Review Committee on Diet, Nutrition and Cancer, National Research Council, 1980-1983
J. B. Storer	-	Scientific Committee 1 on Basic Radiation Protection Criteria, National Council on Radiation Protection and Measurement Council Member, National Council on Radiation Protection and Measurements
R. L. Ullrich	-	Scientific Committee 40 on the Biological Aspects of Radiation Protection Criteria, National Council on Radiation Protection and Measurements
H. R. Witschi	-	Scientific Review Panel for Health Research, Office of Research and Development, Environmental Protection Agency, 1980-present Toxicology Study Section, National Institutes of Health, 1980-1984.

Committee for the Characterization of the Status of Toxicity Data Elements for a Select Universe of Compounds, National Research Council, 1980-1983
Safe Drinking Water Committee, National Research Council, 1981-1982
Review Committee (Chairman), Inhalation Toxicology Division, Health Effects Research Laboratory, Environmental Protection Agency, 1982-present

W. K. Yang - Experimental Virology Study Section, National Institutes of Health, 1982-present

4. Editorial Boards

H. I. Adler - Radiation Research, 1980-1983

D. Billen - Radiation Research (Editor-in-Chief), 1979-present

J. S. Cook - American Journal of Physiology, 1981-present
Cell and General Physiology, American Physiological Society Handbook Series, 1982-1984

R. B. Cumming - Risk Analysis (Editor-in-Chief), 1981-present

J. L. Epler - Mutation Research, 1977-present
Environmental Mutagen Society Newsletter, 1980-present

W. M. Generoso - Mutation Research, 1975-present
Teratogenesis, Carcinogenesis, and Mutagenesis, 1979-present

F. C. Hartman - BioScience, 1980-1986
Journal of Protein Chemistry, 1982-1987

A. W. Hsie - Mutation Research, 1976-present

J. W. Longworth - Biophysical Journal, 1979-present
Comments on Molecular and Cellular Biophysics, 1980-present

P. Mazur - Cryobiology, 1967-present
Cryo-Letters, 1979-1982
Revue Francaise de Transfusion et Immunohématologie, 1979-present

R. J. Preston	-	Environmental and Experimental Botany, 1979-present Mutation Research Letters, 1980-present Teratogenesis, Carcinogenesis, and Mutagenesis, 1980-present
L. B. Russell	-	Mutation Research, 1976-present Environmental Mutagenesis, 1978-present
J. K. Selkirk	-	Cancer Research Carcinogenesis
D. M. Skinner	-	Growth, 1979-1982 Biological Bulletin, 1981-1984
T. J. Slaga	-	Cancer Research Chemical-Biological Interactions Cancer Surveys
H. R. Witschi	-	Toxiology and Applied Pharmacology, 1978-present Toxicology, 1978-present Environmental Health Perspectives, 1981-present

5. Awards

R. K. Fujimura	-	Fellow, Japan Society for the Promotion of Science, 1981
F. C. Hartman	-	Fellow, American Association for the Advancement of Science, 1981
J. Y. Kao	-	Diplomate, American Board of Toxicology, 1981
W. E. Masker	-	Fellow, American Association for the Advancement of Science, 1981
S. Mitra	-	NSF U.S./India Scientists Exchange Fellowship, 1982
S. K. Niyogi	-	Fellow, American Association for the Advancement of Science, 1981
A. L. Olins	-	Naito Foundation of Japan Award, 1982
D. E. Olins	-	Alexander von Humboldt Senior U.S. Scientist Award, 1979-1980

		Naito Foundation of Japan Award, 1982 Fellow, American Association for the Advancement of Science, 1980
P. B. Selby	-	E. O. Lawrence Memorial Award, 1981
D. M. Skinner	-	Fellow, American Association for the Advancement of Science, 1981
A. Stevens	-	Fellow, American Association for the Advancement of Science, 1981
H. P. Witschi	-	Diplomate, American Board of Toxicology, 1980 Diplomate, Board of Toxicological Science, 1982

ABSTRACTS FOR TECHNICAL MEETINGS HELD
JUNE 1, 1980 – JULY 31, 1982

Adams, L. M. and R. L. Ullrich. Survival of mammary epithelial cells from virgin female BALB/c mice following *in vivo* gamma irradiation. Radiation Research Society, Salt Lake City, Utah, April 18-22, 1982.

