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OAK RIDGE
NATIONAL
LABORATORY

MARTIN MARIETTA

Biology Division Progress Report

For Period of October 1, 1985-September 30, 1986

OPERATED BY
MARTIN MARIETTA ENERGY SYSTEMS, INC.
FOR THE UNITED STATES
DEPARTMENT OF ENERGY

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BIOLOGY DIVISION
PROGRESS REPORT

For Period of October 1, 1985 - September 30, 1986

Date Published: February 1987

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R. J. Preston, Comparative and Cellular Genetics
L. B. Russell, Mammalian Genetics and Reproduction
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ORNL-3267	Period Ending February 15, 1962
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ORNL-3498	Period Ending August 15, 1963
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ORNL-3700	Period Ending August 15, 1964
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ORNL-3922	Period Ending January 31, 1966
ORNL-3999	Period Ending July 31, 1966
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ORNL-4740	Period Ending June 30, 1971
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ORNL-4915	Period Ending June 30, 1973
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ORNL-5195	Period Ending June 30, 1976
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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. In keeping with the role of DOE national laboratories in research, the work in Biology is fundamental and the new knowledge is intended to form the basis both for understanding and for applications by industry to biotechnology, medicine, and agriculture. Almost all the research 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 without harm from humans.

Among the possible adverse effects of environmental substances on the health of humans, the Division has concentrated its efforts on mutagenesis, heritable genetic effects, and carcinogenesis all of which involve molecular, cellular, and organizational studies of the consequences of damage to genetic materials. To carry out this program, the Division's scientists are organized in multi-disciplinary teams.

Unique to the Biology Division is a large facility for genetic studies in mice, the mammal of choice for assessing genetic risks to people. Unique, too, among DOE laboratories is the application of genetic engineering methods to the exploration of enzyme structure and function and to the tailor-making of new enzymes. Biology also has been assigned the major responsiblity by DOE for the investigation of the carcinogenic effects of external high LET radiation (neutron and heavy ion radiation).

A major administrative change in April this year incorporated the research component of Martin Marietta Energy System's information activities, under the direction of Tim Ensminger, into the Biology Division. This group of 90 information scientists brings expertise in data systems analysis to Biology. Many of the activities of the eleven Information Centers they brought with them were already being used in collaborative studies by staff members in Biology and other life sciences divisions. The new alliance should intensify research in complex systems analysis to the mutual benefit of both laboratory bench scientists and data analysts.

The resources available for the Division's activities during the report period included a staff of 60 at the doctorate level, 208 support personnel, 35 predoctoral students, 12 postdoctoral students, and an average of 90 other visiting professors, students, and scientists. The Division occupies 327,000 sq. ft. of laboratory space at the Y-12 site and

8,700 sq. ft. at the X-10 site with specialized facilities for 250,000 animals, a collection of 2,000 stocks of mutant 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. Remodeling of space for a User Facility for biotechnology is nearly completed. The facility will include two 500-liter fermenter vessels and associated equipment. The Division also makes extensive use of resources in other Divisions of the Oak Ridge National Laboratory including the biostatisticians in the Engineering Physics and Mathematics Division and the chemists in the Analytical Chemistry Division where collaboration in research has been especially fruitful.

In 1986, several of our staff members received special recognition from the Laboratory's contractor, Martin Marietta Energy Systems, Inc. Drs. John Kao, Steve Kennel, Ray Popp, and Hanspeter Witschi received awards for their outstanding publications. Dr. Sankar Mitra received an award for technical achievement for his research on the mechanisms by which alkylating agents cause mutations and cancer.

Among the other awards to Biology Division staff members, I wish to give special recognition to Dr. Liane Russell for her election to the National Academy of Sciences. Dr. Peter Mazur, who had been author of the year for all of Martin Marietta Energy Systems, Inc. last year, was promoted to Corporate Fellow by Martin Marietta Energy Systems, Inc. Dr. Dorothy Skinner served as National Lecturer for Sigma Xi, and Dr. Po Yung Lu received the Cameron Award from the National Safety Council. Notable, too, was Dr. Hanspeter Witschi's role as Chairman of the DOE chemical mixtures program.

The body of this report provides summaries of the aims, scope, and progress of the research by groups of investigators in the Division during the period of October 1, 1985, through September 30, 1986. At the end of each summary is a list of publications covering the same period. For convenience, the summaries are assembled under Sections in accordance with the organizational structure of the Biology Division, each Section beginning with an overview. It will be apparent, however, that cross-currents run throughout the Division and that the various programs support and interact with each other.

In addition, this report includes information on the Division's educational activities, Advisory Committee, seminar program, and international interactions, as well as the extramural activities of staff members, abstracts for technical meetings, and funding and personnel levels.

With this report, The Biology Division completes its 40th year of operation.

Superscripts - Division Staff

[1]Loan from Solid State Division

[2]Guest Assignment

[3]Postdoctoral Investigator

[4]Leave of Absence

[5]Loan from Computing & Telecommunications Division

[6]Loan to Environmental Health Sciences Division

[7]Loan from Maintenance Division

[8]Loan from Finance and Materials Division

[9]Loan from Information Division

[10]Loan from Engineering Division

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[4]Student, Southern Colleges University Union

[5]Loan from Solid State Division

[6]Consultant

[7]Loan to Environmental Health Sciences Division

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. There is such a vast array of potentially damaging agents that to attempt to assess each of their consequences singly and in combinations is likely doomed to failure. A more logical approach is to develop sufficient fundamental understanding of the structure, biochemistry, and physiology of cells and of cellular repair and defense mechanisms to permit conclusions about classes of action and classes of cellular responses. 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 some birth defects share common origins that entail modifications 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, mechanism and regulation of replication and transcription of DNA, enzymology of repair of DNA damaged by chemicals or radiation, and molecular genetics. The following capsule reports typify recent notable findings in these general areas of molecular biology.

(1) A chimeric gene, containing the DNA coding for human epidermal growth factor (EGF) and that for the signal peptide of *E. coli* alkaline phosphatase, has been synthesized and cloned into a bacterial plasmid under the control of the *E. coli tac* promoter. Expression of EGF and its secretion into the bacterial periplasmic space has been demonstrated. Questions regarding the molecular mechanisms by which EGF triggers the cascade of events leading to cellular proliferation will be addressed by site-directed mutagenesis.

(2) Promutagenic O^6-alkylguanine residues in DNA are repaired by an unusual and ubiquitous repair enzyme, DNA-O^6-methylguanine methyltransferase. This highly regulated protein accepts an alkyl group on a cysteine residue in a stoichiometric, suicide reaction. The *E. coli* protein (Ada) also accepts a methyl group from phosphotriesters on a separate cysteine residue, and the methylated protein is an inducer of its

own gene and some other genes involved in DNA repair. Site-directed Ada mutants have been produced that show differential behavior for induction and O^6-methylguanine methyltransferase activity.

(3) The REV1 gene of yeast, which encodes a component of misrepair mutagenesis, has been cloned. The primary sequence of the REV1 gene and the rev1-1 mutant have been determined. The REV1 transcript has been identified and has been shown to be induced by UV damage.

(4) By use of a synthetic oligonucleotide linker/adapter, the cloned gene for ribulose bisphosphate carboxylase from Rhodospirillum rubrum has been modified to permit juxtaposition of E. coli ribosomal binding sites at positions -3 and -10 from the translation initiation codon. Expression of this gene, under control of the tac promoter, is thereby enhanced such that its gene product represents 5% of the total cellular protein.

(5) A domain that is rich in alternating purines and pyrimidines (Pu/Py) has been subcloned from a cloned variant of a very complex satellite DNA that has a repeat unit of 2089-bp. Three independent methods demonstrate that the Pu/Py-rich domain adopts a Z-conformation under torsional stress: (1) inhibition of cutting at a restriction enzyme site within the Z-DNA region, (2) binding of polyclonal and monoclonal anti-Z-DNA antibodies, and (3) antibody stabilization of the Z-region and subsequent relaxation. The Pu/Py-rich domain is of particular interest because changes in sequence occur at specific locations nearby. These data provide further evidence that specific segments of DNA that undergo alterations in secondary and tertiary structure are hotspots for mutations. This conclusion is reinforced by the fact that other than these changes the domain and several hundred nucleotides surrounding it are 92 and 98% conserved.

(6) The 5S RNA macronuclear gene from Euplotes eurystomus has been cloned and is being sequenced. The gene probe will be employed to monitor soluble macronuclear chromatin subfractionation, and to determine nucleosome positioning and the binding of specific macronuclear proteins. An in vitro DNA replication system with isolated macronuclei incorporates biotinylated dUTP into the replication band with high efficiency.

(7) Three-dimensional reconstruction by electron microscope tomography of thick sections of actively transcribing Balbiani Ring gene from Chironomus has been accomplished with data collected on a Zeiss 902 microscope equipped with electron spectroscopic imaging. This has permitted the collection of image data on the 0.75 μm section at 80 kV; previously, bright-field imaging dictated examination of structures at 300 kV.

(8) The structure of the nucleosome core particle, the fundamental building block of chromatin, has been determined by X-ray diffraction methods to a resolution of 8 Å. The path and properties of the superhelical DNA, the location of the histone domains, and the nature of interactions between histones and the DNA can be seen. Extending this work

to high resolution involves crystallizing reconstituted nucleosomes constructed from purified histone octamer and cloned 146 base pairs specific sequence DNA fragments. Computerized methods for selecting or designing DNA fragments which facilitate superhelical bending of DNA and proper phasing on the histone core have been developed. The application of these methods has thus far led to several fundamental discoveries about the bendability properties of DNA, mechanisms for nucleosome phasing, and identification of several useful DNA fragments for reconstitution. These studies are providing important new information about the function and dynamic mechanisms of chromatin.

Membrane biology is also of prime relevance to the Section. Cell membranes can be considered interfaces between the metabolic machinery of the cell and its milieu, while sheets of epithelial membranes are interposed between the organism and its environment. An example of the latter are the kidney epithelial which regulate the composition of the blood plasma, secreting waste solutes into the urine and retaining by reabsorption such essential solutes as sodium and glucose. Failure of glucose reabsorption is a form of diabetes. The regulation of several of the essential renal transport systems is being studied in cultured proximal tubule cells that have been cloned here in Oak Ridge. A recent investigation has probed the structure:function relations of a series of diuretics known to be effective against sodium reabsorption and calcium secretion and has demonstrated that, independently of these activities, the diuretics are also effective against the Na-dependent reabsorption of glucose. That they act directly in competition with Na for its binding site on the cotransporter is clearly demonstrable because of the clonal cell system used in the study. Further modification of the diuretics has the potential of specifically labeling the Na binding site on the glucose transporter.

Another aspect of cellular-environmental interactions and membrane physiology that receives emphasis is cryobiology. Recently, the long-held view that injury to cells subjected to slow freezing is due to the increased solute concentration and osmotic dehydration resulting from ice formation has been challenged. Data on human erythrocytes and 8-cell mouse embryos indicates that injury is due more to the narrowing of the unfrozen aqueous channels in which the cells lie. Two factors appear to be involved. One is damage from rheological forces that develop in the gradually constricting channels. The other is the development of cell-cell contacts as the cells become increasingly crowded together in the narrowing channels. The latter phenomenon is being studied in more detail by subjecting erythrocytes to hyperosmotic solutions at subzero temperatures in the absence of ice formation.

Given the absolute dependence of life processes on catalysis and the adverse consequences of altering catalytic events, our long-standing interests in enzyme mechanisms and metabolic pathways continue. Major activities are the design of affinity labels for the characterization of catalytic sites and characterization of enzymes that are involved in regulatory aspects of growth and development. Recent advances include (1) demonstration that the catalytic site and regulatory site (i.e., the

thioredoxin binding site) of phosphoribulokinase, a light-regulated enzyme of the Calvin cycle, are contiguous, and (2) mapping the distance between two active-site residues of ribulose bisphosphate carboxylase with covalent cross-linking reagents.

As a logical extension of our historical strengths in enzymology and molecular biology, a new initiative in protein engineering (site-directed mutagenesis) has evolved. This program is designed to integrate and enhance ongoing endeavors through focusing on a common interdisciplinary theme having both fundamental and applied significance. Current activities include mutagenesis studies of enzymes, polypeptide growth factors, and DNA repair proteins as well as development of more facile procedures for introducing site-specific mutations into cloned genes.

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 necessitated our securing funds from other agencies to provide postdoctoral positions and other supportive personnel so essential to maintaining productive programs of high quality.

MOLECULAR MUTAGENESIS AND PROTEIN ENGINEERING

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Initiated in April 1984 with partial support from the Laboratory Director's R & D Fund, this program represents a collaborative effort that involves about one-half of the Section's senior staff and integrates activities in biochemistry and molecular genetics. Our interests include the use of site-directed mutagenesis to elucidate mechanisms of protein function and the development of methods to facilitate applications of site-directed muagenesis. Currently, we are addressing structure/function correlations of three proteins: ribulose bisphosphate carboxylase, DNA-$\underline{O}^6$-methylguanine methyltransferase, and epidermal growth factor.

Ribulose Bisphosphate Carboxylase (Rubisco)

Ubiquitous among photosynthetic organisms, Rubisco is essential for net conversion of atmospheric CO_2 into carbohydrates. Thus, this enzyme is a major cornerstone of living processes and is highly relevant to the production of biomass for energy and to the global CO_2 issue (i.e., the greenhouse phenomenon). The enzyme is bifunctional. In addition to

catalyzing the carboxylation of D-ribulose-1,5-bisphosphate to yield two molar equivalents of D-3-phosphoglycerate (the CO_2-fixation reaction), it also catalyzes the oxidation of ribulose bisphosphate by molecular oxygen to yield one molar equivalent each of phosphoglycolate and 3-phosphoglycerate. Although multiple substrate specificities among enzymes are not unusual, the bifunctionality of Rubisco is perhaps unprecedented in that the two reactions catalyzed are the initial steps in competing metabolic pathways -- photosynthetic assimilation of CO_2 and photorespiration, the latter an energy wasteful process without a known function which results in the release of previously fixed CO_2.

The objectives of site-directed mutagenesis studies are 1) to understand the mechanism of this complex enzyme, especially the roles of specific amino acids and domains in interaction with substrates and in mediating the discrete catalytic steps, and 2) to evaluate the feasibility of improving the carboxylase/oxygenase activity ratio and thereby provide an approach to enhancing biomass yields.

In contrast to Rubisco from higher plants which is comprised of two gene products (eight large and eight small subunits), the functionally analogous enzyme from the purple, non-sulfur photosynthetic bacterium *Rhodospirillum rubrum* represents a single gene product (a homodimer of large subunits) and is thus more amenable technically to *in vitro* mutagenesis. Because the original clone (Nargang *et al.*, Mol. Gen. Genet. 193: 220, 1984) of the gene (*rbc*) for *R. rubrum* carboxylase expresses a *lacZ* fusion protein in *E. coli*, we reconstructed it and obtained a clone (plasmid pFL34) which expresses wild-type enzyme. A restriction fragment from pFL34 that contains the *rbc* gene has been subcloned into single-stranded M13 DNA to provide a template for directing *in vitro* mutagenesis by single-primer extension. Some site-specific mutations have also been introduced directly into plasmid pFL34 by a novel procedure described below (see "Techniques for Effecting Site-Directed Mutagenesis").

One issue being addressed is the identity of the active-site base of the carboxylase that abstracts the C3 proton from ribulose bisphosphate to generate the corresponding enediol intermediate and thereby initiates the catalytic pathway. Based on our affinity labeling studies (see following report on "Protein Chemistry"), Lys-166 may be the essential base that initiates catalysis. However, publications from other laboratories have invoked His-291 in this same role. To resolve this disagreement, we have used site-directed mutagenesis to replace His-291 with alanine and Lys-166 with glycine, alanine, serine, glutamine, arginine, cysteine, or histidine. All mutant proteins were purified by a single-step, immunoaffinity chromatographic procedure and shown to be dimers like the wild-type enzyme (i.e., the single amino acid substitutions do not interfere with association of subunits to give normal quaternary structures).

The Ala-291 mutant is highly active; its V_{max} is 40% as great as that of the wild-type enzyme. Thus, His-291 is excluded as the base that initiates catalysis. In contrast, all substitutions for Lys-166 result in essentially total abolishment of catalytic activity. Some of these

position-166 mutants, however, are functional in substrate binding. These observations verify a catalytic function for Lys-166 and are consistent with Lys-166 serving as the essential base.

DNA-O^6-Methylguanine Methyltransferase

The DNA-O^6-methylguanine methyltransferase gene (ada) of E. coli has been cloned by several laboratories (Demple et al., PNAS 82: 2688, 1985; Nakabeppu et al., J. Biol. Chem. 260: 7281, 1985; Lemotte and Walker, J. Bacteriol. 161: 888, 1985). Its gene product, a 39-kDa protein called Ada, acts in a suicide reaction in which Cys-69 and Cys-321 accept methyl groups from phosphotriesters and O^6-methylguanine, respectively, in DNA. It was shown that methyl transfer from phosphotriesters activates Ada such that it acts as a positive regulator of transcription of its own gene and of a number of other genes coordinately regulated during the adaptive response of E. coli to alkylating agents.

We have initiated a program to systematically alter the ada gene by site-directed mutagenesis in order to study the mechanisms of reaction and induction of the Ada protein.

(i) Overproduction of Ada: We introduced an HpaI site between the regulatory region and the structural sequences of the ada gene via oligonucleotide-mediated mutagenesis. The structural sequence of the gene excised at this site was inserted in a λP_L-based expression vector, pTK23 (Court, D., unpublished). The recombinant plasmid pKT-1 expresses Ada protein following induction of plasmid bearing host WPS18 carrying λcI repressor at 42° to about 5% of total cell protein, facilitating its purification.

(ii) Mutations of Cys-321 region: Demple et al. (loc. cit) suggested that the -Pro-Cys-His- sequence (positions 320-322) may be important in the reaction involving transfer of the methyl group of O^6-methylguanine to Cys-321. Because His-322 may play a role as a nucleophile, we generated mutant ada genes containing (A) -Pro-His-His- and (B) -Pro-His-Cys- coding sequences for positions 320-322 of the protein. Preliminary experiments indicate that both proteins coded by A and B are inactive as methyltransferase for O^6-methylguanine. However, the mutant B protein, but not A protein, can accept a methyl group from a DNA phosphotriester, and is inducible.

Epidermal Growth Factor (EGF)

The involvement of extracellular protein factors in the regulation of DNA replication, specific RNA synthesis and cell division has long been recognized. Several factors have been isolated with the ability to stimulate the growth of cells and the expression of specific genes believed to be involved in the entrance of mammalian cells into and progression through the cell cycle. One of the most highly studied among these is epidermal growth factor (EGF), a 6-kDa single polypeptide chain with three internal disulfide bonds, which is known to initiate its action through

high-affinity binding to the specific cell surface EGF receptor. In response to EGF, the receptor undergoes autophosphorylation on tyrosine residues by its intrinsic protein kinase besides phosphorylation of exogenous substrates.

Despite the variety of functions elicited in response to EGF, little is known about the biochemical mechanisms by which EGF activates the receptor's tyrosine kinase activity, stimulates the internalization of the EGF•EGF-receptor complex, and eventually stimulates DNA replication and specific RNA synthesis and cell division.

Our major objectives are: Identification of the amino acid residues in EGF that are ncessary for 1) its binding to the EGF receptor, 2) stimulation of the EGF receptor protein kinase activity, and 3) stimulation of growth of quiescent cells. These studies will involve site-directed mutagenesis or protein engineering using the human EGF gene cloned in E. coli as a secretion vector.

Fragments of the 159-base pair DNA coding for human EGF and those of the 63-base pair DNA coding for the signal peptide of E. coli alkaline phosphatase were synthesized in an automated DNA synthesizer. Each oligonucleotide was purified by gel electrophoresis and phosphorylated at the 5' end with T4 polynucleotide kinase. Subsequent annealing led to the formation of six double-stranded DNA fragments with single-stranded termini that enabled the ligation of the DNA fragments using T4 DNA ligase. The resultant chimeric gene, purified by gel electrophoresis, was placed under the transcriptional control of the trp-lac (tac) promoter contained in a plasmid vector by using standard recombinant DNA techniques, and then transformed into E. coli. The correct orientation of insertion was checked by diagnostic restriction enzyme analysis followed by direct sequence analysis to confirm the cloning of the chimeric gene.

Following induction with isopropylthiogalactoside, the secretion of EGF into the E. coli periplasmic space was confirmed by its specific binding to the EGF receptor isolated from A431 cells (derived from a human epidermoid carcinoma) and stimulation of the EGF receptor tyrosine kinase activity. Current efforts are directed towards optimizing the induction and secretion of the EGF product. This will be followed by studies of structure/function relationships by specific alterations of targeted amino acid residues in the EGF molecule by site-directed mutagenesis.

Techniques for Effecting Site-Directed Mutagenesis

Design and Construction of New Expression Plasmids for High-Level Synthesis of Cloned Proteins: Using a synthetic oligonucleotide linker/adapter, the 5' untranslated region of the rbc gene from Rhodospirillum rubrum was modified to permit juxtaposition of the Escherichia coli ribosome binding sites -3 and -10 nucleotides from the translation initiation codon. During this construction, a new restriction endonuclease cleavage site for the enzyme NcoI was introduced. This site overlaps the initiator ATG and allows the mobilization of a module which contains the

tac promoter, ribosome binding site, and the initiation codon. Proteins which are expressed from such a construction constitute approximately 5% of the cellular protein. Ten previously constructed rbc mutants (6 at position 166, 4 at position 291) have been placed in this high-level expression plasmid. This expression plasmid along with the rbc transcription terminator have also been used for the expression of a synthetic EGF gene.

Further Development of the Bandaid Mutagenesis Technique: Bandaid mutagenesis has been used to introduce a number of mutations at seven different sites of the gene for ribulosebisphosphate carboxylase. Briefly, this technique takes advantage of the fact that a gap in a double-stranded DNA molecule which is flanked by one protruding 3'- and one protruding 5'-end can be bridged by ligating a synthetic oligonucleotide across the gap. Since this oligonucleotide may be of any sequence, provided that its ends are complementary to the protruding ends flanking the gap, it can be used to introduce a wide variety of mutations into the gene of interest.

Two regions of the R. rubrum ribulosebisphosphate carboxylase gene have been modified for bandaid mutagenesis. The region between amino acid residues 163 and 168 was made available for bandaid mutagenesis by introducing a KpnI and a HindIII site into the gene. Asp-166, Cys-166, Gln-166, Gly-166, and Gln-168 mutants have been made by bandaid mutagenesis. Two new restriction sites, a KpnI and a XhoI site, were created by primer-extension mutagenesis to make a vector for bandaid mutagenesis of the region of the enzyme between amino acid residues 325 and 333. In order to construct this vector it was necessary to make a mutant, Asp-333, which can be reverted to the wild-type, Glu-333, by the 36 base oligonucleotide used for mutagenesis. Bandaid-induced mutants in this region include Arg-329, Ser-329, Cys-329, Gly-329, and Leu-325.

Besides introducing mutations at amino acid residues which are implicated in the active site by affinity labeling studies, namely Lys-166 and Lys-329, bandaid mutagenesis has been used to study the effects of changing residues in the neighborhood of the active site residues. In one case, we have simultaneously changed a group of six amino acids near the Lys-166 residue so that they are now homologous to the residues found in the carboxylase from spinach at this position. Changing a number of residues simultaneously is readily accomplished with bandaid mutagenesis, while it is very difficult with the primer extension methods.

Validation of mutants which have been made by bandaid mutagenesis has been facilitated by sequencing the double-stranded DNA of bandaid constructions using reverse transcriptase (Zagursky et al., Gene Anal. Techn. 2: 89-96, 1985).

Creation of Chimeric Proteins: It may be possible to gain insight into the functional domains of an enzyme by creating chimeras between enzyme molecules that are evolutionarily distant. In other systems, domains from distantly related molecules have been combined and functional enzymes were generated (Mas et al., Science 233: 788-790, 1986). We have

begun studies of this kind by creating a chimeric protein which contains the first 324 residues of the ribulose bisphosphate carboxylase from R. rubrum and the last 147 amino acids from the carboxylase of the cyanobacterium Anabaena 7120. The gene for this chimera was constructed by using a KpnI site which was introduced into the R. rubrum gene during a bandaid vector construction and a naturally occurring KpnI site at the homologous position of the Anabaena gene. Though the carboxylase activity of the chimeric enzyme is low (<1% in crude extracts), its gene can be a starting point for other novel chimeras.

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PROTEIN CHEMISTRY AND ENZYME MECHANISMS

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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 localized high concentration of reagent within the substrate binding site and thus increases the likelihood of modification of a residue within this site as compared to other positions of the protein molecule.

Many of our recent investigations have centered on two Calvin cycle enzymes that are major determinants of plant growth and yield: ribulose bisphosphate carboxylase and phosphoribulokinase.

Ribulose Bisphosphate Carboxylase (Rubisco)

This enzyme mediates the photosynthetic assimilation of CO_2 by catalyzing the carboxylation of ribulose bisphosphate to yield 3-phosphoglycerate. This enzyme also possesses inherent oxygenase activity which accounts for photorespiration -- a nonessential, energy-wasteful process that reduces net CO_2 fixation. There is general agreement that preferential abolishment of the oxygenase activity would elevate by 50% the yields of C3 plants (plants in which ribulosebisphosphate is the initial acceptor of atmospheric CO_2 in contrast to C4 plants in which phosphoenolpyruvate is the initial acceptor). 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 as an energy source.

As one facet of elucidating the mechanism of Rubisco and subsequently evaluating the feasibility of altering the carboxylase/oxygenase ratio, we have focused on active-site characterization with affinity labels. Collective data from our laboratory provided by several different affinity labels have revealed the presence of two distinct lysyl residues at the active site. In the hetero-hexadecameric enzyme from spinach, these lysines occupy positions 175 and 334 of the large subunit; and in the homo-dimeric enzyme from *R. rubrum*, they occupy positions 166 and 329. The absolute species invariance of both lysines and the sequence homologies of adjacent regions among the carboxylases from evolutionarily diverse organisms (e.g., spinach and *R. rubrum*) suggest that the two active-site lysines are directly or indirectly required for proper enzyme function.

Judicious application of affinity labeling followed by cautious interpretation of results can identify active-site residues but cannot reveal their function. Three approaches are being taken to bridge the gap between assignment of residues to the active-site and defining their

function (if any): (a) a determination of their pK_a values based on the pH-dependencies of chemical modification; (b) a determination of their proximity based on covalent cross-linking with bifunctional reagents; and (c) a determination of the consequences on catalytic functionality of their selective replacement with other amino acids by site-directed mutagenesis (see preceding report under "Molecular Mutagenesis and Protein Engineering").

pK_a Values of Active-Site Lysines: During the screening of lysine-selective reagents for their ability to preferentially modify the active-site lysines of ribulose-P_2 carboxylase, we observed that 2,4,6-trinitrobenzenesulfonate (TNBS) arylates Lys-166 of the *R. rubrum* enzyme and Lys-334 of the spinach enzyme with a very high degree of specificity. Hence, a single reagent, applied to the carboxylase from two different organisms, can provide the pK_a values for both active-site lysines. Based on the pH-dependencies of arylation, the following pK_a values and intrinsic reactivities (k_o) have been obtained: acetyllysine, pK_a = 10.8 and k_o = 1250 $M^{-1}min^{-1}$; Lys-166 of the *R. rubrum* enzyme, pK_a = 7.9 and k_o = 670 $M^{-1}min^{-1}$; and Lys-334 of the spinach enzyme, pK_a = 9.0 and k_o = 4500 $M^{-1}min^{-1}$. Particularly noteworthy are the enhanced nucleophilicities of the two protein ε-amino groups compared to acetyllysine, despite their stronger acidities. These unusual properties are suggestive of, but do not prove, catalytic functionality of the active-site lysines.

Lys-166 (pK_a = 7.9) of the *R. rubrum* enzyme may serve as the essential base that enolizes ribulose-P_2, thereby initiating catalysis. An examination (Van Dyk *et al.*, Biochemistry 25: 5145, 1986) of the *R. rubrum* enzyme for the pH-dependencies of both V_{max}/K_m and the deuterium isotope effect, with [3-^{2}H]ribulose-P_2 as substrate, reveals that this essential base (as the conjugate acid) has a pK_a of 7.5. The pK_a is insensitive to the dielectric constant of the solvent, which suggests a cationic acid, i.e., a lysyl or histidyl residue.

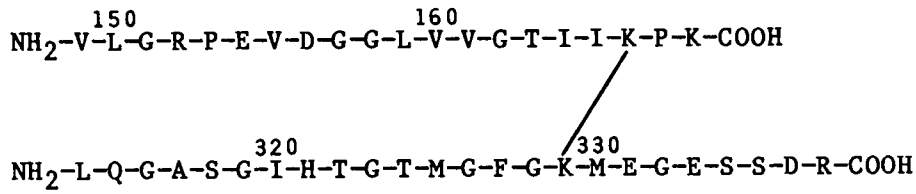
Although the unusual acidity and nucleophilicity can account for the specificity of TNBS for Lys-166 and Lys-334, their similar reactivities at pH 8.0 raise the question as to why only one of them is modified in each species of carboxylase examined. A likely explanation is that the two lysines are juxtaposed within the catalytic site so that derivatization of both is precluded on steric grounds. This consideration prompted cross-linking studies, because a high-resolution three-dimensional structure of ribulose-P_2 carboxylase from any species is not yet available.

Distance Between Active-Site Lysines: To challenge the postulate of proximity of the two active-site lysines, we have explored the reactions of the *R. rubrum* enzyme with several chemical cross-linking agents. One reagent that inactivates the carboxylase with a high degree of specificity is 4,4'-diisothiocyano-2,2'-disulfonate stilbene (DIDS), which spans 12 Å.

Rubisco from *R. rubrum* is a dimer so that any cross-links introduced could be intrasubunit, intersubunit, or intermolecular. As only intrasubunit cross-links are pertinent to distances between residues within

the active site, the DIDS-inactivated enzyme, subsequent to carboxymethylation of sulfhydryls, was subjected to gel filtration in the presence of urea, thereby permitting the isolation of the monomeric polypeptide. Samples of the isolated monomeric fraction were digested with trypsin; these digests were inspected by HPLC to ascertain the degree of reagent specificity. Based on the $\underline{A}_{342nm}$ (the major visible absorbancy band of DIDS), over one-half of the incorporated reagent was associated with a single peptide. This peptide was purified by preparative ion-exchange chromatography on DEAE-cellulose followed by gel filtration on Sephadex G-25.

The amino acid composition and sequence of the purified peptide demonstrated that it is comprised of two chains encompassing position 149-168 and 314-337 of the original protein subunit:



The location of the covalent cross-link between Lys-166 and Lys-329 was revealed by the complete absence of phenylthiohydantoin-lysine at their respective positions during Edman degradation.

These results are compatible with proximity of Lys-166 and Lys-329 as dictated by their purported catalytic functionalities.

Phosphoribulokinase (PRK)

PRK catalyses the ATP-dependent synthesis of ribulose bisphosphate in the illuminated, chloroplast stroma. The enzyme is regulated *in vivo* by thioredoxin-mediated oxidation/reduction of sulfhydryls/disulfides. One sulfhydryl group of the kinase from spinach is preferentially alkylated by bromoacetylethanolamine phosphate, leading to complete loss of enzyme activity, concomitant with the incorporation of only one molar equivalent of reagent. The tryptic peptide containing the alkylated cysteine has been purified, sequenced, and observed to be derived from the amino terminus of kinase. The highly reactive cysteinyl residue occupies position 16 of the primary structure. The protection against alkylation afforded by ATP corresponds to a dissociation constant of ~30 μM, similar to the $\underline{K}_m$ for ATP and hence supporting an assignment of cysteine-16 to the active site. Several lines of evidence suggest that the active-site sulfhydryl group is involved in the thioredoxin-mediated regulation of the kinase. The oxidatively, deactivated kinase does not react with bromoacetylethanolamine phosphate. ATP, which protects against alkylation, also protects against oxidation with an apparent dissociation constant of ~25 μM. The pH dependencies of both alkylation and oxidation reflect a $p\underline{K}_a$ of ~7.8, consistent with the involvement of the same sulfhydryl in both processes.

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STUDIES ON RENAL TRANSPORT SYSTEMS IN A LINE OF CULTURED KIDNEY CELLS, LLC-PK_1

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Inhibition of Amiloride Analogs of Na^+-Dependent Hexose Uptake in LLC-PK_1/C14 Cells

Amiloride is a synthetic loop diuretic developed for its potency in blocking Na^+ reabsorption in the distal proximal tubule of the kidney and thereby generating an osmotic diuresis. Further exploration has shown that amiloride inhibits several Na^+-dependent transport functions in many cells types, including the surface Na^+,K^+-ATPase. Certain amiloride analogs developed by E. J. Cragoe, Jr. (Merck, Sharp and Dohme Research Laboratories, West Point, PA) have been shown to have both greater potency and/or

greater specificity than the parent compound against specific cellular functions. In our investigations of Na^+-dependent hexose transport in cultured renal cells, it was of interest to determine whether these drugs were effective against this transporter. Amiloride and four analogs of amiloride were shown to inhibit the Na^+-dependent, phlorizin-sensitive hexose uptake by a clone of pig kidney cells, LLC-PK_1/C14. The analogs tested, and the membrane functions they primarily affect, were: 5-(N-ethyl-N-isopropyl)amiloride [EIPA] and 5-(N-methyl-N-isobutyl)amiloride [MIBA], both potent inhibitors of Na^+/H^+ exchange; 3',4'-dichlorobenzamil, an inhibitor of Na^+/Ca^{2+} exchange; and phenamil, a Na^+ channel blocker. The hexose transport substrate was the nonmetabolizable glucose analog α-methyl-D-glucoside. Blockade of Na^+-K^+ transport with ouabain at the basolateral membranes or removal of divalent cations from the assay medium had little effect on the initial rate of hexose uptake, while MIBA remained an effective inhibitor under both conditions. The inhibitions by EIPA of Na^+/H^+ exchange and hexose-dependent Na^+ uptake could be distinguished by appropriate choice of concentrations of the inhibitor; exchange was blocked at EIPA concentrations below those affecting hexose transport. Hexose-transport inhibition does not appear to be secondary to other known effects of the amilorides. Inhibition by all analogs is enhanced when they are tested in low (2 mM) Na^+ medium, where they show half-maximum inhibition in the range of 100-300 μM. More detailed kinetic analysis of inhibition by EIPA shows it to be competitive with Na^+ with a Ki of 70-110 μM. It is concluded that the amilorides are acting directly and not secondarily on the hexose transporter.

Retrieval of Protein Kinase C from a Cytosolic Fraction of LLC-PK_1 Cells by TPA-Treated Cell Membranes and Synthetic Liposomes

Tumor-promoting phorbol esters and certain diradylglycerols elicit changes in Na^+-dependent amino transport in the renal epithelial cell line LLC-PK_1, which changes are one-to-one correlated with changes in the cellular distribution of protein kinase C (PKC), an enzyme activated by both the tumor promoter TPA and by the diradylglycerols. In homogenates of confluent LLC-PK_1 cells centrifuged at 100,000 x g for 1 h, PKC is found in the "soluble" supernatant. If the supernatant is further fractionated PKC activity does not appear evenly distributed but is concentrated toward the meniscus, possibly in low density cytoplasmic particles. To study further the redistribution of PKC in the cells we measured the clearance of PKC from the cytosolic fraction by isolated LLC-PK_1 cell membranes. Untreated membranes had no effect on supernatant PKC activity but membranes treated with ^{3}H-TPA or diradylglycerols and then washed until no free ^{3}H-TPA or lipid was left in the medium were capable of quantitatively removing PKC from the "soluble" compartment. When synthetic liposomes were used in clearance experiments only those liposomes containing phosphatidylserine were capable of removing PKC from the fraction. Liposomes constructed with phosphatidylcholine, phosphatidylcholine and diacylglycerol, or phosphatidylcholine and TPA had no effect on "soluble" PKC activity. Finally, although Ca^{2+} is required for enzymatic activity of PKC, the redistribution of PKC into TPA-treated LLC-PK_1 cell membranes or into synthetic liposomes occurs in Ca^{2+}-free medium containing EDTA (2.0 mM)

and EGTA (0.5 mM). We conclude that the TPA receptor is not PKC, as is commonly assumed, but a membrane-PKC complex.

In whole cells, PKC translocation in response to either TPA or diradylglycerols always accompanies the stimulation of amino acid transport, and both are blocked in parallel by cytochalasin B. The translocation occurs within 1-5 min, but the enhanced amino acid uptake is seen only after 20-40 min. The two phenomena can nevertheless be separated with the use of cycloheximide which does not affect translocation but does prevent the augmented amino acid uptake. Thus PKC translocation appears to be a necessary but not sufficient prerequisite to transport modulation.

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THEORETICAL AND APPLIED CRYOBIOLOGY

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The purpose of the Theoretical and Applied Cryobiology program is to determine the responses of cells to the major cryobiological variables involved in freezing and thawing such as cooling rate, and to the physical state of the cell and its surroundings. Besides freezing studies per se, our research involves a combination of experimental and mathematical approaches to determine the permeability and osmotic responses of cells to critical solutes and water. The cells currently under study are mouse embryos, Drosophila embryos, and human erythrocytes.

The program is related to DOE's energy missions in the following way. First, animals and their tissues are used to assess health effects of effluents. Freezing can help insure that the assay systems remain invariant with time. It can also reduce the "noise" from genetic heterogeneity by allowing the storage of many tissue samples from a single donor. Second, freezing permits the storage of animal and plant germplasm in an unchanged state. The ability to freeze mammalian embryos (first reported by this laboratory) is proving important to basic and applied geneticists. It is providing the former with an economical means of preserving mutant strains of mice. It can help the latter accelerate the development of breeding strains of livestock that are more energy efficient, or strains that can adapt to the altered climatic conditions that might attend the massive use of fossil fuels. Third, environmental insults are less serious if their effects can be reversed by medical therapy. There are a number of medical applications to cryobiology, especially in the transplantation of cells, tissues, and organs.

Mechanisms of Slow Freezing Injury in Human Red Cells

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 thought that slow freezing injury is the result of either excessive salt concentration or excessive cell shrinkage. But we have found recently that the survival of human red cells is predominantly 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. When the unfrozen fraction drops below 10%, a high percentage of the cells are damaged regardless of whether the salt concentration in that unfrozen fraction is 1 molal or as high as 2.8 molal.

The above results apply to cells frozen at low hematocrit (0.4 to 8%). At high hematocrit (40 and 60%), survival is affected by both the unfrozen fraction and the salt concentration in that fraction. (Mazur and Cole, Cryobiology 22: 505, 1985).

Our experiments the past year have concentrated on mimicing the events to which cells are exposed during freezing under conditions where no ice at all is present, i.e., the unfrozen fraction is 1.0. Cell suspensions are initially suspended in a solution of 0.5 m glycerol in 0.15 m NaCl and then in the usual experiment supercooled to -5° to -8°C (supercooled because the temperature is 4 to 7 degrees below the freezing point of the solutions). The solute concentration is then raised to 5.5 molal glycerol and 1.6 molal NaCl, the concentrations that would be present in the unfrozen channels when the intitial solution is frozen to -16°C. The consequence of this rise in solute is that the cells undergo extensive osmotic shrinkage, but with no ice present. If the cells are in suspension in the supercooled 0.5 m glycerol- 0.15 m NaCl at the time the 5.5 m/1.6 m solution is added, some 95% survive. But if the cells are in a pellet in the supercooled 0.5 m glycerol- 0.15 m NaCl at the time the cold concentrated solution is added, only 20% survive. In both cases the cells see the same concentration in the extracellular medium and therefore should have undergone the same extent of osmotic shrinkage, yet survival depends greatly on the "path" taken to reach the same chemical potential in the external medium. In freezing a decrease in the unfrozen fraction and an increase in the initial cell hematocrit both produce cell crowding as the cells are shrinking, and they produce damage. In the present experiments, the pelleted cells are also crowded together as they undergo shrinking, and they too are extensively damaged.

This past year we have also examined a number of variations on this basic experiment.

1. Effect of <u>time in contact with concentrated glycerol/NaCl</u>: Survival was found to drop progressively with time of exposure to the test solution at -5°C from some 70% at 5 min to some 20% at 75-90 min. A still longer exposure of 120 min, however, produced a significant upturn in survival.

2. The <u>type of sample container</u> affected the results. The above results applied to samples pelleted in standard 15 ml conical centrifuge tubes. When the samples were pelleted and further treated in tubes with narrower bore stems (Kolmer tubes), the minimum survival occurred earlier (45-60 min), and the upturn with longer exposure times was much more pronounced.

3. Survival was affected by the <u>centrifugal acceleration</u> used to pellet the cells prior to their being cooled to -5°C and exposed to 5.5 m glycerol/1.6 m NaCl for 75 min. Survival dropped from 70% at 3 g to about 30% at 200 g and higher.

4. <u>Holding temperature</u>. The basic experiment was repeated with pelleted cells held in contact with 5.5 m glycerol/1.6 m NaCl at 37, 22, 15, 10, 0, or -12°C rather than at -5°C. The results fall basically into two groups. More than 70% of the cells survived 5 to 120 minute exposure at 37 and 22°C. But at the four lower temperatures the cells responded

like those at -5°C, although the lower the temperature the greater was the time taken to attain the minimum in survival.

5. Maximal damage requires high concentrations of both glycerol and NaCl. Cells in contact with 5.5 m glycerol/0.15 m NaCl undergo little damage. Cells in 0.5 m glycerol/1.6 m NaCl undergo considerable damage, but not as much as in 5.5 m glycerol/1.6 m NaCl. Attempts to substitute equiosmolal sucrose (also nonpermeating) for the NaCl were unsuccessful because the high density of the sucrose prevented pelleting.

6. Concentration of glycerol/NaCl. We have now exposed pelleted cells at -5°C to the following molalities of glycerol/NaCl: 0.5/0.15, 1.07/0.31, 2.1/0.61, 3.44/1.0, 5.5/1.6, and 8.3/2.4 molal. These solutions in which the mole ratio of glycerol to NaCl is 3.44 correspond to the concentrations produced by freezing a 0.5/0.15 molal solution to -3, -6, -11, -16, and -26°C respectively. The minimum survivals in the six solutions were 98, 95, 90, 80, 20, and 15% respectively.

Our conclusions to date are that the human red cells are damaged if while in close cell to cell contact at +10 to -12°C they are made to shrink extensively by exposure to a solution containing >1.6 molal NaCl, a nonpermeating solute, and are held in that shrunken state for the order of an hour. Damage is far less if any of these components is absent; i.e., if the cells are not in close contact with each other, the temperature is not low, or they are not extensively shrunken.

To further test the role of cell-to-cell contact, we are currently conducting experiments in which the pellet consists of a mixture of red cells and six times as many yeast cells. The idea here is prepare pellets in which a given red cell is on average in close contact with yeast cells -- not other red cells. The preliminary results are that the damage is lessened substantially.

Ideal Osmotic Response of Mouse and Bovine Embryos

The steps involved in freezing subject cells to major changes in osmotic pressure that produce major changes in cell volume. The cells shrink abruptly when initially exposed to molar solutions of cryoprotectant, and then slowly return close to their isotonic volume as the cryoprotectant permeates. They shrink again to a third of their isotonic volume or less as slow freezing progressively increases the concentration of the external medium to 30 osmolal and above. Their volume returns to isotonic during thawing. Finally, the cells undergo major osmotic changes in volume as they are returned to physiological saline. The equations we have used to predict these reponses assume that the volumes of the cells respond in an ideal fashion to changes in external osmotic pressure both at above zero temperatures and during freezing. Basically, ideal osmotic reponse means that cell volume is a linear function of the reciprocal of the external osmolality.

We have measured the osmotic response of mouse and bovine embryos at different developmental stages at room temperature and found that they do behave ideally over a broad range of external osmolalities (0.5 to 4 × isotonic) (Mazur and Schneider, Cell Biophys. 8: 259, 1986). We have also computed the volume decrease of bovine morulae during slow freezing under the assumption that they behave ideally, and compared these predicted volumes with those observed by others. The agreement is excellent (Mazur, Cryobiology 23, 1986).

Relative Contributions of Salt Concentration and Unfrozen Fraction to the Survival of Slowly Frozen 8-Cell Mouse Embryos

In a paper in press in Cryobiology, we (Schneider and Mazur) have investigated whether unfrozen fraction plays as important a role in the survival of a slowly frozen nucleated mammalian cell system (mouse embryo) as in the human erythrocyte. The study reports survival of some 3300 mouse embryos as a function of the unfrozen fraction and the concentration of salt in that unfrozen fraction. Also varied in the study was the weight percent ratio of glycerol to salt. The concentration of embryos in these experiments (i.e., the cytocrit) was so low that cell-cell contacts should have been rare during the freezing. As in the case of the red cells at low hematocrit, we find that the survival of slowly frozen 8-cell embryos is not affected by the high concentrations of salt produced by freezing, at least up to 3.3 molal NaCl and therefore is not affected by the extent to which the cells shrink below their isotonic volume. Nor in general is survival influenced by the temperature at which given salt concentrations and unfrozen fractions are attained, or by the glycerol concentration at those temperatures. On the other hand, the attainment of low values of the unfrozen fraction (U) is damaging, but the damage appears in part to be due to the fact that low values of U had to be achieved by placing embryos in solutions hypotonic with respect to NaCl, which caused their volume to be greater than isotonic prior to freezing.

Cryobiology of Drosophila Embryos

With support from the National Science Foundation and in collaboration with K. B. Jacobson and A. Mahowald we have initiated a study on the effect of low temperatures and dehydration on Drosophila embryos (eggs) with the hope of being able to devise a means of preserving them by freezing. The ability to preserve them would be of substantial benefit to Drosophila geneticists who currently have to maintain some 10,000 mutant strains by monthly transfer.

Our efforts to date have been three-fold: (1) Determine the water content and rate of water loss of both intact eggs and eggs with the chorion removed at room temperature and below 0°C. The water content is about 75%. The rate of water loss in intact eggs is 15%/h, 0.6%/h, and 0.1%/h at 22, -6, and -15°C respectively. In dechorionated eggs, the rate of water loss is about 50% higher. (2) Develop a method to assess viability of the eggs; namely, the percentage that develop into larvae. (3) Determine the sensitivity of the eggs of various developmental stages

to being cooled to 0°C at various rates and being held at 0°C for various times. Early stage eggs survive the initial cooling but succumb over a period of hours at 0°C.