Adler, H. I. and W. D. Crow. A novel approach to the growth of anaerobic microorganisms. Third Symposium on Biotechnology in Energy Production and Conservation, Gatlinburg, Tennessee, May 12-15, 1981.

Adler, H. I. and W. Crow. Factors influencing the development of spontaneous mutant colonies in the Salmonella mutagenicity assay. American Society for Microbiology, Atlanta, Georgia, March 7-12, 1982.

Adler, H. I., W. D. Crow, and J. S. Gill. Evidence for a division-promoting factor associated with the cytoplasmic membrane of *E. coli*. Radiation Research Society, New Orleans, Louisiana, June 1-5, 1980.

Allen, B. E., T. K. Rao, J. T. Cox, and J. L. Epler. Promotion of mutagenicity by oral contraceptive steroids. Southeastern Cancer Research Association Annual Meeting, Atlanta, Georgia, November 13-14, 1980.

Amsler, Kurt and John S. Cook. Induction of Na^+-dependent hexose transport in LLC-PK_1 cells in culture. Society of General Physiologists, Woods Hole, Massachusetts, September 4-7, 1980.

Amsler, Kurt and John S. Cook. Na^+-dependent hexose transport in a cultured epithelial cell line: Characterization and effect of various treatments on the induction of this transport process. American Society for Cell Biology, Cincinnati, Ohio, November 14-18, 1980.

Amsler, Kurt and John S. Cook. Reciprocal regulation of Na^+-dependent hexose and Na^+-dependent amino acid uptake in LLC-PK cells: Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA). Thirteenth Miami Winter Symposium on Cellular Responses to Molecular Modulators, Miami, Florida, January 12-16, 1981.

Amsler, Kurt, Ellen R. Weiss, Carolyn Shaffer, and John S. Cook. Initiation and irreversibility of expression of Na^+-dependent hexose transport in LLC-PK_1 cells. American Society for Cell Biology, Anaheim, California, November 9-13, 1981.

Archibald, A., A. Miles, and N. W. Revis. The effect of rat strain variation on cadmium metabolism and blood pressure. Federation of American Societies for Experimental Biology, Atlanta, Georgia, April 12-17, 1981.

Armitage, W. J. and Peter Mazur. The response of granulocytes to osmotic shrinkage. Society for Cryobiology, Houston, Texas, June 27-July 1, 1982.

Armitage, W. J., Peter Mazur, and K. W. Cole. The detrimental effect of glycerol on granulocytes. Society for Cryobiology, Houston, Texas, June 27-July 1, 1982.

Ashley, Terry, N. L. A. Cacheiro, and L. B. Russell. Assessment of factors involved in initiation of synapsis in three X-7 translocations in male mice. 7th International Chromosome Conference, Oxford, England, August 26-30, 1980.

Ashley, Terry, N. L. A. Cacheiro, and L. B. Russell. Synaptonemal complex analysis of three X-7 translocations in male mice: Assessment of factors involved in initiation of synapsis. Second International Congress on Cell Biology, Berlin, West Germany, August 31-September 5, 1980.

Au, William W. and Henry E. Luippold. Sister chromatid differentiation analysis of cell cycle kinetics in mouse bone marrow cells. Environmental Mutagen Society, Boston, Massachusetts, February 27-March 2, 1982.

Au, William W., Henry E. Luippold, and James A. Otten. Development of an RFM mouse myeloid leukemia model. Symposium on Genetic Mechanisms of Carcinogenesis, Gatlinburg, Tennessee, April 11-15, 1982.

Au, William W., James A. Otten, and Henry E. Luippold. Mouse myeloid leukemia as an experimental model for monitoring progression of leukemia and chemotherapy. American Association for Cancer Research, St. Louis, Missouri, April 28-May 1, 1982.

Banerjee, P., D. P. Allison, and R. C. Bates. Electron microscopic studies of sequence homology of single-stranded genomes of mammalian parvoviruses. American Society of Biological Chemists, St. Louis, Missouri, May 31-June 4, 1981.

Barnes, D. S. and N. K. Clapp. Differences in effects of dietary brans before and after tumor initiation by 1,2-dimethylhydrazine (DMN) in rats. 4th International Symposium on the Prevention and Detection of Cancer, London, England, July 26-31, 1980.

Beck, Anton K., Jeffrey F. Lemontt, Frank W. Larimer, and Edward G. Bernstine. Isolation of genes involved in mutagenesis and DNA repair in yeast. Symposium on Molecular and Cellular Mechanisms of Mutagenesis, Gatlinburg, Tennessee, April 6-9, 1981.

Beck, A. K., J. F. Lemontt, F. W. Larimer, and E. G. Bernstine. Gamma-ray-induced nonrevertible *ade2* mutation in *Saccharomyces cerevisiae* carries a large insertion as revealed with cloned *ADE2* probel. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, August 11-16, 1981.

Beck, A. K., J. F. Lemontt, F. W. Larimer, and E. G. Bernstine. Isolation of genes involved in mutagenesis and DNA repair in the yeast Saccharomyces cerevisiae. Third International Conference on Environmental Mutagens, Tokyo, Japan, September 21-27, 1981.

Bednar, Rodney, A., Fred C. Hartman, and Roberta F. Colman. 3-(R,S)-3-bromo-2-ketoglutarate: Inactivator and substrate of pig heart DPN-dependent isocitrate dehydrogenease. American Chemical Society, Minneapolis, Minnesota, August 31-September 3, 1981.

Billen, Daniel. Selective suppression by cysteamine of DNA lesions produced by ultraviolet light exposure of toluene-treated Escherichia coli containing 5-bromouracil in their DNA. Radiation Research Society, Minneapolis, Minnesota, May 31-June 4, 1981.

Billen, D. and P. Furnad. The relationship between DNA repair synthesis terminated by DNA ligase and strand breaks in toluene-treated E. coli exposed to X rays. Radiation Research Society, New Orleans, Louisiana, June 1-5, 1980.

Bingham, G. A., N. W. Revis, R. L. Nelson, and J. R. Wells. The effects of pipe materials on hyperchlorinated water. 32nd Annual Session of the American Association of Laboratory Animal Science, Salt Lake City, Utah, September 20-25, 1981.

Birchler, James A. A unifying genetic principle for dosage compensation, aneuploid inviability and sexual dimorphisms in Drosophila. Genetics Society of America, Boulder, Colorado, August 18-20, 1980.

Birchler, James A. The dosage rules of gene expression in higher eukaryotes. Genetics Society of America, Raleigh, North Carolina, June 15-17, 1981.

Bonnewell, V., R. F. Fowler, M. S. Spann, and D. M. Skinner. Themes and variations on a repeat unit for a complex satellite DNA. American Society for Cell Biology, Anaheim, California, November 9-13, 1981.

Boone, L. R., F. E. Myer, M. Yang, J. O. Kiggans, C. Koh, R. W. Tennant, and W. K. Yang. Analysis of recombinant DNA clones of the endogenous BALB/c murine leukemia virus WN1802N: Variation in LTR size. Symposium on Genetic Mechanisms of Carcinogenesis, Gatlinburg, Tennessee, April 11-15, 1982.

Boone, L. R., F. E. Myer, M. Yang, J. O. Kiggans, C. Koh, R. W. Tennant, and W. K. Yang. Analysis of recombinant DNA clones of the endogenous BALB/c murine leukemia virus WN1802N: Variation in LTR size. RNA Tumor Virus Meeting, Cold Spring Harbor, New York, May 26-30, 1982.