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ENZYME REGULATION

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Mechanism(s) of Resistance to Toxic Metals

In higher organisms several metals are toxic. In some cases naturally occurring resistance has been acquired. In the case of Drosophila, cadmium-resistant and sensitive strains are readily identifiable. Earlier studies have shown that the resistance trait could be located on the X-chromosome. This year we have performed further genetic studies by cross-over experiments involving genetic markers on the X-chromosome and

have located the cadmium-resistance (Cdr) gene to a region where the forked locus occurs. These studies can be pursued further since additional markers in this region of the chromosome are available; we intend to identify as closely as possible the site(s) that determines resistance. The location of the cadmium-resistance trait on the X-chromosome results in an interesting dilemma since others have shown that the structural locus for Drosophila's metallothionein is on the third chromosome. It has been assumed that metallothionein plays a major role in determining resistance. A hypothesis to explain the function of Cdr is that this locus regulates the activity of the metallothionein gene of chromosome 3.

Measurements of Drosophila metallothionein in cadmium-resistant and sensitive strains were also made. When both strains were raised on sub-lethal concentration of cadmium chloride they both contained metallothionein at comparable concentrations. Since no difference in the amount of metallothionein was found, we initiated experiments to determine if the rate of synthesis of metallothionein differed between the resistant and sensitive strains. The initial results do indicate such a difference and we shall pursue this approach.

Efforts to purify metallothionein from the resistant and sensitive strains have made some progress. We showed that C18-reversed phase chromatography can produce a significant purification but the final state of purity has not been determined.

A third approach to determination of the mechanism of cadmium resistance was to learn whether the resistant and sensitive strains were able to acquire resistance by being exposed to cadmium during their growth and development. The experiment was to grow both strains on media that contained 0, 0.02, 0.06 and 0.12 mM $CdCl_2$ and collect adults at one day of age. The LC_{50} of these adults was measured in the standard 4-day toxicity test. For the resistant strain, the LC_{50}s were 7.6, 9.9, 10.4 and 12.4 mM respectively for the above growth conditions whereas for the sensitive strain they were 2.2, 2.6, 2.6 and 3.2 mM respectively. This experiment in collaboration with W. M. England (Health and Safety Research Division) is currently being repeated for the third time. It seems to indicate that the resistant strain acquires resistance to a greater extent than the sensitive strain. This is an important point with regard to the prediction of biological response to a toxic substance.

Another metal project involved a physical chemical evaluation of the effects of Zn^{2+} on the tRNA molecule. These studies were described in a previous report and the project has been completed. The thesis and publications on this work have been written.

Effect of Alzheimer's Disease on Elemental Composition of the Brain

Alzheimer's Disease is prevalent in the age group of 60-90, but the cause of this mental-behavioral disorder is not established. Six possible causes being considered include genetic inheritance, an abnormal protein, an infectious agent, a toxin, inadequate blood supply to critical areas of

the brain and abnormal acetylcholine function. Metal ions have been examined as possible toxins and Crapper and colleagues (Brain 99: 67-80, 1976) have found that increased levels of aluminum are associated with Alzheimer's. Marksberry et al. (Ann. Nerol. 10: 511-516) failed to confirm this finding but reported that the increase in aluminum concentration is related to increased age, but not to Alzheimer:s Disease. Perl et al. (Science 208: 297-299) found Al in localized deposits within the diseased neurons in Alzheimer's brains.

We have entered this study to ask 1) whether aluminum is altered in Alzheimer's and 2) whether any other element is altered in human brains as a function of age and Alzheimer's Disease. We have examined this problem in collaboration with R. C. Switzer, at the University of Tennessee Memorial Research Center, Knoxville, where there is a collection of over 100 brains and spinal cords obtained at autopsy that has been created over the past five years; about 20 brains are added each year. From selected Alzheimer and control brains we have dissected eleven areas from the brain and three from the spinal cord. These tissues were digested in acid and analyzed for aluminum and 37 other elements by J. S. Stewart, S. Bobrowski, and J. Hackney in the Analytical Chemistry Division using the inductively coupled plasma instrument. Data were obtained from eight normal and four Alzheimer's Disease brains. We found that the cortex contained aluminum at higher than normal concentration of age matched controls. The cortex is one of the primary areas that display neuritic plaques that are characteristic of the pathology of Alzheimer's Disease. Further samples are currently being analyzed. If the first results are supported by the current analysis the association of aluminum with Alzheimer's Disease will be confirmed. We found a remarkably high concentration of aluminum in the spinal cord of one of the four Alzheimer's patients. Since only one of four had the high spinal cord aluminum levels, it seems unlikely that it is directly associated with Alzheimer's Disease. There is no precedent for high aluminum in the spinal cord. We shall examine this matter further with the data that are being collected currently.

Localization of metal deposits to certain structures in the cells and tissues provide insight into function. A type of mass spectroscopy, called ion microprobe mass analysis (IMMA), is extremely sensitive for a number of elements including aluminum and iron. W. H. Christie is performing this analysis in the Analytical Chemistry Division. Brain slices (30 μm) have been examined to determine if certain elements are deposited in specific sites or if there is a correlation between the localized presence of one element and another. Our analyses have met with the difficulty of contamination from extraneous sources, presumably dust. The IMMA is very sensitive and detects a dust particle and determines the elements present in it. Using techniques that avoided contamination, we examined samples of human brain and the brain of a rat that was maintained on 50 μM $AlCl_3$ in its drinking water for six months. The IMMA detected Al and Fe in highly localized deposits that were mutually exclusive. There was reason to think that these two elements would be found together, but the exclusion of one in the area of the other is surprising. This work is in a preliminary

stage, but it demonstrates that the IMMA has much to offer in biological studies.

As an adjunct to the analyses of human brain we have initiated administration of $AlCl_3$ to mice to examine the uptake of Al into the brain. Samples of the first experiment are currently being analyzed by inductively coupled plasma, by atomic absorption and by IMMA.

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MOLECULAR MECHANISMS OF MUTAGENESIS AND DNA REPAIR

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Most organisms including man have evolved ways to handle damage produced in DNA by environmental agents including chemical mutagens and carcinogens. The process of repair of some types of damage is highly regulated in a tissue and cell line-specific fashion and varies from organism to organism. Thus, the ultimate biological effects of the lesions depend not only on the extent of their formation but on the efficiency of their removal as well. The research objectives of this laboratory are to elucidate the mechanism and regulation of repair of damage in DNA produced by simple alkylating mutagens and carcinogens, as well as the mutagenic changes in DNA produced as a result of persistence of unrepaired lesions. Specifically, the current topics of our research are (1) to elucidate the enzymatic mechanism of the human repair enzyme, DNA-O^6-methylguanine methyltransferase, and to determine the molecular mechanism of its regulation and (2) to study the nature of mutations induced by the presence of alkylated bases and ionizing radiation-damaged bases in DNA using

shuttle plasmids that replicate both in human cells and *E. coli*. The following report on last year's experiments bear upon the first objective.

Purification and Characterization of Human DNA-O^6-Methylguanine Methyltransferase (MGMT). MGMT is an unusual repair enzyme because of its ubiquitous presence in bacteria and mammals and its suicide reaction with promutagenic and procarcinogenic O^6-alkylguanine lesions in DNA, in which the alkyl group is stoichiometrically transferred to the protein. The methyltransferase is present at a low level (~10 μg/100 g tissue) in human placenta. Up to 90% of the activity can be recovered in a crude nuclear fraction of the tissue, and we have purified the protein from this fraction using ammonium sulfate precipitation, gel filtration, affinity chromatography on DNA-cellulose, and hydroxyapatite chromatography. The MGMT preparation was approximately 1-2% pure, and was used for preliminary characterization of the activity. Like the bacterial and other mammalian methyltransferases, the human MGMT reacts stoichiometrically with O^6-methylguanine in DNA. The reaction follows apparent second-order kinetics with a calculated rate constant of approximately 1.5×10^8 liter $mol^{-1}min^{-1}$ at 37°C. The energy of activation, determined from the temperature dependence of the reaction rate, was found to be about 18 kcal. The reaction rate was optimal at pH 8.5, possibly indicating that the methyl-acceptor cysteine-thiol group in the protein must be ionized in order to react. Like the other methyltransferases, the human protein has no known cofactor requirements and the reaction is not significantly effected by low concentrations of Mg^{++}, Mn^{++}, spermidine, or EDTA. The reaction is inhibited by both single-stranded and double-stranded DNA, presumably due to nonspecific binding of the methyltransferase. Interestingly, the activity of the protein is also significantly reduced in the presence of physiological concentrations of NaCl.

Metabolism of O^6-Methyldeoxyguanosine Triphosphate (m^6dGTP). It has been proposed (Topal and Baker, Proc. Natl. Acad. Sci. USA, 79: 2211, 1982) that *in vivo* alkylation of the cellular nucleotide pool may play an important role in chemical mutagenesis via incorporation of modified DNA precursors. In collaboration with Dr. A. W. Hsie and L. F. Stankowski of this Division, we have previously shown that m^6dGTP introduced into Chinese hamster ovary (CHO) cells is demethylated prior to incorporation of the base into DNA and therefore does not cause detectable mutations in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene locus (Ref. 3 below). We have now found that incubation of radiolabeled m^6dGTP with CHO cell extracts results in rapid degradation of the modified nucleotide to free guanine. Presumably the free base in converted to dGTP *in vivo* by a nucleotide salvage pathway. Adenosine deaminase, an enzyme known to catalyze demethylation of O^6-methylguanine nucleosides, has been implicated in the degradation by the fact that deoxycoformycin, a powerful inhibitor of the enzyme, causes significantly slower formation of guanine *in vitro* as well as lower incorporation of the demethylated base into DNA *in vivo*. These findings suggest that nucleotide alkylation may be a much less significant factor in mutagenesis than was proposed earlier.

Correlation of DNA-O^6-Methylguanine Methyltransferase with Resistance to DNA-Methylating Antineoplastic Drugs in Human Gliomas. Dr. S. C. Schold of Duke Medical Center has observed variations in sensitivity of human glial tumors to the drug procarbazine, whose tumoricidal effect is presumably due to the formation of O^6-methylguanine in DNA. We have collaborated with Dr. Schold on the correlation of procarbazine-sensitivity of the tumor cells, clonally amplified in nude mice, with their MGMT level. All drug-resistant tumors have MGMT levels in excess of 100 fmol/mg protein. The drug-sensitive tumor cells have at least 10-fold less MGMT activity.

The Use of Somatic Cell Hybrids to Map the Gene for Human DNA-O^6-Methylguanine Methyltransferase. We have measured MGMT activity in a number of clones of hybrids produced by the fusion of rodent cells with no measurable MGMT activity (Mex^-) and human cells which are proficient in repair of O^6-methylguanine (Mex^+). These hybrids selectively lose human chromosomes, thus a loss of MGMT activity in Mex^-/MEX^+ hybrids should indicate the loss of the human chromosome carrying structural and/or regulatory genes for MGMT. We had previously found that 1) hybrids of Mex^- mouse rag cells and Mex^+ human lymphocytes possessed no MGMT activity regardless of the presence of any specific human chromosome, 2) hybrids of Mex^+ mouse and Mex^+ human cells show no additive effect in MGMT activity and that 3) hybrids of Mex^- mouse and Mex^- hamster cells lack MGMT activity and thus do not complement each other. Using hybrids of CHO cells (Mex^-) and human xeroderma pigmentosum fibroblasts (Mex^+) we have seen various levels of MGMT activity dependent upon the human chromosome makeup of the cells. The levels of MGMT activity in the hybrids ranged from zero to about 80% of the level in the human parent cell. So far, we have reduced the number of candidate chromosomes to five (3, 5, 6, 12 and 22), by eliminating from consideration those which are absent from two or more Mex^+ hybrids. Since hybrid clones may contain as little as 15% of cells positive for a particular human chromosome marker, we are attempting to isolate subclones of hybrids lacking one or more of the five remaning suspect chromosomes to analyze human chromosomal content and MGMT activity. The results of these determinations should allow us to finally determine which human chromosome(s) carry the structural and/or regulatory gene sequences for MGMT. (Work performed in collaboration with Dr. Larry Thompson, Lawrence Livermore National Laboratory, and with Dr. Peter Lalley, Institute for Medical Research, Bennington, Vermont.)

Comparison of Two Human Cell Lines Differentially Regulated for DNA-O^6-Methylguanine Methyltransferase. Two human fetal fibroblast lines, GM 010 and GM 011, show significant differences in their MGMT level as a function of cell doubling. While the enzyme level remains constant in GM 010 following subculture, it declines rapidly in GM 011 and is undetectable by passage 15. To determine whether this differential regulation of MGMT can be correlated with an observable cytogenetic parameter, G-banded metaphase chromosome spreads were prepared from the cell lines at passage number 15, when the MGMT activity in GM 011 is undetectable. A number of variations in the prefixation procedures of the chromosomes were attempted to improve the resolution of the banding

pattern. The results, so far, indicate no significant difference in the complement of chromosomes or their banding patterns in the two cell lines. (Work performed in collaboration of R. J. Preston of this Division).

The extinction of MGMT activity in GM 011 in the absence of detectable loss or attention of a chromosome is puzzling. Because several agents, notably, 5-azacytidine, α-retinoic acid and phorbal esters (TPA) are known to induce expression of selected genes in specific cell lines, we attempted to see whether MGMT activity can be restored in GM 011 cells following their exposure to these agents. So far, following several experimental protocols, we have been unsuccessful in detecting MGMT activity in the late passage GM 011 cells.

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GENETIC CONTROL OF MUTAGENESIS AND DNA REPAIR

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In the yeast Saccharomyces cerevisiae, three major epistasis groups of genes controlling the processing of DNA damage have been recognized: the RAD52 group is required for the repair of double-strand breaks in DNA via a post-replication repair process; the RAD3 group directs excision-repair of pyrimidine dimers and other bulky (e.g., chemical) lesions; the RAD6 group is required for a second form of post-replication repair and is responsible for nearly all induced mutagenesis.

In recent years the RAD6 epistasis group has occupied our interest, in particular those mutants such as rev1 and rev2 which have allele-specific and mutagen-specific effects on mutability. Several lines of evidence suggest that these are mutants of structural genes involved in misrepair mutagenesis. We have sought to clone the REV genes to facilitate 1) the study of their regulation, and 2) the identification of their gene products.

1. Isolation of REV1 by complementation. A cosmid library of S. cerevisiae DNA was used to transform XL68-3C rev1-1 lys1-1 ura3-52 to uracil-independence. 770 Ura^+ transformants were obtained of which 440 were screened for complementation of rev1-1. Two Rev^+ transformants were identified. Cosmids were rescued from each transformant, and restriction mapping of the two cosmid inserts showed a 15.5 kbp overlapping segment. Subcloning of HindIII and BglII fragments and testing for Rev complementation showed that the Rev^+ segment was confined to an 8.0 kbp BglII fragment and possessed an internal HindIII site. Deletion analysis of the 8.0 kbp BglII fragment extended the limits of the Rev^+ segment to greater than 1100 bp.

2. Disruption of the REV1 gene. The subcloning results indicated that a 750 bp HindIII-BamHI fragment was wholly contained within the complementing region. Therefore, this fragment should be internal to the gene. To test this argument, the HindIII-BamHI fragment was cloned into the integrating plasmid YIp5, creating pFL44. Integration of a plasmid containing an internal fragment of a gene into the chromosomal copy results in a disrupted gene interrupted by the vector sequences. The Rev^+ strain XL70-1B REV1 lys1-1 ura3-52 was transformed with plasmid pFL44, which had been linearized by partial digestion with the restriction endonuclease EcoRI to create free ends to direct integration by homologous recombination. The transformants obtained displayed the Rev^- phenotype. These disruption strains were crossed to the rev1-1 mutant, and the resulting diploids were shown to be Rev^-. The disruption was crossed to a Rev^+ strain, and after the resulting diploid was sporulated and tetrads dissected, the disruption (Rev^- Ura^+) segregated 2:2. These results indicate that the HindII-BamHI fragment is internal to the REV1 gene.

3. Direction of transcription and size of the REV1 transcript. An EcoRI fragment which contained the BamHI-EcoRI REV1 internal fragment was cloned into M13 phage mp19. Both orientations of the fragment with respect to the phage were obtained. Radioactively labeled single-stranded DNA probe from each phage was used in two separate hybridizations to total RNA and poly(A)$^+$ RNA from a Rev$^+$ strain. Hybridization was obtained only with the phage priming the Rev fragment 3'-EcoRI-BamHI-5', indicating that the direction of transcription of REV1 is from BamHI toward HindIII. Hybridization to an approximately 2.3 kbp transcript was observed.

4. Nucleotide sequence of the REV1 gene. The dideoxynucleotide chain termination method was used to determine the nucleotide sequence of REV1 DNA. The sequence of 2969 bp corresponding to the mRNA contains a single long open reading frame (ORF) of 2193 bp, which can encode a protein of 731 amino acid residues. This reading frame is preceded by the sequence TATATAAAT at nucleotides -45 through -37, which resembles the canonical 'TATA' box found upstream of the transcription initiation site of eukaryotic genes. Primer extension experiments suggest that the 5' end of the mRNA begins at the T at position -9. The end of the ORF is immediately followed by the sequence TAG . . . TAGT . . . TTT, which closely resembles the canonical yeast transcription termination/polyadenylation signal sequence.

Multicopy plasmids bearing REV1 are being constructed to study dosage effects, and we will attempt to identify the protein product by its overexpression in bacteria and/or yeast. Gene fusions will be utilized to facilitate study of the regulation of REV1.

STUDIES OF RNA PROCESSING AND TURNOVER AND EGF STIMULATION OF TRANSCRIPTION

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In the last ten years, it has become apparent that the transcription process does not produce mature RNA molecules. Precursors of tRNA, rRNA, and mRNA are produced with extra nucleotide sequences at both the 5' and 3' ends and with extra sequences (introns) in the middle of the RNA. Trimming of RNA molecules at both ends as well as splicing to remove introns must occur before the final mature RNA is produced. Turnover of RNA is also a vital reaction in cells. mRNA molecules undergo turnover in order to modulate their levels and their respective protein products. Removal of RNA primers from the 5'-end of DNA strands during DNA replication occurs, and introns and other RNA "extra" sequences must be degraded. RNases are the key enzymes involved in both the maturation and turnover reactions. The integrity of each cell is dependent on it having the proper array of RNA molecules. Any environmental agent that interferes with the maturation

or turnover reactions may be very deleterious to the cell. Studies of RNases and their possible involvement in processing and turnover of RNA continue in this laboratory.

Exoribonucleases which hydrolyze RNA by a 5'→3' mode yielding 5'-mononucleotides have been investigated with respect to cellular location and substrate specificity. Such a yeast exoribonuclease, purified to near homogeneity, has been found to hydrolyze RNA•DNA hybrids. Poly(A)•poly(dT) with the poly(dT) lacking a 5'-phosphate end group is hydrolyzed at 50% of the rate of poly(A), and an RNA•M13 DNA hybrid is hydrolyzed at a rate similar to that of the heated hybrid. The mode and specificity of the enzyme suggest that it could be involved in RNA primer turnover during DNA replication. A second exoribonuclease fraction of yeast has also been found to hydrolyze by a 5→3' mode and to copurify with an mRNA decapping activity. Association of the RNase and decapping activity is found on hydroxylapatite, heparin agarose, and Sephacryl S-300 chromatography of enzyme fractions prepared in the presence of protease inhibitors. The two 5'→3' exoribonucleases and the decapping activity differ in heat sensitivity. The decapping enzyme has previously been shown to yield m^7GDP and 5'-pRNA as products, and since m^7GMP is not also a product and UDPG is not a substrate, it appears to be a specific pyrophosphatase. The copurification of the two activities suggests that they may be involved in the turnover of mRNA. Further purification of the complex is in progress.

An analysis of 5'→3' exoribonuclease activity in cytoplasmic and nuclear fractions of human placenta and a purification of such an enzyme from human placental nuclei were carried out. The purpose of the investigations was to determine if normal higher eukaryotic cells contain the enzyme, to determine if it is predominantly a nuclear enzyme, and to compare it directly with the yeast enzyme.

Human placenta (full-term) were homogenized and nuclear and cytoplasmic fractions were isolated by centrifugation. Nuclei were then further purified by centrifugation through sucrose solutions and disrupted to produce a nuclear sap fraction. The nuclear fraction was found to contain 60-70% of the poly(A) hydrolytic activity at pH 9.5. This activity is found in one peak on DEAE-cellulose column chromatography and the active peak has been further purified with an excellent yield of 5'→3' exonucleolytic activity. Studies to determine if the activity is found predominantly in the nucleoplasm or nucleoli have shown that about 70% of the activity is recovered in the nucleoplasm.

The purification scheme for the enzyme involves DEAE-cellulose, heparin agarose, and Sephacryl column chromatography of an ammonium sulfate precipitate of the nuclear sap and the overall purification is about 800-fold. Most of the features of the mechanism of hydrolysis which were investigated with the yeast enzyme have also been investigated with the placental enzyme. Using poly(A) as a substrate, the studies show that 5'-AMP is the only product, that the enzyme is a processive exoribonuclease, and that it has 5'→3' exonucleolytic activity. The apparent molecular weight, determined by measurements of both the Stokes radius and

the sedimentation coefficient, is 115 kDa. The enzyme does not hydrolyze DNA. Unlike the yeast enzyme, it has only low hydrolytic activity with [^{3}H]poly(A)•poly(U) [10-20% of the rate with poly(A)] and no detectable activity with poly(A)•poly(dT) or an RNA•DNA hybrid. Capped mRNA is not hydrolyzed. Poly(A) with a 5'-triphosphate end group is hydrolyzed at 20% of the rate of poly(A) with a 5'-phosphate end group.

The results of our studies of the highly purified exoribonucleases of yeast and human placenta show that the enzymes are predominantly nuclear enzymes and are dominant nuclear RNases. The specificity studies suggest that the enzymes may be involved as 5'-trimming reagents in the processing of rRNA precursors, in turnover of intron and other "extra" sequences, or as deprimases. The association of the second yeast exoribonuclease with an mRNA decapping activity suggests a role of both activities in mRNA turnover.

Recent studies indicate that the expression of genes for several proteins, for example, actin, prolactin, lactate dehydrogenase and other glycolytic enzymes, are stimulated by EGF. The protein factors that mediate the stimulation are not known. Studies of the specificity of the EGF receptor tyrosine kinases of human placental membranes and A431 cells have been initiated to identify possible protein substrates which may be involved in the stimulation of transcription of certain genes by EGF. The EGF receptor kinase has been purified by wheat germ lectin-agarose chromatography and its activity measured using the synthetic polymer $(Glu_4Tyr_1)_n$ as a substrate. Analysis of the activity of different tyrosine-containing synthetic polymers as substrates has led to the interesting finding that tyrosine-rich polymers inhibit the EGF receptor kinase. At concentrations of less than 5 μM, $(Glu_1Tyr_1)_n$, $(Glu_1Tyr_1Ala_1)_n$ and $(Lys_1Tyr_1)_n$ inhibit the phosphorylation of $(Glu_4Tyr_1)_n$, the phosphorylation of the src peptide, and the autophosphorylation of the EGF receptor by 80-90%. Kinetic studies have been carried out on the inhibition of phosphorylation of $(Glu_4Tyr_1)_n$ by $(Glu_1Tyr_1)_n$ and $(Lys_1Tyr_1)_n$ and of the src peptide by $(Glu_1Tyr_1)_n$. The results suggest that the inhibition is of a mixed type. Low levels of the insulin receptor kinase are also present in the purified receptor fraction; the activity and autophosphorylation of this kinase are also inhibited by the tyrosine-rich polymers. The inhibitory polymers may be useful in altering the EGF and insulin responses in both permeabilized cells and in cell extracts. Since the inhibition appears to be specific for tyrosine phosphorylation by receptor kinases, it will be useful in identifying protein substrates of tyrosine kinases in crude extracts.

The phosphorylation of calf thymus RNA polymerase II by the EGF receptor kinase of A431 cells has also been studied. These studies show that the polymerase is, at best, a low-level substrate, being phosphorylated on the IIo/IIA, IIB, and IIC subunits at less than 5% of the rate of the autophosphorylation of the receptor.

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MOLECULAR STUDIES OF TRANSCRIPTION BY EUKARYOTIC RNA POLYMERASE II

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The elucidation of the molecular mechanisms of accurate transcription initiation by eukaryotic RNA polymerase II requires knowledge of the properties of the enzyme, the DNA regions that serve as transcription control signals, and the protein factors that mediate correct initiation through their interaction with these DNA signals and/or the RNA polymerase II enzyme. We, along with other investigators, have studied the nature of the protein factors and their interaction with the template DNA. Our studies have been described in previous annual reports and documented in publications. During last year, we turned our attention to the molecular structure of RNA polymerase II, a protein composed of different subunits, in an effort to determine the sites on the enzyme that are targets for physiological modifications, e.g., phosphorylation, and for interaction with possible transcription specificity factors.

Purified RNA polymerase II consists mainly of two forms, IIA and IIB, and a third rare form called IIO. The three forms, which are composed of 8-10 different subunits, apparently differ only in the size of their largest subunit, designated IIo, IIa, and IIb (220-240, 200-210, and 180 kDa, respectively) and appear to be related in part by limited proteolysis. They also differ in their ability to be phosphorylated both *in vivo* and *in vitro* by casein kinases I and II; only subunit IIa(IIo) are phosphorylated. Subunits IIo and IIa are closely related; the differences between them are unclear. Several lines of evidence have suggested that subunit IIa(IIo), but not IIb, is involved in specific transcription in a eukaryotic cell-free transcription extract. Subunit IIa(IIo) has been implicated in DNA binding during promoter recognition and RNA chain elongation. IIb appears to arise from IIa by cleavage of a unique C-terminal repeating structure -- a heptapeptide repeated 26-52 times depending on the species from which the polymerase is isolated, and containing serine, threonine and tyrosine as possible phosphorylation targets.

We have modified the widely used method of Hodo and Blatti (Biochemistry 16: 2334-2343, 1977) which yields predominantly (>98%) the

IIB (nonspecific) form of the enzyme, by adding various protease inhibitors during the isolation of RNA polymerase II from calf thymus. We have obtained preparations containing about equal amounts of the IIA (specific) and IIB forms. As expected, the subunit composition correlates well with the relative amounts of the largest subunits IIa and IIb. Interestingly, we observe (particularly on 5% polyacrylamide gels) doublets not only for IIa (possibly a mixture of IIa and IIo) but also for IIb (not reported before). In collaborative studies with Audrey Stevens of our Group, incubation with [γ-^{32}P]ATP leads to phosphorylation only of the presumably IIo subunit by the endogenous casein kinase II, as revealed by electrophoresis on 5% polyacrylamide gels.

The protease inhibitors tried include Leupeptin, Pepstatin A, APMSF, TPCK and Soybean Trypsin Inhibitor. By using various combinations of these inhibitors, we hope to further increase the content of form IIA in our enzyme preparations. Since the separation of the two forms of the native enzyme can be achieved by non-denaturing polyacrylamide gel electrophoresis, we hope to test the IIA form of the enzyme for binding to transcription specificity factors.

The phosphorylation of RNA polymerase II by the epidermal growth factor receptor tyrosine-specific protein kinase is described separately in this Progress Report — see Stevens et al.

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THE STRUCTURE AND ORGANIZATION OF THE EUKARYOTIC GENOME WITH SPECIAL EMPHASIS ON SATELLITE DNAs AND THE PHENOMENOLOGY OF THE CRUSTACEAN MOLT CYCLE

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We are working with a model system to characterize hotspots for mutations in the eukaryotic genome. The model system is a very complex

satellite DNA of a land crab Gecarcinus lateralis. We have selected this system because of the unusual characteristics of the subject DNA coupled with the biology of the animal, which has a cyclic pattern of growth and can regenerate appendages. The satellite has an average repeat unit of 2.1 kilobase pairs and is characterized by domains that are highly conserved among individual copies of the satellite. The primary sequence of these domains is not remarkable in either base composition or arrangement. These conserved domains are interspersed with domains that are hypervariable, undergoing deletions as large as 108 base pairs. The hypervariable domains are characterized by unusual sequences. These sequences are either (i) highly repeated ones [i.e., $(CCT)_{15}$, (AAG/C), $(AGG)_{10}$, or $(C{\cdot}G)_{8-23}$] that can adopt altered secondary or tertiary structures or (ii) alternating purines and pyrimidines that have the potential for adopting a Z-conformation.

Eukaryotic DNA Diverges at a Long and Complex Pyrimidine Purine Tract That Can Adopt Altered Conformations. Segments of DNA with marked strand compositional bias toward either pyrimidines or purines (Py•Pu) exhibit unusual, and as yet uncharacterized, helical structures. Py•Pu tracts are dispersed throughout the genomes of higher organisms; they are especially abundant in moderately repeated DNAs. Interest in Py•Pu tracts has increased since they have been found upstream from a number of eukaryotic genes. Nuclease sensitivity in Py•Pu tracts has been correlated with transcription; left-handed (but non-Z) conformations are among the structures proposed to account for the nuclease sensitivity.

A domain exhibiting major sequence divergences among three cloned repeat units of a complex satellite DNA of the Bermuda land crab Gecarcinus lateralis contains a repetitive Py•Pu segment consisting of a long C•G tract embedded between runs of CCT•AGG and CGCAC•GTGCG and their variations. The domain adopts at least two types of altered conformations that are markedly affected by pH and negative superhelical density; only one is sensitive to ionic strength. Supercoil-dependent distortions in helical structure are most pronounced at points of interruption in compositional bias in this domain nearby. Since the domain is the site of major sequence divergences among individual satellite repeat units, the altered conformations may be involved in site-specific recombination between repeat units, either those arranged in tandem or those scattered throughout the genome.

A Domain That Assumes a Z-Conformation is Associated with Specific Deletions in a Complex Satellite DNA. Deletions of ten base pairs from one cloned variant of the complex satellite and twelve base pairs from the homologous site of another cloned variant occur immediately upstream from a domain that is rich in alternating purines and pyrimidines (Pu/Py) and that adopts a Z-conformation. A three bp deletion occurs about thirty bp upstream from these major deletions in three of the twelve variants in which this region has been sequenced. We have used a subclone containing the Pu/Py-rich domain (Domain V) from a satellite variant (RU, repeat unit length, 2089 bp) that has suffered the three bp deletion but not the major deletion to demonstrate the presence of Z-DNA and to determine the length

distribution within the population of Z-DNA conformers over a range of superhelical densities by studying (1) inhibition of cutting at a restriction enzyme site within the Z-DNA region, (2) binding of polyclonal and monoclonal anti-Z-DNA antibodies, and (3) antibody stabilization of the Z-region and subsequent relaxation. Interspersed with highly conserved domains that are not repetitive in full-length repeat units of the satellite are four other domains (Domains I-IV), each containing a species of repetitive DNA distinctly different from that of the other three repetitive domains and the Z-region. Domains I-IV are also sites of major sequence changes. As with the Pu/Py-rich Domain V, Domains I through IV undergo alterations in secondary and tertiary structure, albeit of different characteristics from Z-DNA. In general, regions susceptible to alterations in secondary and/or tertiary structure are also susceptible to modification of the primary sequence.

The Molt Cycle of the Bermuda Land Crab is Characterized by Stage-Specific Protein Synthesis. Sheets of epidermis and associated integumentary tissues, which synthesize and degrade the crustacean exoskeleton, were removed from animals at different stages of the molt cycle and labeled *in vitro* by incubation in the presence of ^{3}H-leucine or ^{3}H-tyrosine or ^{35}S-methionine. Tissues were solubilized and electrophoresed on gels which were sliced and counted or exposed to X-ray film. Molt-specific proteins are synthesized in a stage-dependent manner. Three distinct periods of protein synthesis occur: one prior to apolysis, another late in proecdysis when epi- and exocuticle are being synthesized, and a third in stage B of metecdysis when the exoskeleton is thickened by the addition of endocuticular layers. Autoradiographs of tissue sections show newly synthesized proteins in tegumental glands in early proecdysis. Later, label occurs in the newly synthesized exoskeleton. Some exoskeletal proteins are similar to cuticular proteins of the tobacco hornworn, as shown by western analyses, with both pupal and larval antibodies.

Proteinases Involved in Cyclic Degradation of Crustacean Exoskeleton. In experiments to characterize the molecular basis of apolysis and degradation of the crustacean exoskeleton, we are studying enzymes of integumentary tissues of the branchiostegites; these cover the gill chambers of the animals. Both acidic proteinase (AP) and alkaline cysteine proteinase (ACP) activities have been identified. On hydrophobic chromatography, ACP activity separated into 2 peaks (H1 and H2). On gel filtration, AP sized at ~15 kD, H1 at 45 kD, and H2 separated into two peaks at ~45 and ~145 kD. Following loss of 5 or more legs, most *G. lateralis* enter proecdysis; after ~9 months in the lab, some "blocked" crabs do not. ACP activity was lower in an extract from "blocked" crabs compared to extracts from autotomized crabs that entered proecdysis; levels of AP activity were not affected. H1 activity was reduced by ~75% while H2 activity was absent from "blocked" crabs extracts. Autoradiograms of SDS PAGE gels showed that H1 and to a lesser extent, H2, selectively digested a ~17 kD protein extracted from membranous layer. Whether degradation of this protein is the first essential step in apolysis is under investigation.

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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 year our laboratory has concentrated its efforts in two major directions: (1) chromatin structure in the hypotrichous ciliated protozoa, and (2) 3-D reconstruction of a transcriptionally-active gene by electron microscope tomography (EMT).

1. Chromatin Structure in the Hypotrichous Ciliated Protozoa. All ciliated protozoa exhibit nuclear dualism, i.e., the existence of transcriptionally-active macronuclei in the same cytoplasm with inactive micronuclei. The hypotrichous ciliated protozoa possess two distinct nuclear features that distinguish them from other ciliates: (1) macronuclei consist of a "bag" of high polyploid (ca. 10^4-fold), short (ca. 2-3 kbp), linear DNA molecules of low sequence complexity - each fragment probably corresponding to an individual structural gene and its regulating flanking sequences; and (2) macronuclear DNA replication is localized exclusively in a Replication Band (RB) that migrates along the nucleus during S phase. Both of these features are unique in biology and offer considerable advantage compared to typical eukaryotic nuclei. During the past year significant advances have been accomplished concerning both of these nuclear attributes. With regard to the gene-size DNA molecules, we have generated a macronuclear genomic library which has been screened with a heterologous 5S gene probe from Tetrahymena to yield clones of the homologous gene. These 5S clones are currently being sequenced as a first step towards understanding their structure and function as chromatin molecules. Soluble macronuclear chromatin can now be readily prepared with the absence of any proteolytic or nucleolytic degradation, and stable to storage for at least one week. Purified macronuclei need only be dialyzed against low-ionic strength buffer to generate soluble chromatin molecules. These soluble gene-chromatin molecules can then be subfractionated by isokinetic sucrose gradient ultracentrifugation into molecules of varying DNA length. There is a strict correlation between DNA size (and number of nucleosomes/molecule) and sedimentation coefficient. When the DNA from individual fractions was probed with various heterologous gene-probes (i.e., rDNA, α-tubulin and 5S RNA), a clear enrichment of specific macronuclear genes was demonstrable in the different fractions. The 5S gene-chromatin molecules (DNA length, 1 kbp) sedimented near the top of the gradient and were distributed in an asymmetric peak. We intend to repeat this type of analysis using homologous 5S gene-probe. Recentrifugation should lead to considerably greater enrichment of the 5S gene chromatin molecules. It will be of interest to determine whether these chromatin molecules support transcription, exhibit defined nucleosome positioning, can be differentiated into transcriptionally active- and inactive forms, and whether protein differences can be demonstrated. With regard to chromatin proteins, we have completed a study describing the H1-type protein from Euplotes macronuclei. It is about 17-18 kDa, very rich in lysine and threonine plus serine, and can be phosphorylated. Further work is planned in characterizing the structure and properties of this protein. Information on the replication band has been obtained in several fronts. An *in vitro* replication assay has been developed for isolated macronuclei that incorporated thymidine precursors only into the replication band. This assay involves the use of biotinylated dUTP and specific antibodies,

and can be examined in the light and electron microscope. In addition it offers the possibility of a handle for the isolation of replication DNA and chromatin molecules. The unique cytochemistry and ultrastructure of the replication band has been described previously. This past year resulted in one additional shred of evidence on the uniqueness of this nuclear region. The RB appears to be a site of highly concentrated protein phosphorylation. We are actively engaged in trying to identify these molecular targets, which we hope will play an important role in the chromatin reorganization, replication and/or band movement.

2. Three-Dimensional Reconstruction by EMT. We have developed a method for defining the 3-D ultrastructure of sectioned and stained chromosomal preparations. This method does not require internal regularities or a lattice arrangement of identical structures. It is strictly analogous to tomography. The resulting reconstructed images can be viewed as 2-D slices or built into solid models by stacking balsa wood slices. Employing EMT, we have completed 3-D reconstructions from a number of chromosomal structures. Most attention has been focused upon a chromosomal region of RNA synthesis, the Balbiani Rings (BR) of Chironomus salivary gland cells. This gene is present on highly polytene chromosomes (ca. 10^4 endoreplicated), and when active generates a "puffed" region in the chromosome body. In the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. Using EMT, we have analyzed the 3-D arrangement *in situ* of these RNP around the chromatin, as well as the patterns of folding the nascent RNP. During the past year we have concentrated on the reconstruction of thick (0.75 μm) sections of BR transcription loops. One reconstruction required tilt projections every 2-1/2° over an angular range of ±60°, an initial digitization of 1 pixel = 75 Å, and reconstruction of a volume 512^3 pixels. This yields twice the resolution that was previously obtained on 0.75 μm sections. We also collected a tilt series on a thick section with a Zeiss 902 electron microscope equipped with electron spectroscopic imaging. Employing this data we reconstructed the BR loops at an initial digitization of 1 pixel = 150 Å in a volume of 256^3 pixels. Most of our earlier studies on thick sections were performed with a Philips 430 intermediate voltage electron microscope operated at 300 KV. With the Zeiss 902 we were able to collect equivalent data at 80 KV, and with greater monochromicity. Also, during the past year, we have devoted time to the development of a number of image processing programs for the varifocal mirror (an interactive 3-D display). These programs permit viewing and analysis of the reconstruction in 3-D, and in real time. They include: intensity windowing, depth windowing, image zoom, cursor overlay, and image rotation.

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NUCLEOSOME AND CHROMATIN BIOPHYSICS

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Our goal is to analyze and understand the structure and function of the key macromolecular components of genetic material. The macromolecular components under investigation are involved in the major functions of chromatin: DNA packaging, regulation and transcription, and replication. It is our intent to define the role these macromolecules play in facilitating these processes and how exposure to mutagenic or carcinogenic environmental agents may affect necessary genetic functions. Our approach is to use a number of biophysical and biochemical techniques to provide detailed structural models of these important macromolecules. We have concentrated our effort in two major directions: (1) X-ray crystallography of avian erythrocyte mononucleosomes; (2) small-angle neutron scattering studies of nucleosomes, HMG-nucleosome complexes, nucleosome oligomers, and related materials.

1. X-Ray Crystallography of Nucleosomes: Two crystal forms of avian erythrocyte nucleosome core particles (i.e., 145 bp DNA and the histone octamer) have been discovered in this laboratory. One is in the space group P21 and contains two nucleosomes in the asymmetric unit. Diffraction data have been collected on this form to a resolution of 6.1 Å. The second crystal form, in space group C2, with one-half nucleosome per asymmetric unit has similar diffraction quality.

The structure of the nucleosome core particles was initially determined by X-ray diffraction methods to a resolution of 15 Å and has been extended to a resolution of 8 Å (4). The path and properties of the superhelical DNA, the location of the histone domains, and the nature of the interactions between histones and DNA can be observed in the electron density map. Interpretation of the 8 Å map and extension of the resolution to 6.1 Å are planned.

Extending the crystallography to high resolution (3 Å) will involve crystallizing reconstituted nucleosomes constructed from purified histone octamer and cloned 146 base pair specific sequence DNA fragments. Toward this goal we have developed computerized methods for selecting or designing DNA fragments which facilitate superhelical bending of DNA and proper phasing on the histone core. The application of these methods has thus far led to several fundamental discoveries about the bendability properties of DNA, mechanisms for nucleosome phasing, and identification of several useful DNA fragments for reconstitution. These DNA fragments are currently being cloned.

These studies are providing important new information about the structure and dynamic mechanisms of chromatin. The crystallographic structure determination of the nucleosome may also allow detailed modeling of how carcinogenic agents are likely to affect nucleosome function and genetic expression. Experimental structure determinations of nucleosomes which have been modified by carcinogenic agents may also be possible in the future.

2. Dynamical Studies of Nucleosomes and Related Materials. Transcription of chromatin necessarily requires conformational changes at the level of the nucleosome. Small-angle neutron scattering is being used to elucidate the conformation of the chromatosome (nucleosome with H1 and 175 base pairs of DNA). The detailed modeling from this study will provide new information about the way in which DNA exits from one nucleosome and enters the next nucleosome in the chromatin fiber. This will provide a basis for distinguishing between the several models for chromatin fibers in the literature.

Small-angle scattering studies were completed on the histone octamer in several solution environments. The shape analysis demonstrated that in 2M NaCl the octamer is similar in size and shape to the histone component of crystallized core nucleosomes. In contrast, in 2.2 M $(NH_4)_2SO_4$ the octamer is elongated, resembling an ellipsoid with approximate dimensions of 114 by 62 by 62 angstroms. These results indicate that the elongated conformation seen in the 3.3 Å electron density map of the histone octamer crystalized in $(NH_4)_2SO_4$ buffer is due to the particular salt conditions used.

3. National Center for Small-Angle Scattering Research. The facilities of the National Center for Small-Angle Scattering Research have been used by more than a dozen groups from external laboratories and my group from within ORNL. Several of the external groups have made more than

one trip to the facility. The major part of sample preparations and data analyses were carried out within the Biology Division, making the Division a major link in the Biology Program of the NSF-DOE-ORNL sponsored National Center for Small-Angle Scattering Research.

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X-RAY DIFFRACTION

C. H. Wei

The goal of crystal-structure analysis is to elucidate the three-dimensional structure of compounds. The structure can be determined from a knowledge of the intensities and phases of the diffraction spectra. Measurements of X-ray intensity data can be accomplished by the use of computer-controlled diffractometers. If a compound is of biological interest, the analysis of the structure can provide insights about the function of the compound.

It was reported last year that X-ray diffraction data for four different molecules had been collected. The analyses of these data have now been completed and are reported here. (a) Further attempts to crystallize the methanesulfonate salt of an antischistosomal drug, IA-4 N-oxide, have led to an accidental preparation of yellowish thin-plate crystals which are monoclinic with space group C2/m and Z = 4. The results showed that the compound was the ammonium salt of methanesulfonic acid. The correctness of the structure is substantiated by the fact that diffraction patterns for the unknown with those taken from crystals of ammonium methanesulfonate agree completely. The structural results provide

information concerning the hydrogen bonding scheme involving the tetrahedral NH_4^+ and $CH_3O_3S^-$ ions. (b) A compound, which can be considered to be derived by connecting the methyldithiolethione ring and the pyrazine ring of oltipraz (a slowly acting antischistosomal drug) by a vinylene group, was finally crystallized from a solution of 1:1 mixture of toluene and chloroform at -16°C. This compound, $C_{10}H_8N_2S_3$, crystallizes with four formula species in a monoclinic unit cell of symmetry $P2_1/c$. The X-ray analysis showed that the molecule as a whole has a roughly planar configuration. The results also add to more information already known for this family of compounds containing dithiole heterocyclic ring system. (c) Among transition metal-nucleotide complexes that have been structurally characterized, only two of them are those with uridine monophosphate. In an attempt to prepare cadmium-UMP complex, $Cd(NO_3)_2$ was reacted with Na_2(5'-UMP), and the product that showed positive to cadmium test was obtained. After attempts to crystallize the cadmium complex from aqueous solution failed, it was dissolved in water containing pyridine and a small amount of HCl. After the solution was suction dried under vacuum at 60°C, needle crystals were obtained. This unknown compound was eventually characterized as $[C_5H_6N^+ \cdot Cd_2Cl_5^-]_n$ which crystallizes with two basic formula species in an orthorhombic unit cell of symmetry Pmnm. Each cadmium atom is octahedrally surrounded by six chlorines, all of them are shared by the neighboring metals, thus resulting in infinite chains in a and c directions of the unit cell. (d) Crystals of 6,7-dihydro-3-methyl-2-one-pyrimido[5,4-b][1,4]thiazine-7-carboxylic acid were furnished by Dr. Bimal Pal of the Biology Division. The solution of this compound was straightforward. The crystal belongs to monoclinic with an acentric space group $P2_1$, two molcules per unit cell. The most significant results of the analysis is that the molecule possesses a zwitterion structure and that the molecular formula is $C_8H_9N_3S.xH_2O$, where x = 0.857 (8).

The X-ray structure of $[Pt(C_5H_5N)_4]^{2+}.2Cl^-.3H_2O$ was determined some years ago with a discrepancy index of R(F) = 2.10%. Due to the nature of X-ray data the role of water in the crystalline space could not be determined. In order to collect the neutron diffraction data, recrystallization of the compound from pyridine-water solution was carried out and usable crystals of approximately 1.2 × 1.5 × 4 mm size was obtained. The collection of neutron diffraction data to unravel its hydrogen bonding scheme has been under way at the High Flux Isotope Reactor (HFIR) of ORNL.

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Comparative and Cellular Genetics Section

SECTION OVERVIEW - R. JULIAN PRESTON

The aim of the research of the Comparative and Cellular Genetics Section is to use in vivo and in vitro model systems to study the mechanisms of DNA repair, mutation induction, and the production of chromosomal alterations, and use the information to attempt to determine potential genetic and somatic risks in man from exposure to radiation and chemical agents. Our aim has been similar for several years but the approaches currently being used represent quite a change from previous years and are reflective of the recent advances in the field of genetics. In addition, we have established collaborative programs with the other Sections of the Biology Division that have broadened our perspectives and have proved to be mutually beneficial.

A selection of examples is presented to provide an overview of the research of the component groups of the Section:

(1) The breakpoints of the deletion causing β-thalassemia in mice have been characterized. The 5' end is in a hexanucleotide sequence that is common to several other deletions that have been described. A 66 base pair insertion bridges the deletion with homology to chromosome 3.