Bostick, W. D., J. Kao, J. M. Holland, and J. E. Mrochek. Induction of ethoxyresorufin and ethoxycoumarin-o-deethylase activity as an index to exposure to coal derived products and trace environmental pollutants. 13th Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, Gatlinburg, Tennessee, April 23-24, 1981.

Braslawsky, G.R., S. J. Kennel, and P. Nettesheim. Phenotypic changes in epithelial cell population undergoing neoplastic transformation in vitro. Symposium on Genetic Mechanisms of Carcinogenesis, Gatlinburg, Tennessee, April 11-15, 1982.

Brent, Thomas P. and Ronald O. Rahn. UV action spectrum for production of DNA lesions sensitive to a damage-specific human endonuclease. American Society for Photobiology, Vancouver, British Columbia, Canada, June 27-July 1, 1982.

Brimer, P. A., E. L. Tan, and A. W. Hsie. Effect of metabolic activation on the mutagenicity and cytotoxicity of ethylene dibromide in the CHO/HGPRT system. Environmental Mutagen Society, San Diego, California, March 5-9, 1981.

Brockman, Herman E., D. DeMarini, F. J. deSerres, A. Katz, T-M. Ong, and R. S. Stafford. Mutation tests in Neurospora crassa: A report for the "Gene-Tox" Program. Genetic Toxicology (Gene-Tox) Meeting, Environmental Protection Agency Conference, Washington, D.C., December 3-5, 1980.

Broyde, S. and B. Hingerty. Minimized conformational potential energy calculations for the major adduct of N-acetoxy-2-acetylaminofluorene with dCpdG. 2nd SUNYA Conversation in Molecular Stereodynamics, Albany, New York, April 26-29, 1981.

Broyde, S., S. Stellman, and B. Hingerty. DNA backbone conformation in cis-syn pyrimidine — pyrimidine cyclobutane dimers. Biophysical Society, New Orleans, Louisiana, June 1-6, 1980.

Bruns, G., P. S. Gerald, P. Lalley, U. Francke, and J. Minna. Gene mapping of the mouse by somatic cell hybridization. 5th International Workshop on Human Gene Mapping 5, Edinburg, Scotland, July 9-13, 1979.

Butler, A. P. and D. E. Olins. Interaction of high mobility group proteins with DNA and chromatin. American Society of Biological Chemists/ Biophysical Society, New Orleans, Louisiana, June 1-6, 1980.

Cacheiro, N. L. A. and E. L. Russell. Male sterility in the mouse caused by translocations involving the Y chromosome. 7th International Chromosome Conference, Oxford, England, August 26-30, 1980.

Cacheiro, N. L., J. M. Russell, and E. W. Russell. Nature of sterility in male mice derived from X-irradiated spermatogonia. Environmental Mutagen Society, San Diego, California, March 5-9, 1981.

Carrasco, Alicia, H. I. Adler, and W. D. Crow. Cytoplasmic membrane and cell-septation. Kentucky-Tennessee Branch of the American Society for Microbiology, Gatlinburg, Tennessee, November 13-15, 1980.

Carrier, W. L. and J. D. Regan. The number and fate of near-UV (FS40 sunlamp) and far-UV (254 nm) induced primidine dimers in the DNA of human fibroblasts. Radiation Research Society, New Orleans, Louisiana, June 1-5, 1980.

Carrier, W. L. and James D. Regan. The induction and excision of pyrimidine dimers in human cells. Symposium on Molecular and Cellular Mechanisms of Mutagenesis, Gatlinburg, Tennessee, April 6-9, 1981.

Carrier, W. L., R. D. Snyder, and J. D. Regan. Pyrimidine dimer excision repair in human cells and the effect of inhibitors. 8th International Congress of Photobiology, Strasbourg, France, July 20-25, 1980.

Christie, Nelwyn T. and K. Bruce Jacobson. Toxicity of metal ions in Drosophila: Characterization by statistical parameters. Federation of American Societies for Experimental Biology, Atlanta, Georgia, April 12-17, 1981.

Christie, Nelwyn T. and K. Bruce Jacobson. Metal ion effects on queuine-containing tRNAs in Drosophila. International Symposium on the Biological Aspects of Metals and Metal-Related Diseases, Toronto, Canada, October 19-22, 1981.

Christie, Nelwyn T. and K. Bruce Jacobson. Metal ion toxicity in Drosophila. Society of Toxicology, Boston, Massachusetts, February 22-26, 1982.

Christie, Nelwyn T., R. K. Owenby, and K. Bruce Jacobson. Genetic and environmental factors determine the level of Q(+)tRNAs in Drosophila. Genetics Society of America, Raleigh, North Carolina, June 15-17, 1981.

Clapp, N. K., M. A. Henke, T. L. Shock, L. Triplett, S. Nesnow, and T. J. Slaga. Preliminary report of systemic carcinogenic studies on diesel fuel particulate emissions applied to mouse skin. EPA Diesel Emissions Symposium, Raleigh, North Carolina, October 5-7, 1981.

Clapp, N. K., J. F. London, and M. A. Henke. Suggested promotion of 1,2-dimethylhydrazine (DMH) colon carcinogenesis. Symposium on Cocarcinogenesis and Biological Effects of Tumor Promoters. Castle of Elmau, Klais/Bavaria, Germany, October 13-16, 1980.

Clapp, Neal K., Jerry F. London, Marsha A. Henke, and Terry L. Shock. Modification by dietary brans of 1,2-dimethylhydrazine colon carcinogenesis in male BALB/c mice. National Large Bowel Cancer Project Workshop "The Large Bowel Cancer Program: Its Achievements and Future Direction of Investigation," Dallas, Texas, January 8-9, 1981.

Clapp, N. K., J. F. London, M. A. Henke, and T. L. Shock. Enhancement by dietary brans of 1,2-dimethylhydrazine colon carcinogenesis in male BALB/c mice. American Association for Cancer Research, Washington, D.C., April 27-30, 1981.

Cook, J. S. and L. R. Pollack. Turnover of Na,K-ATPase in HeLa cell membranes. XXVIII International Congress of Physiological Sciences, Budapest, Hungary, July 13-19, 1980.

Cook, John S., Emily H. Tate, and Carolyn Shaffer. Uptake of ^{3}H-ouabain from the cell surface into the lysosomal compartment of HeLa cells. Federation of American Societies for Experimental Biology, Atlanta, Georgia, April 12-17, 1981.

Cook, J. S., E. R. Weiss, C. Shaffer, and K. Amsler. A model for the differentiation of Na-dependent hexose transport capacity in a population of $LLC-PK_1$ cells. Federation of American Societies for Experimental Biology (American Physiological Society), New Orleans, Louisiana, April 16-23, 1982.

Crow, W. and H. I. Adler. Membrane preparations used to produce anaerobic conditions in bacteriological media. American Society for Microbiology, Atlanta, Georgia, March 7-12, 1982.

Cumming, R. B., G. A. Sega, C. Y. Horton, and W. H. Olson. Degree of alkylation of DNA in various tissues of the mouse following inhalation exposure to ethylene oxide. Environmental Mutagen Society, San Diego, California, March 5-9, 1981.

Dalbey, W. Formaldehyde and respiratory tract tumors in Syrian golden hamsters. American Industrial Hygiene Association Conference, Cincinnati, Ohio, June 6-11, 1982.

Dalbey, W., S. Lock, R. Holmberg, J. Moneyhun, and M. Guerin. Acute toxicity of an aerosol of diesel fuel. Society of Toxicology, San Diego, California, March 1-5, 1981.