(2) A study of C57BL/10.F mice show that they have lower IgA levels and shorter lifespans than the inbred partner C57BL/10. An ecotropic virus is shed by C57BL/10.F mice and is reintegrated into spleen and lymph node DNA. It appears that this viral reintegration can modulate IgA expression, and perhaps is involved in shortening lifespan.

(3) A specific deletion of chromosome 2 is observed in bone marrow and spleen cells of mice with myelogenous leukemia induced by neutrons. An analysis of neutron-induced chromosome aberrations in bone marrow cells at the first division after irradiation shows that aberrations involving chromosome 2 are three times as frequent as expected. The reasons for this apparent non-random induction are being investigated.

(4) Restriction endonucleases have been introduced into CHO cells and can induce chromosome aberrations. The frequency of aberrations is proportional to the calculated cutting frequency for each enzyme. There is a cell cycle dependency on the frequency and types of aberrations. For enzymes that cut very infrequently (e.g., Not I) the induced aberration are restricted to a small number of specific breakpoints.

(5) Several in vivo/in vitro cell systems have been developed for the study of the progression of specific karyotypic changes that precede transformation or are a consequence of transformation. These studies are

collaborative efforts with other groups. To date there is an indication that in plastic disc induced sarcomas, tracheal cells, and mammary gland cells specific chromosome changes can be identified, and their role in transformation is being investigated.

(6) A UV-induced DNA lesion has been identified that is sensitive to photolysis by 313 nm light in normal human cells. However, in xeroderma pigmentosum (XP) cells, this lesion is processed but not removed, and in cells from XP-variant individuals is neither processed not removed. This could explain why XP-variant cells are proficient at the repair of UV-induced pyrimidine dimers but are UV-sensitive, and the individuals are cancer prone.

(7) Oxygen-sensitive mutants of *E. coli* have been isolated, and 7 of 10 of the most sensitive group have been shown to lack detectable catalase activity, have low peroxidase activity, and low superoxide dismutase activity. These mutants can help in an understanding of the mechanism of oxygen toxicity, and could provide important information on the role of oxygen in toxicity and mutation induction in other species.

There are several other studies that are integrated with these examples and others that are quite separate, as described in the individual progress reports that follow. We feel confident that the research of the Comparative and Cellular Genetics Section will shed light on the mechanism of induction of point mutations and chromosomal alterations that have important consequences in terms of adverse human genetics and somatic effects.

MAMMALIAN CYTOGENETICS

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The Mammalian Cytogenetics Group has continued with its basic program to determine the mechanism of induction of chromosomal alterations in human and other mammalian cells (*in vivo* and *in vitro*), and in particular to understand how non-random chromosome changes can be produced. In addition, the consequences of the induction of non-random (i.e., specific) chromosome aberrations in terms of the production of genetic defects and cancer are being studied. Several mouse tumor models are being utilized for studying whether or not particular chromosome aberrations are involved in the initiation or progression of neoplasia, and whether there are progressive chromosomal changes during the development of tumors that are specific for different inducing agents. We have also recently begun several projects

that are designed to study chromosome aberration and mutation induction by radiation and chemical agents at the molecular level.

1. Neutron-Induced Chromosomal Aberrations in Mouse Bone Marrow Cells and Myelogenous Leukemias. We have completed the first phase of this study and have been able to draw several important conclusions, and, through the course of the work, to develop several methods for improved chromosomal analysis of cells exposed *in vivo*.

A technique was developed that allowed a determination to be made of the cell cycle stage at the time of irradiation for each bone marrow cell analyzed for chromosome aberrations. This same technique was used to obtain a cell proliferation index over a range of sampling times from 6-24 h after irradiation and for unirradiated control animals that allowed us to compare aberration frequencies for cells in very similar cell cycle stages at the time of irradiation, i.e., radiation-induced delay could be taken into account.

Using these two techniques, dose response curves were obtained for the different aberration classes and for the different cell cycle stages. Linear dose response curves were obtained over the 0-40 rad range studied for all aberration classes (i.e., for G_1 and S/G_2 irradiated cells). An RBE value of approximately 6 was calculated from the ratio of the slopes of the neutron dose response curve for dicentrics or deletions for G_1 treated cells and an X-ray dose response curve, also for G_1 irradiated cells obtained in a separate study. A similar value for RBE was obtained for chromatid-type aberrations in S/G_2 irradiated cells.

A deletion of chromosome 2, either interstitial or terminal but always involving band C1, was shown to be present in bone marrow and spleen cells of almost all neutron-induced myelogenous leukemias. A loss of the Y chromosome was associated with the remainder. This specific deletion has been observed in "spontaneous" and X ray-induced leukemias. Thus, it could be argued that differences in sensitivity to leukemia induction by X rays and fission neutrons is a reflection of differences in sensitivity to the induction of deletions of chromosome 2. To this end it is important to determine if neutron-induced chromosome aberrations were distributed randomly or non-randomly in bone marrow cells.

In order to determine the distribution of aberrations, banded karyotypes were obtained for unselected cell samples at three fixation times (6, 10 and 14 h) after 20 or 40 rads of neutron radiation. A total of 473 cells were analyzed (260 at 20 rads, 213 at 40 rads), and the frequency with which each chromosome was involved in an aberration was determined. These values were compared to the expected frequency that was calculated on the assumption that there was a random distribution of aberrations based upon the relative proportion of the genome that each chromosome represented. For the 20 rad dose, the X^2 for deviations from a random distribution was 42.5 (p 0.05-0.1) suggesting no significant deviation from random involvement for the total genome. However, one-half of this X^2 value was due to a large excess of chromosome aberrations

involving chromosome 2 (10 translocations and 8 deletions). For the 40 rad dose, the X^2 was 26.83 (p 0.5-0.7). There was no excess of aberrations involving chromosome 2 for this dose. It is possible that at 40 rads the much higher overall frequency of aberrations masks any specific involvement of chromosome 2, or that the apparent specificity of involvement at 20 rads does not occur at 40 rads. More data are required to determine the validity of our observations and the hypothesis presented.

2. Chromosome Aberration Induction by Restriction Endonucleases. Restriction endonucleases have been used to study the involvement of specific types of DNA damages in the production of chromosome aberrations. Previous investigators have reported that cohesive-end cutters are ineffective or less effective than blunt-end cutters at inducing aberrations. In this study restriction endonucleases were introduced into viable CHO cells using osmolytic shock of pinocytic vesicles. We compared two cohesive-end cutters, Msp I (C ▼ CGG-2-base overlap) and Sau3A I (▼ GATC-4-base overlap) with two blunt-end cutters, Alu I (AG ▼ CT) and Rsa I (GT ▼ AC). All four enzymes were effective at inducing aberrations. The 4-base overlap cohesive-end cutter Sau3A I was approximately as effective as the blunt-end cutter Alu I. We have shown that cutting frequency rather than cut end-structure is important in determining efficiency of aberration induction. There is over-dispersion of the distribution of dicentrics and rings among cells, and the data could be fitted to a Neyman Type A distribution, a modified Poisson, that indicates that there is a probability distribution both for the entry of the enzyme into a cell nucleus and for the induction of aberrations once the enzyme has entered a cell nucleus. In addition, we used Alu I to determine the sensitivity of cells to aberration induction in the different stages of the cell cycle. Alu I induced aberrations in all stages of the cycle, chromatid-type in S/G_2 and chromosome-type in G_1. There were variations in sensitivity with cycle stage and changes in the proportions of the different aberration classes for chromatid-type aberrations.

In order to use restriction enzyme induced DNA damages as a means of understanding the mechanism of production of chromosome aberrations and as a model for radiation and chemically-induced aberrations, we studied the potential for blunt-ends and cohesive ends, produced by Rsa I and Msp I, respectively, to interact to produce greater than additive frequencies of aberrations. The initial results showed quite clearly that the two types of cut ends could interact to produce aberrations. Such an interaction could arise by removing the cohesive ends produced by Msp I to give blunt ends that could simply ligate with the Rsa I blunt ends, or the cohesive ends and blunt ends could ligate, and the short gap in the unligated DNA strands could be filled in. In order to determine the mechanism involved, we have used two inhibitors of DNA replication/repair resynthesis, ara-C and aphidicolin, that cause their effects by inhibiting the action of polymerase α. Cells were treated with either Rsa I (blunt-end) or Sau 3A I (cohesive-end) and then incubated with ara-C or aphidicolin until fixation 1, 2, or 3 h later. The aberration frequency was increased substantially (twofold - threefold) in cells incubated with ara-C or aphidicolin after Rsa I and Sau 3A I treatment compared to enzyme alone. A similar increase

was observed for all three sampling times, for both enzymes, and for both inhibitors. These data suggest that the double-strand breaks induced by restriction enzymes can be repaired in 1 h, and that for both blunt-end and cohesive-end cuts some polymerase activity is required. Further studies will determine the mechanism involved and how these specific breaks induced by restriction enzymes are similar to those induced directly or indirectly by radiation and chemical agents. Also, it should be possible by comparisons of mechanism of induction and distribution of aberrations induced by restriction enzymes and radiation and chemical agents to determine if specific DNA sequences are involved in the production of chromosome aberrations (e.g., short or long repeated sequences).

In order to study the distribution of aberrations induced by restriction enzymes, it is simplest to use an enzyme that cuts very infrequently. Not I with an 8-base recognition sequence and producing 4 base overlap cohesive ends cuts once every 9×10^6 base pairs in the Chinese hamster genome. Treatment of cells in G_2 did produce chromosome aberrations and there was a marked non-random distribution with the majority of the aberrations being located at four specific chromosomal locations. These studies will be continued to attempt to determine if repeated sequences are located in the vicinity or at the points of aberration formation. This sort of approach could be considered as a potential model for the induction of aberrations by very low doses of radiation or chemical agents.

3. Induction of Chromosome Aberrations by Chemical Agents: Increasing the Sensitivity of Assay Systems. The majority of chemical agents induce chromosomal aberrations during the S-phase irrespective of the cell stage treated. The aberrations produced are of the chromatid-type. This considerably reduces the sensitivity of the lymphocyte assay for G_0 cells. We have utilized the DNA excision repair inhibitor, cytosine arabinoside (ara-C), to attempt to increase the sensitivity. The approach, based on our previous studies, was to treat G_0 lymphocytes with triethylenemelamine (TEM) or N-acetoxy-acetylaminofluorene (NAcAAF) and then incubate them with ara-C following mitogenic stimulation of the lymphocytes with phytohemagglutinin. Ara-C should accumulate gaps in the DNA by inhibiting repair resynthesis, and these can be converted into aberrations on reversing inhibition with deoxycytidine. The aberration types expected will be those produced in G_1-namely chromosome type - not the chromatid-type normally produced by the same agents as G_1 treated cells pass through the S phase. TEM and ara-C produced high frequencies of dicentrics (~0.5 per cell) whereas TEM alone produced only a few chromatid aberrations. A less dramatic but clearly significant effect was produced with NaCAAF + ara-C. Thus the sensitivity of the assay is markedly enhanced in this way, and the aberration types produced are the easiest to accurately detect and are present at a very low background frequency. If ara-C is added at longer times after treatment with TEM, there is, as expected, a gradual decrease in aberration frequency with time, as repair of the TEM-induced DNA damage takes place. When the interval between TEM treatment and ara-C addition is greater than 3 days no enhancement of aberration frequency compared to TEM alone was observed. This suggests

that it is unlikely that the lymphocyte assay will be able to detect an exposure to an individual or group if samples are taken a few days after the exposure.

4. DNA Repair Kinetics and Sensitivity to Chromosome Aberration Induction. We have conducted a series of experiments with a mouse leukemic cell line that has an increased sensitivity to the induction of chromosome aberrations by X rays compared to normal mouse bone marrow cells. By using cytosine arabinoside and aphidicolin as repair inhibitors, it appears that the increased sensitivity is due to a slower resynthsis of the gaps produced during excision repair of radiation-induced base damages. In addition, the leukemic cells are very sensitive to aberration induction by ara-C alone in the G_2 stage of the cell cycle, and relatively sensitive to aphidicolin, indicating some fragility of the chromosomes.

We have also selected an ara-C resistant mutant of CHO cells that appears from preliminary experiments on radiation-induced cell survival to have a similar polymerase defect to that indicated for the mouse leukemic cells.

The most interesting aspect of these studies is the preliminary observation that several ataxia telangiectasia cell lines show radiation responses for killing and aberration induction that are similar to those for the leukemic line and ara-C resistant mutant. It is hoped that these studies will help identify a mechanism for increased radiation sensitivity for some cell types, particularly AT cells. We have previously shown that an increased rate of incision during excision repair of base damages could account for an increased X ray sensitivity of G_0 lymphocytes from individuals with Down syndrome to chromosome aberration induction.

5. Background Frequency of Chromosome Aberrations and Sister Chromatid Exchanges in Human Lymphocytes. In conjunction with Dr. Michael Bender of Brookhaven National Laboratory, we have continued a very large cytogenetic study of a sample of persons employed at BNL. To date more than 500 samples have been analyzed for chromosome aberrations and SCE. The population was in part selected such that each decade of age was well represented, males and females about equal, and that several races were sufficiently well represented for statistical analysis of their possible influence on the cytogenetic end-points to be feasible. Since the last Progress Report we have included persons in the older age range (retired persons) and young age range (children). The only statistical analysis so far performed indicates that in our study group there is no increase or decrease in aberrations or SCE with age, and there is no sex difference. The age response does not yet include data from the upper and lower extremes. The final intent of this study is to determine background frequencies and ranges for aberrations and SCE, and attempt to identify sources of variation between individuals and/or groups.

6. Studies of Chromosome Changes and Tumor Progression: Are There Identifier Chromosomes? We have studied three different tumor models that allow us to follow karyotypic changes and progression to neoplasia.

In studies with Dr. R. J. M. Fry, plastic discs are inserted into mice and sarcomas develop on the disc. The discs can be removed at different times after insertion, even before a sarcoma is visible, and the cells can be karyotyped. In this way, changes in chromosome number and structure can be followed. In addition, the mouse can be irradiated or treated with chemical carcinogens at different times after disc insertion to determine if different karyotypic changes occur or whether the time of appearance of specific alterations is reduced. To date, we have analyzed some 20 sarcomas from animals that contained discs alone or discs plus additional treatment. The chromosome numbers vary from about 45 to over 200, but with much less variation in the additionally treated animals. It is difficult to ascertain whether there are any specific alterations, except that double minutes are frequently present. We intend to determine what gene(s) is amplified in these tumors. We have analyzed a small number of cells from discs prior to the appearance of a sarcoma. The chromosome number is low - close to the normal 40. Banding analysis is currently being performed on these cells to look for specific aneuploidies and structural changes.

In conjunction with Susan McKarns (a graduate student with R. L. Ullrich) we have been analyzing cell lines derived from mammary glands of irradiated animals. After 11 passages *in vitro* the cells are cloned and each clone assessed for chromosome changes and tumorigenicity by injection into cleared mammary fat pads of untreated animals. The cells of tumors that develop are also analyzed for chromosomal alterations. A large number of observations have already been made. In summary, when the cells progress to a neoplastic state they very frequently acquire metacentric chromosomes; the chromosomes involved in these is being determined by banding. Cells acquire double minutes prior to being clearly tumorigenic; homogeneously staining regions (HSR) rather than double minutes are observed once cells become neoplastic. The origin of the double minutes and HSRs will be determined. Additional chromosome instabilities are observed with increasing *in vitro* passage.

In collaboration with Dr. M. Terzaghi-Howe, we are analyzing tracheal cells that were isolated from treated animals and grown *in vitro*. The cells can be assayed *in vivo* for their ability to produce tumors at different *in vitro* passages. There is a characteristic change in DNA content of cells from different clones from 2.4°C to about 3.4°C at a very similar passage number. This increase in DNA content is, as expected, the consequence of an increase in chromosome number from about 60 - 100. It is also quite clear that for several different clones this DNA increase occurs at the same time as a large metacentric (or metacentrics) is observed. The consequences of metacentrics, if there are any, are yet to be determined, but we have the impression from additional observations that these chromosomes are indicators of a specific change, rather than simply markers.

These different in vivo/in vitro tumor systems appear to be very promising for attempting to determine if specific chromosome changes are involved or associated with the initiation or development of solid tumors.

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THE REPAIR OF HUMAN DNA

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It is essential that the information contained in the DNA base sequences remain intact. Most organisms have evolved molecular mechanisms for the detection, removal and repair of DNA lesions. These DNA repair mechanisms find their most sophisticated form in man and are responsible for maintaining the fidelity of the DNA. They are therefore critical in the prevention of mutations and carcinogenesis. Our research objectives

are to analyze the various components of the human repair system and determine their impact on the carcinogenic event in man. To these ends we employ both normal, repair-proficient human cells and cells derived from patients with the hereditary, cancer-prone disease, xeroderma pigmentosum (XP) which are deficient in DNA repair. Repair in other cell types is studied whenever such experiments can contribute to understanding of the DNA repair process in human cells. Our primary objective is to elucidate the molecular events in human cells when DNA is damaged by radiation or chemical agents. We study and characterize the sequence of DNA repair events, the various modalities of repair, the physiological inhibition of repair due to biochemical inhibitors, and the genetic basis of repair. Our ultimate goals are to isolate and analyze the repair component of the mutagenic and/or carcinogenic event in human cells, and to evaluate the 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 the belief that mutagenic and carcinogenic events may arise from nonrepaired lesions or the operation of error-prone repair systems, and the clear association of repair defects and highly carcinogenic disease states in man (XP).

Correlation of the Rate of Excision Repair with Structural Composition of Ultraviolet-Induced Pyrimidine Dimers. The excision repair process in human cells after ultraviolet (UV) irradiation is largely, but not wholly, concerned with the removal of UV-induced pyrimidine dimers. We have derived average excision repair curves which describe in detail the kinetics of this excision process. The shape of excision repair curves depends on base ratio, wavelength, and dose. Excision repair curves, while usually drawn as single component "average" curves, are generally considered to be two-component curves. The excision repair system removes all the pyrimidine dimers from the DNA but these dimers actually consist of three distinct molecular species: thymine-containing dimers (T-T) and cytosine containing dimers (C-T, C-C). Through specific radiochromatography we have determined that C-containing dimers are excised twice as fast as T-containing dimers. Thus while normal human cells can remove 10^6 of these photoproducts in 24 h, the C-dimer removal system is apparently the source of the fast component of the excision process.

Studies of a UV-Induced Photosensitive Lesion in the DNA of Normal and Cancer-Prone Human Cells. We have previously shown the existence of a UV-inducible lesion in the DNA of human cells which is sensitive to photolysis by large doses of 313 nm light. Repair of the lesion can be followed by measuring the single strand breaks resulting from 313 nm photolysis of the DNA. This lesion is processed and then removed in normal cells in 3 - 5 h. In excision repair deficient xeroderma pigmentosum (XP) group A cells, it is processed but not removed and in excision proficient XP-variant cells, it is not processed nor removed. This lesion might, therefore, explain the cancer-prone nature of the XP-variant individuals. When UV-induced pyrimidine dimers are studied by chromatography, we often observed a slight increase in the apparent number of dimers which occur in the first hour after UV irradiation. There has been no satisfactory

explanation for this phenomenon. The photosensitive lesion occurs in the right magnitude and time frame to account for this increase provided that its formation involves some post-irradiation chemical or enzymatic modification or activation resulting in an isolatable chemical lesion which co-chromatographs with the pyrimidine dimers. To test for this possibility, we irradiated cells with UV light while the cells were kept for 10 min at ice temperature and compared the numbers of lesions observed with those seen when the cells were irradiated and kept for the same period at 37°. In both cases the same number of photosensitive lesions were observed. This is consistent with the formation of the photosensitive lesion being entirely photodynamic and therefore is not involved in the increase seen in dimers after UV irradiation. Repair of the photosensitive lesion was examined at 37°C and at 3°C. We observed little change in the number of lesions in cells kept on ice over the 5 h period, whereas the lesions rapidly disappeared from the DNA of the cells kept at 37°. Repair of the lesion thus appears to be an enzymatically catalyzed event and therefore genetically controlled. The action spectra of the lesion's formation and its photolysis were measured. We found that the formation of the lesion occurs with approximately the same spectrum as for the formation of pyrimidine dimers. Photolysis of the lesion occurs with wave lengths from 300 nm - 420 nm with a maximum at about 313 nm. However, the induction frequency for the photosensitive lesion appears to be about 1/25 of that reported for the 6-4 product.

Further Quantitation of Non Pyrimidine Dimer DNA Damage After Ultraviolet Radiation. While a major DNA damage from ultraviolet (UV) radiation both quantitatively and in terms of biological significance is certainly the 5,6 pyrimidine dimer, other UV damages, while not quantitatively numerous, may have high biological significance because of their critical placement in the genome. For experiments with non-dimer UV damages it is useful to have a vertebrate cell line which has high levels of both photoreactivation (PR) and excision ("dark") repair. However, vertebrate cells generally have either high levels of PR and very low excision (fish up to marsupials) or high excision and no PR (primates). Rattlesnake skin cells have both repair systems intact and highly efficient. Thus, we can perform experiments in which we are able to UV irradiate these cells, reverse the pyrimidine dimers and then measure the dark repair of non-dimer damage by the bromodeoxyuridine photolysis assay. In these experiments we have found ~50% of the dark repair activity associated with non-dimer lesions. Thus UV-induced non-dimer DNA damage apparently plays a far greater role in DNA damage and repair (and by implication in mutagenesis and carcinogenesis) than was previously supposed.

Metabolic Activation and Structure/Activity Relationships in Chemical Carcinogenesis. The purpose of these experiments with chemically induced damage to DNA was to establish a system of *in vitro* metabolic activation using human cell DNA repair as a biological end point. Although our assays are sensitive and capable of resolving low levels of damage to the genetic material, such a system of activation of promutagens and procarcinogens becomes necessary in order to simulate actual human exposure. As an extension of work with polyaromatic hydrocarbons, a group of substituted

benzo(a)pyrene [B(a)P] derivatives was selected. For metabolism, a number of approaches were considered: (1) the direct application of conditions for the bacterial microsomal activation assays, (2) the modification of conditions used in the mammalian cell CHO assay for gene mutation, and (3) the utilization of isolated rat hepatocytes in association with human fibroblast cells. Our preliminary work has suggested that the application of CHO methodology with rat liver enzymes is feasible. Two end points of DNA damage are assayed: DNA repair inhibition by ara-C in growth arrested cells and the dBrU photolysis assay. Development of the *in vitro* activation system has concentrated on 10-aza B(a)P as a continuation of our collaborative effort with Dr. C. H. Ho of the Analytical Chemistry Division. Using the ara-C assay, the aza-derivative and the parent B(a)P have been shown to require metabolic activation. Optimum conditions and requirements for activation and dose-response utilizing both variation in concentration and treatment time have been determined. Additionally, the time of repair necessary for optimal response has been established. Treatment times of 2 - 3 h at concentrations of 10^{-6} - 10^{-5}M with a 24-h repair period result in excess single-strand breaks of up to 2.1 breaks/10^6 daltons in the ara-C assay. Resolution of damage with the dBrU assay, however, has been complicated by photodynamic effects. Further studies with a group of three hydro B(a)P derivatives have shown that only the dihydro-derivative induces repair in the fibroblast cells while the tetra- and octo-derivatives are not active. Additionally, the 9-methyl, 10-aza-derivative has been shown to be less active than the 10-aza or parent B(a)P. In analogy with previous work on active metabolites (diol epoxides) damage induced by the 10-aza B(a)P cannot be excised by cells from excision deficient human mutants (xeroderma pigmentosum). Current experiments are concerned with other families of structurally related derivatives (e.g., aromatic amines) and the use of additional sources of metabolizing enzymes (e.g., bladder cells).

Large Discrepancies in DNA Repair Capacity in Closely Related Species: Evolution, Longevity or Ploidy? We have confirmed the finding by other investigators that cells cultured from the white-footed mouse, *Peromyscus leucopus*, are able to repair 2 - 3 times as many UV-induced DNA lesions as other rodent cells. Peromyscus lives 2-3 times longer than *Mus musculus* and the increased longevity of Peromyscus has been attributed to increased DNA repair capacity. However a previously unreported aspect of Peromyscus cells in culture is that they rapidly (perhaps in the first or second *in vitro* divisions) become tetraploid. In collaborative experiments with Dr. Julian Preston's group, we have investigated the possible interelation of these two unusual properties. The number of UV-induced DNA lesions repaired in 24 h has been measured in various cultures of Peromyscus cells and compared to the percent of tetraploid cells in each culture. We observed a linear increase in the number of repaired sites with the percent of tetraploid cells.

Early cell cultures contain a few cells, about 10%, which are smaller and morphologically different from the rest of the cells. These cells were isolated and found to be mostly diploid; however, they contained a large marker metacentric chromosome whose origin is probably due to Robertsonian

fusion. The number of repaired DNA lesions measured in these cells was about 20% less than in the tetraploid cells. The molecular basis for this DNA repair difference has not yet been determined; however, there clearly appears to be a correlation of DNA repair capacity and ploidy in these cells.

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MAMMALIAN BIOCHEMICAL GENETICS

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The principal aim of our studies is to evaluate hazards to humans of exposure to nuclear and chemical by-products of energy production. Mice are used as experimental animals but the data obtained are analyzed with respect to potential hazards to man. Techniques of molecular biology and genetic engineering are used to understand the mechanisms of mutations and the effects of specific mutations on health and reproduction. Below are summaries of our studies during the past year.

An ethylnitrosourea-induced hemoglobin mutation (Hbb-s2) has been used during the past two years to study beta-globin gene expression in the mouse. We reported last year that the differential expression of the two beta-globin genes during development is regulated at the level of transcription. Erythrocytes formed during development differ in the tissue of origin, cell size, nucleation, and type of hemoglobin synthesized. Thus, the larger nucleated cells derived from the visceral yolk sac blood islands and the smaller non-nucleated cells of fetal liver origin were separated by centrifugal elutriation to study the expression of the two beta-globin genes in each cell population. On the same day of gestation,

relatively greater amounts of beta-s minor globin are found in the large nucleated cells of yolk sac origin and relatively greater amounts of beta-s2major globin are found in the small non-nucleated cells of fetal liver origin. Such changes in the expression of identical genes in erythrocytes that develop in two different tissues suggest that the two tissues produce different types or different amounts of transacting factors that regulate globin gene expression in these tissues.

We have recently initiated studies on the expression of the two alpha-globin genes during development. Preliminary studies show that the switch in expression of the two alpha-globin genes occurs earlier than beta-globin gene expression switching. It would appear that the 3' of the duplicated alpha-globin genes is expressed at a higher level early in development in mice, which is also true for the 3' alpha-globin gene in the human fetus. We propose to use the switch in the expression of the two alpha-globin genes during development to order the 5' and 3' genes of mice of several alpha-globin haplotypes and to confirm this order in a few cases by recombinant DNA methods.

Unstable hemoglobins have been described for humans but none have been reported for the laboratory mouse. During some studies on the synthesis of alpha- and beta-globins in mice with a combination of alpha-thalassemia and an induced mutation Hbb-s2 at the beta-globin locus (Hba-b(th)/Hba-b; Hbb-s2/Hbb-s2), we found an equal recovery of labeled alpha- and beta-globin chains when reticulocytes were incubated in [^{3}H]leucine *in vitro* rather than the expected deficiency of alpha-globin synthesis usually observed in alpha-thalassemic mice. We proposed that the mutant beta-s2major globin might be partially unstable when it is present in excess of alpha-globin, as would be expected in alpha-thalassemic mice. Denaturation of mouse hemoglobins in solutions of isopropanol indeed showed that the mutant beta-s2major globin is less stable than other beta-globins which are identical except for a Val → Glu substitution at position 60. This substitution alters the charge within the heme pocket and makes the molecule less stable, probably in a manner similar to the unstable human hemoglobin Milwaukee which has a Val → Glu substitution at position 67.

The nucleotide sequence at the deletion breakpoints for the spontaneous mutation causing beta-thalassemia in mice has been fully characterized. The 5' end of the deletion begins in the G^GTTTC hexanucleotide that is common to several deletions previously described. The gap is bridged by a 66-base pair deletion-associated insertion comprised of 43 bp at the 5' end identical in sequence to a region of DNA that maps to chromosome 3 and a string of 23 A's at the 3' end. The structure of the deletion-associated insertion suggests that it might be a reverse transcript of the genetic sequence that maps to chromosome 3.

Adult beta-thalassemic mice produce a larger than normal number of erythrocytes to compensate for the reduced amount of hemoglobin per cell and for the reduced lifespan of these defective red cells; however, newborn beta-thalassemic mice are paler than heterozygotes and normal littermates. The Till and McCulloch colony forming unit-spleen (CFU-S) assay was used to

determine whether the anemia observed at birth might be due to a difference in the number of hematopoietic stem cells in the fetal livers of normal and beta-thalassemic mice. At daily intervals between 12.5 days of gestation and 5 days after birth, the number of CFU-S in the livers of beta-thalassemic mice was fewer than in normal mice, which probably accounts for the obvious anemia in newborn beta-thalassemic mice. Expansion of the CFU-S population occurs primarily in the spleen of older beta-thalassemic mice; in adults splenic CFU-S is about fourfold higher in thalassemic than in normal mice. Perturbation of more differentiated erythroid populations, such as BFU-E and CFU-E, in the spleen and bone marrow of beta-thalassemic mice is being studied.

Mammalian aldehyde dehydrogenases are an important complex of enzymes that oxidize a variety of aldehyde substrates in the body, including products of the metabolism of alcohols and biogenic amines and the peroxidation of polyunsaturated fatty acids in membranes. Roger Holmes and his associates have shown that the multiple isozymes of aldehyde dehydrogenase are uniquely distributed in mouse tissues and are encoded by several structural genes. Ahd-1 and Ahd-2 are located on mouse chromosomes 4 and 19, but the location of the linked Ahd-4 and Ahd-6 was not known. In a collaborative study with Holmes and VandeBerg, we have found that Ahd-4 is linked with the hemoglobin alpha-globin locus, Hba, on mouse chromosome 11. The recombination frequency of Hba and Ahd-4 is approximately 20 percent. A 4-point cross involving Hba, Ahd-4, Tr and Re is being made to locate the position of Ahd-4 more precisely.

Efforts have continued to determine the chromosome location of the locus that controls the differential sensitivity of different strains of mice to ethylene oxide. The EtO sensitivity of BALB/c mice was studied because BALB/c was one of the parental strains used to establish strain SEC/R1 that is highly sensitive to EtO. That BALB/c is sensitive to EtO permitted us to use the BXC/By recombinant inbred stains to extend coverage of the genome in search of the single locus controlling EtO sensitivity. Two of the BXC/By R1 strains are sensitive and five are resistant to EtO; however, no known genetic marker in these R1 lines clearly segregates with EtO sensitivity. We want to determine the genetic basis for EtO sensitivity in mice because we believe that EtO sensitivity in humans might be associated with homologous genetic factors.

Compared with their inbred partner, C57BL/10.F mice have low IgA levels and shortened lifespans. We recently learned that C57BL/10.F mice also shed an ecotropic virus that is reintegrated into DNA of spleen and lymph node cells. Although the inbred partner C57BL/10 does not actively shed virus, injection of a filtrate from C57BL/10.F spleen into C57BL/10 mice suppresses IgA expression. Moreover, viral reintegration into the DNA of these C57BL/10 mice was established by Southern hybridization for ecotropic viral sequences. The observation suggests that an etiological role for the ecotropic virus in the modulation of IgA expression. The

sites of viral reintegration into the mouse genome are being investigated using gene cloning and somatic cell hybrid techniques.

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GENETICS OF ANAEROBIC ORGANISMS

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Degeneration in *Clostridium acetobutylicum*. We have continued studies of the rapid genetic changes that take place spontaneously in cultures of the solvent producing anaerobe, *Cl. acetobutylicum*. These genetic changes can be recognized and studied by isolation of cells from morphologically distinct regions of colonies of the parental wild type. We have now characterized many such isolates with regard to their growth rates, ability

to form spores, ability to produce solvents, cell morphology and colony morphology.

Most of the isolates are genetically unstable and continue to degenerate, finally producing a stable, non-solvent producing form. This stable form produces large quantities of hydrogen, CO_2, acetic and butyric acids. It does not produce spores. Cells from liquid cultures of this final organism tend to be longer and thinner than cells of the parental type or the intermediate isolates. Determinations made with flow cytometric techniques establish that cells of the stable type have, on the average, twice the volume of the parental type. In addition, the range of volumes of individual cells is greater for the stable derivative.

The intermediate isolates are, in general, less efficient spore formers and solvent producers than the parental type. However, the morphology of cells and spores from these types is indistinguishable from the parental type. It was of interest to determine if spores of the intermediate organisms would give rise to their respective types of colonies or produce colonies of the parental type. For this reason, air dried spores of representative intermediate types were germinated and allowed to form colonies. They did not give rise to colonies of the parental type but produced colonies characteristic of the isolate from which they came. This establishes that these spores either contain a different genome from the parental type or are expressing a different set of genes. We have also established that the spores of the intermediate isolates have essentially the same sensitivity to heat as the parental type (LD50=1 min at 90°C).

The majority of the degenerate organisms isolated came from the morphologically distinct regions that develop at or near the edge of old (3-8 days at 37°C) colonies of the parental organism. These regions begin to develop after most, but not all, cells in the center of the colony have initiated sporulation. In order to determine with greater precision both the temporal and spatial relationship between sporulation and the production of the degenerate clones, we isolated organisms from the central regions of developing colonies of the parental type. These organisms were examined microscopically to determine the presence of sporangia and free spores. They were also plated to determine if any of them give rise to colonies characteristic of the degenerate types. The results of these experiments reinforced the concept that the degenerate types arise at or after the time when most cells are forming spores. Furthermore, they are produced in the center of colonies as well as the edges but do not have the opportunity to replicate extensively when they arise in the former location.

Many thousands of colonies of the intermediate and final stable types have been observed to determine if there is any tendency to revert back to the parental type. No such spontaneous revertants have ever been observed. The genetic changes that do occur always move the organisms away from the spore and solvent forming, parental type towards the stable nonsporulating, acid producing organism. We refer to this cascade of events as

"degeneration" because, in general, it seems to reflect a progressive loss of functions. However, the process might be regarded more generally as a bacterial model for differentiation — a colony initiated from a single cell or spore develops into a structure containing discrete regions with unique properties. In certain environments, the "degenerate" forms have a distinct advantage. For example, in a rich complex liquid medium the stable acid producing organism continues replication long after the parental form sporulates. By this process, it eventually replaces the parental form as the predominant organism.

Continued study of this phenomenon may lead to some understanding of the underlying genetic mechanism involved in this form of differentiation and may also allow us to stabilize Clostridium acetobutylicum in its solvent producing phase — the phase of interest for the commercial production of solvents and fuels from renewable feedstocks.

Characterization of Oxygen Sensitive Escherichia coli. Fifteen mutants of E. coli K-12 AB1157 have been isolated after exposure to ultraviolet light. These mutants, incubated aerobically, will not form colonies on complex agar medium containing glucose. They do form colonies anaerobically. Each of the mutant cell lines retains the genetic markers of the parental strain (AB1157). Survival studies indicate that atmospheric oxygen is bacteriostatic, rather than bacteriocidal. Genetic mapping using oxygen tolerant Hfr strains indicates that the mutations are located in five regions of the chromosome (map min 8.5'-13.5', 42'-47', 47'-51.5', 77'-83', 83-84.5', 88-91.5'). Growth curves indicate that oxygen, introduced during log phase, inhibits further growth of the cultures. The degree of inhibition can be used to classify the mutants into three groups. Group I organisms are the most oxygen-sensitive and are unable to divide after the introduction of oxygen. Group II mutants are less oxygen-sensitive; the cells are able to divide at least once after oxygen exposure. Group III organisms form filaments in response to oxygen. Certain enzymes that may protect cells from the toxic effects of oxygen have been assayed. The results show that seven of the ten Group I mutants lack detectable catalase activity, have low peroxidase activity and low superoxide dismutase activity. Two of these mutants with low enzyme levels map to distinct chromosomal regions. However, the mutations in the other five mutants map to a single chromosomal region (8.5'-13.5') that may control the expression of these enzymes. The remaining three Group I mutants, two of which map to a single chromosome region (83'-84.5'), retain normal levels of the protective enzymes but have a diminished ability to consume oxygen. This indicates that respiratory enzyme deficiencies could be responsible for their inability to grow in the presence of oxygen. Group II organisms are not as oxygen-sensitive and possess wild-type levels of catalase, peroxidase and superoxide dismutase. The reason for oxygen sensitivity remains to be determined. When oxygen is bubbled through a log phase culture of either of the two Group III mutants, the cells stop dividing, but protein and DNA synthesis continue. This results in filamentous cells that are several times the normal length. In some way, oxygen inhibits septation but not growth in these organisms.

These studies have revealed some unique features of the bacterial response to oxygen. The correlations that are drawn will help define the mechanism of oxygen toxicity and should provide a basis for studies in other organisms.

During the isolation of the oxygen-sensitive mutants, an E. coli mutant was discovered that showed an unusual response to carbon dioxide. This organism, unlike the parental strain, requires carbon dioxide for growth. Atmospheric levels of carbon dioxide (0.03%) will not support growth. The mutant grows as well as the parental strain (AB1157) if supplied with 5% carbon dioxide but growth is diminished as the carbon dioxide concentration in its environment decreases. It appears likely that this organism requires carbon dioxide for the initiation rather than the maintenance of growth.

Indirect Action of Ionizing Radiation as the Primary Cause of Potentially Lethal Damage and Its Repair. The radiosensitivity of rec-type E. coli mutants deficient both in recombinational repair and induced-error prone (SOS) repair was measured in the presence of OH radical scavengers. RecA is deficient both in pre- and post-replication recombinational repair and SOS induction. RecB is recombination deficient but does not prevent SOS induction. The study on a DNA polymerase 1 deficient mutant was described in the previous report.

The results to date may be summarized as follows: Indirect X-ray damage via water radicals (primarily OH radicals) is responsible for >90% of potentially lethal damage (PLD). For repair of PLD caused by indirect damage to DNA the major repair pathways are in order of importance: excision repair > recombination repair > S.O.S. repair. Repair of PLD caused by direct action on DNA is accomplished by the three major systems in the following order of importance: recombination repair > S.O.S. repair > excision repair.

PLD caused by direct damage therefore differs in part from that PLD caused by indirect effects.

Based on these findings it would be predicted that increased RBE with increasing LET is a reflection of the increasing direct damage (less PLD) component and a decreasing indirect damage component. As direct damage causes more lethal lesions and relatively less PLD, it becomes apparent that higher LET's should be more efficient in causing damage not mitigated by the repair pathways.

In addition, at different LET's the relative importance of the several repair pathways for PLD should shift with recombination repair becoming more important that excision-repair at higher LET's.

Operation of the Biotechnology User Facility Fermentors. During this reporting period we have continued to grow large quantities of a variety of microorganisms for investigators at ORNL and other institutions. Twenty-two operations were performed. Of these, 13 made use of the

500-liter fermentor. The remainder were carried out in the 30-liter unit. The organisms grown and harvested were Pseudomonas aeriginosa, Escherichia coli, Azotabacter vinelandii and Saccharomyces cerevisiae. Seven operations were performed in support of the Protein Engineering group of the Biology Division, ORNL; five were done for colleagues at The University of Tennessee; and the one was performed for investigators at Louisiana State University. The remainder were in support of other ORNL research groups. The fermentation facility has also produced large quantities of an oxygen-reducing E. coli membrane fraction. This material has been used in various research projects here in the Biology Division, in the Chemical Technology Division, in the Environmental Sciences Division, and in two research projects at The University of Tennessee.

During this year we have also been involved in the extensive renovation of space to which the fermentors and accessory equipment will be transferred. This work is proceeding on schedule and it is anticipated that the equipment will be moved and installed in the November 1986 - January 1987 interval.

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Mammalian Genetics and Reproduction Section

SECTION OVERVIEW - L. B. RUSSELL

The work of the Mammalian Genetics and Reproduction Section lies in the area of mouse germ-line mutagenesis and genetics. This year the research was characterized by the strong emergence of new themes as well as by in-depth studies on continuing themes. We were able to add to our Section a young molecular geneticist, E. M. Rinchik, and are continuing to recruit for additional ones. Further, K. B. Jacobson, a biochemist, has transferred to our Section and has begun to turn his attention to questions of mammalian gene action.

Components of the information needed to understand the mechanisms for, and assess the risk from, the induction of heritable mutational damage include the detection of such damage, the exploration of factors that affect yield, the investigation of organismic expressions of mutations, and the analysis of the structural nature of the damage at the chromosome, gene, or molecular level.

Growing out of (and expanding) the last two components are our newly developing themes in the area of molecular analysis of structure and function of several specific regions of the mouse genome. The altered genes and chromosomes produced in the course of more routine mutagenesis experiments (and subsequently maintained as stocks) supply an unparalleled wealth of genetic tools for these molecular analyses. The reciprocal benefit is that, as knowledge of the molecular structure of a region is ultimately achieved, this will, in turn, elucidate the manner of action of mutagenic agents.

The major strength of molecular chromosome mapping in the mouse is that the outcome need not stand alone as a sterile catalog; it can be readily associated with functional information derived from genetic investigations and with experimental studies that can delve into the earliest development of abnormal phenotypes. Thus, several of the studies reported this year are utilizing series of overlapping deletions derived from past specific-locus experiments, particularly those in which radiation was the mutagen. Genetic complementation studies have generated (and continue to generate) functional maps for regions of considerable length — of the order of 10,000 kb of DNA. A number of molecular methods, described in these reports, are being put to use to derive DNA sequences from the regions in the normal chromosomes that correspond to the various deleted regions. An ordering of these sequences will become possible, as will an association with the mapped functions that have already been (and continue to be) discovered.

Not only deletions but chromosome exchanges are providing unparalleled genetic tools for studies of gene function and chromosome behavior. Particular emphasis is being placed by us on X-autosome translocations, a rare class of rearrangements most of which have arisen in our laboratory over the past decades. These T(X;A)s provide prime material for studies of X chromosome inactivation, X mapping, and meiotic chromosome pairing.

The year's studies in germ-line mutagenesis have led to the exciting discovery of a new type of genetic effect — the induction of high incidences of late fetal death and varied developmental malformations following mutagen exposure during restricted zygote stages. Other mutagenesis studies have been particularly strong in investigating factors that affect mutation yield, both for chemical mutagens and radiations. Unexpected findings have been made in the areas of strain differences in response, dose-rate effects, germ-cell stage, and repair; many of these lead to inferences concerning mechanisms of mutagenic action.

In addition, there has been a continuation of the effort to determine (by various tests) whether certain chemicals are mutagenic in the mammalian germline. Such work serves to enlarge the data base for comparisons with other mutagenesis systems.

The following are brief summaries of selected reports in the various areas outlined.

Investigation of factors that affect mutation yield are feasible only with effective mutagens such as ethylnitrosourea (ENU), ethylene oxide (EtO), and radiations.

- In-depth ENU studies have shown further dose protraction effects indicative of repair. However, pretreatment with MNU, which might have been expected to tie up the available O^6-G alkyl transferase, was found to be ineffective in altering the ENU-induced mutation rate.

- The view that ENU might be mutagenically most effective during DNA replication is supported by two findings:

  1. Exposure of juvenile (12- to 14-day-old) males produces a higher mutation frequency than does exposure of adults, which have a lower mitotic index in spermatogonia.

  2. Non-dividing oocytes are highly resistant to ENU mutagenesis.

- Last year's discovery of an unexpected dose-rate effect of EtO on dominant-lethal induction in postmeiotic male germ cells has been followed up with studies of chromosome breakage (measured by alkaline elution of sperm DNA) and by measurement of unscheduled DNA synthesis induced in spermatids. These indicate that the dose of EtO binding to germ-cell DNA is dependent on the rate at which the EtO exposure takes place.

- The yield of translocations from irradiated spermatogonial stem cells is affected by dose rate, paralleling our earlier findings for specific-locus mutations induced in spermatogonia.

- The yields of "dominant lethals" and of developmental anomalies among offspring of irradiated spermatogonia are affected by strain of irradiated male in a correlated fashion. Both endpoints probably characterize unbalanced segregants of induced translocations. Strain effects on these chromosome-breakage events are, however, not correlated with strain effects on radiation-induced spermatogonial killing (as measured by length of sterile period).

- Induced breakage, as measured by alkaline elution of sperm DNA, had not heretofore been detectable when radiation was the mutagen. Experiments with isolated germ-cell stages now show that radiation-induced breaks can be detected by this method but that they rejoin very quickly.

A number of chemicals were investigated for mutagenicity, using a variety of test systems — specific-locus studies, dominant-lethal and translocation tests, and UDS measurements. In addition to ENU and EtO, which were used for in-depth studies (see above), these include 6-mercaptopurine, urethane (found negative despite claims by others of cancer increases in offspring), platinol, acrylamide monomer (unexpectedly, highly effective in certain germ-cell stages), and two carcinogen/non-carcinogen pairs (benzo[a]pyrene/pyrene, 2AAF/4AAF).

Studies on organismic expression of mutations involved both known genes and as yet unidentified genetic alterations.

- High frequencies of late fetal deaths and varied developmental anomalies result when early zygotes are exposed to any of several chemicals (but not to radiation). This is a totally new syndrome not heretofore found after exposure to any germ-cell or embryonic stage. Investigations of the cytological basis and developmental progression are under way.

- Interactions between two unlinked, homozygous lethal, skeletal mutations have been found to be both of the synergistic and antagonistic types. In human populations, synergistic interactions could be confused with recessive or multifactorial inheritance.

- The effects of *dilute-suppressor* (the only recessive suppressor mutation known in any mammal) on complementing and non-complementing combinations of *dilute* deletions are under study and results, to date, indicate that there is no suppression of lethal factors.

- Some *dilute-opisthotonic* mutants demonstrate a reduction in the rate at which phenylalanine is cleared from the serum.

- Developmental studies with *Steeloid* mutants indicate that at least one may cause death in homozygotes at a stage considerably earlier than that characteristic of lethal *Steel* alleles. This mutation may be a deletion involving nearby factor(s) needed for embryonic survival.