Dalbey, W., S. Lock, R. Holmberg, J. Moneyhun, and M. Guerin. Acute exposures of rats to an inhaled aerosol of diesel fuel. Smoke/Obscurants Symposium V, Adelphi, Maryland, April 28-30, 1981.

Dalbey, W., S. Lock, S. Garfinkel, R. Jenkins, R. Holmberg, and M. Guerin. Effects of repeated exposures to aerosolized diesel fuel. Society of Toxicology, Boston, Massachusetts, February 22-26, 1982.

Dalbey, W., S. Lock, S. Garfinkel, R. Jenkins, R. Holmberg, and M. Guerin. Inhalation exposures of rats of aerosolized diesel fuel. Symposium on Toxicology of Petroleum Hydrocarbons, Washington, D.C., May 11-13, 1982.

Dalbey, W., S. Lock, R. Rice, T. Ross, S. Garfinkel, and L. Balogh. Effects of single exposures to aerosolized diesel fuel. Society of Toxicology, Boston, Massachusetts, February 22-26, 1982.

Dalbey, W. and P. Nettesheim. Influence of nitrogen dioxide or formaldehyde on incidence of diethylnitrosamine-induced tumors in hamster respiratory tract. Society of Toxicology, San Diego, California, March 1-5, 1981.

Das, G. C., P. C. McGray, D. P. Allison, and S. K. Niyogi. Assembly of SV40 chromatin by a host nuclear extract at physiological ionic strength. American Society of Biological Chemists/Biophysical Society, New Orleans, Louisiana, June 1-6, 1980.

DeMarini, D. M. and A. W. Hsie. Cytotoxicity and mutagenicity of five antitumor compounds (ellipticines) in the CHO/HGPRT system. Environmental Mutagen Society, Boston, Massachusetts, February 27-March 2, 1982.

DeMarini, D. M., T. K. Rao, C.-h. Ho, C. Y. Ma, M. R. Guerin, A. W. Hsie, and J. L. Epler. Mutagenicity of PAH-containing fractions of synthetic cells. Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, Chapel Hill, North Carolina, January 25-27, 1982.

DeVault, D., Govindjee, and W. Arnold. Considerations of photosynthetic glow peaks. Biophysical Society, Denver, Colorado, February 22-26, 1981.

Dodson, Lori, Robert Foote, Sankar Mitra, and Warren Masker. *In vitro* site specific mutagenesis of bacteriophage T7 DNA with O^6-methylguanine. *In Vitro* Mutagenesis, Cold Spring Harbor, New York, May 12-16, 1982.

Dodson, Lori A. and Warren E. Masker. *In vitro* host-cell reactivation of alkylated T7 DNA. ICN-UCLA Symposium on Mechanisms of Chemical Carcinogenesis, Keystone, Colorado, February 22-March 1, 1981.

Donohue, T. M., Jr. Inactivation of tyrosine adminotransferase in hepatoma cell extracts. American Society of Biological Chemists/Biophysical Society, New Orleans, Louisiana, June 1-6, 1980.

Dorsett, Dale, J. J. Yim, and K. B. Jacobson. Nonenzymatic synthesis of the sepiapterin synthase intermediate from 7,8-dihydroneopterin triphosphate. American Society of Biological Chemists, New Orleans, Louisiana, June 1-6, 1980.

Dumont, J. N. and C. S. Richter. Osmium-zinc iodide (OZI) reactive sites in the developing oocytes of *Xenopus laevis*. American Society for Cell Biology, Cincinnati, Ohio, November 14-18, 1980.

Dumont, James N. and T. Wayne Schultz. Frog embryo teratogenesis assay, Xenopus (FETAX): A short-term assay applicable to complex environmental mixtures. Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, Chapel Hill, North Carolina January 25-27, 1982.

Dumont, J. N., T. W. Schultz, and S. M. Newman. A frog embryo teratogenesis assay: Xenopus (FETAX)-A model for teratogen screening. Teratology Society, French Lick, Indiana, June 6-10, 1982.