Structural analyses of specific regions of mouse chromosomes by combined genetic and recombinant DNA studies have involved the *c (albino)* and *p (pink eye)* regions in Chromosome 7 (respectively, ca 10,000 and 3,000 kb potentially analyzable), the *d se (dilute-shortear)* region in Chromosome 9 (ca 5000 kb), and the *Sl (Steel)* region in Chromosome 10.

- In the *d se* region (where we have identified at least 27 complementation groups), molecular and genetic maps were found to be generally concordant. *Ashen*, a mimic of *dilute*, was found to be located between *pl-1* and *pl-2*.

- In the *c* region (where we have identified 13 complementation groups), we derived *c*-region-deficient DNA from somatic cell hybrids retaining a deleted mouse 7, from offspring from crosses to *Mus spretus*, and from combinations of complementing deletions. These were probed with clones from Chromosome-7-enriched libraries. The ability of the methods to localize sequences missing from the *c* deletions was demonstrated.

- New approaches for isolating random clones from regions corresponding to long *c* and *p* deletions have been initiated. They include genomic subtractive hybridization, fluorescence-activated sorting of a long X-7 translocation chromosome, and chromosomal microdissection and microcloning, using this same X-7 translocation.

- Using meiotic recombination data for RFLPs, we have mapped nine Chromosome 7 sequences and known genes. Comparison with human 11p suggests a minimum of one rearrangement in the region defined by these loci.

- Genetic analysis of over 60 independent mutations presumed to involve the *Steel (Sl)* locus has revealed a large number of classes having various survival (anemia), fertility, and pigment parameters. At least five of the mutations are reciprocal translocations. The locus is of interest because of the pleiotropic expression of most mutants, with three body systems involved.

X-autosome translocations have been exploited by us for studies of X inactivation (since the rearrangement has distributed the X to two different chromosomes) and chromosome pairing.

- Since the X-linked gene *spf* codes for ornithine transcarbamoylase (OTC), we have used OTC activity measurements to investigate the hypothesis that one or more inactivation centers are located in a specific region of the X and that inactivation spreads from the center(s) in both directions to contiguous parts of the chromosome. Results confirm the hypothesis of contiguity, but there are indications (still to be confirmed) that there may be more than a single inactivation center.

- Synaptonemal complex whole mount analyses of several X-autosome translocations (as well as other rearrangements) have led to the conclusion that the location of a chromosome break relative to chromosomal substructure — as expressed by G banding — determines whether synapsis will be homologous or nonhomologous, and whether or not there will be crossover inhibition.

- Whether or not sterility of all X-autosome translocation males results from reactivation of X-chromosomal material by contiguous autosomal segments is being tested by studying transcriptional activity (^{3}H-uridine incorporation) in the synaptonemal complexes formed by the translocation chromosomes.

In addition to the ongoing work of which the following abstracts provide a sample, we have undertaken a number of collaborative studies with other laboratories, including the following: analysis of mutations in the a-locus region (Siracusa, Jenkins, and Copeland at NCI, Frederick Cancer Research Program); use of X-autosome translocations for molecular mapping of the X (Avner, Pasteur Institute); use of T(X;A) males in studying expression of an oncogene in sterile males (Propst, NCI Frederick Cancer Research Program); use of *Steeloid* translocations to aid in molecular analysis of the *T-t* region (Lehrach, European Molecular Biology Laboratory); analysis of time of death of early-acting recessive lethal *c* region deletions (Magnuson, Case Western Reserve University); search for *T-t* region deletions through study of tail mutants (Shin, Whitehead Institute, MIT). Within the Biology Division, collaborative studies are in progress with Drs. Kenney (*c*-locus mutations), Mitra (O^6-G alkyl transferase), and Yang (mobile elements in mutagenesis).

IN-DEPTH STUDY OF CHEMICAL MUTAGENESIS IN MOUSE GERM CELLS USING N-ETHYL-N-NITROSOUREA (ENU) AS A MODEL MUTAGEN

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The great effectiveness of ENU as a germline mutagen in the mouse (Russell et al., Proc. Natl. Acad. Sci. USA 76: 5818-5819, 1979) has, for the first time, made it feasible to study the biological and treatment factors that can affect the rate of chemically induced mutations in mammalian germ cells. Several of these factors have already been studied and reported on. Additional subjects addressed during the past year are summarized below.

Repeated ENU Exposure to Measure Very-Low-Dose Effect in Stem-Cell Spermatogonia. This experiment was completed during the past year. Twenty doses of 5 mg/kg of ENU (total dose 100 mg/kg) were injected intraperitoneally at 3- or 4-day intervals over a period of 10 weeks. The totals obtained are 15 specific-locus mutations in 40,983 offspring. The induced mutation rate (experimental minus control) is 59% of the induced rate obtained earlier in the 10 × 10 mg/kg fractionation experiment. Although the reduction is significant only at the P = 0.1 level, the difference is in the direction expected if repair is greater with the smaller dose fractions. Evidence for repair comes primarily, of course, from the fact that the induced mutation frequency is only 9.6% of that obtained in experiments using a single 100 mg/kg exposure, and from Sega's demonstration that DNA ethylation in the testis after intraperitoneal injection of tritium-labeled ENU is directly proportional to injected dose over the range from 5 to 100 mg/kg. However, the mutation rate in this experiment is still significantly higher than that of the historical control, showing that, although repair is extensive, it is not complete. Thus, if there is a threshold dose for ENU in stem-cell spermatogonia it must be below 5 mg/kg.

Test of Effect of Age of Adult Males on ENU-Induced Mutation Frequency. This is the continuation of a test to find out if age at injection of ENU has any effect on mutation frequency. Earlier results had suggested the possibility of such effect. Two groups of males, aged 10-12 weeks and one year, respectively, were injected intraperitoneally with 100 mg/kg of ENU and mated for a mutation test. Offspring obtained after the induced temporary sterile period (which was longer in the older group) were scored for specific-locus mutations induced in stem-cell spermatogonia. The results are: 75 mutations in 29,607 offspring from males treated at the younger age, and 48 mutations in 15,638 offspring from males that were older at treatment. The mutation rates are not significantly different (P = 0.3).

Relation of ENU Effect to DNA-Replication Process: Exposure of Very Young Males. Oakberg has shown that in male mice aged 0 to 18 days (postnatal), the cell-cycle time in spermatogonia is shorter, and the mitotic index (and thus the frequency of cells in S phase) higher than in older juvenile or adult males. To test whether the ENU-induced mutation rate might be increased in this period of high mitotic index, we have started an experiment in which 12- to 14-day-old males were injected intraperitoneally with 100 mg/kg of ENU. To date, 21 specific-locus mutations have been obtained in 4,591 offspring. In a parallel experiment in which males were injected from the same batch of ENU at 10-12 weeks of age, 75 mutations were obtained in 29,607 offspring. The induced mutation rate (experimental minus control) for the juvenile males is 1.8 times that for the adults. The increase is statistically significant ($P = 0.016$, one-tailed test). Thus, the results support the view that ENU is most effective at the time of DNA replication.

Possible Relation of ENU Effect to O^6-G Alkyl Transferase. In independent investigations, an O^6-G alkyl transferase has been identified in several cell systems, including mammalian somatic cells *in vitro*. There is some evidence that this transferase is more effective in removing methyl than ethyl groups, which could account for the very much higher mutagenicity of ENU than MNU in spermatogonia. Furthermore, there is evidence that the transferase changes irreversibly on accepting the alkyl group from the O^6-G position. We have started an experiment to test the assumption that this O^6-G alkyl transferase may be active in spermatogonial stem cells. Young adult males were injected intraperitoneally with 50 mg/kg of MNU and, 6 h later, with 100 mg/kg of ENU. If most of the available transferase is tied up (and thus inactivated) by removing the O^6-G methyl groups resulting from the MNU exposure, little or no active transferase will remain to remove ethylations resulting from the subsequent ENU exposure. (The interval between the two exposures is short in order to minimize the possibility for new transferase being formed.) If the assumptions are correct, the mutation rate resulting from the ENU exposure will be greater than would have been the case had this exposure not been preceded by an MNU exposure. The contribution to the mutation rate from the MNU exposure will be negligible, since spermatogonia are involved. To date, 18 specific-locus mutations have been obtained in 6,952 offspring. From an extensive parallel experiment in which 100 mg/kg of ENU only was injected, we would have pedicted 17.7 mutations from ENU in the current experiment. Therefore, the prior injection of MNU apparently had no effect. If alkyl transferase is involved, it would appear that there may be different transferases for the methyl and ethyl groups.

Results with Females Exposed to ENU. This is a continuation of the experiment to test whether the failure of 100 mg/kg of ENU to induce mutations in female mice might be the result of oocyte selection (i.e., that the only oocytes that survive might be those in which the nucleus received a lower-than-average ENU concentration). In the current experiment, oocyte killing is greatly reduced by using a dose of only 50 mg/kg. A total of 13,713 offspring have been scored and 0 specific-locus mutations obtained. The total includes 5,898 offspring from matings started at the

beginning of the third week after injection, thereby increasing the sample size from ooctyes exposed in an intermediate stage of maturation. The results continue to confirm the conclusion that oocytes are extremely resistant to mutation induction, even to the chemical that has proved to be the most effective mutagen in spermatogonia. It has already been noted that ENU appears to be most effective at the time of DNA replication. The results in females correlate with the fact that there is no DNA replication occurring in oocytes.

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DOSE-RATE EFFECT OF ETHYLENE OXIDE INHALATION EXPOSURES ON DNA BREAKAGE IN MOUSE SPERMATIDS

G. A. Sega E. E. Generoso

The work of Generoso et al. (Environ. Mutagen. 8: 1-7, 1986) showed that there was a dose-rate effect on the frequencies of dominant-lethal mutations induced in germ cells of male mice exposed to ethylene oxide (EtO) gas. In view of this finding, we have carried out experiments to determine if the amount of DNA breakage in mouse spermatids exposed to EtO is altered by different rates of exposure.

To assay for DNA breakage, an alkaline elution procedure was used. For each experiment, one group of (C3H × BL10)F_1 male mice had their germ-cell DNA pre-labeled with ^{3}H-thymidine and were subsequently exposed to EtO. A second group of males had their germ-cell DNA pre-labeled with ^{14}C-thymidine and served as controls.

Our earlier alkaline elution studies with injected EtO had shown DNA breakage to be greatest in sperm recovered in the second week posttreatment (mid- to late-spermatid stages at the time of treatment). These are the same germ-cell stages in which the peak incidence of dominant-lethal mutations occurs after EtO injection. Because of this evidence for high sensitivity in late spermatids, a constant time point of 9 days post-exposure was used for the present dose-rate studies.

Nine days following EtO exposure, spermatozoa from the vasa deferentia of treated and control animals were place together on a polycarbonate filter and lysed. DNA was eluted through the filter overnight, using an

alkaline buffer. Any small pieces of single-stranded DNA resulting from breakage by the EtO pass rapidly through the filter, while normal-sized DNA takes considerably longer to pass through. The amounts of treated and control DNA eluted were then determined using liquid scintillation counting techniques.

The mice were given inhalation exposures of EtO at three different dose rates, but the total exposure was fixed at 1800 parts per million (ppm) -h. The rates of exposure were: 1800 ppm for 1 h, 900 ppm for 2 h, and 450 ppm for 4 h. The results showed that with an exposure of 1800 ppm for 1 h the amount of DNA eluted from the sperm cells of EtO-exposed animals was 36% (after subtraction of the control level). With an exposure of 900 ppm for 2 h, 22% of the treated sperm DNA eluted; and with 450 ppm for 4 h, 16% of the treated sperm DNA eluted. Thus, the amount of DNA breakage in mouse spermatids after inhalation exposures to EtO is clearly affected by the rate at which the animals are exposed to the chemical.

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DOSE-RATE EFFECT OF ETHYLENE OXIDE INHALATION EXPOSURES ON UNSCHEDULED DNA SYNTHESIS IN MOUSE SPERMATIDS

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P. A. Brimer

To further assess the relationship between DNA damage in mammalian germ cells and the rate at which a fixed total chemical exposure is administered, a study was made of the unscheduled DNA synthesis (UDS) induced in mouse spermatids by different concentrations and times of exposure of ethylene oxide (EtO) gas.

Male mice were given inhalation exposures of EtO gas at dose rates of 1800 parts per million (ppm) for 1 h, 900 ppm for 2 h, or 450 ppm for 4 h. In all cases, the total EtO exposure was 1800 ppm.h. Immediately after exposure, and at times up to 24 h following exposure, groups of EtO-treated mice were given testicular injections of ^{3}H-thymidine ([^{3}H]dThd). Early spermatids, a DNA-repair competent stage, were used to study the effects of the different EtO exposures.

Sixteen days following EtO exposure and testicular injection of [^{3}H]dThd, sperm were recovered from the caudal epididymides (these sperm were in early spermatid stages at the time of treatment). Upon recovery,

the sperm were assayed for the unscheduled incorporation of [^{3}H]dThd (UDS), using liquid scintillation counting.

The results of the experiments showed that the highest UDS response induced in the spermatids was obtained with an EtO exposure of 1800 ppm given for 1 h. The response in this case was 125 disintegrations per minute (DPM)/10^6 sperm recovered. With an EtO exposure or 900 ppm for 2 h, the UDS response was 72% of that obtained with 1800 ppm for 1 h, and with an exposure of 450 ppm for 4 h, the UDS response was down to 40% of that obtained at 1800 ppm for 1 h.

The germ-cell UDS response to inhalation exposures of EtO clearly shows a dose-rate effect. This result — combined with our finding that EtO-induced DNA breakage in spermatids also shows a dose-rate effect (Sega and Generoso, this Annual Progress Report), and the finding of a dose-rate effect on the frequency of EtO-induced dominant-lethal mutations (Generoso et al., Environ. Mutagen. 8: 1-7, 1986) — strongly indicates that the dose of EtO reaching the germ cells and binding to the DNA is dependent on the rate at which the EtO exposure takes place. To directly verify this hypothesis, work is planned in which male mice will be given inhalation exposures of [^{3}H]-labeled EtO at different dose rates. The actual number of DNA lesions formed in the germ-cell DNA under these different rates of exposure will then be measured.

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DOSE-RATE EFFECTS ON THE RESPONSE OF MOUSE SPERMATOGONIAL STEM CELLS TO X-RAY INDUCTION OF HERITABLE TRANSLOCATIONS

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C. V. Cornett
L. A. Hughes[1]

The primary objective of the present study is to estimate the magnitude of risk per unit exposure - i.e., the number of heritable translocations expected per rad per gamete - from irradiation of stem-cell spermatogonia, the male germ cells most important for genetic risk considerations. In order to make this estimate, it is essential to determine how the rate of induction is affected by the conditions of radiation exposure such as dose, dose fractionation, and dose rate. We have already completed studies that determined the shape of the dose-response curve and the effects of dose fractionation at high dose rate. These studies, (which are by far the most extensive, to date, for heritable

translocation induction at high dose rate in this germ-cell stage) for the first time allow the estimation of the magnitude of risk. The expected increase in heritable translocations per rad of acute X rays is 3.89×10^{-5} per gamete.

We are currently conducting a large project to determine the effect of varying dose rate on the induction of heritable translocations in spermatogonial stem cells. The experimental strategy for this study was based on the earlier specific-locus studies of W. L. Russell. Comparing the effects on spermatogonia of dose rates of 90 R/min and 0.009 R/min, he found that the lower dose rate produced about one-third as many specific-locus mutations as the higher rate. Furthermore, there was no significant change in mutation frequency over a dose-rate range from 0.8 to 0.0007 R/min.

Two ^{137}Cs sources were used in the present heritable translocation study. One group of mice was exposed to 600 rad gamma rays at 30 R/min, the other to either 150, 300, 600, or 1200 gamma rays at 0.7 R/min. Each group consisted of 72 $(101 \times C3H)F_1$ males, each of which was caged individually with an unexposed $(SEC \times C57BL)F_1$ female 42 days after the end of the irradiation. Male offspring of matings made 60 days or longer postirradiation are being collected and tested for the presence of translocations.

The study is still in progress, but the data collected up to this point already show not only an effect of varying the dose rate, but also a clear departure at low dose rate from the humped dose-effect curve we observed at high dose rate. Comparison of the frequencies of partially sterile translocation carriers in the two 600 R groups shows that the lower dose rate of 0.7 R/min produced about 3.5 times fewer translocations than the higher rate of 30 R/min (13/1928 or 0.67×10^{-2} vs 46/1945 or 2.37×10^{-2}). The dose-effect study at low dose rate (0.7 R/min) produced the following interim frequencies: 1/1278 or 0.08×10^{-2}, 10/2342 or 0.43×10^{-2}, 13/2143 or 0.61×10^{-2}, 13/1928 or 0.67×10^{-2}, and 33/1634 or 2.02×10^{-2} for, respectively, 0, 150 R, 300 R, 600 R, and 1200 R doses. Since the ongoing study of Cacheiro and Cornett (see this volume) indicates that the fully sterile sons produced are rarely translocation carriers, the frequencies based only on partially sterile progeny should provide accurate estimates. Thus, the degree of dose-rate effect in this heritable translocation study seems to be similar to that for specific locus mutations. Also parallel is the finding that the dose-effect curve at low dose rate differs from the humped-shaped curve observed for both specific-locus mutations and heritable translocations at high dose rate; at low dose rates, increasing frequencies are apparent with increasing dose.

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NATURE OF STRAIN DIFFERENCES IN SPERMATOGONIAL RESPONSES TO RADIATION

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In the course of an earlier study, we observed a clear-cut strain difference in embryonic mortality following exposure of the father's spermatogonial stem cells to 4 × 500 R acute X rays. Most of this death is presumed to represent the unbalanced segregants of induced reciprocal translocations. The overall frequency of embryonic mortality was about 23% for irradiated (C3H × 101)F_1 or (101 × C3H)F_1 males, and about 7% for irradiated (SEC × C57BL)F_1. The strains differed in the same direction, but to a lesser degree, with regard to length of the sterile period resulting from killing of stem-cell spermatogonia. The average interval between the last exposure and birth of first litters was 132.1 and 117.9 days for (C3H × 101)F_1 and (SEC × C57BL)F_1 males, respectively. This parallelism seems to agree with the belief of a number of investigators that the nature of radiation damage that leads to stem-cell killing is the same as that which leads to induction of chromosomal aberrations.

To obtain some clues as to the nature of the strain differences and check further the possible relationships between cytotoxic and cytogenetic responses, we initiated experiments in which the condition of X-ray exposures was varied. In the earlier study described above, the four fractions of 500 R were separated by four-week intervals. In the new study, males from the two strains were exposed simultaneously to either of the following regimens: 500 R, single dose; 2 × 500 R, 24 h between doses; 4 × 500 R, 8 weeks between doses; and 100 R + 900 R, 24 h between doses.

The average lengths of the interval between the last exposure and birth of first litters for (C3H × 101)F_1 and (SEC × C57BL)F_1 males, respectively, are 122 and 129 days in the 2 × 500 R experiment, 105 and 117 days in the 4 × 500 (8 weeks between doses) experiment, 139 and 129 days in the 100 R + 900 R experiment, and 77 days for both strains in the 500 R experiment. The corresponding incidences of embryonic mortality among offspring resulting from irradiated spermatogonia (males were mated within 3 months after the recovery of fertility) are 12% and 8%, 30% and 17%, and 17% and 13%. No embryonic mortality data are available for the 500 R single dose exposure. Cytogenetic analysis of diakinesis-metaphase I spermatocytes is still in progress.

These results show that the length of the sterile period for the two strains may differ in opposite directions depending upon the exposure regimen used, but the corresponding incidence of embryonic mortality is either similar in the two strains or clearly higher when fathers are (C3H × 101)F_1. Furthermore, within each of the two strains, the two endpoints exhibit no correlation whatsoever. Thus, these results not only are at variance with the hypothesis that there is a relation between cytotoxicity and chromosomal aberration effects, but they also raise new questions regarding spermatogonial killing and repopulation.

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DIFFERENCE BETWEEN TWO HYBRID STOCKS OF MICE IN THE INCIDENCE OF CONGENITAL ABNORMALITIES FOLLOWING X-RAY EXPOSURE OF STEM-CELL SPERMATOGONIA

W. M. Generoso K. T. Cain
P. W. Braden L. A. Hughes[1]

Many congenital abnormalities in humans have a genetic basis. They are manifested in a spectrum of varying severity, ranging from early fetal death to minor inconsequential anatomic variations. In mice, recent studies by others have established that exposure to ionizing radiation of either maternal or paternal germ cells leads to an increase in certain congenital abnormalities and to growth retardation among first generation progeny. Furthermore, evidence indicates that certain alkylating chemicals may resemble radiation in their effects in mice and rats. Radiation- or chemically-induced congenital abnormalities following exposure of parental germ cells almost certainly have a genetic basis. However, the nature of genetic damage, as well as the factors that influence the incidence of induced congenital abnormalities, are not well understood. Recently, we reported that the incidence of X-ray induced embryonic lethality differed markedly between two hybrid stocks of irradiated male mice (Mutat. Res. 152: 217-223, 1985). A parallel difference in the frequencies of reciprocal translocations was observed cytologically in the meiocytes of irradiated males. In the present study, we attempted to determine whether or not similar differences between the two stocks exist for congenital defects resulting from genetic damage to stem-cell spermatogonia.

Ninety-six males from each of two stocks of mice, (C3H × 101)F_1 and (SEC × C57BL)F_1, were divided randomly into experimental and control

groups. The experimental groups were simultaneously irradiated with 4 doses of 500 R (89 R/min) X rays given 4 weeks apart. During the interval 152-159 days after the last exposure, each surviving male was mated to three (C3H × C57BL)F_1 females (about 12 weeks old). The pregnant females were killed 17 days after observation of the vaginal plug. Each live fetus was weighed, coded, and fixed individually in buffered formalin. Autopsies, aided by the dissecting scope, were accomplished by a ventral incision with in situ inspection of the body cavities and organs. An extension of the incision cephalad was made in order to examine the neck organs. The palate and part of the brain were examined with an incision through the mouth. All anatomic abnormalities were recorded prior to breaking the code at the end of the experiment.

Results showed a parallelism between the frequencies of congenital abnormalities and of reciprocal translocations. The frequencies of congenital abnormalities in the X-ray groups were significantly higher than those in the respective control groups. And, while the controls of the two stocks were not significantly different from one another, the frequency of anomalies among offspring of irradiated (C3H × 101)F_1 was significantly higher than that among offspring of irradiated (SEC × C57BL)F_1. Growth retardation and neural-tube and abdominal-wall defects were the types of abnormalities most commonly observed. The present results add support to the suggestion that the congenital abnormalities observed are attributable to chromosomal defects, perhaps resulting from unbalanced segregants that allowed survival beyond implantation. While the great majority of unbalanced gametes lead to death at around the time of implantation, it is known that certain cases of partial trisomies do survive through gestation.

This study was conducted in collaboration with Dr. J. C. Rutledge of the University of Texas, Medical Branch, Dallas, Texas.

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ALKALINE-ELUTION MEASUREMENT OF DNA BREAKAGE AND REPAIR IN ISOLATED PACHYTENE SPERMATOCYTES AND SPERMATIDS AFTER EXPOSURE TO X RAYS

G. A. Sega E. E. Generoso

In previous work, we have been able to demonstrate DNA breakage in developing spermiogenic stages of mice exposed to chemical mutagens such as methyl methanesulfonate and ethylene oxide. This has been done using an

alkaline elution technique that detects DNA breaks on the basis of how rapidly a high pH buffer can elute the germ-cell DNA through a filter. However, not all mutagens studied by this technique have been demonstrated to induce DNA breakage in mouse germ cell, a notable exception being X irradiation.

No increase in the elution of DNA from sperm was observed after exposures as high as 1200 R to various spermiogenic stages, even though genetic and cytogenetic evidence has shown X rays to be very effective in causing chromosome rearrangements in meiotic and postmeiotic germ-cell stages of male mice. Furthermore, a number of alkaline-elution studies have shown a high level of DNA breakage after X-ray exposure of somatic cells.

To further study the effects of X rays on the induction of DNA strand breaks in germ cells, single-cell suspensions made from mouse testes were irradiated with 800 R of X rays. Following irradiation, pachytene spermatocytes or early spermatids were isolated from the cell suspensions using centrifugal elutriation. This procedure separates cells on the basis of their size. After isolation, the pachytene spermatocytes or early spermatids were maintained for 1 h at either 33°C (the approximate temperature of the mouse testis) or at 0°C. Following this 1 h period, the cells were lysed on polyvinyl filters, and the alkaline elution procedure was carried out to monitor DNA breakage.

The results of this study showed that there was increased DNA elution (strand breaks) from both pachytene spermatocytes and early spermatids exposed to 800 R of X rays, but this breakage was measurable only when the cells were maintained at 0°C. After 1 h posttreatment incubation at 33°C, no measurable DNA breakage could be detected with our alkaline elution procedures. Thus, our experiments have now shown that DNA breaks do occur in pachytene spermatocytes and early spermatids of the mouse after X-ray exposure, but that the breaks are normally rejoined very rapidly (in less than 1 h). In view of the fact that X rays induce considerable genetic damage in these germ-cell stages, the possibility remains that the broken fragments of DNA have not been correctly rejoined or that events other than DNA single-strand breaks are responsible for the genetic damage.

MECHANISMS FOR INDUCTION OF CHROMOSOME ABERRATIONS IN MALE GERM CELLS BY CHEMICAL MUTAGENS

W. M. Generoso K. T. Cain
P. W. Braden L. A. Hughes[1]

A long-term project has been under way to study the mechanisms by which lesions induced by chemicals in the chromosomes of germ cells are actually converted into aberrations of one sort or another. In trying to

understand these mechanisms, one must keep in mind not only the reaction properties of chemical mutagens but also the biological properties (such as repair, length of cell-cycle time, etc.) of various germ-cell stages. Chromatid deletions and exchanges are the types of aberrations chemicals and ionizing radiations produce. Either of these two major classes of aberration may lead to abnormal chromosome complements in the conceptus that can cause any of a variety of effects (including death) in first- or later-generation offspring. Thus, an understanding of how various chemicals produce the two major classes of aberrations is essential in evaluating genetic risk.

MNU, EMS, MMS, and EtO (ethylene oxide) are effective inducers of both dominant lethals and heritable translocations. ENU and IMS, on the other hand, produce primarily dominant lethals. MNU, MMS, EMS, and EtO are very similar to one another with respect to their reaction with DNA: 65 to 83% of the total DNA alkylations occur at the N-7 position of guanine. IMS and ENU, on the other hand, alkylate primarily the oxygens of the phosphate backbone (about 60% of the total DNA alkylation) and the oxygen positions in guanine, thymine, and cytosine. N-7 alkyl guanine is known to be unstable; it hydrolyses to form an apurinic site. O-6 alkyl guanine, O-2 and O-4 alkyl thymine, and O-2 alkyl cytosine on the other hand, have been shown to be highly stable. Thus, there appears to be a good association between formation of unstable N-7 alkyl guanine products and production of heritable translocations. Because N-3 alkyl adenine products are also unstable, they too could be a target site for the production of heritable translocations. Our hypothesis that stable alkylation products lead primarily to dominant lethals is consistent with the fact that the primary alkylation products of IMS and ENU are at the oxygens in the phosphate backbone and in guanine, thymine, and cytosine.

Further tests of this hypothesis require that more chemicals be investigated with respect to the relationship between the molecular binding property and the induction of dominant lethals and translocations. We have been studying the compound acrylamide (monomer) for this purpose. It is produced on an industrial scale and its production and use involve exposure of many thousands of individuals.

We find that acrylamide induces dominant lethals in early spermatozoa and late spermatids, but not in other stages. The stage-sensitivity pattern observed is bell-shaped, which is similar to that for EMS, MMS, MNU, and EtO. In a recently completed heritable-translocation study, we found acrylamide to have a strong positive effect. Up to 40% of male progeny of acrylamide-treated male parents carried one or, occasionally, more translocations. Thus, acrylamide is an effective inducer of both dominant lethals and heritable translocations, and in this regard too, resembles EMS, MMS, MNU, and EtO. Acrylamide, however, differs markedly from these four compounds in that results from other mutagenicity assays had given no grounds for suspecting it to be such an effective germ-cell mutagen in the mouse. Thus, the chemical is not mutagenic in Salmonella, is only marginally mutagenic in mouse bone-morrow cells, and is negative in the germ-cell unscheduled DNA synthesis test. Furthermore, while the other

four compounds bind readily and primarily to the N-7 position of guanine, acrylamide, according to a published in vitro study, appears to bind to DNA very slowly. The slow rate of reaction of acrylamide with DNA in vitro seems inconsistent with its potent germ-cell mutagenicity and points toward the possibility of the formation of a reactive intermediate in vivo. The mode of DNA binding of acrylamide in vivo is under investigation by G. A. Sega. It should be of interest to find out if acrylamide, like the other four compounds, alkylates primarily at the nitrogen positions of DNA.

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DNA REPAIR STUDIES IN MOUSE GERM CELLS EXPOSED TO TWO "CARCINOGENS" AND TWO "NON-CARCINOGENS"

G. A. Sega J. G. Owens

An in vivo test was used to measure induced unschedule DNA synthesis (UDS) in the germ cells of male mice exposed to the carcinogens benzo(a)pyrene [B(a)P] and 2-acetylaminofluorene (2AAF), and to the noncarcinogens pyrene (PYR) and 4-acetylaminofluorene (4AAF). Early spermatids, a DNA-repair competent stage, were used to test the effects of all chemicals. After chemical treatment and testicular injection of [^{3}H]dThd, sperm were recovered 16 days later from the caudal epididymides (these sperm were in early spermatid stages at the time of treatment) and assayed for the unscheduled incorporation of [^{3}H]dThd using liquid scintillation counting (LSC).

Exposures of 2AAF ranged from 125 to 1600 mg/kg, 4AAF from 125 to 2000 mg/kg, PYR from 100 to 600 mg/kg, B(a)P from 100 to 400 mg/kg. Chemicals were administered both by intraperitoneal (i.p.) injection and by gavage. Methyl methanesulfonate (MMS) was used as a positive control.

MMS produced a strong, exposure-dependent UDS response in the early spermatids, with both i.p. and gavage exposures. Neither the two

carcinogens, 2AAF and B(a)P, nor the two noncarcinogens, 4AAF and PYR, induced a measurable UDS response in the early spermatids with any of the exposures used. As an additional check on the effect of B(a)P on mouse germ cells, a constant, high exposure of 400 mg/kg was given by gavage or i.p. injection, and the testicular injection of [^{3}H]dThd was administered 0, 1, 2, or 4 h after the B(a)P. Since most of the injected [^{3}H]dThd is either used or is removed from the testis within about 1 to 2 h after injection, varying the time of [^{3}H]dThd injection permits a study of any possible delay in the UDS response in the germ cells that might result from delayed formation of DNA lesions. The results of this study indicate that for up to at least 4 h following treatment there is no UDS response to B(a)P in early spermatids.

It appears that the chemicals B(a)P, PYR, 2AAF and 4AAF do not produce repairable-type lesions in germ-cell DNA, and that the germ cells cannot metabolize the chemicals to reactive species that are capable of producing repairable-type DNA lesions.

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SPECIFIC-LOCUS EXPERIMENTS AND RELATED STUDIES WITH FOUR CHEMICALS

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D. A. Carpenter
C. C. Cummings
G. M. Guinn
P. R. Hunsicker
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The mouse specific-locus test (SLT) is currently the definitive test for detecting induction of heritable mutations in mammals and for the quantitative studies needed to estimate risk. It is used as a standard against which the predictiveness of short-term tests may be assessed. An effort is therefore under way to enlarge the SLT data base. The work is being carried out under contract with the National Toxicology Program.

1. 6-Mercaptopurine (6MP). The base analog 6-mercaptopurine (6MP) was administered at the sublethal dose of 150 mg/kg i.p., and various male germ-cell stages were sampled. The overall finding of 3 mutations in 65,376 offspring rules out (at the 5% significance level) an induced mutation rate greater than 122 times the historical control rate. For

spermatogonial stem cells alone, the multiple ruled out is 3.7; and for postspermatogonial stages, it is 0.7. For late differentiating spermatogonia and preleptotene spermatocytes, stages that had earlier been found sensitive to dominant-lethal induction (a result confirmed in the present experiment), the results (0 mutations in 5214 offspring) do not rule out a positive effect; they indicate only that the induced rate is unlikely to be greater than 9.8 times the historical control rate.

There is evidence that 6MP (or an active metabolite) reaches all germ-cell stages of concern. Because spermatogonial stem cells are not killed, the negative mutation-rate result cannot be attributed to cell selection.

2. Urethane. To aid in the interpretation of mutation-rate data from experiments completed last year we have, this year, attempted to obtain evidence on whether urethane kills male germ cells. Urethane is a rodent carcinogen that has given widespread, though erratic, evidence of mutagenicity. Of greatest interest is the claim (Nomura, T., Nature 296: 575-577, 1982) that mutations induced in male germ cells by urethane lead to an increased tumor incidence in the first descendant generation. Since there existed no independent evidence - positive or negative - on the ability of urethane to induce transmissible gene mutations in mammalian germ cells, there was a clear need to obtain data from a specific-locus experiment. Altogether 32,828 offspring were examined from successive weekly matings of males exposed to the maximum tolerated i.p. dose of 1750 mg urethane/kg. The combined data rule out (at the 5% significance level) an induced mutation rate greater than 1.7 times the historical control rate. For spermatogonial stem cells alone, the multiple ruled out is 3.2, and for poststem-cell stages, 3.5.

Litter sizes from successive conceptions made in any of the first 7 weeks give no indication of induced dominant lethality, confirming results of past dominant-lethal assays. On the other hand, urethane may induce some spermatogonial killing, as indicated by a slight drop in litter number during the eighth and ninth post-exposure weeks, as well as by cell counts performed at 4 dose levels in seminiferous tubule cross sections. (We thank Dr. E. F. Oakberg, a former staff member, for carrying out the histological studies for these experiments.) These findings, as well as the induction of SCE in spermatogonia demonstrated by other investigators, suggest that urethane (or an active metabolite) reaches the target cells. The negative results for induction of gene mutations as well as clastogenic damage are at variance with Nomura's reports of dominant effects (F_1 cancers and malformations) produced by urethane.

3. Acrylamide (AA). This chemical is of interest in that its mutagenic properties have become apparent only in mammalian tests. The monomer acrylamide, enters into the production of polyacrylamides and receives wide use in the paper industry, in water treatment, and in research laboratories (for gel electrophoresis). The chemical is negative in the Ames test, but cytogenetic damage has been reported in mouse bone marrow as well as germ cells. Clear-cut dominant-lethal and translocation

induction has been demonstrated at our laboratory. (See report by Generoso et al., p. 77, this volume).

We have administered AA by 50 mg/kg i.p. injection on five successive days (total exposure, 250 mg/kg). A specific-locus experiment with spermatogonial stem cells has been completed this year. No mutants were found among 17,112 offspring, ruling out (at the 5% probability level) an induced rate that is 2.3 times the historical control rate. The results for postspermatogonial stages, while still incomplete, are potentially more interesting. Since AA exerts most of its dominant-lethal effect on mature spermatozoa and late spermatids, only a greatly reduced number of offspring derived from these stages is scorable in the specific-locus test; thus the number of first- and second-week offspring scored was only about 2 and 30%, respectively, of the average number of third- and fourth-week offspring scored. Despite this, five out of six mutations observed were found among offspring conceived in the second and third weeks. The mutation rate for the second week is about 3×10^{-4} per locus, almost 40 times the historical control rate. Furthermore, all three of the second-week and one of two third-week mutations were at the same locus, s, suggesting a possible "hot spot". We are planning to explore this question further.

4. Platinol (PLA). Cis-diaminedichloroplatinum (II), abbreviated platinol (PLA), is a chemotherapeutic agent used for the treatment of testicular and other cancers. In specific-locus experiments with this agent, we exposed males by i.p. injection at a level of 10 mg/kg (somewhat below the maximum tolerated dose).

In sampling from exposed spermatogonial stem cells, no mutations were observed in 15,583 offspring, ruling out (at the 5% probability level) an induced mutation rate greater than 2.6 times the historical control rate. To sample exposed poststem-cell stages, each male received two fresh females per week, except that during the 7th week posttreatment, males were also remated in the middle of the week. This procedure was adopted on the basis of preliminary results that indicated a high frequency of nonfertile mating during the 7th week. The fertility disturbance was, in fact, lower than expected. No mutants were observed among 18,848 offspring derived from exposed poststem-cell stages. This rules out an induced mutation rate of 2.0 times the historical control rate. For weeks 6 and 7 alone, the absence of mutations among 6,181 offspring rules out a multiple of 8.1.

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EXPOSURE OF FEMALE MICE TO ETHYLENE OXIDE WITHIN HOURS AFTER MATING LEADS TO FETAL MALFORMATION AND DEATH

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The zygotic stage, which covers the period from sperm entry to first cleavage division, lasts for almost a day in the mouse. It is characterized by a series of complex developmental changes, including the formation of male and female pronuclei and pronuclear DNA synthesis. Early studies by L. B. Russell have shown that the sensitivity of the zygotes, as well as of the individual male and female genomes, to X-ray induction of chromosome breakage, varies with the developmental stage exposed. Lethal chromosome damage in the zygotes and early cleavage stages is expressed near the time of implantation, leaving the embryos that survive to develop without anatomical or physiological abnormalities. There are few studies on the response of zygotes to noxious chemicals. However, exposure of early cleavage and predifferentiation stages to various chemicals is generally known to give response patterns resembling those observed with X ray. It was generally believed that it is only beginning with the period of organogenesis that abnormalities in surviving embryos can be induced by chemicals or radiations. Our new finding changes this long-standing belief.

Effects of EtO were studied in conceptuses of mothers that were exposed by inhalation either before they were mated (treatment of preovulatory oocytes) or after mating (treatment of zygotes and two-cell embryos). The types of female mice used were (C3H × C57BL)F_1, and (SEC × C57BL)F_1 hybrids. Females were about 12 weeks old at the time of the study; males were 12- to 24-weeks-old (C3H × C57BL)F_1 hybrids.

In experiments in which the females were exposed to EtO after they had been mated, the duration of the mating period was restricted to 30 min. Each experimental group was given a single exposure to a chamber concentration of 1200 ppm EtO for 1.5 h (total exposure, 1800 ppm.h), beginning either 1, 6, 9, or ca 25 h after the end of the 30-min mating period. For (C3H × C57BL)F_1 females, these postmating intervals correspond, respectively, to the time of sperm entry, early pronuclear stage (before DNA synthesis), pronuclear DNA synthesis and early two-cell stage. In experiments that tested the effects of EtO on preovulatory oocytes, one group was exposed to 1200 ppm for 1.5 h each day for four consecutive days, and another group to 300 ppm for 6 h per day for a total of 10 exposures spread over a 14-day period. These exposure conditions were used in order to maximize the dominant-lethal response of preovulatory oocytes. All females were killed for uterine analysis on the 17th day after observation of the vaginal plugs. Dead embryos were classified into three categories: resorption bodies (embryos that died near the time of implantation), midgestation death (characterized by the presence of placenta and embryonic mass that lacked eye pigment), and late fetal death (death that occurred on

day 11 postmating, or later, with at least some eye pigment visible). In certain experiments, fetuses were fixed in buffered formalin and examined for external and internal congenital abnormalities.

Exposure of females prior to mating resulted in increased numbers of resorption bodies primarily. This response is probably attributable to dominant-lethal mutations induced by EtO in ovarian oocytes. Exposure administered 1 or 6 h after mating greatly increased the numbers of midgestation and late fetal deaths. These effects dropped to marginal levels in females that had been exposed beginning 9 h postmating, even though the incidence of resorption bodies remained high. In females exposed 25 h postmating, the incidences of midgestation and late fetal death were no longer significantly different from control levels, and the incidence or resorption bodies was only marginally increased. Since the incidence of late fetal deaths was greatly increased in females exposed 1 or 6 h postmating, the condition of these fetuses, as well as of the surviving ones, was examined. Fetuses that died several days before uterine analysis were difficult to examine; autolysis and postmortem deformation prevented detailed dissection. Nevertheless, among dead fetuses that were analyzable, as well as among survivors, a high rate of congenital abnormalities was observed. These abnormalities were grouped into several major classes. Cursory gross examination of living fetuses from females that had been exposed 1 or 6 h postmating easily identified the obvious abormalities, such as omphalocele, hydrops, open thorax, and tail and limb defects. These defects were not found among fetuses in the 9- and 25-h groups.

The remarkable stage-dependent sensitivity and the varied nature of congenital defects suggest a genetic basis for the response. To what extent this is true is currently under investigation. Whatever the underlying mechanisms might be, this result promises to be of major interest, both as a new concept for consideration in chemical safety evaluation and as a new area of study on the etiology of congenital abnormalities. From a practical standpoint, it raises questions regarding the vulnerability of human zygotes when the mother is exposed to environmental chemicals.

This study was conducted in collaboration with Dr. J. C. Rutledge, University of Texas, Medical Branch, Dallas, Texas.

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CYTOGENETIC AND DEVELOPMENTAL STUDIES OF EMBRYONIC ABNORMALITIES INDUCED BY EXPOSING MOUSE ZYGOTES TO CHEMICAL MUTAGENS

M. Katoh[2] W. M. Generoso

The zygotic stage is generally believed to be refractory to effects of noxious agents that would be manifested during the fetal stages. Results from a limited number of agents studied, including ionizing radiation, have led to the general understanding that exposure of the zygote leads to early embryonic death but that the surviving fetuses are free of developmental abnormalities. Recently we made a significant observation that broke this dogma (see preceding abstract). When previously mated female mice were exposed to inhaled ethylene oxide (EtO) at about the time when the sperm enters the egg, or during the early pronuclear stage of the zygote, we observed not only a high incidence of mortality among conceptuses, but also congential abnormalities among both the dead and the surviving fetuses. The developmental stage at which death occurred ranged from near the time of implantation to day 17 of gestation, when examination of the uterine contents was performed. By comparison, when exposure was given either to females before mating, or to conceptuses in later zygotic stages (pronuclear DNA synthesis) or in the early two-cell stage, midgestation and late fetal deaths were absent or minimal. The effects differ, both from genetic damages induced in premating germ cells, which lead only to death near the time of implantation, and from teratogenic damage, which leads to malformations only when exposure of embryos occurs during the period of major organogenesis. The pronuclei in zygotes are the ultimate stage in male and female gametogenesis, since the respective genetic contributions are not yet united in the egg.

We have, subsequently, observed the inductions of congenital defects from exposure of the zygotic stage to three other clastogens (EMS, ENU, and mitomycin C), but not to a non-clastogen (hycanthone).

We have begun a study designed to analyze the nature of these developmental anomalies, using EMS. Like EtO, EMS produced resorption moles and midgestation and late fetal deaths following exposure of early zygotic stages (6 h after mating). The early cleavage embryos (up to the 8-cell stage) appear normal as examined under phase-contrast microscope except for a slight delay (of about two hours) in the division of blastomeres. By the time control embryos reached early blastocyst stage, many embryos in the EMS group were abnormal. They failed to form the blastocoele, and the blastomeres were uneven in size. It is likely that these abnormal embryos are responsible for the decidual reaction that formed the resorption moles. This developmental study will be carried out also for the postimplantation embryos in order to analyze midgestation and

late fetal deaths. An accompanying cytogenetic study of the developmental anomalies is also under way.

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SYNERGISTIC AND ANTAGONISTIC INTERACTIONS OF TWO RADIATION-INDUCED DOMINANT SKELETAL MUTATIONS IN MICE

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The study of the interaction of mutations Ccd (Cleidocranial dysplasia) and Dsh (Dominant short digits), both of which are congenic on the C57BL/10 genetic background, was completed. Double heterozygotes were constructed to determine whether these mutations might together cause malformations that neither one can cause by itself. When acting alone, each mutation produced 14 different abnormalities, with very different degrees of penetrance. We earlier showed that both mutations are homozygous lethal and unlinked. Homozygotes for Dsh are short-limbed dwarfs that always lack forefeet and hindfeet, and usually also lack eyes, ears, external genetalia, and a mouth. The head is greatly reduced in size and has a long trunk. On the C57BL/10 genetic background, many of these homozygotes live at least until 12 days p.c., and on the mixed C3H-102 genetic background, they often live until late pregnancy.

Because these mutations influence many developmental pathways severely enough to cause abnormal morphology, it is likely that they influence still other developmental pathways to a lesser degree. Since one abnormality (a hole between the frontals) is found in both syndromes, it seems especially likely that Ccd and Dsh may influence some common developmental pathways and that, if both mutations are present in the same mouse, some of their combined influences might exceed thresholds for anomalies that neither mutation can produce alone.

Offspring from crosses of the two types of single heterozygotes were classified on the basis of the invariant parts of the syndromes, and were analyzed for variations that might indicate interaction effects. Seven different synergistic interactions were demonstrated, including one which yielded an entirely new abnormality (detached, or weakly attached, ventral tubercle on the atlas) not predicted from any abnormalities found in either of the single heterozygotes. Synergistic interactions of the type demonstrated might, in human populations, be confused with recessive or multifactorial inheritance. The results also raise questions about the

often-made assumption that mutations influencing quantitative or threshold characters have equal and additive effects. If synergistic interactions turn out to be common, they will have to be taken into account in genetic risk estimation for chemicals and radiation.

Interactions were not always in the direction of enhancement. Thus three antagonistic interactions were also found.

Ccd shows striking homology with cleidocranial dysplasia in humans, and it provides an opportunity for studying the timing and importance of interacting connective-tissue events in the development of skeletal form. Because homozygotes for Dsh can survive so long in spite of their extreme malformations, it will be possible to obtain DNA from them. This will facilitate studies of the molecular basis of this fascinating mutation. For both Dsh and Ccd attempts will be made to learn (1) what DNA changes result in such dominant mutations causing serious health effects, and (2) how these mutations lead to so many pleiotropic and interaction effects.

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PHENYLALANINE CLEARANCE RATE IN MICE: STUDIES ON DILUTE-OPISTHOTONIC AND OTHER MUTANTS

K. B. Jacobson P. R. Hunsicker
E. B. Wright

A new program in mammalian regulation has been initiated. Several subjects in the literature have been investigated so that the ones showing greatest promise can be identified. One project currently in progress is reported here. (See also report by L. B. Russell et al., Investigation of the Process of X-Inactivation Through Analysis of OTC Activity in X-Autosome Translocations).

One of the goals in biochemical genetics is to characterize the biochemical defect that results from a mutation. It is seldom possible to predict the biochemical lesion from observation of the phenotype of a mutant. However, if there are two traits, the predictability should be improved. Accordingly, we have sought mutants that have a defect in

melanin pigment and that have some neurological defect. Since phenylalanine is utilized for both melanin and adrenalin synthesis, we have examined a number of mouse strains and mutants that have altered pigmentation and neurological traits to measure the rate at which they clear phenylalanine from the serum. A defect in phenylalanine oxidation could be due either to the phenylalanine hydroxylase or the tetrahydrobiopterin cofactor used by this enzyme.

Mouse strains and neurological mutants were obtained from the research colony of Dr. L. B. Russell. Each animal was fasted overnight. L-phenylalanine solution containing 25 mg of L-phenylalanine/20 g of body weight was injected intraperitoneally. Blood samples were obtained at 30 min intervals for 210 min. Each sample of plasma was screened for phenylalanine by a one-dimensional TLC-Cellulose procedure that uses n-butanol : acetone : acetic acid : water = 7:7:2:4 for solvent (M. L. Cabella, La Fe Hospital, unpublished). The time to remove 50% of the phenylalanine was recorded to indicate the rate of clearance.

Mice at ages from 38 to 168 days showed a slight and statistically insignificant decrease with age in the rate of clearance of phenylalanine from the serum. Of 16 strains tested the rate of removal of 50% of the phenylalanine varied from 60 to 105 min; the median time was 82 min.

The mutant microphthalmia (82UT Mi^{OR}) has a defect in melanin and abnormal eye structure. This animal cleared phenylalanine at a normal rate (72 min).

Four independently occurring mutations to dilute-opisthotonic (d^{op}), a mutant with defective melanocytes and a neurological abnormality, were examined. The d^{op} mutant in strain 61 DENUg lost no phenylalanine in 3.5 h and was clearly very slow in clearing phenylalanine. Possibly, 7 MNUR also shared this slow trait. On the other hand, $19ENUR_k$ and 4R75M were only slightly slower than controls. In each case, the three types of littermates were compared: d^{op}/d^{op}, d^{op}/d, and d/d. Only the d^{op} homozygote of $16DENU_g$ and 7MNUR showed the very slow rate.

Two other mutations that exhibit a nervous-system disorder were also examined. These were provided by W. M. Generoso. One is a newly obtained mutant blind (BLD), and the other is a translocation involving Chromosomes 2 and 14 (the "diver" mutation). In both cases, the clearance rate for phenylalanine was normal.

The d^{op} mutants are the same as d^1, a designation used in earlier literature. Coleman (Arch. Biochem. Biophys. 91: 300, 1960) had examined a d^1 mutant and concluded that the liver phenylalanine hydroxylase activity was decreased. However, Zannoni et al. (Genetics 54: 1391, 1966) re-examined this claim and concluded that there was no deficiency in this enzyme in three stocks that carry d^1. It is clear from the results reported here that the d^{op} mutants vary in their ability to clear phenylalanine from the serum. This same test is used to detect human

heterozygotes of PKU mutants. No attempt has yet been made to measure phenylalanine hydroxylase in 61 $DENU_g$ and $19ENUR_k$.

STRUCTURAL AND FUNCTIONAL ANALYSES OF THE DILUTE-SHORTEAR REGION: GENETIC AND MOLECULAR STUDIES

L. B. Russell C. S. Montgomery
E. M. Rinchik

In earlier genetic experiments with a large number of radiation-induced mutations in the dilute-shortear (d-se) region of Chromosome 9, we had been able to characterize a series of presumed deletions that overlap at one or both marker loci, extending to various distances on both sides. Complementation mapping yielded a functional map encompassing at least 12 functional units, including some that control prenatal survival (6 factors, pl-1 through pl-6), neonatal survival (nl-1 and nl-2), neurological or neuromuscular development (op and sv), cartilage development (se), and melanocyte morphology (d). At least 27 complementation groups could be identified that encompassed two or more of these functional units (Russell, Mutat. Res. 11:107-123, 1971; Rinchik et al., Genetics 112: 321-342, 1986).

Subsequently, molecular entry into this region was facilitated by the discovery that the original dilute mutation was associated with the integration of an ecotropic murine leukemia virus genome. Several unique sequence cellular DNA probes flanking this provirus were identified and were used to investigate the organization of wild-type and mutant chromosomes. Molecular and genetic maps were found to be in general concordant, and the identification of a deletion breakpoint fusion fragment made it possible to orient the physical map with respect to the genetic complementation map. Furthermore, it became possible to discriminate among members of specific complementation groups.

This year, we initiated further genetic experiments to refine the complementation map. Deletion mapping has been carried out with the gene ashen (ash), a closely linked mimic of dilute (d). Experiments with 34 mutants have been completed, and crosses with an additional 9 are in progress. Of 22 Df(d se) mutants tested, 9 were found to be deficient also for ash, while 13 were not. Only one of 9 d^{pl} (prenatally-lethal dilute) and none of 3 se^{l} (lethal shortear) mutations tested was deficient for ash. On the basis of these results and the prior complementation map, ash appears to be located between pl-1 and pl-2; and several of the mutations that previously were in the same complementation group can now be demonstrated to be distinct from each other. The region covered by the deletions is now known to be at least 3.3 cM in length, extending from proximal to ash to distal to sv, thus, presumably, involving approximately 5000 kb of DNA.

Recently, we have obtained from Dr. Josephine Peters an electrophoretic variant at the Pk-3 locus, which is thought to be 2 cM proximal to ash. As soon as that stock has become established, deletion mapping will be carried out with those Df(d se) and d^{p1} mutants that we have now shown to be deleted for ash. The electrophoretic procedures are being refined by Dr. Jacobson in our Section.

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THE EFFECT OF DILUTE-SUPPRESSOR ON COMBINATIONS OF DILUTE-LETHAL MUTATIONS

L. B. Russell C. S. Montgomery

The only recessive suppressor mutation known in any mammal is dilute-suppressor (dsu), identified by Sweet (J. Hered. 74: 305-306, 1983). This gene is located on Chromosome 1 and thus unlinked to dilute (d) on Chromosome 9. Suppression of the pigment (melanocyte morphology) phenotype of d/d has been shown not to be the result of excision of the Emv-3 provirus associated with d (Jenkins and Copeland, unpublished). Although this was not reported by Sweet, we find the suppression to be incomplete.

We have undertaken a study to determine the effect of dsu on complementing and non-complementing combinations of d deletions (see preceding abstract). For this purpose, it was necessary for us, first, to construct a stock that was dsu/dsu; d se/d se. We have now made over a dozen crosses of the type $d^{p\ell 1}$ +/d se; +/dsu x $d^{p\ell 2}$ +/dse; +/dsu, where $d^{p\ell 1}$ and $d^{p\ell 2}$ represent any two different $d^{p\ell}$ (prenatally-lethal dilute) mutations. In the absence of dsu, we have earlier shown some of these $d^{p\ell 1}/d^{p\ell 2}$ combinations to be prenatally lethal, while others produce a dilute-opisthotonic phenotype. In the presence of dsu/dsu, some combinations in the latter class now appear to be non-dilute opisthotonic, i.e., the pigment phenotype has been suppressed. There is, to date, no evidence that dsu/dsu can suppress opisthotonus or lethality.

EXPLORATION OF METHODS TO LOCALIZE DNA SEQUENCES MISSING FROM c-LOCUS DELETIONS

L. M. Albritton[3] C. S. Montgomery
L. B. Russell

We have earlier characterized a large number of radiation-induced mutations at the c locus (on Chromosome 7) through genetic analysis, including extensive complementation tests (e.g., Russell et al., Genetics 100: 427-453, 1983). Based on this work, we have postulated that many of these mutations are deletions of various lengths, overlapping at c (the marker used in the mutation-rate experiments that generated the mutants). It was possible to apportion these deletions among 13 complementation groups and to fit them to a linear map of 8 functional units. Collectively, the deletions extend from a point between tp and c to one between sh-1 and Hbb, i.e., a genetic distance of from 6 to 10 cM, corresponding to at least 10^4 Kb of DNA.

This year, we completed a pilot study designed to explore methods for finding DNA sequences that map to the region covered by the various c-deletions (see abstract by Rinchik et al., p. 93, this volume, for ongoing studies in this area). The general plan was to probe DNA with clones derived from Chromosome-7-enriched libraries or with sequences known (or suspected) to reside in Chromosome 7. Three methods were explored for deriving the c-region-deficient DNA: (a) from mouse-hamster somatic-cell hybrids retaining a deleted mouse Chromosome 7, but no homologue; (b) from F_1 hybrids of M. musculus domesticus (carrying a c-locus deletion) by M. spretus; and (c) from F_1 hybrids of M. domesticus stocks carrying complementing deletions.

Mouse-hamster somatic cell hybrids were constructed using X-7 translocations that have a long X^7 chromosome carrying the selectable Hprt locus on the X portion, and the c locus on the Ch 7 portion. Into one of these translocation chromosomes, we had introduced by genetic recombination the second longest one of the c deletions (26DVT), using a series of two crosses. To verify that the somatic cell hybrid had retained X^7 but not 7 (which would cover the deletion), the cell lines were tested for Gpi, Pep-4, Ldh-1 and Hbb (Ch 7 has all four, X^7 only the last two). A problem with the somatic cell hybrids is that cell lines can lose various portions of the X^7 chromosome (except for Hprt), a situation that can falsely lead one to assume that a particular DNA sequence failing to hybridize maps to the deletion when, in fact, it may map to the lost part of the chromosome. Not all of such losses could be recognized by appropriate isozyme tests.

The crosses to M. spretus were made with several c deletions of which four were found to have been transmitted to some of the F_1 hybrids. Finally, four different complementary combinations of c deletions were constructed — c^A/c^A, c^A/c^C, c^B/c^E, and c^C/c^E. All of these various types of animals and cell lines provided DNA for a mapping panel. This was probed with three anonymous sequences selected from Chromosome-7-enriched

recombinant DNA libraries. Two came from a flow-sorted Is(7;X)1Ct chromosome (sorting gave only 40% purity); the third was derived from a somatic cell hybrid carrying Ch 7 among four mouse chromosomes, and was selected by using mouse-specific repeats. Also tested were a number of sequences from human Chromosome 11p, which, on the basis of other known homologies, were thought to have a good chance of being located on mouse Ch 7.

None of these various sequences were found to map to c locus deletions. However, clone 12A (supplied by Dr. C. Disteche) did map to the region. The hybridization pattern with mapping-panel DNAs indicates that 12A is located in a segment marked by the "juvenile survival" functional unit.

This result demonstrates the ability of the methods to localize sequences missing from c-locus deletions.

We appreciate the collaboration of Dr. E. G. Bernstine, formerly a staff member in this Section, and Dr. D. Housman, Massachusetts Institute of Technology, who provided some laboratory facilities, as well as advice.

GENETIC MAPPING IN MOUSE CHROMOSOME 7, USING MEIOTIC RECOMBINATION DATA FOR RFLPs; COMPARISON WITH HUMAN 11p

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L. B. Russell

One method explored for localization of DNA sequences to c-locus deletions involved crossing some of these deletions to *Mus spretus* (see preceding abstract). Male progeny from such crosses is invariably sterile, but female progeny was crossed to a c^{ch}/c^{ch} stock, or to C57BL/10, to generate meiotic recombination data for RFLPs. This procedure (reported previously by Benoit *et al.*, Nature 314: 181-183, 1985), relies on the high degree of polymorphism between *M. musculus* and *M. spretus*.

Altogether 44 backcross offspring were studied to analyze five previously unmapped Chromosome-7 sequences for which RFLPs had been identified by us, as well as three sequences and one phenotypic marker (c^{ch}) that have earlier been mapped to specific loci on Ch 7. The most probable gene order was inferred by arranging the sequences in all possible permutations and determining which order would minimize the number of double crossovers that would have to be postulated. The following order requires no doubles:

Centromere - *Ldh-1* - *Hras-1* - *c* - 12A - *Hbb* - (*Ct*,*Pth*) - CLX18 - H19

[*Ldh-1*, lactate dehydrogenase-1; *Hras-1*, Harvey murine sarcoma virus; *c*, albinism; 12A, clone otained from Dr. C. Disteche, which we mapped to a

segment marked by the c-region "juvenile survival" functional unit (see preceding abstract); Hbb, hemoglobin, beta-chain; Ct, calcitonin; Pth, parathyroid hormone; CLX18, an anonymous sequence obtained from a flow-sorted Is(7;X)1 Ct chromosome; H-19, an anonymous cDNA coding for a protein subject to the same regulation as alpha-fetoprotein, obtained from Dr. S. Tilghman.]

This makes possible the following gene-order comparison for mouse Chromosome 7 and human 11p.

Mouse 7: Ldh-1 - Hras-1 - Hbb - Ct, Pth
Human 11p: LDHA - CALC1 - PTH - HBBC - HRAS1

(corresponding loci are Ldh-1 and LDHA; Hras-1 and HRAS1; Hbb and HBBC; Ct and CALC1; Pth and PTH)

This comparison suggests that a minimum of one rearrangement has occurred between the two species in the region defined by these 5 loci.

(We appreciate the collaboration of Dr. D. Housman, Massachusetts Institute of Technology.)

MOLECULAR GENETIC ANALYSIS OF REGIONS OF THE MURINE GENOME ASSOCIATED WITH RADIATION-INDUCED MUTATIONS

E. M. Rinchik D. K. Johnson[3]
C. C. Cummings

The complexity of the mammalian genome is realized both in terms of its organization and structure at the DNA level, and in terms of the myriad different functions and diverse developmental processes that the genome must specify and then control throughout the life cycle of the organism. Heritable mutations of the mouse allow the visualization of specific developmental pathways that have gone awry, and can provide valuable insights into the structure and function of the mammalian genome. We are exploiting the unique resource of the Section's large array of radiation-induced mutations to develop both detailed molecular and functional maps of selected small "model" regions (as opposed to specific genes) within the mouse genome. Through the integrated use of recombinant DNA technology (which allows both the identification and analysis of expression of single genes), and classical genetic and cytogenetic analysis (which allows a correlation between basic DNA defects and the organismic consequences of such defects), we hope to relate the structure and function of these regions to the study of both normal and abnormal mammalian development.

Over the years, the Section's germ-line mutagenesis program has generated (and continues to generate) a valuable array of induced mutations

at several specific loci scattered throughout the murine genome. Many of these mutations (particularly those induced by radiations) are multilocus deletions of chromosomal DNA. Genetic analysis of these types of lesions has detected passenger mutations of wide-ranging effect and severity, and has generated gross functional maps of entire chromosomal regions. We have initiated a program to expand the molecular analysis of these types of deletion mutations. This program exploits the deletion mutations and other chromosomal rearrangements to obtain molecular clones of wild-type DNA that map to regions absent in mutants carrying the (often deleterious) deletions. These clones will then be used to correlate the resultant molecular/physical map of the chromosomal region with the genetic/functional map in both mutant and wild-type individuals. Such correlations are essential to a strategy for identifying as many of the genes as possible in a particular region of the genome and for ascertaining their role(s) in the normal development of the mouse.

We have selected the c (albino) and p (pink-eyed dilution) regions of mouse chromosome 7 as model genomic regions of substantial length on which to develop this type of genetic structure-function analysis. We have defined the c- and p- "regions" as those segments of chromosome 7 that are missing in the radiation-induced deletion mutations c^{26DVT} and $p^{46DF10D}$, respectively. The 26DVT deletion is anywhere from 6 - 10 cM in length (perhaps 6000 - 20,000 kilobases of DNA), whereas the 46DF10D deletion is at least 2 cM in length (perhaps 2000 - 4000 kilobases or more). Complementation analyses of a large series of c-region deletions (Russell et al., Genetics 100: 427, 1982) have shown that a large variety of genes (all critical for normal development of the mouse) is deleted in the 26DVT mutation. An on-going complementation analysis of a series of p-region mutants is yielding similar results.

In our first year at Oak Ridge, we have concentrated on solving technical and logistical problems encountered in obtaining, and then subsequently mapping, large numbers of random DNA genomic clones specific for the c- and p-regions. We have, where genetically possible, generated banks of DNA derived from individuals who are compound heterozygotes for various independently-derived radiation deletion mutations. Certain combinations of c-deletions are viable or cause death postnatally; hence, DNA can be obtained from certain compound heterozygotes carrying overlapping deletions within the c-region. These homozygously-deleted DNAs have then been utilized in pilot genomic subtractive hybridization (GSH) experiments. The GSH technique allows one to enrich for genomic sequences present in a wild-type genome that are absent in a homozygously-deleted genome. The analysis of clones obtained in this pilot experiment is ongoing; in fact, the homozygously-deleted DNAs are being used to ascertain which, if any, of the subtraction clones derive from the c-region. Because a large series of independent deletion mutations has been used in deriving these banks of compound deletion-heterozygote DNAs, the DNAs can subsequently and ultimately be used to map random clones derived from GSH experiments [as well as from other procedures (see below)] with respect to the many deletion breakpoints carried in these mutants. This will

facilitate the ordering of molecular clones within the comparatively large c-region with a minimum of transmission genetic analysis.

Because several of the longest deletion mutations also delete genes required for embryo survival at implantation, it is impossible to obtain DNA from deletion-homozygotes; hence, mapping of clones with respect to these deletion breakpoints is more difficult. We have therefore initiated a series of genetic crosses between c- and p-deletion heterozygotes carrying "early-lethal" (preimplantation or implantation) deletions and Mus spretus, a different species of Mus. Again, as a part of resource-building that will aid in the mapping and characterization of DNA clones, these crosses will balance a c- or p-deletion opposite a spretus chromosome; DNAs obtained from these F_1's will be of crucial importance for accurate mapping of clones, by RFLP analysis, with respect to deletion breakpoints carried in some of the longer c- and p-radiation mutants.

We are also attempting to develop a chromosome-7-enriched DNA library to aid in obtaining random c- and p-region clones. We are collaborating with R. E. Hand, Jr. of the Cancer Section to isolate a pure population of the T(X;7)5R1 translocation chromosome by fluorescence-activated sorting. This translocation chromosome, which carries the wild-type c- and p-regions, is 1.4 times the length of the longest mouse chromosome, and thus should be amenable to efficient chromosome sorting based on DNA content. We have developed an SV40-transformed cell line that carries the translocation, and are currently working out conditions for isolating the T(X;7)5R1 chromosome.

As a third approach to isolating random clones from these regions, we are collaborating with Dr. T. Magnuson at Case Western Reserve University, Cleveland, in microdissection and microcloning of the c- and p-regions from the T(X;7)5R1 translocation chromosome, and will correlate any microclones obtained by his laboratory with our emerging physical and functional maps of the c- and p-regions.

Though this project is at a very young stage, we hope that gaining molecular access to a large number of sites within a given chromosomal region will provide a basic framework on which to build and extend a detailed analysis both of the constitution and structure of specific areas of the murine genome and of the role(s) of identified, as well as hitherto unidentified, genes in mammalian development.

GENETIC ANALYSIS OF MULTIPLE CLASSES OF *STEELOID* MUTATIONS: PRELIMINARIES FOR DEVELOPMENTAL ANALYSIS OF PLEIOTROPIC EFFECTS

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The original *Steel (Sl)* mutation discovered at this laboratory three decades ago (Sarvella and Russell, J. Hered. 47: 123-128, 1956) is expressed in the heterozygote by slight lightening of coat color and a small white head (and sometimes belly) spot. Homozygotes are severely anemic and die in late fetal stages. Since the original report, over 60 presumed mutations at the *Sl* locus have arisen at Oak Ridge and have been provisionally named *Steeloids*. We have found some of these *Steeloids* to be apparently inseparably associated with reciprocal translocation, having one breakpoint in chromosome 10, presumably at the *Sl* locus. Four of these were studied more extensively and found to be T(5;10)9R1, T(10;12)10R1, T(10;17)11R1, and T(10;18)12R1 (Cacheiro and Russell, Genet. Res., Camb. 25: 193-195, 1975).

In the past year, we have analyzed data (comprising about 100,000 offspring) from our numerous crosses involving the remaining 60+ *Steeloid (Sld)* stocks. About 10% of the mutations occurred spontaneously; the remainder occurred among offspring of male or female mice exposed to various types of radiations (X rays, gamma rays, neutrons, bomb tests, internal emitters), or chemicals (ENU, MNU, EMS). At least one (and, possibly, two) additional translocations were found, one of these being of spontaneous origin.

Although several intercrosses are being made among *Steeloids*, our first analysis involves three standard crosses made in each stock: (a) *Sld*/+ (of either sex) x +/+ ($C3H_f$); (b) *Sld*/+ x *Sld*/+ (sibs); and (c) *Sld*/+ x Sl^{1XY}/+ (one of the translocation mutants, chosen because it is homozygous viable, black-eyed white). The last of these crosses is essentially on allelism test. At least four of the *Steeloids* have, to date, failed this test and may be mutations at one or more other loci. Homozygotes of all four of these mutants survive well past birth and have very little or no hair pigment but probably fully pigmented eyes.

Another 55 of the *Steeloids* have produced black-eyed whites in combination with Sl^{1XY} and are thus assumed to involve the *Sl* locus. These 55 mutants encompass a variety of classes. Some are fully viable in the homozygous state, others are of reduced viability, still others die neonatally, and one group dies prenatally. Wherever homozygotes are viable, they have very little or no hair pigment but probably fully pigmented eyes. Where viability is reduced, and particularly in the neonatally lethal class, anemia is evident. When survival is compatible with breeding tests, again various classes are found, both among

homozygotes and in combinations with Sl^{1XY}: males fertile, females sterile (many stocks); both sexes fertile (one translocation mutant); both sexes barely fertile; males poorly fertile, females sterile.

Steeloid mutations have clear pleiotropic expression, involving at least three systems: hemapoeitic, reproductive, and hair (but not eye) pigment. The wealth of different types of *Steeloids* provides considerable opportunities for studies in developmental genetics (see following abstract).

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1. Russell, L. B. Information from specific-locus mutants on the nature of induced and spontaneous mutations in the mouse. In: Genetic Toxicology of Environmental Chemicals, Part B., ed. by C. Ramel. Alan R. Liss, Inc., New York, 1986, pp. 437-447.

ANALYSIS OF TIME OF DEATH OF PRENATALLY LETHAL STEELOID MUTATIONS

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Deletion mutations have been extremely useful in initiating the functional and molecular dissections of regions of the mouse genome. For the d-se and c regions, for example, it was observed that radiation mutations carrying lethal factors separable, by complementation analysis, from the primary d, se, or c mutation itself, could often be associated at both the genetic and molecular levels with multilocus chromosomal deletions (Russell, Mutat. Res. 11: 107, 1971; Rinchik et al., Genetics 112: 321, 1986; Gluecksohn-Waelsch, Cell 16: 225, 1979; Russell et al., Genetics 100: 427, 1982). Since many of the Oak Ridge *Sld* mutations arose in radiation mutagenesis experiments (see preceding abstract), a substantial number may carry chromosomal deletions that involve the *Sl* locus in chromosome 10. Because of the great value of deletion mutations for the genetic and molecular analysis of chromosomal regions and complex genetic loci, we have initiated a series of experiments designed to test whether radiation-induced *Sld* mutations carry other lethal factors, in addition to the lethality caused by "severe" alleles of the *Sl* locus itself, as one prescreen for identifying *Sld*'s that are caused by deletions.

The macrocytic anemia observed in *Sl/Sl* homozygotes and other *Sl* compound heterozygotes is evident from day 14 of gestation, and produces a recognizably "pale" fetus that possesses a pale, underdeveloped fetal liver. In *Sl/Sl* homozygotes, the anemia is severe enough to cause death before birth; hence, *Sl/+* x *Sl/+* crosses never produce black-eyed white *Sl/Sl* live-born offspring, but these homozygotes can be detected as anemic

fetuses late in gestation. On the other hand, some *Sld* alleles are less severe, thus allowing the birth of (usually) black-eyed white offspring from crosses between *Sld*/+ heterozygotes. Still other *Sld* mutations mimic the effect of *Sl* in that no recognizable *Sld/Sld* offspring are born in crosses between *Sld*/+ heterozygotes.

We have begun an analysis of 15 radiation-induced *Sld* mutations that comprise a part of this last class (no recognizable *Sld/Sld* offspring born). We are examining the uterine contents of Sld^{x}/+ females crossed by Sld^{x}/+ males (where "x" represents a specific radiation-induced *Sld*) at 16.5 day post coitum for fetuses manifesting the typical *Sl*-like anemia. Four such mutations have been analyzed to date: Sl^{7DThWb}, Sl^{FxFr}, Sl^{24FrS}, and Sl^{32HATh}. The Sl^{7DThWb} and Sl^{32HATh} mutations are congenic on the C3Hf background (N60 and N13, respectively); the Sl^{FxFr} and Sl^{24FrS} mutations (as all *Sld* mutations not already congenic on C3Hf) are currently being backcrossed to C3Hf to create congenic strains. As indicated, these four *Steeloid* mutations are all alleles of the *Sl* locus: each mutation, when crossed to Sl^{1XY}/+ heterozygotes, gives black-eyed white (presumably Sld^{x}/Sl^{1XY}) heterozygotes (see preceding abstract). Fertility tests (still ongoing) of these compound heterozygotes can be summarized at this time as follows: Sl^{7DThWb}/Sl^{1XY}, 5/8 males, 0/7 females fertile; Sl^{32HATh}/Sl^{1XY}, 10/14 males, 0/12 females fertile; Sl^{FxFr}/Sl^{1XY}, 5/6 males, 0/2 females fertile; Sl^{24FrS}/Sl^{1XY} 2/2 males, 0/4 females fertile. Results of the final allelism confirmation crosses (Sld^{x}/Sl^{1XY} x +/+) are 0 wild-type/ 127 offspring (7DThWb), 0/200 (32HATh), 0/82 (FxFr), and 0/39 (24FrS).

Three of the mutations (Sl^{7DThWb}, Sl^{FxFr}, Sl^{24FrS}) produce obviously anemic fetuses at 16.5 days from Sld^{x}/+ x Sld^{x}/+ crosses, and, as expected, these comprise anywhere from 15-28% of the total embryos examined, depending on the mutation. One mutation (Sl^{32HATh}), however, has failed to yield any obviously anemic 16.5 day fetuses in these crosses, and we have observed a slight increase in number of absorption moles relative to both the number normally found in the background strain (C3Hf) and the number found in the C3Hf-Sl^{7DThWb} congenic.

Thus, at least three radiation-induced *Sld* mutations do not appear to cause death before the typical lethal period of the original *Sl* allele, and it is likely that these particular *Sld/Sld* fetuses are dying from anemia during late gestation, much like *Sl/Sl* homozygotes. Thus, the nature of these mutations must await further molecular genetic analysis, since there is yet no evidence for mutation of another function (besides *Sl* itself) in these mutants.

The Sl^{32HATh} mutation, however, may carry an additional recessive lethal factor(s) that causes embryonic death earlier than the typical "late" *Sl/Sl*-like death. This result is consistent with, but does not prove, deletion of the *Sl* locus, along with a portion of chromosome around it. Alternatively, the Sl^{32HATh} mutation could be a simple mutation within the *Sl* locus itself that results either in an "ultra-severe" *Sl* allele that causes death earlier in gestation or in an allele that causes death in late gestation without the typical anemia syndrome. This latter of two

alternatives appears unlikely at present because of the increased number of absorption moles seen in the C3Hf - Sl^{32HATh}/+ intercrosses as compared to C3Hf crosses.

We shall continue this type of analysis on the remaining radiation-induced *Sld* mutations that give no obvious *Sld/Sld* offspring. In addition, we will initiate deficiency-mapping experiments with the closely-linked (3 ± 2 cM) Pep-2 gene, as well as other genetic and cytogenetic experiments, to ascertain how many (if any) of the *Steeloid* mutations are indeed deletions. This information, in turn, will be utilized to develop a gross genetic deletion map of the region of chromosome 10 that includes the pleiotropic *Steel* gene(s) as well as other gene functions necessary for normal development of the embryo.

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INVESTIGATION OF THE PROCESS OF X INACTIVATION THROUGH ANALYSIS OF OTC ACTIVITY IN X-AUTOSOME TRANSLOCATIONS

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Though the single-active-X-chromosome hypothesis is now 25 years old, the question of how any signal for activity/inactivity received by an X comes to affect an entire chromosome is still unresolved. X-autosome translocations [T(X;A)s] provide potentially excellent tools for an approach to this problem, since the rearrangement has distributed the X to two different chromosomes, the X^A and the A^X (where A is any autosome). In studying XX^AA^XA females, the question can be asked whether, in those cells in which the intact X is active, both X^A and A^X are inactive or only one (or part of one). Using this approach in the past with a group of T(X;A)s discovered in our laboratory, we concluded that an inactivation center or region was located in the central portion of the X chromosome, somewhere between the R2 and R6 breakpoints [(R2 and R6 designating different T(X;A)s], and that inactivation spread from there in both directions to contiguous parts of the chromosome. (See review by Russell, 1983, Progress and Topics in Cytogenetics 3A: 205-250. Note: no existing translocation had given evidence for more than one center, but it is at least possible that a future one might do so, if the X breakpoint were located between the centers, which both would have to lie between R2 and R6.)

For all T(X;A)s, except R2, all meaningful evidence on inactivation vs noninactivation is confined to that translocation product (X^A or A^X, in different cases) that includes the R2-R6 region of the X. In R2, evidence is, in addition, available for the reciprocal chromosome (i.e., the one lacking the R2-R6 region). This evidence was based on studies with three autosomal (Chromosome 7) loci, (c, Mod-2, and sh-1), all of which were shown not to be subject to inactivation. It was of interest, therefore, to obtain evidence also on an X-linked locus in that chromosome, as well as on other T(X;A)s. The locus chosen was sparse-fur, spf, the wild-type allele of which codes for ornithine transcarbamoylase, OTC (DeMars et al., Proc. Natl. Acad. Sci. USA 73: 1693-1697, 1976), an enzyme whose activity can be readily quantitated. The quantitation procedures were refined in Dr. Jacobson's laboratory.

For this first study, we chose two T(X;4)s (namely, R1 and R8) and two T(X;7)s (namely, R5 and R2). The first-named translocation in each set (R1 and R5) has a long X^A and short A^X, and the second-named (R8 and R2) has a long A^X and short X^A. In all four T(X;A)s, the long translocation product includes some or all of the R2-R6 region and has given evidence for inactivation of autosomal and/or X-linked loci. The spf locus is on the long X^A chromosome in R1 and R5, and on the short X^A chromosome in R8 and R2.

Crosses to spf were made for all four of the T(X;A)s. In R1 and R8, these crosses were of the type R(+b/b ♀ x b/b; spf/Y ♂; in R5 and R2, they were R(+p+c)/p c^{ch} ♀ x p c^{ch}/p c^{ch}; spf/Y ♂. With these mating schemes, all progeny classes can be identified by external phenotype. (Possible crossovers between breakpoint and the closely-linked autosomal marker, which might lead to misidentification of a small proportion of the male progeny, were identified by weighing the testes of all sons; testes weight is greatly reduced in translocation heterozygotes). In addition, genotypes not obtainable from the cross (e.g., spf/Y males) were analyzed in the parental stocks. Over 40 animals have been tested to date.

Enzyme activity, stated as micromoles of citrulline per mg of liver, averaged only 1.0 for spf/Y males. In the +spf/+spf, +spfY and R(+spf)/+spf groups, activities averaged between 27 and 29, and the average for R(+spf)/Y was only slightly lower (ca 24). As expected on the single-active-X hypothesis, +/spf females were more variable than the other groups; the average activity was slightly above half of the normal level namely ca 16. The critical class is R(+spf)/spf. In the case of R1, R8, and R5, the activity level for this class resembled that for +spf/sfp; while, in the case of R2, full activity was observed.

The R2 result confirms the earlier findings for autosomal genes located on the short X^A chromosome (see above): in the absence of a contiguous R2-R6 region, neither X-linked nor autosomal genes appear to be subject to inactivation. (This evidence is our first one for an X-chromosome locus). Similarly, the R1 and R5 results are as expected: there is inactivation of spf on the long X^A chromosome that includes the R2-R6 region.

What is unexpected on the assumption of a single inactivation center is the R8 result. If the X breakpoint is, in fact, located distal to spf, this would mean that both the short X^A (carrying the spf locus) and the long A^X (carrying the b locus, known to have mosaic expression in females) are subject to inactivation. This would, in turn, imply either that an inactivation region has been split into two parts, or that there are at last two inactivation centers, one located between R2 and R8 and one between R8 and R6. (Note that both would still lie within the R2-R6 region).

It must be noted, however, that the present localization of the R8 breakpoint (by genetic and cytogenetic means) is not yet as accurate as that for the other T(X;A)s used in this study. There is at least a slight possibility that R8 could be proximal to spf, and this would nullify the dramatic conclusion. To obtain evidence on this point, we will check the spf location in R8 by means of in situ hybridization with a mouse OTC DNA probe recently developed at Yale. If the probe is found to hybridize to the short chromosome, the conclusion will be justified that there is more than a single X-inactivation center, or that there is an inactivation region that can be divided by rearrangement.

STUDIES ON T(X;7)18Rℓ, AN X-7 TRANSLOCATION IN WHICH NON-INACTIVATION OF ONE TRANSLOCATION PRODUCT IS DEMONSTRABLE

L. B. Russell J. W. Bangham

We have this year continued studies with the X-autosome translocation T(X;7)18Rℓ (abbreviated R18), which in several respects resembles R2, previously unique in providing evidence of non-inactivation in one of the translocation products (see preceding abstract). R18, the seventh T(X;7) to arise at our laboratory, was found in a specific-locus experiment among offspring derived from ENU-exposed spermatonia. Since T(X;A)s are unable to pass through male meiotic divisions, it is likely that R18 arose spontaneously in a postmeiotic stage.

R18 resembles other T(X;A)s in the following general features: some autosomal genes are inactivated in females but not in males; females are semisterile or infertile, small, of reduced prenatal viability, and with slightly reduced ability to raise their offspring; males are totally sterile, but otherwise normal. We have this year weighed all offsprings at weaning. Paired comparisons show that R18 females weigh only 75.1% as much as their non-R female littermates, while the weight of R18 males is 101.0% that of their non-R male littermates.

Although production of offspring in the R18 stock is very slow, we have added considerably to information needed for breakpoint mapping by obtaining testes weights on almost all male offspring in the stock. The

testes of translocation heterozygotes are only about one-fourth normal weight and male segregants can therefore be simultaneously classified for translocation genotype and color markers. To map the Chromosome-7 breakpoint, we have carried out a 3-point cross with c^{ch}, p, and *R18* (the italicized symbol is used to designate the breakpoint). Among 427 classified offspring of both sexes, we have observed no *R18*-c recombinants. The c-p (or *R18*-p) recombination frequency is 10.1%. Since, as in R2, the c locus in R18 is not subject to inactivation, while the p locus is, we assume that the order is centromere - p - *R18* - c. R18 appears to cause slight crossover inhibition.

We have observed two p-variegated males in the R18 stock. While cytogenetic analysis is still incomplete, it is likely that these males are AA^XX^AXY. If so, they presumably resulted from 3:1 segregation in R18 oocytes. A relatively high 3:1 or adjacent-2 segregation may, additionally be inferred from the finding that the average litter size of R18 females is less than one-third that of their non-R18 sisters. The p-variegated (presumed AA^XX^AXY) males, unlike AA^XX^AY males, are small — only 79% the weight of like-sexed littermates. This indicates that the X is inactivating vital autosomal genes in these two-X males, as it does in AA^XX^AX females.

SYNAPTIC BEHAVIOR OF A NEW TRANSLOCATION T(X;7)18Rℓ AND THE HYPOTHESIS OF G-SYNAPSIS

T. Ashley N. L. A. Cacheiro[1]
L. B. Russell

The synaptic behavior of T(X;7)18Rℓ (abbreviated R18) has been studied by synaptonemal complex analysis of spermatocytes from translocation heterozygotes. A small amount of nonhomologous synapsis was observed. Varying degrees of nonhomologous synapsis had been found by us in some, but not all, X-autosome translocations we have studied. The R18 results led us to reexamine each of these other translocations in an attempt to determine whether degrees of nonhomologous pairing might be correlated with any variables.

We made the discovery that those translocations that have both breaks in G-light bands (as detected by G-band analysis of mitotic chromosomes) exhibit only homologous synapsis during early meiotic prophase, while those translocations that have one or both breaks in a G-dark band exhibit varying degrees of nonhomologous synapsis. These observations have led us to suggest that if a translocation has meiotically aligned nonhomologous sequences, G-light chromatin has the ability to recognize homology during zygotene and to cease synapsis. However, if a translocation has meiotically aligned G-dark chromatin with G-light chromatin, the G-dark

chromatin does not recognize lack of homology and continues synapsis. We have termed this type of nonhomologous synapsis "G-synapsis".

RELATION OF NON-HOMOLOGOUS SYNAPSIS FOUND IN SYNAPTONEMAL COMPLEXES OF T(X;4)1Rℓ MICE TO LOCATION OF THE TRANSLOCATION BREAKPOINTS

T. Ashley[1] L. B. Russell

The synaptonemal complexes of T(X;4)1Rℓ (abbreviated R1) translocation heterozygotes have been examined by electron microscopy and compared with those of two X-7 translocations: R5 and R6. The X-chromosome breakpoint of R1 is estimated to lie between 78 and 82% from the proximal end of the X, in the same general region as the R5 and R6 breakpoints. The position of the autosomal breakpoint of R1, like that of R6, is about 30% from the centromeric end of the respective autosome, the R5 breakpoint is slightly more proximal.

Despite these general similarities between the three translocations, there are differences in synaptic behavior. In R1 and R6, there is extensive nonhomologous synapsis, both in quadrivalents and in heteromorphic bivalents, while in R5, all synapsis is confined to homology. The translocations also differ in the location of breakpoints relative to the position of the G-bands. These results, when added to those found for other rearrangements, fit the hypothesis summarized in the preceding abstract. Thus, in R5, both breaks of the reciprocal translocation lie in G-light bands, and synapsis is strictly homologous. In R1 and R6, at least one break lies in or immediately adjacent to a G-dark band, and there is nonhomologus synapsis. Comparison of the synaptic behaviors also leads to the conclusion that G-band-related nonhomologous synapsis is different from the "synaptic adjustment" phenomenon previously described by Moses (1977). The G-band-related nonhomologous synapsis is not substage specific, but competes with homologous synapsis during zygotene-early pachytene.

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EXCESSIVE NONHOMOLOGOUS SYNAPSIS IN T(X;16)16H HETEROZYGOUS MALE MICE SUGGESTS A TRANS-CHROMOSOMAL EFFECT OF G-DARK BREAKS

T. Ashley[1]

In examining the pairing behavior of male mice heterozygous for the T(X;16)16H translocation by synaptonemal complex analysis, extensive nonhomologous synapsis was observed (involving 38% of chromosome 16). One of the translocation breakpoints is located in the very distal portion of band XD, on the border of XE, a G-dark band; therefore, on the basis of the hypothesis summarized above, some nonhomologous synapsis was expected. However, the amount actually observed was considerably greater than anticipated. It may be suggested that nonhomologous synapsis of the G-dark chromatin in band XE (on the X chromosome) with G-light chromatin on chromosome 16 proceeded into the G-dark band 16C3 (on chromosome 16) and triggered a second round of nonhomologous synapsis of the dark 16C3 band with G-light chromatin on the X. The data suggest that nonhomologous "G-synapsis", triggered by a G-dark-band break in one of the translocated chromosomes, can cascade from one chromosome to another with which it is meiotically paired.

RNA SYNTHESIS IN SPERMATOCYTES OF STERILE X-AUTOSOME TRANSLOCATION MALES

T. Ashley[1] L. B. Russell

All X-autosome translocations studied to date produce sterility in male heterozygotes, with spermatogenesis ceasing in pachytene. It has been suggested (Lifschytz and Lindsley, 1972) that this sterility is caused by the reactivation of X-chromosomal material (normally inactive in spermatocytes) by the contiguous autosomal segments. Thus, reactivated X transcription could result in translation of a "nonpermissible" gene product in the spermatocytes.

During normal spermatogenesis, extensive transcriptional activity (as measured by ^{3}H-uridine incorporation into RNA) can be detected in autosomal bivalents, but little or none in the sex chromosomes. Testing of the above hypothesis about the sterility of T(X;A) males requires a high-resolution technique that can distinguish X-chromosomal from autosomal portions of the translocated chromosomes with regard to ^{3}H-uridine incorporation. The microspreading technique routinely used for electron microscopic analysis of synaptonemal complexes should provide sufficient resolution. However, this procedure does not preserve the chromatin in sufficient quantity for study.

We have been able to modify the technique so as to increase the retention of chromatin. We can now demonstrate ^{3}H-uridine incorporation in

the autosomes and little incorporation (above background) in the sex chromosomes of nontranslocation mice. Although the technique still requires modifications, it is already evident that it will provide sufficient resolution to distinguish between incorporation over the X and autosomal portions of translocation chromosomes.

(We acknowledge the technical assistance of F. Shrode, University of Tennessee.)

CYTOLOGICAL STUDIES OF STERILITY IN MALE PROGENY OF MUTAGEN-EXPOSED MALE PARENTS

N. L. A. Cacheiro[1] C. V. Cornett

We have found that genetic male sterility results primarily from certain types of balanced reciprocal translocations, and, more rarely, from certain numerical sex-chromosome anomalies. Sterile male offspring sired following exposure of male meiotic or postmeiotic stages to radiation or chemical mutagens are not uncommon and are predominantly carriers of balanced translocations. The chromosome breakpoints of these translocations are generally located near the ends of autosomes or anywhere in the sex chromosomes. Sterile offspring from mutagen-treated spermatogonial stem cells, on the other hand, are not only rare but most of them have no readily detectable chromosomal anomalies. Those that have are either carriers of balanced translocations, or are sex-chromosome trisomics or mosaics (XYY, XXY, XY//XYY, XO//XY, etc.). The nature of the exchange in spermatogonia-derived sterile translocations is not clear and awaits detailed study. In addition, the data base is not yet sufficient for determining the role of induced nondisjunction in the production of genetic male sterility. Accordingly, we have an ongoing project that attempts to study associations between the nature of genetic fertility impairments and the detailed chromosomal genotype, and between chromosomal causes of sterility and the nature of mutagenic treatments that produce them.

In studying sterile males found as offspring in mutagenesis experiments, we check epididymal sperm and testis weight, after which testes are prepared for cytogenetic studies. If meiotic stages are present, diakinesis or metaphase-I configurations are analyzed for the presence of multivalents, and these are classified as rings, chains, III + I, etc. If blockage occurs prior to meiosis, testicular mitotic metaphases are checked for the presence of excessively long or excessively short chromosomes as a first indicator of unequal translocations. Numerical chromosome anomalies can also be recognized in such preparations. Finally, kidney cultures are initiated, and cells are harvested for the study of banded chromosomes in order to identify breakpoint locations.

From a large study to determine the effect of radiation dose rate in the induction of heritable reciprocal translocations in stem-cell spermatogonia (see Generoso et al., p. 72, this volume), we have, so far, analyzed 188 sterile male progeny — 175 from experimental groups and 13 from the control group. Among the 175 sterile males from experimental groups, 157 appeared karyotypically normal, 4 carried balanced translocations, and 14 were sex-chromosome aneuploids. All of the 13 steriles from the control group appeared normal. A detailed karyotype study of the 4 translocations is currently underway.

More sterile male progeny from the experimental group are expected to be generated in the ongoing dose-rate project. Completion of these studies would add to our understanding regarding (1) the nature of chromosomal exchange among sterile translocations, and (2) whether induced nondisjunction significantly contributes to the male-sterile pool, as seems to be indicated by the data to date.

REGULATION OF PTERIDINE BIOSYNTHESIS AND OF PTERIDINE FUNCTION

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E. B. Wright

The pathways for the biosynthesis of biopterin, the cofactor for synthesis of mammalian neurotransmitters and of tyrosine, and for the biosynthesis of drosopterins, the red and yellow eye pigments of Drosophila, share common intermediates in the first three enzymatic steps. These intermediates are dihydroneopterin triphosphate, 6-pyruvoyltetrahydropterin and 6-lactoyltetrahydropterin. An investigation of the mammalian enzymes in the pathways for biopterin synthesis has been initiated as the study of the Drosophila system is being phased out.

Two technical developments have been accomplished that will facilitate studies on pteridine biosynthesis. First, a method was developed for the isolation and stable storage of dihydroneopterin triphosphate. This compound is the substrate for the enzyme that produces 6-pyruvoyltetrahydropterin. The highly purified enzyme from E. coli GTP cyclohydrolase is used to convert GTP to dihydroneopterin triphosphate, this product is extracted from the reaction mixture with two solid phase cartridges C18 Bond Elut (Analytichem) and Silica Sep Pak (Waters). In earlier studies, we had prepared the dihydroneopterin triphosphate in 0.5-1 ml batches daily, but now we can perform a large-scale reaction (50 ml) in an Amicon cell, filter off the product to be extracted as described above, and reutilize the enzyme for 6-10 successive reactions. The dihydroneopterin is then stored at -80° and has proved to be stable for at least four months. This large-scale preparation of the substrate will be a marked improvement in the way we conduct studies on pteridine biosynthesis.

A second technical accomplishment was the evaluation of a variety of pteridines for their affinity for C18 Sep Pak cartridges. We found that certain pteridines, e.g. sepiapterin, 6-acetyldihydrohomopterin and "compound X", have a high affinity for C18 Sep Pak and they can be isolated from complex mixtures. Other pteridines, such as biopterin, 6-formylpterin, 6-hydroxymethylpterin, 7.8-dihydrobiopterin, isoxanthopterin, pterin and 7.8-dihydroneopterin have an intermediate affinity; and others have a weak affinity, e.g. neopterin and xanthopterin. Furthermore, the high affinity can be altered by causing the pterin to acquire a positive or negative charge. For example, when sepiapterin is at pH > 11, it becomes negatively charged and does not adsorb to C18 Sep Pak. For another example, when a complex with Ni^{2+} is formed, the pterin fails to adsorb to C18 Sep Pak. Since the affinity of sepiapterin for Ni^{2+} is 0.001 that of 6-acetyldihydrohomopterin, the separation of these two pterins can be accomplished by adjusting the Ni^{2+} concentration. The C18 reversed-phase cartridge is therefore quite useful for fractionation of mixtures of pteridines.