Easley, J. R. and J. M. Holland. Percutaneous acute and chronic renal toxicity induced by shale oil and petroleum derived jet and diesel fuel. Society of Toxicology, Boston, Massachusetts, February 22-26, 1982.

Epler, J. L. Bioassay of complex samples. Gordon Research Conference on Genetic Toxicology Bioassays, New London, New Hampshire, June 29-July 3, 1981.

Epler, J. L. Health effects. Electric Power Research Institute Workshop on Coal Gasification, Palo Alto, California, July 28-30, 1982.

Epler, J. L., F. W. Larimer, M. P. Maskarinec, and C. W. Francis. Ecological and health effects bioassay results from coal conversion solid wastes. EPA Symposium on Environmental Aspects of Fuel Conversion Technology VI, Denver, Colorado, October 26-30, 1981.

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Epler, J. L., T. K. Rao, and M. R. Guerin. Distribution of mutagenic activity in neutral subfractions of synthetic fuels. Environmental Mutagen Society, San Diego, California, March 5-9, 1981.

Ethier, S. P. and R. L. Ullrich. Altered growth potential of cells from mammary tissue of virgin female BALB/c mice exposed to 7,12-dimethylbenz[a]anthracene (DMBA) or γ-ray irradiation. American Association for Cancer Research, Washington, D.C., April 27-30, 1981.

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Ethier, S. P. and R. L. Ullrich. Induction of mammary tumors in virgin female BALB/c mice by single low doses of 7,12-dimethylbenz[a]-anthracene (DMBA). American Association for Cancer Research, St. Louis, Missouri, April 28-May 1, 1982.

Farmerie, W. G., S. H. Chang, and W. E. Barnett. Nucleotide sequence of the Euglena cytoplasmic aspartic acid tRNA. American Society of Biological Chemists/Biophysical Society, New Orleans, Louisiana, June 1-6, 1980.

Filler, R. An in vitro/in vivo coupled prescreen to identify teratogens requiring metabolic activation. Teratology Society, Portsmouth, New Hampshire, June 8-12, 1980.

Filler, R. The metabolism of xenobiotics by embryonal carcinoma cells. Second International Congress on Cell Biology, Berlin, Germany, August 31-September 5, 1980.

Filler, R. Developmental onset of xenobiotic activation capability in preimplantation embryos from genetically "responsive" and "nonresponsive" mice. EPA-NCI Symposium on Organ and Species Specificity in Chemical Carcinogenesis, Raleigh, North Carolina, March 2-4, 1981.

Filler, R. Onset of embryonic capability to activate proteratogens by mixed function oxidase (MFO) system during the preimplantation period. 21st Annual Meeting of the Teratology Society, Stanford, California, June 21-25, 1981.

Filler, R. The presence of metabolites in early embryos. Teratology Society, French Lick, Indiana, June 6-10, 1982.

Filler, R. and Linda Spurling. Embryonal carcinoma cells as a model system for characterizing the embryonic aryl hydrocarbon hydroxylase (AHH) enzyme system. American Association for Cancer Research, Washington, D.C., April 27-30, 1981.

Filler, R. and L. S. Woods. Cell specific activation and detoxification of benzo[a]pyrene by two cloned murine testicular cell lines. Society of Toxicology, Boston, Massachusetts, February 22-26, 1982.

Fischer, S. M. and G. Furstenberger. An epidermal in vitro model for the in vivo TPA induced prostaglandin synthesis, ODC activity and DNA synthesis. ICN-UCLA Symposium on Mechanisms of Chemical Carcinogenesis, Keystone, Colorado, February 22-March 1, 1981.

Fischer, Susan M., G. D. Mills, and T. J. Slaga. Modulation of prostaglandin synthesis and tumor promotion. International Conference on Prostaglandins and Cancer, Washington, D.C., August 31-September 3, 1981.

Fischer, S. M., G. D. Mills, and T. J. Slaga. Modulation of skin tumor promotion by inhibitors of arachidonic acid metabolism. V International Conference on Prostaglandins, Florence, Italy, May 18-21, 1982.