Several years ago, we discovered that a chemical reaction occurs when dihydroneopterin triphosphate is heated at 80-100° in Tris pH 8.5. Several products are formed, all of which resemble a yellow pterin-like substance. These are called X, X1, X2 and X3. Although we have been unable as yet to establish their structure, a number of properties are known: they have a pterin-like UV spectrum and a greenish-yellow fluorescence at neutral pH. Further study in collaboration with W. Pfleiderer (University of Konstanz, W. Germany) established that further modification of the spectra occurs for X and X3; when first dissolved in 0.1 N HCL, the absorption maximum is at 450 nm, but within 30 min that peak disappears and is replaced by one with a maximum at 390 nm. The peak of X1 is at 381 nm immediately, on the other hand, and the material degrades to pterin-6-carboxylic acid. The relative polarity was judged by their chromatographic mobility on C18 reversed-phase column and is a follows X2 < X < X1 < X3. Another indication of relative stability is seen during storage; in water, X is converted to X1, whereas in 10% methanol, X is stable. This may indicate the presence of an unstable carbonyl group. The new high resolution mass spectrometer that was recently installed in the Analytical Chemistry Division will be available for further studies on these compounds and may help resolve the structure.

The biosynthesis of biopterin and other pterins begins with the formation of dihydroneopterin triphosphate from GTP and its subsequent conversion to 6-pyruvoyltetrahydropterin. Tetrahydropterins are unstable in that they are rapidly oxidized in the presence of oxygen to form dihydropterins. Therefore, their study requires anaerobic conditions. We have exploited the lactate oxidase system of bacterial membranes to achieve an oxygen-free solution in which tetrahydrobiopterin, for example, is stable. This enzyme is part of the inner membrane of E. coli, and the membrane fragment has been purified by ammonium sulfate precipitation and chromatography on BioGel A-50m, a matrix that fractionates very large molecules and small particles by a sieving mechanism. The pH optimum, substrate specificity, temperature stability and storage properties of this

purified membrane have been evaluated. Also, the effectiveness for stabilization of tetrahydrobiopterin has been examined as a function of the membrane concentration and the time required to de-oxygenate a solution. We conclude that this purified membrane fraction is effective in producing anaerobic conditions and its state of purity is favorable for it application in biochemical studies.

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Carcinogenesis Section

SECTION OVERVIEW - R. J. M. FRY

The Carcinogenesis Section is concerned with studies of (1) the mechanisms of carcinogenesis at the molecular, cellular, tissue, and whole-animal level, and (2) both the fundamental and the pragmatic aspects of risk estimates for radiation-induced cancer. Our studies in this latter area, a special responsibility of DOE, involve members of other sections and divisions and collaborative studies in other laboratories. The wide range of expertise and special radiation facilities required for some of these studies can only be found in the national laboratories.

The exciting advances in molecular biology have raised hopes of revealing, in the near future, the mechanisms involved in carcinogenesis. However, as is so often the case, it has become clear that the mechanisms are complex, not only at whole animal level, but also at the molecular and genetic levels. These complexities are well illustrated by the work of the Molecular Genetics of Carcinogenesis group (W. K. Yang). The possibility is being investigated that retrotransposable genes inherited in the germ line of the mouse may be activated and be associated with the instability of the genome of neoplastic cells. This work has established that the sequences related to MuLV found in the DNA of RMF/Un mice are proviral in nature and are a distinct endogenous proviral gene family. The examination of the DNA of various members of the Mus species indicates that MuLV-related proviruses date back to before the development of inbred laboratory strains. The various studies are aimed at investigating what elements of the genome are involved in carcinogenesis and what role transposition plays.

Normal development growth and differentiation depend on orderly control of gene expression. Abnormal expression can lead to various lesions. The Regulation of Gene Expression group's (F. T. Kenney) research is concerned with the mechanisms of gene expression and its control at the molecular level. The genes of developing and adult livers provide an appropriate model. The expression of the genes of developing hepatocytes can be modulated by agents such as the demethylating agent 5-azacytidine. Thus, the mechanisms of gene expression and its control can be probed. Expression of some hepatocyte genes is regulated by hormones. For example, insulin accelerates the rate of transcription of the relevant genes. The question is, how does insulin which is bound to a membrane receptor increase the specific gene transcription? The search is on for the regulatory elements that respond to insulin.

A new approach to the assay of mutations is being developed. The assay depends on using hybridization probes to analyze restriction-fragment length polymorphisms. The probes are moderately repeated elements. The

assay, that is still undergoing development, is being tested on mice with a deletion involving the c locus on chromosome 7 that was induced by radiation in the studies of the Mammalian Genetics and Reproduction Section. This is a most appropriate test system because the assay is being developed to increase the ability to detect radiation-induced mutations which characteristically involve deletions.

There are marked tissue- and species-dependent differences in susceptibility to the induction of cancer by chemical carcinogens and also by ionizing radiation. Currently there is little understanding of the genetic basis of such differences. Since no metabolism is essential in the mechanism of radiation carcinogenesis, there must be important factors other than metabolic differences that are involved in species and tissues differences in carcinogenesis. However, it is also clear that there are all important sex-, tissue-, and species-dependent differences in the metabolism of chemical carcinogens. The associated differences in tumor induction are marked, or even complete. Another group is studying the differences in the metabolic pathway of benzo(a)pyrene [B(a)P] in order to identify the relationship of the pathways and their kinetics to the susceptibility to malignant transformation. Identification of subpopulations of cells *in vitro* that differ significantly in the metabolism of B(a)P and subpopulations that differ significantly in susceptibility to transformation is providing appropriate model cell systems to test associations, and, hopefully, causal relationships. The cost in time in identifying all the relevant enzyme systems and the metabolites is high. Thus, a major effort has been devoted to the development of what is termed computer-assisted quantitative two-dimensional gel electrophoresis. An enormous quantity of data can not only be obtained, but winnowed to identify, with relative ease, the pertinent differences in proteins produced by different treatments.

A major effort of the Section is devoted to radiation carcinogenesis, an area of research that is recognized as an important part of the mandate of DOE. Two very different aspects of these radiation studies are important. First, radiation provides the most appropriate agent for studying the mechanisms of induction of cancer. The knowledge of the initial biophysical changes, gene mutations, deletions, and rearrangements that result from exposure to radiation is unequaled in comparison to other agents. Furthermore, dose can be measured and the distribution of energy deposition can be determined with precision. This precision makes it possible to determine the size of the targets involved in biological effects, such as cancer induction. Second, certain essential radiation studies can be carried out only in special facilities. Some of these facilities are unique and almost all of them are in national laboratories. For example, we have the only facility in the world in which it is possible to undertake studies of the effects of fission spectrum neutrons protracted over a complete day or longer. Such facilities make it possible not only to carry out experiments to answer essential pragmatic questions related to risk estimates but also incisive studies on mechanisms. Thus, the Radiation Carcinogenesis group has attempted to build on two assets, namely, the broad range of expertise available in the division and the

special radiation facilities. One good example of the use of these assets has been a collaborative study between the Radiation Carcinogenesis group and the Comparative and Cellular Genetics Section to investigate the role of specific chromosome aberrations in the induction of myeloid leukemia by neutrons. The first of the publications reporting this work is in press and part of the study is included in the Comparative and Cellular Genetics Section of this report. The association between specific chromosome aberrations and certain types of leukemia is now accepted but the important question of whether or not the induction of specific chromosome aberrations is associated with the induction of leukemia remains unanswered. Radiation is the inducing agent of choice, and the RFM mouse, developed many years ago in this division, is a suitable model system for the study. In fact, the possibility of an association between a chromosome alteration and the myeloid leukemia found in this strain was considered by Wald and Upton long before the idea of such associations had become popular. The studies are strengthened by parallel investigations of the molecular basis of the myeloid leukemia in the RFM mouse by the Molecular Genetics of Carcinogenesis group.

It is obvious that initiation is essential for cancer induction but what has not been so obvious is that initiation is not the major determinant of whether or not a cancer develops and becomes of clinical significance. The communication between cells and their ability to suppress growth of transformed members in a tissue may also involve factors lumped into the term "growth factors." Normal tracheal cells inhibit the growth of neoplastic cells when mixtures of normal and neoplastic cells are used to reseed tracheas. The possibility is being investigated that the inhibition is, in part, mediated by transforming growth factor type B. Clearly cell-cell communication is an important aspect of the control of tumors by host factors.

The use of mammary and tracheal *in vivo*/*in vitro* systems is proving very useful for investigating the changes and processes involved in the development of cancer. The changes occurring in the long latent period between exposure and tumor appearance are difficult and expensive to study in the whole animal and the use of the available *in vitro* systems is inappropriate. Sequential changes in the cellular phenotype are being detected in both the tracheal and mammary cells that have been exposed to radiation. In the case of the mammary cells there is some evidence of reversion of the initial events. Obviously an understanding of reversion would be very important, especially if therapeutic interference becomes a possibility.

The association of various growth factors with oncogenes and their normal cellular counterparts has brought together two facets of cell and tissue growth that until recently have been disparate.

The study of growth factors and their receptors has become an essential part of the investigation of the mechanisms of cancer. S. J. Kennel's group has identified a protein that is found on the surface of murine cancer cells. This tumor surface protein (TSP) with a molecular

weight of ~180,000, and thus is called TSP-180, has some of the characteristics of known growth factor receptors. Monoclonal antibodies to TSP-180 have been used to determine whether the protein, and at what level, occurs in various tissues, and in benign and malignant tissues. A protein that is very similar to TSP-180 has also been identified on cells of human carcinomas. Currently, the studies are aimed at establishing whether or not the protein is a specific receptor.

The expertise in monoclonal antibody technology of this group is not only applied to the studies of other groups but also to the identification and quantitation of cells with certain surface markers, especially macrophages. The assay of dioxins which is difficult at the low levels, that may be of biological importance, has also been developed using monoclonal antibodies.

Although the studies of the various groups may appear to be diffuse there are common features that are salient to the objective of determining the mechanisms of cancer induction. The mutual benefits of the various independent but related studies are considerable.

MOLECULAR GENETICS OF CARCINOGENESIS

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Two aspects are considered to be of importance in our studies on the molecular genetic mechanisms of carcinogenesis. One is the gene rearrangement event frequently observed in neoplastic cells and the other is the genetic control of neoplastic phenotype expression initiated by an oncogene alteration. To investigate the first aspect, our working hypothesis is that the genomic instability of neoplastic cells is associated with the activation of mobile gene elements in the cell genome; and the subject of our experimental work is the retrotransposable genes inherited in the germ line of the mouse. To investigate the second aspect, our initial efforts have been to develop an *in vitro* cell system for demonstrating the dominant control of neoplastic phenotype expression involving ras oncogenes. In the past year, progress has been made mainly on molecular and functional characterization of murine leukemia virus-related (MuLV-related) proviral sequences and MboI-repeat LTR-containing (MRL) elements isolated from mouse chromosomal DNA and also on exploiting the DNA transfection approach for the detection of controlling factor(s) of neoplastic phenotype expression.

MuLV-Related Proviruses in the Mouse Genome (Characterization of a molecular clone of RMF/Un mouse chromosomal DNA that contains a full-length

endogenous murine leukemia virus-related proviral genome). Although there are 30 to 50 copies of MuLV-related proviral sequences per haploid genome of the mouse, previous attempts to isolate these LTR-containing proviral sequences by molecular cloning were unsuccessful either due to the choice of restriction enzymes that cut within the proviral structures or due to apparent selection of clones with deletions in the proviral structural genes. In this work, a lambda phage recombinant library was constructed using HindIII chromosomal DNA fragments of RFM/Un strain mouse. Of the 20 molecular clones isolated by screening with an LTR molecular probe, one of them, designated pRFM#6, was found to harbor an apparently intact endogenous MuLV-related proviral genome. The proviral genome, including two long terminal repeats (LTRs) and sequences in between, was approximately 9.3 kilo-base-pair long and marked by restriction enzyme sites known to be common to many mouse endogenous MuLV-related proviral genomes. Two structural features, a distinct 170-200 bp sequence segment in the LTR U3 region and a specific binding site implicating the use of glutamine tRNA as the primer for reverse transcription of minus strand DNA, were also found. The 5' and 3' LTRs, respectively 699-bp and 700-bp long, were of identical nucleotide sequences except three base changes in the U3 "enhancer" area. At the cell-provirus DNA junctions, 4-bp direct repeats were present. The presumed transcriptional regulatory signals in U3 as well as the sequences and sizes of U5 and R found in pRFM#6 LTR were similar to those in the LTR of an endogenous ecotropic MuLV isolate of the RFM/Un mouse, but the middle U3 regions were highly divergent. Nucleotide sequence analysis of the 3' pol gene predicts an encoded endonuclease of 390 amino acids, which is 15 amino acids shorter than the deduced sequence for AKV. With respect to nucleotide sequences in the 5' portion of the env gene, pRFM#6 provirus is closely related to the MCF type MuLV, but not to the xenotropic MuLV. The same chromosomal DNA site in BALB/c, AKR, C3H and CBA strains of mice, but not in NSF/N and C57BL/6, was found to harbor this proviral genome, suggesting its integration into the mouse germ line after speciation of the mouse. This work provides the first conclusive experimental evidence for the provirus nature of MuLV-related sequences in the mouse chromosome and also for establishing them to be a distinct endogenous proviral gene family.

MuLV-Related Proviruses in the Mouse Genome (Nucleotide sequence analysis of other pRFM molecular clones of chromosomal DNA harboring endogenous MuLV-related proviral sequences). Using the pRFM series clones, we performed nucleotide sequencing analysis to answer two additional specific questions: (a) What characteristic features may be present in the env gene of the MuLV-related proviral gene family, which contain 750-800 bp size LTR and can be represented by pRFM#12? (b) What is the nature of sequence deletion(s) in MuLV-related proviral genomes, which appear to be frequently isolated by molecular cloning and may be represented by pRFM#16 and pRFM#17?

(a) From previous restriction enzyme and nucleotide sequence analyses, it is evident that the MuLV-related proviral genome in mouse chromosomal DNA can be divided into two major gene families, one possessing 700 bp LTR and the other possessing 750-800 bp LTR. As demonstrated from analysis of

pRFM#6 and pRFM#16, env gene sequence of MCF type specificity (MCF 247 virus isolate) is present in the MuLV-related proviral genome family that contain the 700 bp size LTR. In the pRFM clone series, the 750-800 bp size LTRs are found in pRFM#1, pRFM#9, pRFM#12 and pRFM#17. An HindIII restriction site is present at the 3' end of pol in this provirus family; hence, pRFM#12 is the only clone that harbors the env and 3'LTR sequences whereas the other 3 clones harbor the 5' portion of the proviral genome. We therefore determined the nucleotide sequences at the 5' region of env gene that presumably specifies the envelope type specificity. Nucleotide sequences elucidated in pRFM#12 env gene are nearly identical to those found in pRFM#6 and the MCF 247 virus isolate. This result clearly indicates that the env gene sequences that determine the MCF type specificity can be found in both the two major families of endogenous MuLV-related proviral genomes in mouse chromosomal DNA.

(b) The proviral DNA sequences in pRFM#16 and pRFM#17 are interesting mainly in two aspects. First, restriction enzyme mapping results suggested that there were apparent sequence deletions in the gag and pol gene regions of these clones. Second, many investigators, including us, have frequently isolated molecular clones of apparently defective endogenous MuLV-related sequences from mouse chromosomal DNA. It is not known whether this reflects whether the content of these defective proviral sequences in the mouse germ line or the molecular cloning conditions favor their selection. To answer this question, we subcloned the BglII fragments containing the deletion (698 bp from pRFM#16 and 569 bp from pRFM#17) and determined their nucleotide sequences. A common deletion of 1487 bp starting from the 3' end of gag and ending in the 5' portion of pol gene was found; in known proviral DNA nucleotide structures (e.g., Moloney MuLV and AKV), this 1487 bp sequence is flanked by 7 bp direct repeats, ACTGCCC. An additional 113 bp deletions was found downstream in the pol region of pRFM#17 and it was also deleted from a distinct 7 bp direct repat, CAGGCAA. These results indicate that sequence deletion of endogenous MuLV-related proviral genome occurred from specific segments flanked by 7 bp direct repeats. These sequence deletions apparently alter the reading frame of proviral structure genes. By the use of the BglII 698 bp gag-pol sequence fragment as a molecular probe for Southern gel analysis, it is possible to detect corresponding gag-pol fragments with the deletions in mouse chromosomal DNA; however, the 698 bp and the 569 bp fragments detected represented the minor population of the homologous sequence fragments found in the mouse chromosomal DNA. The major MuLV and related BglII gag-pol fragments detected were apparently intact and could be distinguished in three kinds (i.e., 2.2 kbp, 1.2 + 1.0 kbp and 3.7 kbp). Detection of 3.7 kbp in many mouse strains but not in the NFS/N strain suggests that this 3.7 kbp fragment is likely derived from the ecotropic MuLV provirus. Examination of various Mus species chromosomal DNA preparations revealed that the defective proviruses, as represented by the 0.7 and 0.6 kbp BglII gag-pol fragments, were present in all strains of *Mus musculus domesticus* and *Mus spraetus*, but not in *Mus musculus musculus* CechII nor in other *Mus species* while the apparently intact 2.2 kbp BglII gag-pol sequence fragments were prominent in *Mus musculus domesticus*, *Mus musculus musculus* CechII and *Mus spraetus*. In DNA preparations from *Mus cervicolor papeus*,

Mus caroli, and Mus pahari, DNA fragments of less hybridization intensity and different sizes were observed. These results indicate that the majority of endogenous MuLV-related proviruses were stably integrated into the mouse germline before the establishment of inbred laboratory mouse strains but after speciation of Mus musculus.

MRL Elements of the Mouse (Molecular cloning of an internal sequence fragment of a unique male-specific MRL retro-transposable gene element from chromosomal DNA of NFS/N mouse). Examination of mouse chromosomal DNA by Southern gel analysis using molecular probe of the "D" fragment has revealed that male mice possess unique MRL elements not found in female mice. A few attempts were made, but in vain, to molecularly clone an EcoRI fragment of NFS/N mouse DNA that harbors the male-specific element. Comparison of male mouse DNA and female mouse DNA, using different restriction endonucleases and molecular probes from various structure gene region of a MRL element clone, pIS23, suggested that a 1.5 kbp male-specific SstI DNA fragment could be generated from the 3' end of a male-specific MRL element, and also gave an erroneous impression that this SstI DNA fragment would likely include the 3' flanking cellular sequence. A NFS/N mouse chromosomal DNA fraction enriched in this SstI fragment was inserted into a lambda phage vector, and subsequent screening was successful in isolation of a desirable recombinant phage clone that contained the male-specific SstI fragment. The insert of this phage clone was transferred into the plasmid vector pUC12 and the resultant recombinant plasmid was designated pNFS10. Restriction enzyme mapping and nucleotide sequence analyses of the insert, NFS10, were performed. It was found that NFS10 (or the 1.5 kbp SstI fragment) is an internal segment of the male-specific MRL element rather than an element-cell junction fragment. Its nucleotide sequence could be matched at low (i.e., 50-60%) homology levels to the 3' portion of pol gene of MuLV genome, except a non-homologous block of about 150 bp corresponding to the 5' end of the endonuclease-coding sequence. This non-homologous block is distinctive in two aspects: (a) it is present in the male-specific MRL element but not in other MRL elements, and (b) it consists of four repeating units of short sequences that bear similarity to the "enhancer"-like consensus sequence. Further Southern gel analysis of male and female mouse DNA preparations, using various restriction enzymes, revealed that the unique sequence unit, called A2, or a homologous sequence is also present elsewhere in the mouse Y chromosome.

Retroviral Endonuclease (Insertase) Proteins (Construction of plasmid expression vectors to express the pol 3' sequences of ecotropic MuLVs and endogenous MuLV-related proviral DNA clones). Recently it has become known that 3' portion of pol gene in the provirus structure encodes a functional proterin, presumably with endonuclease activity, which plays an essential role in the proviral DNA integration process. We have determined the nucleotide sequence of this region in several molecular clones of endogenous MuLV-related proviruses and found open reading frames that are highly homologous, especially in the middle part of the coding sequence, to that of ecotropic MuLV. Thus, these endogenous MuLV-related proviral genomes appear to have the potential of producing functional endonuclease or insertase that may cause DNA breakages in the cell. In view of the fact

that one of the possible outcomes of this enzyme expression is the instability of cellular genome, we have initiated a systematic study on this protein. The presumed endonuclease-coding segments in ecotropic MuLV clones, pWN41, pWB5 and pG104, and in endogenous MuLV-related proviral DNA clones, pAL10 and pRFM#6, were constructed into a lac-Z-promoter-carrying plasmid expression vector in correct reading frame and introduced into E. coli hosts. Upon induction, the bacterial hosts were found to produce a 46 kd protein from the ecotropic MuLV sequence and a 45 kd protein from the pAL10 proviral sequence. These proteins could not be solubilized under non-denaturation condition but could be purified by SDS polyacrylamide gel electrophoresis for the purpose of immunizing rabbits and mice. A potent rabbit antiserum preparation has been obtained, which is suitable for Western gel blot analysis purpose. Works to produce monoclonal antibodies are also in progress.

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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 mechanisms operate during differentiation, and how they are regulated by hormones and other specific effectors. We focus primarily on liver and on genes that are both developmentally and hormonally regulated in that tissue. During this year we have also worked toward development of a general assay for mutations of the deletion or translocation type, by direct analysis of DNA. Significant progress has been made in a number of experimental approaches.

Differentiation. Earlier analyses of mRNA or protein gene products have established that a number of genes that are expressed in adult liver are essentially silent in fetal liver, expression rising abruptly to the adult level shortly before or after birth. To proceed further in analyses of the mechanism of these activations it was necessary to determine at what

level of the readout of genetic information the developmental change occurs. We assessed the rate of gene transcription in isolated nuclei in livers from fetal and newborn rats for three such genes for which cDNA probes are available: tyrosine aminotransferase (TAT), p-enolpyruvate carboxykinase (PEPCK), and an unusually regulated gene that we have isolated and characterized but whose protein product is not yet identified (gene 33). Rates of transcription of each of these increased in extent and at times during development that are consistent with earlier measurements of the gene products; hence, the developmental activation of their expression is a transcriptional phenomenon.

We have established that full expression of these genes can be induced precociously by treatment of late term fetuses *in utero* with the DNA demethylating agent, 5-azacytidine. The mRNAs of all three genes were increased to levels equal to or greater than those of adult liver. We have now measured the rate of transcription of these genes after treatment of fetuses with 5-azacytidine, and find that transcription is only slightly enhanced, the increases being inadequate to account for the large increases in levels of the respective mRNAs. These data suggest, then, that the acceleration by this drug of liver differentiation must be attributable to effects other than, or in addition to, its capacity to bring about demethylation of DNA.

Mutant mice designated c^{14CoS} carry a radiation-induced deletion on chromosome 7 that results in aberrant differentiation of the liver; current data indicate that the deleted region, normally produces a *trans*-acting product that is required for normal developmental activation of genes such as TAT and PEPCK (gene 33 is not affected). Since expression of TAT and PEPCK can be precociously activated in rat fetuses by 5-azacytidine, we asked if this drug might bypass the requirement for the *trans*-acting regulatory gene in development of the liver in mutant mice. In two such experiments we have observed activation of TAT expression in mouse fetuses treated with the drug; the gene is activated in homozygous mutants (albino) as well as in heterozygotes or homozygous normal fetuses, albeit to a somewhat lesser extent. Activation of TAT expression was also increased in several experiments with newborn mutants. Thus it is clear that the hypomethylating agent can bring about the activation of TAT expression that normally requires the product of the putative regulatory gene on chromosome 7. Whether this is due to demethylation of DNA and/or other effects of the drug remains to be determined. Progress on this project has been disappointingly slow, owing to intolerance of these mice to the surgical procedures required for treatment of fetuses *in utero*. Other approaches to circumvent this problem are being explored.

Hormonal Regulation. We have established that expression of both TAT and gene 33 in adult liver is rapidly and markedly enhanced by insulin, via acceleration of the rates of transcription of these genes. These effects of insulin are known to be initiated by interaction of the hormone with its membrane-bound receptor, but there is no understanding of how this interaction at the cell membrane becomes manifested in the control of transcription of specific nuclear genes. Gene 33 has been characterized by

restriction mapping and partial sequencing, and we have subclones containing defined regions of the gene and ca. 5 kb of 5'-flanking rat DNA. To aid in finding and defining the putative insulin-responsive regulatory element(s) of this gene, we have initiated a search for DNase I hypersensitive sites that may be related to its control by insulin. These experiments have not yet progressed to the point where definitive data are available. If one or more insulin-responsive sites are detected, we will proceed to construction of fusion genes to define the putative insulin regulatory element(s) and then search for cellular components that may interact with the DNA to enhance transcription.

In a related approach to this question we are collaborating with Drs. J. Larner (Univ. Virginia) and A. Larner (NCI). These investigators are using a cloned fragment of gene 33 that contains the 5'-transcriptional start site, a short transcribed region downstream from the cap site, and ca. 1.5 kb of 5'-flanking sequences that include TATA and CAAT promoter elements in their typical positions. This fragment is incubated in a cell-free transcription system that is capable of initiation. Results to date are promising; transcription of the fragment proceeds well. Analysis of putative intracellular mediators of insulin action (discovered by J. Larner and colleagues) to determine if any of these can enhance the rate of transcription is currently under way.

Preparation of Reagents. A simplified and inexpensive method for synthesis of large double-stranded cDNAs was developed, using cloned reverse transcriptase, RNase H, and DNA polymerase I. Large mRNAs from rat liver were selected by sucrose gradient fractionation and used as template, followed by cloning of the cDNA products in *E. coli* using conventional procedures. Two percent of the clones tested contained cDNAs specific for TAT, and of these most were full-length (2.4 kb). A smaller but significant fraction consisted of gene 33 cDNAs, the largest of which (as yet uncharacterized) appeared to be a few hundred base pairs short of a full-length copy of this mRNA (3.4 kb). About 1000 bp of gene 33 cDNA were sequenced, and we aim to sequence the complete cDNA in order to aid in identification of the protein gene product. We also intend to introduce the complete cDNA into an expression vector that produces large amounts of protein in *E. coli* and then prepare antisera to the purified protein to characterize its expression as well as its structure. Other objectives of this project include the introduction of the complete cDNAs into mammalian cells via expression vectors in order to study the structural features of TAT and gene 33 mRNAs that may be responsible for their rapid intracellular turnover. Earlier data suggesting the involvement of liver-specific factors in rapid turnover of both mRNA and protein products of these genes will also be evaluated by introduction of the "pseudogenes" into various types of cultured mammalian cells.

Mutation Assay. Our objective here is to develop a general assay for mutation anywhere in the genome by analyzing for restriction fragment length polymorphisms using as hybridization probes moderately repeated elements known to be scattered throughout the chromosomes. For this developmental/feasibility study we are using well-characterized mutant

mice carrying radiation-induced deletions involving the c (albino) locus on chromosome 7; these mutants were developed and characterized in the Biology Division's mouse genetics facility.

We have developed a "2D" gel electrophoretic method to improve sensitivity of the assay. Genomic DNA is cut with one restriction enzyme, and the fragments separated in a narrow agarose strip using a device of our design. This gel is then incubated with a second restriction enzyme and the fragments of the first digestion are cut again in situ. This is then used as the origin for a second electrophoresis, resulting in a wide array of fragments. After Southern transfer to nylon membranes, hybridization is done with cloned mouse DNAs labeled by nick translation. This results in a reproducible pattern of labeled spots and increases sensitivity about tenfold. We are also currently exploring the feasibility of using periodic electric field inversion methods (Carle et al., Science 232: 65, 1986) to separate large DNA fragments such as those generated by "infrequent cutter" restriction enzymes.

Our second immediate goal has been to characterize cloned probes that could be expected to hybridize to a large number of DNA fragments but not so many as to yield a meaningless blur. At present we are working with four probes that meet this requirement, all from retroviral or retroviral-like mouse DNA elements. Each of these yields a reproducible, but different, array of labeled bands (1-D gels) or of spots (2-D gels) upon hybridization after cutting genomic DNA with judiciously chosen restriction enzymes.

Our efforts to this point have been aimed almost entirely at methods development and we are just beginning to focus on differences in patterns that can be traced to radiation-induced deletions. Preliminary results using c^{14CoS} mutant mice are encouraging; there seems to be little doubt that these methods will be effective. When we have a confirmed positive result with this strain, we will extend the observations to other c locus mutants and to mutants at other chromosomal loci. A secondary but not unimportant outcome of this experimentation relates to our studies on regulatory genes controlling differentiation that are described above. Identification of genomic DNA elements that are deleted in the c^{14Cos} mutant can be expected to lead to cloning and characterization of the trans-acting regulatory genes.

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DETECTION OF PROTEIN PERTURBATIONS IN METABOLICALLY ACTIVATED CELLS

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Incomplete combustion of carbonaceous material appears to be the major source of the carcinogenic polycyclic hydrocarbons that are prevalent in the environment. Upon entering a biological system, this class of chemicals is recognized as foreign and the cell begins processing them for excretion. This processing is necessary because environmental carcinogens are often chemically non-reactive and cannot be excreted readily. The first processing step, or biological activation, increases the chemical reactivity of the carcinogen so that in the second, or detoxification step, the reactive metabolites can be made into the more polar dihydrodiol derivatives or metabolized further by conjugation with cellular sulfate, glucuronide and glutathione moieties for eventual excretion. It appears that the normal cellular processing of parent carcinogens and mutagens results in the formation of highly reactive electrophilic intermediates which in addition to conjugation for excretion can also bind to nucleophilic sites in the cell such as DNA. Such metabolites are assumed to be the carcinogenic and mutagenic species of the parent molecule. Metabolite profiles for various chemicals from tissues and cells in many species have been determined. For any given carcinogen tested, all animal species form the same family of oxygenated metabolites and show similar patterns and relative magnitude of DNA binding. Therefore, the enzymatic array of activation and detoxification is common to most species. However, for a given carcinogen, there is a great variation in susceptibility to malignant transformation between species. It is critical to understand the genetic and biochemical mechanisms for this paradoxical situation. Why does a given carcinogen that is metabolized to produce the same putative reactive intermediate in two species exhibit a dose-dependent malignant transformation response in one species but no transformation in the other? It is important to understand if species variance to chemical carcinogenesis is a function of the genetic regulation of the array of enzymes involved in activation and detoxification of the carcinogen. In the past it has only been possible and practical to monitor a few enzymes in the metabolic scheme in any one experiment. Enzyme assays are sufficiently labor intensive so as to prohibit large experiments that would include many timepoints and various chemical treatments. However, high resolution two-dimensional gel electrophoresis (2-DGE) has the ability to simultaneously and reproducibly separate a large number of cellular proteins. This technology is currently being used in an attempt to enhance our understanding of the potential role of each component in the enzymatic pathways responsible for carcinogen processing. By demonstrating which cellular proteins are altered by chemical treatment, we may discover which components of the metabolic machinery play a role in determining the cascade of events required for cellular commitment to malignant transformation. Susceptible and resistant cells may differ by demonstrating perturbations or kinetic differences in the enzyme pathways responsible for

carcinogen processing so that we can predict which cells are at greater risk to chemical carcinogenesis.

Computer-Assisted Quantitative Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis is uniquely sensitive in its ability to separate polypeptides in the first dimension by isoelectric focusing as a function of polypeptide net charge and in the second dimension as a function of molecular weight. We have developed the capability of generating reproducible 2-DGE polypeptide spot patterns that make it possible to carry out computer image analysis and comparison of multiple images with ease and consistency. The enormous amount of data contained on these two-dimensional (2-D) gels is analyzed using sophisticated computing systems to systematically store, retrieve, compute, and compare data from multiple gels. We have developed, tested, and refined a series of computer algorithms to accurately detect polypeptide spots. The percent of total gel image radioactivity contained in each spot is determined and compared with corresponding qualitative and quantitative data for polypeptides in gel images obtained from electrophoresis of samples from different treatments.

In recent experiments we have used this 2-DGE system for detection of polypeptide profile differences between untreated, non-differentiated, Friend erythroleukemia cells (FELC) and FELC which had been chemically induced to differentiate into erythroid cells. Out of a total of 211 polypeptides found on the 2-D gels analyzed in this experiment, 114 polypeptides were found to be common to both treated and control cells and 65 polypeptides were unique to the control (untreated) cells and thereby repressed by treatment. The de novo synthesis of 32 polypeptides, including hemoglobin was shown to be induced in the treated FELC cells. Additionally, quantitative differences were found among 5 of the 114 corresponding control and treatment polypeptides found in the 2-DGE profiles. Three of these polypeptides were shown to be increased twofold to threefold over the control while two of these polypeptides decreased to about one-half of the control levels. We also followed the percent of total radioactive amino acid incorporation into hemoglobin throughout induction of differentiation utilizing the 2-DGE technique. The hemoglobin synthetic rates throughout the induction period, as measured from 2-D gel data, were corroborated by the standard benzidine staining assay for hemoglobin. Therefore, this system has proven to be sensitive to kinetic changes in individual polypeptides and capable of detecting qualitative polypeptide differences between treated and control cells.

Metabolic Variants of C3H 10T1/2 Cells Selected by Flow Cytometry. We have used flow cytometry, in collaboration with the Cytometrics group, to select two subpopulations of C3H 10T1/2 cells that differ in metabolism of benzo(a)pyrene [B(a)P]. B(a)P fluorescence has been used to identify and select cells that metabolize B(a)P rapidly and cells that do so significantly more slowly. The cells that metabolize B(a)P more rapidly produce within 24 h a greater amount of 9,10 and 7,8 diols, the precursors to the putative carcinogenic intermediate diol epoxides, than the slow metabolizing population. The fast metabolizing population produced 17.6%

and 7.6% of the 9,10 diol and 7,8 diol, respectively; the slow metabolizing population produced 11.3% and 1.3% of the 9,10 diol and 7,8 diol respectively. Each metabolite is expressed as a percent of total metabolism. Studies are under way to determine the transformation potential of these two cell populations. If transformation susceptible and transformation resistant C3H 10T1/2 subpopulations can be identified, then differences in cytoplasmic B(a)P processing will be studied using 2-D gel electrophoresis.

Benzo(a)pyrene: DNA Adduct Formation in Normal Human Mammary Epithelial Cell Cultures and the Human Mammary Carcinoma T 47D Cell Line. B(a)P/DNA adducts formed in normal human mammary epithelial cell cultures and human mammary carcinoma T 47D cell line were examined in collaboration with Donna Pruess-Schwartz and William Baird of the Department of Medicinal Chemistry and Pharmacology, Purdue University. Human mammary epithelial cell cultures and human mammary carcinoma T 47D cells were exposed to ^{3}H B(a)P for 24 h and the levels of binding were 81 and 182 pmol B(a)P/mg DNA in normal and T 47D cultures, respectively. Analysis of B(a)P:deoxyribonucleoside adducts resolved by immobilized boronate chromatography and reverse-phase HPLC demonstrated the presence of three B(a)P:DNA adducts in both cells: M2, MS1, and MS2 (adducts identified on reverse phase HPLC elution profiles). MS2 was identified as the (+)-anti-B(a)P diol epoxide (DE):deoxyguanosine (dGuo) adduct. MS1 was identified as a (-)-anti-B(a)PDE:dGuo adduct. Thus, both adducts that bound to the immobilized boronate column were formed from (±)-anti-B(a)PDE. One major adduct, M2, also found in both cells, was formed from (±)-7b,8a-dihydroxy-9b,10b-epoxy-7,8,9,10-tetrahydro-B(a)P or (syn-B(a)PDE). This work gave additional evidence that (-)-anti-B(a)PDE is formed in cell systems and reacts with DNA in cells to form (-)-anti-B(a)PDE dGuo adducts. The similarities between the B(a)P:DNA adducts formed in both cell types suggests that the T 47D cell is an excellent model for studies on metabolic activation of polycyclic aromatic hydrocarbons (PAH) to DNA binding intermediates in human mammary cells.

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RADIATION CARCINOGENESIS

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The radiation carcinogenesis research program encompasses studies of the carcinogenic effects of ionizing radiations as well as ultraviolet radiation. Research continues to emphasize neutron carcinogenesis, dose response and time-dose relationships, the dynamics of the carcinogenic process after exposure to radiation and/or chemical, and the role of host factors (e.g., cell-cell communications, growth factors, genetic background, and the immune system) in tumor development. These problems are being approached by the *in vitro* and *in vivo*/*in vitro* methods. The scope of the research is greatly facilitated because of numerous collaborative

research efforts with other investigators within the division and with investigators outside the division. The examination of mechanisms and the establishment of general principles which may allow an understanding of risks to humans from radiation exposure is a common emphasis among all the research projects described. Data from these studies are also being used to try to develop methods of extrapolation of animal data to human risks.

Neutron Carcinogenesis. A question of major interest in neutron carcinogenesis is the shape of the dose response for tumor induction after low dose neutron exposures. Two alternative models have been suggested, but current data do not allow either model to be rejected. The models are (1) a continuously bending response in which the effect increases as a function of the square root of the dose, or (2) a linear response. An experiment has been under way using fractionated exposures which is designed to clearly distinguish between these alternative dose-effect models. This is because these alternative models predict markedly different results following multiple low dose fractions. If the curve is continuously bending, then small dose fractions separated by sufficient time to preclude interaction should produce effects very much greater than those seen following a single exposure. If the curve is linear at low doses, however, then fractionation should produce the same result as a single exposure. We are conducting an experiment with 5600 mice to determine the shape of the dose response curve at low total doses by using single or fractionated exposure. The neutron exposures are now complete and we are obtaining complete autopsies on the mice at death.

Ullrich has previously reported that neutrons delivered at a low dose rate are more effective in producing mammary tumors in mice than are neutrons delivered at a high dose rate. His cellular studies also seem to support a conclusion of greater effectiveness at the low rate. Because of the great importance and potential impact of this finding, it is imperative that the animal experiment be repeated. Accordingly we are repeating the original mammary tumor induction experiment with the same mouse strain. The neutron exposures are nearly complete and we expect to obtain preliminary results during the coming year.

In vivo/in vitro methods are being used by Drs. Ullrich and Terzaghi to compare the effects of high- and low-LET radiation on mouse mammary epithelial cells and rat tracheal epithelial cells, respectively. Initial studies with mouse mammary cells suggest dose and dose rate dependent effects on the *in vitro* growth potential of these cells following *in vivo* exposure to both neutrons and gamma rays with neutrons being markedly more effective. These results differ somewhat from those obtained by Dr. Terzaghi with rat tracheal cells in that no dose rate effects have been obtained in the tracheal system (see below). Studies are currently under way further characterizing sequential cellular changes produced with respect to subculturability of these cells and their tumorigenic potential.

Studies by Dr. Terzaghi to compare the effects of low-LET (X rays) and high-LET radiation (neutrons) on rat tracheal epithelial cells have used cells which are irradiated either in cell culture or as part of the intact

tissue. Following irradiation, cells were seeded onto feeder layer cells and maintained for 1 wk in medium containing 0.2% fetal bovine serum. After 1 wk in low serum, the purpose of which is to limit the growth of fibroblasts, cultures were switched into medium containing 5% serum. At 4-6 wks the number of cells with altered growth capacity were quantitated. In previous experiments the data indicated that low-LET radiation did not induce populations with altered in vitro growth characteristics typical of chemical carcinogens exposed intact tracheal epithelium. Over the past year we have further evaluated radiation effects on tracheal epithelium. Tracheal cells irradiated in culture with both neutrons and X rays yielded cells with altered in vitro growth capacity. In contrast, irradiation of the intact trachea with low-LET radiation was without effect. High-LET radiation, however, appeared as effective in inducing populations with altered in vitro growth capacity in the intact tissue as in cell culture. These data suggest that low-LET radiation-induced damage is effectively repaired in the intact tissue while high-LET radiation-induced damage is apparently not repaired. Preneoplastic lesions were observed by 5-6 months after high-LET radiation. No such lesions have been observed following low-LET radiation of intact tracheas. We are currently further investigating the mechanism by which radiation-induced alterations are modified in the intact tissue.

In an effort to develop a better understanding of dose and dose-rate effects following neutron irradiation, efforts have begun to examine mutagenic effects of neutrons in collaboration with Dr. Katz, a postdoctoral fellow in Dr. Hsie's laboratory. These studies utilize a pSV2gpt transformed Chinese hamster ovary cell line designated AS52. Initial studies have shown that this system can provide data on dose and dose-rate effects over a wide dose range. Particularly important are results showing that this system will provide dose-response data at relatively low doses following exposure to either neutrons or gamma rays. These studies hae also shown that the AS52 cell system is sufficiently sensitive, reproducible, and flexible to examine dose-rate effects.

Time-Dose Relationships. Previous studies on the effects of low-dose rate and fractionated exposures to gamma rays indicated a reduced carcinogenic effect at low dose rates or with low doses per fraction. Current studies are aimed at determining the extent to which these effects are related to a reduction in the frequency of initiated cells or to effects related to progression. These studies utilize techniques which allow the examination of altered growth potential of irradiated cells. Altered growth potential has been examined in vivo by dissociation of mammary glands at various times after irradiation and injecting cells into gland free fat pads of 3-wk-old recipients. Alterations in the in vitro growth potential of these cells have been examined by culturing these dissociated cells. Using these methods, we have recently described several sequential phenotypic changes which appear to be involved in mouse mammary tumor development. These results are described more fully later in this report. With respect to dose-rate studies, results thus far suggest that certain of the radiation-induced alterations observed may be reversible in some instances. In addition, the results suggest a lower frequency of

subculturable epithelial foci following low-dose rate exposure compared to that obtained following high-dose rate irradiation.

Host Factors in Radiation Carcinogenesis. Numerous studies have demonstrated that radiation is able to transform cells. Evidence is also available which supports the hypothesis that host factors in the intact animal can play a significant role in determining whether these transformed cells progress toward the neoplastic phenotype and whether these neoplastic cells express their tumorigenic potential. Experiments are currently being conducted which are designed to investigate the relationship of natural incidence of specific tumors and their susceptibility to induction by exposure to radiation. These experiments are meant to provide information of both a fundamental and a practical nature. Whether the natural incidence of specific cancers determines the response to carcinogens has fundamental implications for the role of inherent host factors in tumor development. On the practical side this investigation will provide information required to determine whether relative risk estimates provide a reasonable basis for extrapolation across species. In these experiments tumor induction in two strains of mice, C57BL/6 and C3Hf/He, are being compaared following gamma irradiation. These strains were chosen because they differ widely in the spectrum of tumor types that develop spontaneously. Interest in the role of host factors is not confined to this experiment. Studies have recently begun which are examining underlying reasons for difference in susceptibility to mammary tumor development between BALB/c and C57BL/6 mice using the mammary tumor model described below. The modulation of tumor expression is also of interest in the tracheal carcinogenesis studies and in the UV skin carcinogenesis studies described in this report.

Mechanisms of Mouse Mammary Tumor Development. These studies have been directed toward the determination of sequential cellular changes in mammary epithelium after exposure to radiation and/or chemical carcinogens. These studies have used two techniques to try to examine phenotypic changes which occur with time following *in vivo* exposure. The first approach has examined mammary outgrowths produced from cells which were removed from animals at various times, ranging from 24 h - 1 year, after carcinogen exposure. This involved the injection of dissociated cells into gland-free fat pads of 3-wk-old recipient mice. Altered growth potential of cells in this cell dissociation (CD) assay could be detected as ductal dysplasias or as tumors. The second approach involved culturing dissociated cells which had been removed from animals at various times after carcinogen exposure. Altered *in vitro* growth potential could be characterized using this epithelial focus (EF) assay. Under the growth conditions used, carcinogen-exposed cells with altered growth potential were detected as proliferative epithelial foci, while non-altered cells senesced. These foci could be further characterized by examining their ability to be subcultured and their growth potential following injection into mammary fat pads.

Initial studies in this laboratory used the CD technique to examine early cellular changes of relevance to mammary tumorigenesis following

carcinogen exposure. In those studies, it was shown that within 24 h after carcinogen exposure mammary cells acquired the ability to yield ductal dysplasias in mammary outgrowths following injection into gland-free fat pads. Dysplastic outgrowths derived from cells injected 24 h after carcinogen exposure regressed in the absence of a growth stimulus. In outgrowths derived from cells obtained 1-16 wks after carcinogen, an increasing frequency of dysplastic lesions was found which were able to persist in the absence of a growth stimulus. These results suggested that the ability to autonomously maintain the altered phenotype was an event subsequent to the initial carcinogen exposure. The ability to fully characterize the cellular changes occurring with time after carcinogen exposure is limited with the CD technique because of the difficulties associated with identifying dysplastic lesions in viable mammary glands and isolating cells from these lesions. Studies usng the EF assay were initiated because this assay allows the characterization and identification of carcinogen-altered cells, based on their growth capacity *in vitro* in a manner analogous to the CD technique. Furthermore, the assay makes it possible to study biochemical changes in isolated carcinogen altered cell populations.

With the EF assay, cells which had acquired an altered *in vitro* proliferative potential (EF) were detected fairly early after carcinogen exposure and, with time *in vivo*, cell populations emerged which had acquired the ability to maintain that phenotype, i.e., EFs. While the EF frequency peaked at 4 wks, the percentage of EF which could be subcultured (EFs) increased as a function of time in both following carcinogen exposure. The decline in EF frequency may indicate that these carcinogen-induced alterations in proliferative potential are reversible in some instances. Differences with respect to altered proliferative potential *in vitro* and altered morphogenic potential *in vivo* are suggested by results obtained following injection of the EFs derived 1-16 wks post carcinogen treatment. Using the CD assay altered morphogenic potential expressed as persistent dysplastic lesions were found over this time period. However, injection into fat pads of EFs derived from these same cell preparations resulted in normal ductal outgrowths at 10 wks following injection. Subsequent studies with one of these cell lines (designated EF 42) have shown that tumors eventually develop from these normal appearing outgrowths but not until approximately 1 year after injection. Clearly many more studies are needed to develop a better understanding of the factors which control the expression of altered phenotypes in the mammary gland.