Fishman, J. B. and J. S. Cook. Recycling of cell surface glycoconjugates. American Society for Cell Biology, Anaheim, California, November 9-13, 1981.

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Fraij, Bassam M. and Fred C. Hartman. A new affinity label for ribulose-bisphosphate carboxylase/oxygenase. American Chemic.l Society, Minneapolis, Minnesoto, August 31-September 3, 1981.

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Francis, Andrew A., R. D. Snyder, W. C. Dunn, and James D. Regan. Measurements of DNA repair in human cells following exposure to various chemical agents. Environmental Mutagen Society, San Diego, California, March 5-9, 1981.

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Fry, R. J. M. Experimental radiation carcinogenesis. American Chemical Society, Atlanta, Georgia, March 29-April 2, 1981.

Fry, R. J. Michael. Radiation oncogenesis: From mouse to man. Conference on Normal Tissue Effect, Bethesda, Maryland, April 13-15, 1981.

Fry, R. J. M. Radiation carcinogenesis. First Conference on Radioprotectors and Anticarcinogens, Gaithersburg, Maryland, June 21-24, 1982.

Fry, R. J. M., R. D. Ley, and D. Grube. Experimental ultraviolet radiation carcinogenesis. 8th Inernational Congress on Photobiology, Strasbourg, France, July 20-25, 1980.

Fry, R. J. M., R. D. Ley, and D. Grube. Ultraviolet radiation skin carcinogenesis. American Association for Cancer Research, St. Louis, Missouri, April 28-May 1, 1982.

Fry, R. J. M., R. D. Ley, D. Grube, and E. Staffeldt. Studies in skin and Harderian gland on the multistage nature of tumorigenesis. Symposium on Cocarcinogenesis and Biological Effects of Tumor Promoters, Castle of Elmau, Klais/Bavaria, Germany, October 13-16, 1930.

Fry, R. J. M., R. L. Ullrich, and E. L. Alpen. High LET-radiation carcinogenesis. Committee on Space Research XXIV, Ottawa, Canada, May 27, 1982.

Fujimura, R. K. and B. C. Roop. Initiation of phage T5 DNA replication. ICN-UCLA Symposium on Structure and DNA-Protein Interactions of Replication Origins, Keystone, Colorado, March 8-13, 1981.

Fuller, Ron and Linda Spurling. Embryonal carcinoma cells as a model system for characterizing the embryonic aryl hydrocarbon hydroxylase (AHH) enzyme system. American Association for Cancer Research, Washington, D.C., April 27-30, 1981.

Gaertner, F. H., A. K. Beck, K. W. Cole, and F. W. Larimer. The ARO cluster-gene of N. crassa and S. cerevisiae: Molecular biology of the gene and catalytic properties of the pentafunctional product. Iowa Genetics Symposium in "Genetic approaches to the study of metabolic processes," The University of Iowa, Iowa City, Iowa, October 16-17, 1981.

Generoso, Walderico M., J. B. Bishop, D. G. Cosslee, G. W. Newell, C. W. Sheu, and E. S. Von Halle. Heritable translocation test in mice. Genetic Toxicology (Gene-Tox) Meeting, Environmental Protection Agency Conference, Washington, D.C., December 3-5, 1980.

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Financial Summary and Personnel Distribution

FY 1982

Funding Source	Funding in thousands	% of total budget	Person-years
Department of Energy	14,610	69.4	193.0
National Cancer Institute	2,150	10.2	28.0
Environmental Protection Agency	1,625	7.7	23.0
National Institute of Environmental Health Sciences	1,100	5.2	14.0
Department of Defense	1,080	5.2	14.0
National Institute of General Medical Sciences	118	0.6	1.3
Food and Drug Administration	36	0.2	0.2
Department of Agriculture	29	0.1	0.2
Miscellaneous	302	1.4	6.3
TOTAL	21,050		280.0