Both the CD and EF data suggest that cells continue through new phenotypic phases with time *in vivo* and *in vitro* following carcinogen treatment. EFs derived 1-16 wks post treatment required many *in vitro* passages before they displayed neoplastic growth *in vivo* while those derived 52 wks post treatment required only a relatively few *in vitro* passages. The continued acquisition of new phenotypes is further supported by additional results at the 52 wk time point. With the CD technique a new cell population was detected in cells dissociated 52 wks post carcinogen treatment which had acquired the ability to elicit a host response *in vivo*. The ability to elicit such a host response following injection was also

detected in the EFs derived from the same donor mouse cells using the EF assay. These observations, coupled with the fact that such responses are also associated with many of the preneoplastic ductal dysplasias which develop in the intact mammary gland 12-14 months after carcinogen treatment, suggest that the ability to elicit such a host response including angiogenesis may be an important characteristic for tumor development. Such a concept is not new and has been pursued by several investigators. The present results do, however, provide data suggesting that this is a late change in the process of neoplastic development in the mouse mammary gland.

Cell lines obtained using the EF assay have also provided the opportunity for the morphological and biochemical study of isolated carcinogen altered cell populations. One cell line designated EF42 is being extensively studied by S. C. McKarns, a graduate student in The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences. This cell line was derived from mammary tissue of a mouse exposed to 100 rad of γ rays 4 wks prior to cell dissociation. At low passage these cells contain a near 2C DNA content and produce normal ductal outgrowths *in vivo* 10 wks after injection (by leaving these cells in animals we have recently observed that tumors [5/16] develop from these normal appearing outgrowths approximately 1 year after injection). With time in culture these cells undergo several characteristic changes including loss of contact inhibition, an increase in DNA content (2C→4C), and rapid tumor formation (8-10 wks after injection). Because this cell line appears to offer opportunities to study neoplastic progression, a number of different parameters are being examined with this system. Of particular interest are results obtained from chromosomal analyses in collaboration with Dr. R. J. Preston. These results are discussed in Dr. Preston's section. Studies are also being conducted with this cell line and others which are attempting to study the possible role of oncogenes and of growth factors during tumor progression. These studies have required collaborative studies with Drs. Yang, Kennel, and Terzaghi within the Division and outside the Division with Dr. Walter Gunzburg of the Medical College of Georgia and Dr. Daniel Medina from Baylor Medical School.

Effect of Cell Interactions on Neoplastic Development. We have evaluated (1) the influence of cellular communications (requiring direct cell contact) or intercellular interactions (e.g., diffusible factors) between normal and carcinogen-altered rat tracheal epithelial cells (RTEC) cocultured *in vivo* or *in vitro*, and (2) whether carcinogen altered cells lose the capacity to alter or be altered by other populations as they progress from a normal to a neoplastic state. Normal and carcinogen-altered RTEC are co-cultured *in vitro* on irradiated 3T3 feeder layers or *in vivo* in denuded tracheal grafts. Following coculture *in vivo* or *in vitro* the relative proportions of diploid (normal) and aneuploid (carcinogen-altered) cells are determined. Repopulated tracheas and mixed cell cultures are evaluated histologically or mixed populations are harvested enzymatically and the proportion of normal to altered populations evaluated by flow cytometry or in cell culture.

Normal cells clearly exert a strong inhibitory effect on both preneoplastic and neoplastic cell in vivo and in culture. In vivo inhibition is observed when a small fraction (e.g., 10%) of normal tracheal or esophageal cell is inoculated with preneoplastic or neoplastic tracheal cells. However, when a comparable number of preneoplastic or neoplastic cells occupies a contiguous region of the reconstituted tracheal mucosa, no inhibition by normal cells is observed. In cell culture, neoplastic and preneoplastic tracheal cells are inhibited when cocultured with normal tracheal or esophageal epithelial cells. The inhibitory effect of normal cells on carcinogen altered cells in culture appears to be at least in part mediated by transforming growth factor type β. We are currently investigating the possible relevance of TGF-β to our observations of normal cell mediated inhibition of preneoplastic and neoplastic cells in the intact or reconstituted tissue.

One reported effect of TGF-β is the induction of terminal differentiation in human bronchial epithelial cells. We have characterized two monoclonal antibodies (3BG8, 9BG8) which appear to recognize differentiation-specific antigens on normal and carcinogen-altered epithelial cells. These antibodies are being utilized to evaluate the possible induction of differentiation in carcinogen-altered cells by normal cells and/or TGF-β in vivo and in vitro.

Ultraviolet and Ionizing Radiation Carcinogenesis in the Skin. We are investigating the following aspects of ultraviolet radiation carcinogenesis: (1) Dose-response relationships for the induction of skin cancer by ultraviolet radiation (UVR) and how they relate to the mechanisms involved; (2) dose-rate effects in the induction of skin cancer with UVR; (3) the basis of strain-dependent differences in susceptibility; (4) the role of the immune system in skin cancer, in particular, the response of Langerhans cells to UVR, X rays and PUVA and the effects on delayed-type hypersensitivity (DTH); and (5) the interactions of ionizing radiation with other agents such as UVR, PUVA and the well-known promoter 12-O-tetradecanoylphorbol-13-acetate (TPA).

We have almost completed a study of the effects of dose rate of UVR exposures on the induction of skin carcinomas. The experiment was designed to test the following hypothesis. There is good evidence that the degree of some biological effects of ionizing and ultraviolet radiation are cell-cycle stage dependent. For example, cells in DNA synthesis are more susceptible to UVR-induced cell killing than in other stages. There is suggestive, but not unequivocal, evidence that the initiation of cancer may also be influenced by the stage of the cell cycle in which cells are exposed. If there is a particularly sensitive stage, the duration of this stage in relation to the cell cycle time will determine the number of cells at risk for cancer initiation in any one acute exposure. Assuming all other factors being equal, a reduction in dose rate should increase the numbers of cells passing through the window of susceptibility and therefore, the cancer induction rate. In the case of low-LET radiation, of course, the ability to repair induced lesions is thought to be greater with low dose rates and therefore, results in a reduced carcinogenic effect. In

the case of the mouse, UVR-induced pyrimidine dimers, which are thought to be the lesions of interest in UVR carcinogenesis, are not repaired by simple excision repair. We have established that with exposure to the low dose rates used in the cancer studies the efficiency of pyrimidine dimer induction is the same as at high dose rates. Therefore, any increase in cancer rate by reduction in exposure dose rate might be attributed to an increased number of cells being exposed in a susceptible stage of the cycle. This hypothesis has been involved as an explanation of the finding that protracted exposures to neutrons cause more malignant transformation than single exposures.

The high dose-rate exposures were given in 6.25 min compared to 6 h, 15 min for the low dose rate. In UVR carcinogenesis it is necessary to use multiple exposures and it is possible that dose rate might increase or decrease the "promoting" effect of the later UVR exposures. In order to maximize the expression the initiated cell groups exposed to either high or low dose-rate exposures were treated with TPA.

In the low dose-rate group, the cumulative incidence of papillomas was 55% and of carcinomas 48%. In the case of high dose-rate exposures the incidences of papillomas and carcinomas were both 68%. The average time of appearance of neither papillomas or carcinomas appeared to be influenced by dose rate. These findings do not suggest a marked difference in susceptibility among stages of the cell cycle.

We have initiated a collaborative study of the induction of subcutaneous sarcomas by various agents. The intention is to determine changes at a molecular, chromosomal, and cell surface level in sarcomas induced by radiation, chemical carcinogens, and plastic discs. The findings should identify both common and distinct changes that may result from the differences in the mechanism of induction. For example, R. J. Preston and his colleagues are identifying the chromosomal abnormalities associated with the various initiating agents, and W. K. Yang and K. N. Noghrei-Nikbakht are identifying the oncogenes that are activated. We are sampling subcutaneous tissues at various times after treatment and establishing cultures of the fibroblasts in the preneoplastic and early neoplastic time. Cytophotometric assessments and dilution assays are used to track nuclear changes and immunological responses.

It is hoped that as a result of these experiments, not only will certain common end distinct features of sarcomagenesis become clear but that we will have profiles of the various initiated tumor cells. It seems possible that these profiles may make it possible to distinguish tumors on

the basis of the causative agent. Currently there are very few tumors for which the causative agent can be identified with reasonable success.

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MONOCLONAL ANTIBODIES FOR DIAGNOSIS AND THERAPY

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Basic Carcinogenesis

Cell surface proteins mediate interaction between cells and their environment. In addition to uptake of nutrients, cells receive specific stimuli from growth factor-receptor interaction at the cell surface. Growth factors affect specific cell types in different ways, and it is now clear that aberrations of the normal factor-receptor function can lead to malignancy. Studies on growth factors and receptors identified as proteins analogous to oncogene products are under way in numerous laboratories. We have been studying a protein which has several characteristics in common with known growth factor receptors, but differs from those described to date.

A tumor surface protein of 180,000 Mr (TSP-180) has been identified on cells of several lung and mammary carcinomas of BALB/c mice. TSP-180 was not detected on normal lung tissue, embryonic tissue, or reticulum cell or other sarcomas, but it was found on lung carcinomas and a melanoma from other strains of mice. Considerable amino acid sequence homology exists among TSP-180s from several cell sources, indicating that TSP-180 synthesis is directed by normal cellular gene(s) although it is not expressed at high levels in normal cells. The regulation of synthesis of TSP-180 and its relationship to normal cell surface proteins are being studied with the hypothesis that it may represent a receptor molecule for growth regulation factors.

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 from those with the original MoAb. Two MoAbs were used to develop a "two site" assay for TSP-180. This assay allows quantitation of low levels (ngs/mg protein) of TSP-180 in normal and neoplastic tissue. Results indicate that tumors have 10-100 times more TSP-180 than do normal tissue. Of the normal tissues, trace amounts of TSP-180 can be detected in lung and leg muscle, but not in heart, liver, kidney or spleen. Benign adenomas have small amounts of TSP-180 and the amounts increase as the adenomas become larger or progress to adenocarcinomas. All murine lung carcinomas tested to date, including primary, spontaneous, or chemically induced, and from different strains of mice, contain high levels of TSP-180.

Similarly, induced and spontaneous mammary carcinomas express large amounts of TSP-180. Correlation of TSP-180 concentration with malignant potential in this system is difficult due to multiple phenotypic changes

that cultured mammary cells undergo. TSP-180 concentration correlates with the metastatic potential of Lewis lung carcinoma. Lewis lung cell lines that are capable of forming many spontaneous or artificial metastases have high TSP-180 levels while variant cell lines of low metastatic potential have lower TSP-180 levels. A similar observation has been made for variant lines of B16 melanomas.

Recently, TSP-180-like molecules have been identified on four different human carcinomas (lung, breast, embryonal, and colon). Evidence indicates that the human TSP-180 complex is analogous to, but distinct from, that on mouse cells. Efforts to produce more MoAb to the human protein are under way.

Molecular characterization of TSP-180 has been complex. At least five molecular forms of the molecule exist. All forms are glycosylated but only the largest, i.e. Mr 204 kd is phosphorylated. Differential labeling experiments indicate that the larger forms (Mr 204 kd and Mr 185 kd) are external while a large membrane bound, but internal pool of smaller molecules (Mr 150 kd, Mr 135 kd and Mr 116 kd) represents the majority of cell-associated forms detected by the two-site assay.

Current work is focused on molecular characterization of TSP-180 and attempts to identify a specific receptor function for the complex. Molecular characterization is being studied by molecular cloning. A c-DNA library of Line 1 cells in λgt11 has been screened with a newly prepared rabbit antiserum to TSP-180. Two phage isolates are being characterized which appear to contain fragments of TSP-180 specific DNA. Specific DNA will be used to analyze mRNA sizes and synthesis as well as the gene's size and position in normal and neoplastic cells. The effect of known factors on the phosphorylation of TSP-180 and growth of Line 1 cells is being analyzed to establish a function for the TSP-180 complex.

Monoclonal Antibody for Lung Toxicology. Rat MoAbs to mouse lung proteins have been isolated from rats immunized with several different lung protein immunogens. These antibodies are currently being analyzed for their binding specificity and for specific binding to lung proteins or particular cell types.

One MoAb recognizes murine type I epithelial cells. Toxic injury to these cells can be monitored by immunohistochemistry and by flow cytometry. Early studies indicate differences in lung fibrosis induced with butylated hydroxytoluene or with bleomycin.

Another MoAb recognizes phagocytic cells in various organs. Specifically, it can be used to quantitate macrophages in paraffin sections of lung tissue. This is an advantage over standard histochemical staining which does not detect small or irregularily shaped or positioned cells.

Finally, two MoAbs recognize a 112 kd protein on lung endothelial cells as demonstrated by electron microscopy with gold particle labels. In addition to staining these cells in paraffin sections, the MoAb are

being used to quantitate this protein using a two-site MoAb assay. It is hoped that levels of this protein detected in serum or lung lavage fluid will be diagnostic of the extent of certain types of lung damage.

Monoclonal Antibodies to Dioxins. Dioxins are toxic in many animal species at extremely low levels. Chronic exposure toxicity to 2,3,7,8 tetrachlorodibenzo-p-dioxin is seen at levels as low as 1 μg/kg in monkeys. Levels as low as 10-20 parts per trillion (ppt) in body tissue may be of concern. Presently the only means for establishing dioxin presence in humans is by mass spectral analysis of gram quantities of adipose tissue removed by surgical excision. This procedure is costly, painful, and potentially disfiguring. A simple method is needed to determine the presence of dioxin so that exposed individuals can be identified and the necessity of adipose biopsy can be judged on a more scientific basis. In this manner the development of antibody to TCDD serves several immediate needs: (1) antibodies can be used to develop sensitive, possibly isomer specific assays for dioxins; (2) antibodies may aid in clearance of dioxins from actively exposed individuals; and (3) antibodies may be useful in studies on the cell surface receptors for dioxins.

We have immunized BALB/c mice with a propionic acid adipamide thyroglobulin conjugate of dioxin and have identified 43 hybridomas secreting MoAb which reacts with a bovine serum albumin conjugate of dioxin. During these studies we have developed a sensitive, solid phase radioimmunoassay for antibodies to dioxins. In the future, competition radioimmunoassays will be used to determine the affinity constants and the isomer specificities of the MoAb's on hand.

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CYTOMETRICS

R. E. Hand, Jr.

The Cytometrics group concentrates its activities mainly on work that is related to the operation of a state-of-the-art multi-user facility for flow cytometry (FCM) and cell sorting. FCM is unique in its ability to measure a set of features (such as size, DNA content, amount of antibody bound) of extremely large samples of cells or other biological particles at great speed. This is achieved by measuring the intensity of the light that is scattered by the cells at different wavelengths as they are made to pass single file through a highly focused laser beam. Particles with certain features of interest can be sorted out of the original population for further analyses. FCM has become a powerful technique in basic and applied biomedical research that is being used in many programs in the Biology and other Divisions.

Experiments with mouse bone marrow and ML-1 myelogenous leukemia cells were performed in collaboration with M. J. Aardema and R. J. Preston, Biology Division. After X-ray irradiation, phase fractions of the cell cycle were determined by FCM and correlated with chromosomal aberrations. ML-1 cells were found to have a greater radiosensitivity, hence more chromosomal aberrations, than the normal mouse bone marrow cells. A delay in progression from the G0/G1- to S-phase in the normal mouse bone marrow allowed for DNA repair and fewer aberrations. This delay was not seen in the ML-1 cells.

In collaboration with W. C. Dunn and S. Mitra, Biology Division, FCM and cell sorting was used in a study to determine the level of 6-methylguanine repair in C3H/10T1/2 cells by measuring the enzyme 6-methylguanine-DNA methyltransferase (MGMT). With FCM the phase fractions of the cell cycle were monitored so that accurate measurements of MGMT activity could be correlated to the phases of the cell cycle. If cells were treated with MNNG to induce lesions in the DNA, there was a significant reduction in the MGMT activity at the onset and during S-phase. Recovery of MGMT activity was delayed until the onset of the G2-phase of the cell cycle.

In collaboration with Jean Solomon, Environmental Sciences Division, FCM and cell sorting was applied to a unique problem involving algae. Certain algae when stressed with nitrogen deprivation begin to manufacture, accumulate, and store lipids in vacuoles in their cytoplasm. When ideal growth conditions return, these lipids are used as an energy source. Nile red, a vital lipophilic dye, dissolved in n-Heptane and added to the culture medium is taken up by the algae thereby staining the internal lipids. When these cells are exposed to 488 nm of laser light in the cell sorter, the Nile red stained lipids fluoresce yellow while the chlorophyll fluoresces a deep red. This two-color analysis has allowed us to monitor and quantify the ratio of lipid to chlorophyll production. Cells which appeared to be producing more lipid than the remaining population were

sorted, grown up, stressed, and re-examined by FCM. These cells may represent a genetically stable, high lipid producing sub-population of algae. These preliminary results were included as part of a report to the Solar Energy Research Institute. With assistance from the ORNL Exploratory Studies Program, these experiments will continue.

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Toxicology Section

SECTION OVERVIEW - H. R. WITSCHI

Staff of the Toxicology Section continue to study the untoward effects of complex mixtures. In parallel efforts, attempts were made to further elucidate mechanisms of disease, particularly mechanisms underlying the development of cancer in the respiratory tract. The research highlights may be summarized as follows:

(1) We had found previously that the different complex mixtures of different composition (matrices) may affect the penetration of an inert paraffin across the skin and distribution within internal organs. The observation suggested that matrices may well play a determining role in the toxicokinetic behavior of model compounds. A systematic study was initiated in which the effects of six different matrices (coal-derived fuels and gasolines with varying carcinogenic potential) on such model compounds as benzo(a)pyrne, aza-benzo(a)pyrene, and several inorganic metal salts was examined. Although no definite conclusions can be drawn yet, it has become obvious that matrix effect may be of key importance in determining acute and probably also chronic toxicity of complex mixtures.

(2) A study on the skin tumorigenic potential of a series of coal-derived fuels was completed. The study strongly suggested that some mixtures would have tumor promoting potential rather than being complete carcinogens. Attempts to separate promoting activity from carcinogenic activity have been initiated in a series of *in vivo* skin painting experiments.

(3) The flow-through tracheal implant (FTTI) developed and perfected during the last few years was put to good use in a study on the subchronic and carcinogenic effect of complex mixtures. Preliminary data show that it is possible to characterize different mixtures by their ability to produce, in a dose-dependent manner, epithelial hyperplasia and transitional plus squamous metaplasia. By and large, conclusions drawn from the FTTI corroborate the studies done on the skin carcinogenic potential of the same mixtures.

The pathobiology of tumor development in the respiratory tract was addressed in experiments covering model compounds or model experimental systems. The following accomplishments can be highlighted:

(1) Studies continued to characterize the carcinogenic process in rat tracheal implants. *In vivo* events have been correlated with suitable *in vitro* markers of neoplastic progression.

(2) The effects of dose fractionation upon development of neoplastic lesions are being characterized, both with 7,12-dimethylbenz(a)anthracene and with benzo(a)pyrene.

(3) Toxicological interactions between benzo(a)pyrene and formaldelyde, an ubiquitous pollutant, are being examined in the FTTI model. Of particular interest is the capability of formaldehyde to produce crosslinks and single-strand breaks in the DNA of rat tracheal epithelial cells. Repair mechanisms appear to be selctive, and this selectivity may relate to cancer. Equally important is the observation that pre-exposure to formaldehyde on to benzo(a)pyrene may modify subsequent disposition of radiolabeled benzo(a)pyrene.

(4) Oxidants (oxygen and ozone) inhibit the development of tumors in the lungs of rats and of mice. When the effect of oxygen on the development of artificial lung metastasis was examined, it was discovered that some tumor cell lines are oxygen resistant, whereas other tumor cell lines are highly susceptible to the cytotoxic effect of oxygen. The observation opened up the possibility to examine whether there exists a direct correlation between tumor cell resistance to oxygen and tumor cell antioxidant defense enzymes. So far, no correlation was found. On the other hand, preliminary evidence suggests that oxygen produces in tumor cells a mitotic delay, similar in nature to the effect produced by ionizing radiation in dividing cells.

It is unfortunate that during the past year the Section lost the program dealing with skin toxicology. On the other hand, collaboration with Dr. Guerin's section of the Analytical Chemistry Division continued to be strong. We wish to thank them for their continued support; without it we could not have accomplished as much in the complex mixtures program as we did.

INHIBITION OF LUNG TUMOR DEVELOPMENT BY HYPEROXIA AND BY OXIDANTS

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During the past 10 years we have studied lung tumors in mice. Particularly, we examined whether the mouse lung tumor system could be used to demonstrate that the principles of two stage carcinogenesis apply not only to mouse skin, but also to epithelial tumors in internal organs. In several experiments, we have shown that strain A/J or Swiss-Webster mice treated with one single injection of a carcinogen and then given repeated injections of the antioxidant butylated hydroxytoluene (BHT) consistently develop more lung tumors than do appropriate controls. BHT also enhances lung tumor formation if it is fed in the diet at concentrations from 0.05% to 0.5%. We have further shown that the time interval between application

of the carcinogen and the first exposure to BHT can be extended up to 5 months and that BHT still will enhance the development of lung tumors. The lung tumor-BHT system thus has many elements of a real initiation-promotion system, although it fails to fulfill all criteria. Nevertheless it can be pointed out that BHT has so-called "promoting" activity not only in mouse lung, but also in many other organ systems.

The sum of the evidence lead us to conclude that BHT "promotes" or enhances the development of tumors. The mechanism of action is unknown.

In the last few years, a new hypothesis was developed to explain the mechanisms of action of tumor promoters. Multiple experiments, most of them done with in vitro systems, suggest that oxygen-free radicals play a crucial role in the promotion of mouse skin tumors.

The hypothesis is contradictory to some of our own experimental findings: BHT is an antioxidant, not an oxidant. More specifically, we also had shown that treatment of mice first with a carcinogen and then with repeated short-term exposures to 100% oxygen did not promote tumor formation in the lung. Therefore, although observations made in mouse skin (where BHT reportedly inhibits tumor promotion) would fit into the oxygen free radical hypothesis of tumor promotion, our observations made with the lung tumor system decidedly do not support it.

It seemed appropriate, therefore, to directly test the oxygen free radical hypothesis in our experimental system. We treated strain A mice with one dose of a carcinogen (urethan or 3-methylcholanthrene). Four days later we placed the animals in an atmosphere of 70% oxygen, where they remained for 4 months. We found that mice tolerated continuous exposure to 70% oxygen for 4 months, but that oxygen profoundly inhibited the development of lung tumors.

The observation raised the question whether other oxidants would have a similar effect. In view of widespread concern about the biological effects of air pollutants, it was decided to examine the effect of ozone on lung tumor development in mice. An appropriately designed experiment was carried out in collaboration with our colleagues at the University of California, Davis. Mice, either strain A/J or Swiss-Webster, were exposed for 18 wks to filtered air, or to 0.4 or 0.8 ppm of ozone for 8 h daily. Subgroups in each test group received a single intraperitoneal injection of 1000 mg/kg of urethan or of the 0.9% sodium chloride vehicle 1 day prior to initiation of the exposure regimen. Tumor incidence in Swiss-Webster mice was 0-3% in groups not receiving urethan and was 61-74% in groups receiving urethan. In A/J mice, the corresponding values were 9-38% and 100%, respectively. Exposure to ozone caused a decrease in the number of tumors per lung in urethan-treated mice of both strains in a dose-dependent manner. There seemed to be a specific decrease in tumors derived from alveolar type II cells in the A/J mice given urethan plus ozone. Most interesting, perhaps, was a significant increase in the number of tumors per lung in A/J mice exposed to 0.8 ppm of ozone without urethan,

confirming a previous report by others. The corresponding ozone effect on lung tumor development was not observed in Swiss-Webster mice.

Once we had made the observation that hyperoxia or ozone prevent the development of lung tumors in mice, we attempted to establish whether lung tumors in species other than the mouse would be oxygen-sensitive. We also took first steps towards development of a system amenable to mechanistic studies. The model selected was squamous cell lung tumors in rats produced by 3-methylcholanthrene (MCA).

We anesthetized a total of 60 male F344 rats and instilled 5 mg MCA directly into the trachea. Instillation volume was 0.1 ml. To prepare the MCA solution, a weighed amount of MCA had been ground to a fine powder with pestle and mortar. We allowed the animals to recover for 7 days in order to avoid the possibility that exposure to oxygen would accelerate clearance mechanisms (e.g., accelerate the elimination of MCA from the lung, a process which would reduce effective dose). After 7 days, 20 animals were placed into a chamber ventilated with 70% O_2, 20 animals were exposed to 40% O_2, and 20 animals served as air controls.

Two months after MCA instillation, the animals were killed and the lungs inflated with neutral buffered formalin. On the surface of the lungs of animals kept in air, numerous whitish masses were seen ranging from 1 mm to 12 mm in diameter. On the surface of the lungs of animals kept in 40% or 70% O_2, considerably fewer such lumps were seen and their average diameter was much smaller. Histologically, the lesions were similar to the lesions described by Schreiber *et al*. (J. Natl. Cancer Inst. 49: 541, 1972) in their original observation: multiple bronchiolo-alveolar squamous metaplasia which had developed into small to large nodular lesions. High proliferative activity and cytoplasmic basophilia was seen in the periphery of the nodules and necrotic areas were often found in the center of the nodule. It was concluded that hyperoxia would have an inhibitory effect upon the development of those lesions. The tumoridical effects of hyperoxia are thus not limited to mouse lung tumors.

We then initiated a series of studies which were designed to elucidate the mechanisms underlying the toxic effects of oxygen on transformed cell populations. For this, we decided to abandon the pure *in vivo* system used so far. The disadvantage of any *in vivo* tumor system is that it takes several weeks to months before the result of an experiment becomes known. As far as mechanistic studies are concerned, it is often difficult to obtain enough material from, say, mouse lung tumors to do biochemical analysis; yet such studies will be necessary in order to elucidate possible mechanisms underlying oxygen sensitivity of tumors.

Transplantable tumor cell lines, i.e., tumor cell lines which can be grown *in vitro* and can be injected into syngeneic animals, offer an advantage. Numerous murine tumor lines are established. If suspensions of such cell lines are injected intravenously into syngeneic animals, metastatic tumor nodules in the lung (and on occasion in other organs) will develop within a comparatively short time period. After intramuscular

injection, tumor nodules may not only develop at the site of injection, but some tumor cells will migrate to the lung where they form metastasis. We, therefore, initiated studies on the effect of hyperoxia on artificial lung metastases.

Such a system has two advantages. (1) Cell replication is continuous and the tumors have a large growth fraction; this will allow a study of growth kinetics of tumors in the lung with autoradiographic methods. (2) The tumor cells are readily grown in large quantities in vitro. This will allow us to examine their level of antioxidant enzymes, to evaluate the effects of oxygen (inducibility of defense enzymes) under in vitro conditions, and also to compare biochemical measurements made on a given cell population before injection into an animal and after the cells have grown to sizable tumors within the lung of the animal.

We have begun to inject the following cell lines intravenously into syngeneic mice: Lines M109, 498, and Line 1. They are cell lines derived from lung adenocarcinomas originally found in BALB/c lines. We also used the well-studied murine melanoma, B16-F1 and F10.

In a first series of experiments we found that with all cell lines used, tumor nodules in the lung develop rapidly within 2 to 3 wks and can easily be counted. However, 70% O_2 did not seem to adversely effect the growth of these tumors.

We then changed the protocol. Instead of keeping the animals in 70% O_2, we exposed them to 100% O_2. Since 100% O_2 produces lung damage, we tried to avoid the possibility that tumor cells would simply become dislodged from damaged pulmonary capillaries, a process which could lead to an apparent decrease in pulmonary nodules. Between intravenous injection and exposure to 100% O_2, we waited a full 48 h. Then we placed the animals into 100% O_2, once a wk, for 48 h. In between oxygen exposures, animals were returned to room air.

In doing this experiment, we observed that intermittent exposure to 100% O_2 failed to affect the growth of Line 1 cells in the lungs of BALB/c mice or the growth of B16-F10 cells in the lungs of B57/BL mice. However, we also used a cell line we had not examined so far: MT7, a mammary tumor cell line derived from a DMBA-induced mammary carcinoma in BALB/c mice (R. L. Ullrich, personal communication). When we injected MT7 cells into female BALB/c mice, we made two observations: all animals injected with 100,000 cells and given the intermittent oxygen treatment survived for 3 wks ($n = 20$). This was in contrast to what we found in controls: animals kept in air began to die after 2 wks and, after 3 wks, only 45% (9 out of 20 animals) remained alive. A second group of animals had received an initial intravenous injection of 25,000 cells. All animals survived for 3 wks. The animals that had been given intermittent treatment with 100% O_2 had an average of 8.0 ± 1.9 tumors per lung ($n = 20$). Air controls had 32.9 ± 5.9 ($n = 29$) tumors per lung, significantly more ($p < 0.05$) than animals given the oxygen treatment. In a second experiment, we injected male BALB/c mice with MT7 cells. Artificial lung metastasis developed as

readily in male mice as they did in female mice. In one such experiment, we exposed the animals to 70% O_2, beginning one day after injection of the cells. We found that the number of artificial lung metastasis was significantly reduced in animals kept in a atmosphere of only 70% O_2.

We had thus available tumor cell lines which we can grow and propagate in vitro. If suspensions of such cells are injected intravenously, multiple tumor nodules develop within 2 to 3 wks. Both the Line 1 and MT7 cell lines are derived from the same mouse strain (BALB/c). One tumor cell line apparently is sensitive to oxygen, the other is not. We began to test directly the hypothesis that oxygen is tumoricidal for certain tumor cell lines because they have an intrinsic lack of antioxidant enzymes or are unable to respond with synthesis of antioxidant enzymes upon exposure to oxygen. So far, we have not found any evidence to support such a hypothesis. We measured total glutathione levels and the activities of several antioxidant defense enzymes in the various tumor cell lines; enzyme activities measured were superoxide dismutase, glutathione reductase, glutathione oxidase, and glucose 6-phosphate dehydrogenase. No correlation whatever was found between biochemical antioxidant defense mechanisms and resistance or sensitivity of a particular cell line to hyperoxia.

One possible clue has emerged, however. In animals carrying artificial lung metatasis and exposed to hyperoxia, an analysis of tumor growth parameters was done. This was accomplished by constructing a "labeled mitosis curve." Animals received one single injection of ^3H-labeled thymidine and then were killed at regular intervals up to 48 h later. Lung section were prepared for autoradiography and the percentage of labeled mitosis was determined for each timepoint. The procedure allows us to estimate the length of the various phases of the cell cycle. It was found that hyperoxia prevented cells from entering mitosis. The production of a mitotic delay is very similar to the effects of ionizing radiation upon dividing cells. The molecular events underlying this phenomenon remain to be elucidated.

In summary, we established that hyperoxia inhibits the development of lung tumors in both rats and mice. Presumably, the mechanism is production of a mitotic delay.

CELL KINETICS AND ACUTE LUNG INJURY

H. P. Witschi M. S. Whitaker

For a considerable length of time it was thought that acute lung injury was followed by a rather stereotype pattern of cell proliferation in the lung. Damage to the cells lining 95% of the alveolar surface, the type I alveolar epithelial cells, was thought to be repaired by an extensive proliferation of type II alveolar epithelial cells.

In order to examine whether such a scenerio would apply to a variety of lung-toxic agents, mice were treated with three cytostatic drugs: cyclophosphamide, busulfan, or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). The alveolar labeling index was measured following drug administration with a pulse of ^{3}H-labeled thymidine and autoradiography. In cyclophosphamide treated animals, peak alveolar cell proliferation was seen 5 days after injection of the drug. In animals treated with busulfan or BCNU, proliferation was even more delayed (occuring 2 to 3 wks after administration). In contrast, with oleic acid, the highest alveolar cell labeling was found 2 days after intravenous administration. In animals exposed to a cytostatic drug, proliferation of type II alveolar cells was never a prominent feature; whereas, in animals treated with oleic acid there was an initial burst of type II cell proliferation. It was concluded that the patterns of pulmonary repair vary between chemicals designed to interfere with DNA replication as compared to agents which produce acute lung damage such as oleic acid.

SKIN PAINTING STUDIES

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Human exposure to crude and refined coal liquids is most likely to occur via skin contact. In order to estimate eventual risks to human health as a consequence of incidental and prolonged skin contact, it is necessary to obtain some information on the potential of coal-derived liquids to elicit skin cancer. This can be done with animals in experimental studies which mimic anticipated human exposure. In addition, it also must be established whether prolonged dermal exposure will produce signs of toxicity not only on the skin but to internal organs. During the past 2 years, we completed a life-long skin painting study with mice designed to answer some of these questions.

The following materials were tested: Raw H-coal blend, containing 5700 ppm N; H-coal blend after low hydrotreatment (2650 ppm N); H-coal blend after high hydrotreatment (0.2 ppm N); H-coal "home heating oil," a devolatilized version of the high-hydrotreatment H-coal blend; and an H-coal reformed naphtha. Two petroleum-derived reference samples were obtained from the American Petroleum Institute: Petroleum No. 2 fuel oil and high catalytically cracked naphtha (gasoline). Benzo(a)pyrene (99% pure; Sigma Chemical Company) was used as reference substance.

Experimental animals were male and female C3H mice, bred and maintained in the barrier facility of the Biology Division, Oak Ridge National Laboratory. The test agents were applied three times weekly to

the shaved skin of the back. The following concentrations were used: 50 µl of undiluted material, 50 µl of a 1:1 or 50 µl of a 1:3 dilution with acetone. Positive controls received 50 µg, 25 µg, or 12.5 µg of benzo(a)pyrene in 50 µl of acetone per application (three times weekly) and negative controls were painted with acetone alone or not painted at all. Twenty-five animals of each sex were used per dose level.

All animals had free access to food and water and were kept five to a cage on hardwood shavings for the duration of the study. They were weighed periodically. The animals were treated on Mondays, Wednesdays, and Fridays. Whenever a skin abnormality became visible at the site of test agent application, painting continued for another 2 wks. When the skin mass was found to persist, the animal was killed, and the skin lesion excised and fixed in neutral buffered formalin. A complete necropsy was performed, all observations were noted and selected tissues were fixed in neutral buffered formalin. All skin lesions were examined on paraffin sections under the light microscope. Animals not showing any skin lesions were continuously painted until found moribund or dead and a complete necropsy was performed on all animals killed or found dead.

Data on overall tumor incidence and can be summarized as follows: In animals treated with 50 µg of benzo(a)pyrene per application, the first skin lesions began to appear 14 wks later. In animals given the lowest dose of benzo(a)pyrene (12.5 µg per application) the first tumors appeared after 19 wks. Tumor incidence then rose dramatically in all animals exposed to benzo(a)pyrene within 5 to 10 wks after appearance of the first skin lesion. All animals exposed eventually developed skin tumors. Histologically, the majority of tumors, 70%, were squamous cell carcinomas. On the other hand, in animals painted with acetone alone or not painted at all, very few skin tumors were observed which were benign papillomas.

Among the coal liquids only the raw H-coal blend produced an almost 100% incidence of skin tumors at all three concentrations tested. The first tumors were seen 10 wks after the first application of 50 µl of the undiluted material (highest dose group). With the lower concentrations, the first tumors were seen after 20 and 33 wks, respectively. Once the first tumor was discovered in any group, it took approximately 1 year until most animals in a given group had developed skin tumors. Of these tumors 46% were malignant squamous cell carcinomas.

Hydrotreatment dramatically reduced the carcinogenic potential. The low hydrotreatment preparation, painted undiluted onto the skin, produced the first tumors after 58 wks, and 92 wks after beginning of the experiment, tumor incidence was only 14%. In animals exposed to the high hydrotreated preparation, tumors began to appear usually after 70 to 80 wks, and the final tumor incidence in all three dose groups was between 16% and 44%. Only two tumors were found in the 150 animals exposed to H-coal reformed naphtha. However, in animals exposed to H-coal "home heating oil," the devolatilized version of the highly hydrotreated H-coal blend, tumors began to appear around 40 to 50 wks of exposure and final tumor incidence in animals exposed to the undiluted material was 30%.

The two petroleum-derived samples had practically no carcinogenic potential; overall only 3 animals with tumors were found in animals painted with gasoline and only a total of 13 animals had tumors 95 wks after beginning of the experiment. An evaluation of the gross necropsy findings showed that many animals suffered from lesions usually expected to develop in a certain percentage of aging animals, such as myocardial calcification, renal abnormalities, liver tumors, ovarian tumors, and lymphomas, among others. However, the gross lesions were not associated with any particular treatment regimen and were also seen in the control and untreated animals.

We concluded that the carcinogenic potential of a raw H-coal blend can be mostly abolished by hydrotreatment; however some carcinogenic potential remains associated with a devolatilized version of a severely hydrotreated sample. Petroleum derived products have considerably less carcinogenic activity, an observation compatible with earlier findings. Finally, we did not find gross signs of toxicity in organs other than the skin, and the compounds treated seemed not to act as systemic carcinogens.

As a direct continuation of these experiments, a new series of studies was begun in January 1986 to determine relative potency of selected coal- and petroleum-derived hydrocarbon fuels with regard to their ability to promote skin tumors in mice. The series consists of three parts, one of which involve C3H mice, and two of which involves SENCAR mice. In the experiment with C3H animals, two petroleum-derived fuels and two H-coal derived fuels are used as correlatives to an earlier lifetime skin painting study with the same four fuels. These fuels are also being tested presently in the more sensitive SENCAR mice. In the third experiment, SENCAR mice are being used to assay five diesel fuels.

The general protocol for all three experiments was the same. A single dose of dimethylbenzanthracene is applied to clipped skin. Seven days later, the neat test substance is applied to the same skin area and is applied thereafter twice weekly for 52 wks. The potent promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is used as a positive control agent, and the vehicle for all substances is acetone. During the test period, mice are examined weekly and the number and location of skin tumors are recorded. Tumor incidence and multiplicity are used as indicators of promotional potency of the test fuels. In all three experiments, all groups consist of 25 female and 25 male mice.

After about 29 wks of application, data are largely inconclusive for experiments with C3H mice, although H-coal gasoline appears to be a tumor promoter for female mice. The petroleum fuel oil and H-coal heating oil seem to give a positive response while the petroleum gasoline is clearly negative. However, after about 23 wks application to SENCAR mice, it is almost certain that the petroleum fuel is a tumor promoter while the H-coal heating oil shows marginal promotion. Neither of the gasolines appear to be promoters for SENCAR animals.

At about 15 wks into the experiment, all five diesel fuels would be judged as promoters for SENCAR mice, male and female, although all also appear to be complete tumorigens for these strains with varying degrees of potency.

In conclusion, we found in a first study that raw H-coal blends have carcinogenicity potential and that hydrotreatment greatly mitigates this effect. It is possible that some of the materials examined have promoting potential rather than being full carcinogens. Data available so far certainly support such a possibility.

Throughout the skin painting studies we were assisted by M. R. Guerin, W. H. Griest and C. H. Ho of the Analytical Chemistry Division.

THE TOXICOKINETICS OF COMPLEX MIXTURES

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Previous studies in our laboratory clearly showed that hydrotreatment of coal-derived oil blend markedly reduces its skin tumorigenicity in C3H mice subjected to life-time dermal application of the test petroleums. Benzo(a)pyrene [B(a)P] is a potent carcinogen for mice; its content varies considerably in the complex petroleum mixtures that we have previously tested for skin tumorigenicity. But how or whether the B(a)P content (or other components) of these mixtures relates to skin tumorigenesis is not clear.

In an attempt to further define more clearly the problems involved in the toxicokinetics of complex hydrocarbon mixtures, we have designed experiments to determine the influence of a variety of dermally applied mixtures on the pharmacokinetics of simultaneously applied marker compounds, i.e., B(a)P, aza B(a)P, and $HgCl_2$.

The basic procedure was to apply the complex mixture and radioactive marker to the skin, and at intervals determine the subsequent tissue distribution and excretion of radioactivity. Male BALB/c Bd mice were shaved on the back and sides, and 10 µl each of the test mixture and of the radiolabeled marker were applied to the animal's back. No more than 40 µCi were applied to each mouse. Nine treated mice were placed together in a metabolism cage that allowed separate collection of urine and feces. At 1, 6, and 24 h, three mice were sacrificed to obtain tissues which were assayed for radioactivity along with the urine and feces. Organs assayed were liver, kidney, thymus, spleen, testis, brain, and lung. As a first approach to analysis of the distribution data, radioactivity of the seven organs was combined and expressed as a percent of the applied dose as were

excretion values. Studies with three markers have been completed - ^{3}HB(a)P, aza ^{3}HB(a)P, and $^{203}HgCl_2$. Tests with $^{63}NiCl_2$ and $^{109}CdCl_2$ are currently under way.

In the first series, the influence of six mixtures on the distribution of radioactivity of [^{3}HB(a)P] was examined. The six mixtures were: (1) a crude H-coal-derived light oil/heavy oil blend, (2) a hydrotreated version of the blend, (3) an H-coal-derived gasoline, (4) an H-coal-derived heating oil, (5) petroleum-derived gasoline, and (6) a petroleum-derived fuel oil. For five mixtures, radioactivity in tissues increased during the first 6 h with little change at 24 h. There was no recognizable differential kinetic patterns within the group related to any physical or chemical properties, including B(a)P content. However, the low volatile blend resulted in the lowest 24-h accumulation of radioactivity (2.7%) in the seven organs and a correspondingly low excretion (urine plus feces) value of 33%. Thus, relative to the other mixtures, the blend which is the most tumorigenic of the group may reflect a retarded accumulation of ^{3}HB(a)P in organs together with a parallel increase in dwell time of this potent carcinogen in the skin.

Four of the same mixtures were tested with aza B(a)P, a compound in which the number 10 carbon is replaced by a nitrogen atom, a substitution reported to reduce its tumorigenic potential. Kinetic patterns of organ distribution and of excretion of radioactivity similar to those observed for the parent compound were noted. Again the blend resulted in the lowest accumulation of radioactivity in the seven organs but in contrast, the highest excretion value (60%) of the four mixtures.

The effects of the six complex mixtures on organ radioactivity from $^{203}HgCl_2$ following dermal application were quite different from those in the B(a)P studies. Over the 24 h period, radioactivity in the seven organs showed a steady increase for all six mixtures albeit at different rates. For the blend, its low hydrotreated derivative, and the petroleum gasoline, rates were relatively low (approx. 0.017) compared to those for the H-coal gasoline and heating oil and the petroleum fuel oil (approx. 0.065). Furthermore, the 24 h organ accumulations of radioactivity were considerably less with the first three mixtures (2.7%) than with the latter three (5.3%). As with B(a)P, however, there appears to be no recognizable physical or chemical similarities among the mixtures of each group that could explain the rate and accumulation data.

In this overview of completed experiments, we have presented only the broad aspects of the distribution data. Should it become of obvious value, further analysis of individual organ data, together with the distribution radioactivity values in fat, muscle, blood, intestine, urine, and feces, can be made. Perhaps the most cogent aspect of our pharmacokinetic studies is that they provide initial differential information on each complex mixture which may be used as leads for more definitive approaches to the toxicology of natural and synthetic petroleums.

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INDUCTION AND PROGRESSION OF NEOPLASIA IN THE RESPIRATORY TRACT

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The respiratory tract of man is continually accosted by hazardous agents in the environment that are potentially carcinogenic, co-carcinogenic, or which may promote the development of lung cancer. Many of these hazardsous agents are introduced into the environment as particulates, gases, aerosols, and in complex mixtures during the refining and utilization of most energy sources. There is a great need to develop mammalian model systems in which these potentially hazardous agents can be

studied in their many physical forms under experimental conditions that are well controlled in terms of target site exposed, dose, and duration of exposure. It is also important to define and use endpoints that are relevant to the human situation. The research program of the respiratory carcinogenesis group focuses on the development of such models using *in vivo*, *in vivo-in vitro* and *in vitro* experimental approaches to carry out the highly controlled exposures and to define, correlate, and quantify morphological, cellular, and biochemical changes induced in airway epithelium as markers of the evolution of neoplasia. As these models are perfected, increased emphasis is placed on identifying the underlying mechanisms involved in the initiation of cancer, and the changes in growth control mechanisms during tumor development that are identified as increased growth autonomy *in vitro* and *in vivo*.

1. *In Vivo-In Vitro Study of Carcinogenesis Induced by 7,12-Dimethylbenz(a)anthracene (DMBA).* *In vivo* models are essentially limited to morphological endpoints. To overcome this limitation a combined *in vivo-in vitro* model of carcinogenesis was developed several years ago, which makes it possible to study a gamut of cellular and biochemical changes in cell populations derived from tracheal tissue at different times after carcinogen exposure *in vivo*. Tracheas from specific-pathogen-free Fischer 344 rats, bred in our own facilities, are used to keep the complications from infection out of the experiments and retain a constant genetic background. The tracheas of rats are used in these studies because of the structural similarity of the epithelium to the human bronchus. The approach is to expose rat tracheal implants to known doses of carcinogen for preselected periods of time. The tracheas are then cut into explants and placed in organ culture for 24 h. A cytopathological diagnosis is then made on the cells that exfoliate into the medium. 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 but not altered cells. As reported earlier, we could show, with this approach a dose response with respect to the number of selected cell populations (SPC) obtained after exposure to 200 μg DMBA-beeswax pellets for 2 wks (135 μg DMBA released), 4 wks (165 μg DMBA released) and pellets left in (all released). Further alterations in the selected cell populations (SPC) 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. In these experiments, directly correlated to increased DMBA exposure were a decrease in time to anchorage-independent growth as well as time to tumorigenicity. As expected, severe atypias were found earliest in explants from tracheas that received the greatest amount of DMBA. Since lesions on the explants are initially identified from cytopathology of exfoliated cells, cellular and biochemical properties of cell populations derived from the specific lesions could be studied and correlated directly with conventional morphological markers of the progression of neoplasia. During the past year the relationship between the types of lesions and the growth properties of the cell populations generated from the pieces of

tracheal implants exposed for different times to the 200 μg DMBA pellets was completed.

We found a close correlation between an increase in severity of the lesions and the aquisition of in vitro markers of progression of neoplasia as well as tumorigenicity of the cells when placed back in vivo into suitable hosts. This provides strong evidence that the lesions identified in vivo are indeed the sites of developing cancers. It also indicates to us that we can select specific stages in the progression of neoplasia for further study.

2. Induction of Carcinogenesis with Split Doses of DMBA. We recently developed an open-ended, flow-through tracheal implant (FTTI) model to study the induction and pathogenesis of lung cancer. In this model the well-defined target site can be exposed to unlimited numbers of exposures of single or multiple test agents of any physical form, e.g., solutions, gases, particles. The FTTI has 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, without sacrificing the animals. Another advantage of the model is that, for the first time, tumor promotion studies can be carried out in the airway similarly to that classically done in skin tumor promotion. With respect to exposure to DMBA, we are now able to expose rat tracheas to repetitive low doses of the carcinogen, thereby reducing acute toxicity. Earlier, the acute morphological responses of the FTTI to twice weekly exposures of different amounts of DMBA were determined. In these studies, it was found that at the lowest amount of DMBA [10 μg given twice weekly for 2 wks (total dose 50 μg)] tested more than 50% of the epithelium was stratified hyperplasia; the remainder had either basal cell hyperplasia or transitional metaplasia. After 8 wks of 10 μg pellets (total dose = 170 μg) 20% of the epithelium had become keratinizing squamous metaplasia. When 20 μg pellets were given twice weekly, 20% of the tracheal lumens were already covered with squamous metaplasia (total dose 100 μg DMBA). When 40 μg DMBA pellets were used, 100% of the tracheal lumens were lined with squamous metaplasia within 2 wks (total dose 200 μg DMBA). With these data as references, two in vivo-in vitro experiments were started to quantitate the induction and progression of carcinogenesis. FTTI were exposed to 20 μg DMBA pellets, twice weekly for 2.25 months (total dose 360 μg). After an additional month without carcinogen, SPC were determined by selecting at 14 days of culture for cell populations surviving in the pyruvate deprived medium. We also questioned whether DMBA had initiated carcinogenesis in some cells which had not reached the stage detected by our selection procedures. Therefore, we also selected for SPC after 63 days of culture in the complete medium. Selecting at 14 days of cultures, we obtained the high value of 4.5 SPC/trachea. The later selection time added 2.2 SPC/trachea indicating that some cells progressed during culture in the complete medium. Our findings show that a high number of these cell populations are tumorigenic when inoculated into the nude mouse. In the second experiment, FTTI were exposed to 5 μg DMBA, twice weekly for 2.5 months (total dose 90 μg). Even at this low dose

considerable numbers of SPC are being detected particularly at the 63 day selection time. Final number of SPC/trachea and tumorigenicity will be determined in these experimental groups. These data will give us a dose response curve of DMBA given as split doses over the same length of time, and will serve as a baseline for future studies of inhibition and promotion of carcinogenesis in the trachea.

3. Induction of Carcinogenesis by Split Doses of Benzo(a)pyrene (B(a)P]. The DMBA exposure studies serve as a prototype for examining the induction of altered cell populations (SPC) by B(a)P, a known carcinogen in the environment. The acute morphological responses of FTTI to B(a)P were determined earlier. For example, it required 160 μg pellets given twice weekly for 2 wks (total dose 640 μg) to induce more than 50% squamous metaplasia in FTTI. At 4 wks (total dose 1440 μg) 30% of the epithelium was squamous metaplasia, and the remainder was transitional metaplasia, stratified hyperplasia, and basal cell hyperplasia. When this exposure regimen was used to determine the induction of carcinogen-altered cell populations, 0.33 SPC/trachea was obtained with a 14-day selection. An additional 1.17 SPC/trachea could be detected by selection at 63 days of culture. When the 1440 μg B(a)P exposure was spread over 4.5 months (40 μg twice weekly, 2.0 SPC/trachea were found by 14-day selection. An additional 2.50 SPC were obtained by selecting at 63 days of culture, again indicating that some cells progressed during culture in complete medium. Experiments are now in progress in which FTTI were again exposed twice weekly to 160 μg B(a)P for 1 month, but the tracheas were placed in culture only after 4.5 months. This will distinguish any effects of split-dose exposure (40 μg, twice weekly, 4.5 months) from the longer duration required before the tracheas were placed in culture. With these more sensitive procedures, we should be able to study agents with low tumor potency.

4. Initiation-Promotion and Co-carcinogenesis with B(a)P and Formaldehyde (HCHO). A major project is in progress to study, in the FTTI model, the carcinogenicity, co-carcinogenicity and promotability of HCHO, an ubiquitous environmental pollutant, when given alone or in various combinations with B(a)P. We have completed a study which showed that HCHO solutions (0.2% twice weekly, 4.5 months) will induce a small number of SPC (0.25/trachea) suggesting that it has very low, but definite tumor potency. Experiments are now in progress to determine the number of SPC induced by, (a) 20 μg B(a)P given twice weekly for 4.5 months (total dose 720 μg), (b) 0.2% HCHO followed 2.5 h later by the B(a)P, or (c) B(a)P followed 24 h later by the HCHO; all given twice weekly over 4.5 months. These studies should indicate whether B(a)P and HCHO act as co-carcinogens and whether the order of exposure has any relevance.

(a) The effects of B(a)P and HCHO on rat tracheal epithelial cells at the molecular level are also being investigated. The induction of DNA-protein cross-links (DPC) and single-strand breaks (SSB) in DNA from primary cell cultures of rat tracheal epithelial cells are determined using the alkaline elution techniques of K. Kahn. We have found that, as expected, HCHO induces a high level of DPC which were repaired within 16 h

after exposure. B(a)P alone did not cause DPC, however, reexposure to B(a)P, 24 h before 90 min exposure to HCHO inhibited the number of DPC without inhibiting the rate of repair. B(a)P or HCHO alone caused SSB and exposure to both agents had an additive effect. A most interesting finding was that a portion of the SSB induced by B(a)P was rapidly repaired while a portion was not. It is proposed that the nonrepaired SSB are important to the induction of cancer. These assays will be carried out next on tracheal cells from FTTI exposed *in vivo* to the test agents to determine whether there are correlations between the molecular lesions found in these cells and the long-term toxic and carcinogenic effects of B(a)P and HCHO. It is hoped that assay for DPC and SSB will be applicable to determining the molecular responses of FTTI to complex mixtures and will help to predict the long-term effects of exposure to the mixture.

(b) Another way in which we are assessing the interaction of B(a)P and HCHO is to determine the utilization of these agents by the FTTI. As a first step, in collaboration with the Analytical Chemistry Division, we have measured the uptake of tritium from [^{3}H]B(a)P-gelatin pellets, from 3 h to 4 days after inserting the pellets. By 3 h 65% of the B(a)P is removed from the lumen, and more than 99% by 24 h. Maximal radioactivity is already found in the FTTI by 3 h, while an early rise in radioactivity could be detected in the blood, urine and feces of the host animals. The distribution of the radioactivity between B(a)P and its metabolites is currently being determined. So far we have found that 90% of the radioactivity in the FTTI is in the form of B(a)P while in most cases, a high proportion of the radioactivity was recovered in water-soluble or organic-soluble metabolites in the host tissues. However, when the FTTI were first preexposed for 4 days to 40 μg B(a)P, 50% of the radioactivity from the [^{3}H] B(a)P found in the FTTI was in the form of organic-soluble and water-soluble metabolites. In the future, the effects of long-term B(a)P preexposure and HCHO preexposure on the utilization of B(a)P by the FTTI will be determined.

5. The Subchronic and Carcinogenic Effects of Complex Mixtures in FTTI. As part of the complex mixtures program, the acute and subchronic responses of FTTI to complex mixtures from fuel oils provided by the Analytical Chemistry Division, ORNL, are being investigated as the first step to determining the tumorigenicity of these complex mixtures in the respiratory system. H-coal products that have been chemically characterized by the Analytical Chemistry Division, ORNL, and have been tested in *in vitro* mutagenesis assays and in skin toxicokinetic and carcinogenesis studies in the Biology Division, ORNL, are now being tested in the FTTI model. The subchronic effects on the morphology of FTTI exposed to fuel oil blend No. 931 and the same fuel blend following low severity hydrotreatment (No. 978) have been determined. First of all, it was found necessary to mix the fuel oil with a vehicle and introduce a constant total volume (30 μl) to show dose effects in the FTTI. Under these conditions, 2.5 μl of 931 (+ 27.5 μl vehicle of 25% acetone in PBS) given twice weekly induces 60% hyperplasia and 30% transitional plus squamous metaplasia after 2 wks. Increasing the dose to 10 μl induces transitional plus squamous metaplasia in 75% of the epithelium lining the

FTTI. Doses of 30 µl are toxic (dead cells) to 19% of the epithelial lining after 2 wks of exposure. In contrast, 84% of the FTTI are still lined with hyperplastic epithelium 4 wks after twice weekly exposure to 30 µl of 978. Additional H-coal products will be tested in this manner and suitable doses will be selected for testing long-term toxic and carcinogenic effects in the FTTI.

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Information Research and Analysis Section

SECTION OVERVIEW - J. T. ENSMINGER

The Information Research and Analysis (IR&A) Section was organizationally moved to the Biology Division in April 1986. This decision was made because of the previous parent division's becoming a three-plant organization and, as such, reporting through the management chain of Martin Marietta Energy Systems, Inc. IR&A has been a part of the R&D environment of the Laboratory since it's inception as the Environmental Information Systems Office in 1971, and has historically maintained it's major scientific strength in the area of biological effects of hazardous materials. The transition, which has gone extremely smoothly, presents new potential for interaction between the bench and information research aspects of new and ongoing biological research initiatives. This possibility is directly supported by the Section objective which is to contribute to the research and development goals of ORNL, DOE, and other federal agencies through integration of expertise in science and technical information management, evaluation, and analysis.

The Section's funding situation is unique within the Division and the Laboratory in that the majority of our projects are classified as work-for-others. Although the difficulty in bringing work-for-others funds into the Laboratory has continued to increase, they have provided a certain amount of stability and even slow growth during the recent times of uncertain budgets. They have also provided to DOE research projects the benefit of direct access to extensive high-quality data resources paid for by other agencies. The Section staff now stands at 90 with plans to add three people in FY 1987.

During the past year, the Section continued the work which has been under way for many years in the development of computerized health effects databases. Funded by the EPA and NIEHS/NTP, the Environmental Mutagens and Teratogens Databases now contain about 61,000 and 41,000 records, respectively, on approximately 18,000 total chemicals. The Environmental Carcinogens Database continues to develop information on chemicals tested for *in vivo* animal carcinogenicity and *in vitro* cell transformation in support of the EPA Gene-Tox Program which is being coordinated by Section staff. The purpose of the Gene-Tox Program is to conduct a systematic review and validation of genetic toxicology studies to establish a registry of test results for chemicals evaluated in selected short-term bioassays. The peer-reviewed and NLM funded Hazardous Substances Data Bank now contains over 4,000 records on individual chemicals. During the past year, Section staff prepared and updated 484 of these records which present a detailed profile of each substance. These resources, along with others, were used by the Section staff for preparation of 39 hazard assessments and review documents in related areas for EPA and the Army.

A further application of these resources, and a new initiative for the Section during the past year, is the development of the computer searchable Materials Safety Data Sheet Database. With support from Martin Marietta Energy Systems, the database has been developed to inform employees at the four plants of hazardous materials with which they may come in contact. This was a critical factor in bringing Energy Systems into compliance with the OSHA Hazard Communication Standard which went into effect in May 1986. The overriding need for high quality health hazard data was the major factor in deciding to use the resources of the Section rather than the commercially available systems or chemical manufacturer's information.

In the energy and environmental area, work has continued on development of computerized databases in support of hazardous waste management, transportation, disposal, and remedial actions. Additionally, staff have participated in radiological surveys of contaminated sites and have prepared 265 Remedial Actions Survey Assessment Reports in collaboration with the Health and Safety Research Division. Additional collaboration with other Laboratory organizations includes Acid Deposition Research with the Environmental Sciences Division; Conservation and Materials with Metals and Ceramics; and Fossil Energy with the Fossil Energy Technology Environmental Program. In conjunction with the latter effort, a very flexible information management system has been developed using the INQUIRE database management system. Features developed have proven transportable to other subject areas. As support for the Fossil Energy Program declines, it now appears that funds for Section activities in this area will be replaced by the Clean Coal Program.

An additional new initiative involves the evaluation and further development of data management systems for the Department of Defense, in collaboration with the ORNL Energy Division. This effort was initiated in FY 1986, and should continue to grow in the coming year. Spinoffs from this work will provide benefits to several other Section projects.

ENVIRONMENTAL MUTAGEN, CARCINOGEN, AND TERATOGEN INFORMATION PROGRAM

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The primary focus of the Environmental Mutagen, Carcinogen, and Teratogen Information Program (EMCTIP) is the establishment of information files that can be accessed readily by research personnel, health administrators, and others interested in the scientific disciplines of genetic and reproductive toxicology, carcinogenicity, and *in vitro* cell transformation. The files maintained by the Program are designed to be responsive to users and to provide accurate information retrieval. This characteristic, coupled with that of having available in-house copies of

all cited documents, makes these files unique. The Program has received considerable recognition for efforts to establish an information service that facilitates research in the disciplines covered.

The EMCTIP staff has also participated in the conduct of special data review and analysis projects for various federal agencies. The most extensive of these is the U.S. Environmental Protection Agency's Genetic Toxicology (Gene-Tox) Program. Highlights from the EMCTIP work areas are shown below.

ENVIRONMENTAL MUTAGEN INFORMATION CENTER (EMIC). EMIC collects and analyzes worldwide literature on genetic toxicology including mutagenesis and related areas such as the effects of chemicals on the following: DNA synthesis, DNA and synthetic polynucleotides, the processes of mitosis, meiosis, and reproduction. This information forms a searchable data base available to persons in research, government agencies, and medical or educational institutions. The EMIC data base consists of representative coverage of the pre-1969 literature and comprehensive coverage of the literature for the period from 1969 to date.

Each record in the EMIC data base contains the usual bibliographic data common to all information services. In addition, EMIC indexes a wide range of technical data allowing investigators to search according to specific interests. As the use of the EMIC data base has grown and the EMIC staff has had the opportunity to interact with its users through personal communication, search requests, special projects, and other media, new needs and new uses of EMIC services have become apparent. EMIC has responded by adding new indexing fields, making the EMIC data base more versatile.

When provided in the article, the following information is indexed and entered in the EMIC data base:

Taxonomic, common name, strain, cell line, and sex of test object
Genetic end point examined in either germ or somatic cells
Whether study was conducted *in vitro* or *in vivo*
Assay system employed
Inducers used in metabolic activation studies
Chemical names or abbreviations
Chemical Abstracts Service Registry Number(s)
Keywords involved
Text abstracts (added on a selected basis)

This indexing scheme provides more complete and more readily accessible information than is possible with conventional text abstracting plus the flexibility to respond to the needs of our users. As of September 30, 1986, the file contained 60,863 (4,128 added FY 1986) references from over 3,444 sources, with information on more than 18,000 unique chemicals. EMIC also maintains a separate file on aneuploidy. This file, as of September 30, 1986, contained 3,800 citations. The aneuploidy file was extensively

used by the U.S. Environmental Protection Agency as part of is symposium on Aneuploidy: Etiology and Mechanisms, held in 1985.

The EMIC data base and its aneuploidy file are accessible on-line through the National Library of Medicine's TOXLINE system and the U.S. Department of Energy's RECON system until 1987. In addition to requesters use of EMIC's on-line files, the EMIC staff also responded to 1,299 information requests made directly to the center during FY 1986.

ENVIRONMENTAL TERATOLOGY INFORMATION CENTER (ETIC). ETIC's purpose is to collect, analyze, and disseminate information on the evaluation of chemical, biological, and physical agents for teratogenic activity. The information in this file is available to individual researchers and physicians, as well as to institutions and government research and regulatory agencies. Easy access to this literature facilitates health assessment, research planning, and prevention of the duplication of effort in the field of environmental teratology.

Information in the ETIC data base is obtained from reports in the open literature relating to teratogenicity testing and to evaluation of dietary deficiencies and chemical, biological, and physical agents in animals. The main focus is on the administration of an agent to a pregnant animal and examination of the offspring at or near birth for structural or functional anomalies. ETIC scans key journals as well as many secondary sources and has on-line access to several computerized data bases.

As of September 30, 1986, the ETIC file contained 41,499 (3,700 added in FY 1986) entries from 3,544 sources in the ETIC file, approximately 16% of which are in languages other than English. There is information on approximately 8,000 unique chemicals. In addition to bibliographic information, technical information is indexed so that the file may be searched in a variety of ways according to the needs of its users. Included in the indexed information, when provided in the article, are the following:

Taxonomic and common name, and strain of test object
Organ type or embryo cultured
End point examined in offspring
Chemical names or abbreviations
Chemical Abstracts Service Registry Number
Text abstracts (added only on a selective basis)
Stage treated in cold-blooded and invertebrate animals
Sex treated

This method of indexing is thorough, flexible, easily searchable, and facilitates adding new fields as the need arises.

The ETIC staff coordinated the U.S. Environmental Protection Agency Consensus Workshop on the Evaluation of Maternal and Developmental Toxicity that was held on May 12-14, 1986, in Rockville, MD.

ENVIRONMENTAL CARCINOGEN INFORMATION CENTER (ECIC). The main focus of ECIC is the establishment of information files on chemicals that have been evaluated for *in vivo* animal carcinogenicity and *in vitro* cell transformation. This information is used to support the work of the Carcinogenicity Work Group of the U.S. Environmental Protection Agency's Genetic Toxicology (Gene-Tox) Program. Records in the ECIC file are indexed in the manner similar to that used by EMIC and ETIC providing a user-friendly system for accessing subject matter in these two fields. The number of papers in the ECIC subfiles and the technical indexing fields used are shown below.

In Vitro Cell Transformation File (2,000 citations)
- experimental description
- activation system used
- chemical inducer of the activation system
- end point measured
- animal source of the target cell
- target cell used in the experiment
- virus name (viral enhancement studies only)
- agent(s) tested
- CAS Registry Number(s) (chemical agents only)

In Vivo Animal Carcinogenicity File (4,000 citations)
- test object, general classification
- test object, specific classification
- sex of treated animal
- agent(s) tested
- CAS Registry Number(s) (chemical agents only)
- solvent and/or vehicle
- route of administration
- experimental design
- type of analysis
- organ(s) examined
- assay
- promoter
- controls

Presently, the complete ECIC file is available through the center. Plans are under way to make the ECIC file available on the National Library of Medicine's TOXLINE system.

GENETIC TOXICOLOGY (GENE-TOX) PROGRAM. The objective of the Gene-Tox Program is to conduct a systematic review and analysis of the genetic toxicology literature and to establish a registry of test results for chemicals evaluated in selected short-term bioassays. The Gene-Tox Program was initiated in 1979 and to date has accumulated a peer-reviewed registry of test results on 4,202 chemicals (1,580 added during FY 1986). A total of 40 papers have been published as part of the Gene-Tox Program, with three of these having been published or submitted for publication during FY 1986. The Gene-Tox Program aids the U.S. Environmental Protection

Agency in establishing standard genetic testing and evaluation procedures for the regulation of toxic substances.

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ENERGY AND ENVIRONMENTAL INFORMATION ANALYSIS PROGRAM

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Activities within the program are highly varied. Emphases follow problem or project lines, such as conservation, fossil energy, and acid rain, as well as tasks to develop total information systems. A number of program staff work with researchers in other Oak Ridge National Laboratory divisions or programs in a matrix mode. Subject areas include: applied earth sciences, engineering, environmental effects, waste management, and computer science.

Significant new involvements this year are in information system planning and design using relational database concepts, and in microcomputer applications and local area computer networking.

HAZARDOUS MATERIALS INFORMATION. Hazardous Materials Information's (HMI's) experience and expertise include data analysis and evaluation, design and development of computerized numeric, and bibliographic information resources to support national radioactive and hazardous materials programs. Current HMI activities are summarized as follows.

Nuclear Facility Decommissioning and Site Remedial Actions: HMI is a focal point for technical information exchange among participants in DOE's Remedial Action program. Significant accomplishments for this activity include: abstracting and indexing 773 documents for inclusion in the database developed for the program and responding to 699 requests for assistance from program staff and contractors.

Legislation Impacting the Transport of Radioactive Materials: HMI collects, analyzes, evaluates, and computerizes federal, state, and local legislation pertinent to the shipment of radioactive materials. This information is made available to program participants by monthly legislative updates, an annual report, and online computer access.

Radioactive Waste Management: HMI performs technical information analysis and evaluation for radioactive waste management activities at ORNL. An online data management system was developed to support site characterization and site selection activities. The data system is used as a resource in the preparation of analytical and evaluative reports related to a proposed high-level radioactive waste repository.

Geochemical Technical Assistance: Technical information support is being provided to the NRC Office of Nuclear Material Safety and Safeguards to assist in its effort to approve licensing of a geological repository for high-level radioactive waste and spent fuel. To support these activities HMI developed a computerized data management system for selected

geological, geochemical, petrological, and hydrological data on the candidate repository sites.

FOSSIL ENERGY INFORMATION. The Fossil Energy Information (FEI) Task Group added new projects, new capabilities, and new staff members in FY 1986. A new database, Environmental Compliance Online System (ECOS), in support of Martin Marietta Energy Systems, Inc., environmental compliance activities, was built and made available online via IBM's Time Share Option (TSO). This database contains summary information from the Federal Register on all regulations (proposed or final) that might impact environmental compliance activities in any of the four plants.

Using programming expertise originally developed for use with the Fossil Energy Environmental, Health, and Safety Information System, FEI developed a series of macros that made the Materials Safety Data Sheets Database accessible to a wide range of users. The same expertise was used to provide access to ORNLDB, a database of databases, and to ECOS.

Substantive contributions were made in risk assessment research, the acid rain inventory project, and the ADDNET Database and Notebook. Work was initiated with the U.S. Customs Service concerning microcomputer applications. Subcontracts were set up and managed for work with the Office of the Inspector General (OIG) of the Department of Health and Human Services, and a subcontract was prepared for work on an automation and microcomputer support center for the same OIG.

ENVIRONMENTAL INFORMATION. For the past fifteen years, members of the Environmental Information (EI) Task Group have been part of a multidisciplinary research team studying the ecology of transuranics in the desert environment of the Nevada Test Site. This year, studies were in the area of analysis of methods used to clean up and to treat radioactively contaminated sites. Two reports (an ORNL report and a journal article) are in progress.

EI Task Group members performed site surveys with the Radiological Survey Activities (RASA) Group of Health and Safety Research Division for both the Formerly Utilized Sites Remedial Action Project and the Uranium Mill Tailings Remedial Action Project. Group members analyzed radiological data and wrote assessment reports on potentially radiologically contaminated sites. A total of 265 reports to DOE (RASA reports) were written by EI Task Group members, and five ORNL/TMs are in draft form.

Staff members are assisting the Navy with projects related to information analysis and information computing. A personal computer-based Engineering Approval Tracking System was designed to meet the needs of major acquisition programs where approval status is crucial. This system, designed for the Airborne Self Protection Jammer Program, automates, accelerates, and increases the accuracy of tracking approvals. For another Navy project, information architecture studies were performed. Organizational goals and objectives were identified, analyzed, and defined as

individual data items, and the role of these items in the information system was determined.

ACIDIC DEPOSITION RESEARCH. IR&A staff conducted research in several areas (environmental effects, data management, and federal program development) of ORNL's program on the ecological effects of acid deposition. Research was conducted jointly with Environmental Sciences Division and was sponsored by the Department of Energy and the Environmental Protection Agency. Highlights of the research include:

- providing a technical analysis of key environmental issues associated with proposed and/or developing emission control options;

- contributing to a report summarizing the scientific literature on the aquatic impacts of acidic deposition;

- preparing proceedings of the Workshop on Regionalization of Aquatic Impacts Using the Adirondacks as a Case Study;

- compiling extant acidic deposition data into a central database (ADDNET), distributing ADDNET, and conducting assessment-related analyses; and

- organizing and coordinating the Watershed Coordination Workshop, which brought together a select group of watershed researchers and program managers to deliberate on the need for facilitating watershed research activities across the United States.

CONSERVATION MATERIALS INFORMATION. Ceramic Technology Newsletter. The Ceramic Technology Newsletter is published bimonthly for the Ceramic Technology for Advanced Heat Engines Project, which is a nation-wide project managed for DOE by the Metals and Ceramics Division. This newsletter, which has a distribution list of more than 1000, disseminates information on the latest technical accomplishments of the project as well as notices of recent publications and upcoming meetings. Advanced ceramics are of great interest to many industries, and the newsletter is an excellent mechanism in the technology transfer process.

ECUT TECHNICAL ASSISTANCE. The DOE Energy Conversion and Utilization Technologies (ECUT) Program is a directed research program aimed at processes and materials that improve the efficiency of energy-using systems and their capability to use alternate fuels. IR&A staff provided support to the materials part of the program which is managed by the Metals and Ceramics Division. Technical assistance to this program is provided by computing and editing quarterly and annual reports from the many subcontractors and in-house researchers, managing technical meetings and program reviews, and constructing and maintaining databases for the program.

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CHEMICAL HAZARD EVALUATION PROGRAM

P. Y. Lu
R. H. Ross
J. G. Pruett
S. M. Hubner
B. W. Kline

The objective of the Chemical Hazard Evaluation Program (CHEP) is to compile, analyze, and disseminate technical information related to health and environmental effects of potential toxic/hazardous substances. The assessment reports and databases produced have been applied to facilitate decision-making processes and enhance research carried out by academia, industries, and governmental agencies.

TOXICOLOGY REVIEW AND DOCUMENT PREPARATION. Under various environmental regulations, such as the Toxic Substances Control Act, Clean Air Act, and Safe Drinking Water Act, Federal and State governments are charged by Congress with protecting public health and preserving environmental quality. The CHEP staff members have assisted the EPA, Army, and the states of New York and Tennessee in developing scientific documents and technical reviews on chemicals potentially subject to these regulations. Data is gathered by performing comprehensive literature searches, acquisition of pertinent documents, evaluation of scientific and technical information, and preparation of comprehensive and concise assessment reports. These reports faciliate the decision-making process of establishing permissible toxic levels for chemical regulation. Types of reports include: Chemical Hazard Information Profiles, Chemical Hazard Assessments, Tier-I Health Assessment Reports, Water Quality Criteria Documents, Air and Water Quality Standards, Methodology Development for Occupational Health Criteria, and Problem Definition Studies.

HAZARDOUS SUBSTANCES DATA BANK. The Hazardous Substances Data Bank (HSDB), formerly Toxicology Data Bank, was established in 1974, with the support of the National Library of Medicine (NLM), to provide information on demand under the Toxic Substances Control Act and Superfund. HSDB is an online interactive database providing peer reviewed numeric/textual

information on chemicals. The records include compounds that are toxic, produced in large volume, found in waste dumps, and/or that have great potential for exposure by large populations. Each unit record has information on 10 categories and up to 145 data attributes. The end product is a "mini-encyclopedia" of useful data available to citizens, researchers, industry and government agencies and international users. This year a total of 484 records have been prepared and updated.

MARTIN MARIETTA ENERGY SYSTEMS MATERIAL SAFETY DATA SHEETS. With the promulgation of the Hazard Communication Standard by OSHA, 14 million workers at more than 320,000 manufacturing facilities were given the right to obtain information about the hazardous chemicals in their workplaces. To assist Energy Systems in complying with the law, IR&A and the Industrial Hygiene Department have developed an online interactive retrieval system for Material Safety Data Sheets. Data contents of the Material Safety Data Sheets come from peer-reviewed databases (HSDB and Gene-Tox), handbooks, as well as MSDS sheets from manufacturers. High data and system quality is assured by having an external peer review committee set standards and provide input on current practices in "real world" situations. The menu-driven system uses the INQUIRE Database Management System which resides on the IBM 3033. The system can be accessed through TSO on-site or off-site and is available to all Energy Systems" employees. Currently, the database contains information on 1300 pure chemicals and 1200 trade name products. The expected goal for next year is 4000 records, and the staff will continue to work toward updating and expansion of the present system.

QUALITY CONTROL - EPA PESTICIDE DOCUMENT MANAGEMENT SYSTEM. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), EPA has the responsibility of releasing all chemical scientific citations in the Pesticide Document Management System (PDMS) database without effecting release of chemical information that is classified as Confidential Business Information. Under FIFRA the CHEP staff members have assisted the Office of Pesticide Programs in developing a prototype chemistry dictionary coding guide to facilitate pesticide document registration and submission of PDMS as well as assure the validity of information on approximately 100,000 scientific studies that have been submitted to EPA by pesticide registrants. Phase I of the project will be completed in October 1986 and it is expected that the project will continue for two additional years.

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18. Opresko, D. M. Summary review of health effects associated with chloroethane: Health issue assessment. Final report, 1986.
19. Opresko, D. M. Literature review of air water quality standards: Formaldehyde. Final report, 1986.
20. Opresko, D. M. Literature review of air water quality standards: Vanadium. Final report, 1986.
21. O'Bryan, T., and R. H. Ross. Chemical scoring system for hazard and exposure assessment. Final report, 1986.
22. Pal, B. C. Chemical hazard information profile: Brominated diphenyl ethers. Final report, 1986.
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24. Ryon, M. G. Water quality criteria for nitrocellulose. ORNL-6179, 1986.
25. Ryon, M. G. Water quality criteria for trinitrotoluene, in press.
26. Ryon, M. G. Chemical hazard information profile: Hexafluoroisobutylene. Final report, 1986.
27. Ryon, M. G. The life history and ecology of Etheostoma trisella. Am. Midl. Nat., 115: 73-86, 1986.
28. Sigmon, C. F. Literature review of air water quality standards: Tetrachloroethylene. Final report, 1986.
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30. Smith, J. G. Water quality criteria for nitroglycerin. ORNL-6180, 1986.
31. Talmage, S. S. Chemical hazard information profile: C. I. basic red 9 monohydrochloride. Final report, 1986.
32. Talmage, S. S. Summary review of health effects associated with phthalic anhydride: Health issue assessment. Final report, 1986.
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34. Thiessen, K. M., and P. A. Lalley. Gene assignments and syntenic groups in the sacred baboon (Papio hamadryas). Cytogenet. Cell Genet., in press.

Educational Activities

Postdoctoral Training Program

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 and other universities. Two sources of funds are a subcontract from Martin Marietta Energy Systems, Inc. and a postdoctoral training grant in Carcinogenesis from the National Cancer Institute. Some appointments are also made through Oak Ridge Associated Universities (ORAU). During the past year, there were 12 trainees enrolled in these postdoctoral programs. After a two- or three-year period of research in the Biology Division, trainees have obtained positions in universities, industries, or other government laboratories.

Doctoral Training Program

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 multidisciplinary 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 Institutes of Health. The School currently has 29 students working toward the Ph.D. degree and 2 in the Master's program. As of October 1986, 131 students have been awarded the Ph.D. or M.S. degree.

1986 is the School's 20th anniversary. W. Edgar Barnett was the Director until July 1, 1986, when R. A. Popp became the Acting Director. The School also has three full-time University of Tennessee faculty members: Daniel Billen, Donald E. Olins, and Cynthia Soumoff. A major portion of the School's teaching and research training is provided by the staff of the Biology Division who serve as part-time faculty.

The students form a very active group of investigators in training, and their names appear on a number of publications each year. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division. In return, the students receive supurb guidance and training by staff members of the Biology Division.

Master of Science Degree Program in Biotechnology

The Master of Science degree program in biotechnology at The University of Tennessee has now enrolled its second class. This program was created with the help of Biology Division staff members who lecture in the program. The students carry out part of their laboratory training in laboratories and in the fermentation plant of the Biology Division.

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. In the past 12 months, there were 16 students, possessing the educational qualifications and the potential for a successful scientific career, who spent 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) performing 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, their colleges, and the members of the Biology Division.

Training Grants

Title	Awarded by	Principal Investigator
Predoctoral Training Program in Genetics	National Institute of General Medical Sciences	R. A. Popp
Predoctoral Training in Carcinogenesis Research	National Cancer Institute	F. T. Kenney
Postdoctoral Training in Carcinogenesis Research	National Cancer Institute	F. T. Kenney
Graduate Training in Radiation Biology	National Cancer Institute	R. L. Ullrich

Appendices

Advisory Committee - FY 1986

Dr. Robert Barker	Provost Cornell University Ithaca, New York
Dr. Verne M. Chapman	Chairman, Department of Molecular Biology and Associate Director for Scientific Affairs Roswell Park Memorial Institute Buffalo, New York
Dr. Robert L. Dixon	Director of Toxicology Sterling-Winthrop Research Institute Rensselaer, New York
Dr. Lorraine Flaherty	Chief, Laboratory of Immunology Wadsworth Center for Laboratories and Research State of New York Department of Health Albany, New York
Dr. Henry C. Pitot	Director McArdle Laboratory for Cancer Research The University of Wisconsin Madison, Wisconsin
Dr. Arthur C. Upton	Director Institute of Environmental Medicine New York University Medical Center New York, New York

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 October 1, 1985 through September 30, 1986.

Speaker	Affiliation	Subject
Adhya, S.	National Cancer Institute Bethesda, Maryland	Novel effects of DNA-protein interaction on gene control
Bunting, Mary	Former AEC Commissioner and former President of Radcliffe College Cambridge, Massachusetts	Using a scientific background to effect social change
Chada, K.	Columbia Medical School New York, New York	Of mice and men: Developmental regulation of global genes in transgenic mice
Cobb, R.	Research Triangle Institute Research Triangle Park, North Carolina	Molecular analysis of Mod-1 mutants
Cook, John	Colorado State University Fort Collins, Colorado	Intracellular pH changes induced by hypothermic treatments
Dorsett, Dale	Harvard University. Cambridge, Massachusetts	Mutational effects of the gypsy element
Edmonds, Mary	University of Pittsburgh Pittsburgh, Pennsylvania	Branched RNA

Fahrig, Rudolf	Fraunhofer-Institut für Toxikologie und Aerosolforschung Hannover, FRG	Mechanism of carcinogenesis and tumor promotion - genetic approach
Giles, Norman H.	University of Georgia Athens, Georgia	Gene organization and regulation in the Qa (quinic acid) gene cluster of Neurospora
Hall, Eric J.	Columbia University New York, New York	*In vitro* transformation following radiation
Hamkalo, Barbara	University of California Irvine, California	*In situ* hybridization in the electron microscope: Studies on chromosomal organization
Harris, Elizabeth	Duke University Durham, North Carolina	Rearrangement of chloroplast DNA in Chlamydomonas
Hemminki, K. J.	Institute of Occupational Health Helsinki, Finland	Benzo[a]pyrene adducts as a measure of exposure in humans
Hernandez-Verdun, D.	Banaras Hindu University Institut Biomedical des Cordeliers Paris, France	Nucleolar organizer regions - chromatin and proteins
Hollaender, A.	Council for Research Planning in Biological Sciences Washington, D.C.	Reflections on the development of Biology
Kovacic, P.	University of Wisconsin Milwaukee, Wisconsin	Unified theory for carcinogens and anti-cancer agents
Longworth, James W.	Illinois Institute of Technology Chicago, Illinois	Fluorescence studies of azurines

Medina, Daniel	Baylor College of Medicine Houston, Texas	Selenium inhibition of mammary tumorigenesis
Moslen, M. T.	University of Texas Galveston, Texas	Oxathiazolidine, a cysteine precursor, decreases activities of some hepatic enzymes involved in glutathione metabolism
Rebar, A.	Purdue University Lafayette, Indiana	Bronchioalveolar lavage profile in Syrian hamster: Effects of nitrogen dioxide
Sarkar, P. K.	Indian Institute of Chemical Biology Calcutta, India	Molecular and cellular aspects of thyroid hormone action in brain development
Singer, John	University of California San Diego, California	Cytoskeletal protein
Sobels, F. H.	Sylvius Laboratories of the State University of Leiden Leiden, The Netherlands	Changing concepts in mutation research
Sollner-Webb, B.	Johns Hopkins University Baltimore, Maryland	Transcription of mouse and frog rRNA genes
Ward, John	University of California San Diego, California	Effects of radiation on DNA
Yim, J.	Seoul National University Seoul, Korea	Preparation of biotin-labeled hepatitis-B virus DNA probes
Yoshida, H.	Armed Forces Radiology Research Institute Bethesda, Maryland	Kinetic flow dichroism study of conformational changes in supercoiled DNA induced by ethidium bromide and the binding of Benzo[a]pyrene diol epoxide

Extramural Activities

1. Society Committees

H. I. Adler	-	Education and Training Committee, Radiation Research Society, 1980-1987
D. Billen	-	Executive Committee and Finance Committee, Radiation Research Society, 1979-present
G. A. Bingham	-	Forum Review Committee, American College of Laboratory Animal Medicine, 1984-present
G. M. Caton	-	Treasurer, Special Information Group on Information Analysis and Evaluation, American Society for Information Science, 1985-1986
J. S. Cook	-	Publications Committee, American Physiological Society, 1985-1988 Publications Committee, Federation of American Societies for Experimental Biology, 1982-1985 Cole Award Committee (Chairman), Biophysical Society, 1984-1985
R. J. M. Fry	-	Awards Monitoring Committee (Chairman), Radiation Research Society, 1982-present History Committee, Radiation Research Society, 1982-present
P. Y. Lu	-	Chemical Section, Executive Committee, National Safety Council, 1983-present
P. Mazur	-	Publications Committee (Chairman), Society for Cryobiology, 1974-present Board of Governors, Society for Cryobiology, 1981-1987
I. C. Miller	-	Communications and Archives Committee, Environmental Mutagen Society, 1984-present
C. J. Oen	-	Special Information Group on Information Analysis and Evaluation (Chairman), American Society for Information Science, 1985-1986 Repesentative to Society Sponsor Group of WATTec Organization, American Society for Information Science, 1985-present

Association for Women in Science (Regional), Vice President, 1985-1986
Special Interest Group on Management, Chair of Nominating Committee, American Society for Information Science, 1985-1986
Jury Member for Award to Outstanding American Society for Information Chapter, 1985-1986

A. L. Olins - Constitution and By-laws Committee, American Society for Cell Biology, 1982-present
Councilor, American Society for Cell Biology, 1984-1986
E. B. Wilson Award Nomination Solicitation Committee, American Society for Cell Biology, 1984-present (Chairperson, 1986)
Minorities Committee, American Society for Cell Biology, 1985-present
Program Committee, Electron Microscopy Society of America, 1986
Oversight Committee, National Science Foundation, 1985

H. A. Pfuderer - Awards and Honors Committee, American Society of Information Science, 1984-1986

R. J. Preston - Education and Training Committee, Environmental Mutagen Society, 1984-present

L. B. Russell - Executive Committee, Environmental Mutagen Society (Chairman, 1984-1985)
Committee on Future Meetings, Environmental Mutagen Society (Chairman, 1986-1987)

R. L. Ullrich - Finance Committee, Radiation Research Society, 1984-present

E. S. Von Halle - Membership Committee (Chairman), Environmental Mutagen Society, 1985-present
Nominating Committee, Environmental Mutagen Society, 1985
Organization and Structure Committee, Environmental Mutagen Society, 1985-present

J. S. Wassom - Communications and Archives Committee (Chairman), Environmental Mutagen Society, 1982-present

H. R. Witschi - President, Inhalation Specialty Section, Society of Toxicology, 1986
Technical Committee, Society of Toxicology, 1986-1988

2. Advisory Committees

H. I. Adler - Joint University of Tennessee-Oak Ridge National Laboratory Committee to Develop Interactions in the Life Sciences, 1984-present
Alumni Advisory Committee, Department of Microbiology, Cornell University, 1986-

M. M. Brown - Gene-Tox Salmonella Work Group, Environmental Protection Agency, 1984-present

J. S. Cook - Member of the Corporation, Mount Desert Island Biological Laboratory
Advisory Committee, Department of Medicine, Baylor College of Medicine, 1984-1986

M. W. Francis - Gene-Tox V79 Mammalian Cell Work Group, Environmental Protection Agency, 1985-present

R. J. M. Fry - Scientific Committee 40, National Council on Radiation Protection and Measurements, 1977-present
Council Member, National Council on Radiation Protection and Measurements, 1980-present
Scientific Committee 75 (Chairman), National Council on Radiation Protection and Measurements, 1983-
Advisory Committee, Radiological Research Accelerator Facility, Columbia University, 1983-present
Committee 1, International Commission on Radiological Protection, 1985-1989
Advisory Committee, Institute of Environmental Medicine, New York University Medical Center, 1985-present
Vice-Chairman, Subcommission F.2 on Radiation Biology, Committee on Space Research, 1986-1988
Committee on Space Biology and Medicine, Space Science Board, National Research Council, National Academy of Sciences, 1986

R. A. Griesemer - Committee on Toxicology, National Research Council, 1983-1986
Joint Graduate Coordinating Committee, Comparative and Experimental Medicine Graduate Program, The University of Tennessee, 1984-

Committee on Toxicology's Subcommittee on Hydrocarbons, National Research Council, 1984-1986
Working Group on Tobacco Smoking, WHO International Agency for Research on Cancer, Lyon, 1985-1986
Science Advisory Board, Environ. Prot. Agency Executive Committee, 1984-
Chairman, Environmental Health Comm., 1984-
Chairman, Dioxin Toxic Equivalency Methodology Subcommittee, 1985-1986
Interagency Task Force on Environmental Cancer and Heart and Lung Disease, 1985-1986
Health and Environmental Research Advisory Committee (HERAC), Department of Energy, 1985-; Chairman, Subcommittee on Radiation Biology, 1985-

K. B. Jacobson - Adviser, The University of Tennessee Memorial Research Unit and Hospital, Metal Analyses in Alzheimer's Disease, 1983-present

S. J. Kennel - Study Section on Health Effects Research, Environmental Protection Agency, 1982-present
Ad hoc member, National Cancer Institute Site Visit Committee, 1986

F. T. Kenney - Ad hoc member, Postdoctoral Fellowship Committee, National Institutes of Health, 1985
Adviser, National Cancer Institute Outstanding Investigator Grants Program, 1984-present

P. Y. Lu - Scientific Review Panel for Small Business Innovative Research Program, National Institutes of Health, 1985-present
Hazardous Waste Information Evaluation Subcommittee, Committee to Coordinate Environmental and Related Programs, Department of Health and Human Services, 1983-present
Information Coordination Subcommittee, Environmental Health and Health Related Programs, Department of Health and Human Services, 1983-present

A. C. Marchok - Special Reviewer, Oral Biology and Medicine Study Section, National Institutes of Health, 1986

K. H. Mavournin - Data Review Committee, Environmental Protection Agency, 1984-1986
Gene-Tox Micronucleus Assay Work Group, Environmental Protection Agency, 1986

I. C. Miller - Gene-Tox Sister Chromatid Exchange Work Group, Environmental Protection Agency, 1986

C. J. Oen - Advisory Committee on Continuing Education (Information Sciences), 1984-present

A. L. Olins - Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-present
Gordon Conference on Chromatin, 1984, 1986 (Co-chairman)

D. E. Olins - Faculty Senate, University of Tennessee, 1984-1986
Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-present
Advisory Committee, Helicon Foundation, LaJolla, California, 1983-present
Faculty Senate, University of Tennessee, 1983-present
Gordon Conference on Chromatin, 1984, 1986 (Co-chairman)

R. A. Popp - Mouse Hemoglobin Nomenclature, 1984-

R. J. Preston - Cytogenetic Adviser to Ethylene Oxide Council and to Health Industry Manufacturers Association, 1981-present
Genetics Working Group, American National Standards Institute, 1983-present
Committee on Genetic Toxicology, American Society for Testing and Materials, 1984-present
Committee on Biological Radiation Dosimetry, International Atomic Energy Agency, 1983-present
Talent Pool for Committee on Radiation Research and Policy Coordination, 1985-
Committee for National Cancer Institute/ Oak Ridge Associated Universities on Radiation Biological Dosimetry in Reference to Atomic Veterans, 1985-present
Gene-Tox Panel on *In Vivo*/*In Vitro* Cytogenetics (Chairman), Environmental Protection Agency, 1986

L. B. Russell	-	International Committee on Standardized Genetic Nomenclature for Mice, 1977-present Coordinating Committee of Gene-Tox, Environmental Protection Agency, 1980-present Committee on Risk Assessment of Gene-Tox (Chairman), Environmental Protection Agency, 1980-present Board on Toxicology and Environmental Health Hazards, National Academy of Sciences, 1981-1986 Board on Environmental Science and Toxicology, National Academy of Sciences, 1986-1987 Distinguished Scientist Committee, University of Tennessee-Oak Ridge National Laboratory, 1983- Advisory Panel for the Assessment of Methods for Measuring Mutation Rates in Human Beings, Office of Technology Assessment, U.S. Congress, 1984-1986 Genetic Risk Assessment Guidelines Review Group, Science Advisory Board, U.S. Environmental Protection Agency, 1984-1985
W. L. Russell	-	Scientific Adviser to U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR)
P. B. Selby	-	Task Group XI of National Council on Radiation Protection Committee 57, Genetic Risk from Internal Emitters, 1984-present Scientific Adviser to U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), 1986
D. M. Skinner	-	Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-present Panel Member, Regulatory Biology Program, National Science Foundation, 1986
A. S. Stevens	-	Biomedical Sciences Study Section, National Institutes of Health, 1985-present
J. B. Storer	-	Scientific Committee 1 on Basic Radiation Protection Criteria, National Council on Radiation Protection and Measurements, 1975- Council Member, National Council on Radiation Protection and Measurements, 1969- Scientific Committee 75, National Council on Radiation Protection and Measurements, 1983-

R. L. Ullrich - Scientific Committee 40 on the Biological Aspects of Radiation Protection Criteria, National Council on Radiation Protection and Measurements, 1977-present
Joint University of Tennessee-Oak Ridge National Laboratory Committee to Develop Interactions in the Life Sciences, 1984-present

E. S. Von Halle - Gene-Tox Drosophila Work Group (Chairman), Environmental Protection Agency, 1984-present
Gene-Tox Salmonella Work Group, Environmental Protection Agency, 1979-present

J. S. Wassom - Gene-Tox Coordinating Committee, Environmental Protection Agency, 1980-present
Coordinating Committee, Environmental Protection Agency Sponsored Consensus Workshop on the Evaluation of Maternal and Developmental Toxicity, 1986

B. L. Whitfield - Gene Tox Mammalian Dominant Lethal Assay Work Group, Environmental Protection Agency, 1986

H. R. Witschi - Scientific Review Panel for Health Research, Office of Research and Development, Environmental Protection Agency, 1980-present
Complex Mixtures Committee, Commission of Life Sciences, BOTEHS, National Research Council, 1985-1986
Environmental Health Science Review Committee, NIEHS, National Institutes of Health, 1986-1990
Ad hoc member, Safety and Ocupational Health Study Section, NIOSH, National Institutes of Health, 1986
Grant Review Panel, Health Effects Institute, 1985, 1986
Scientific Review Panel, National Library of Medicine, National Institutes of Health, 1986-1988
Advisory Board, CRC Critical Reviews in Free Radical Research, 1986-

W. K. Yang - Experimental Virology Study Section, National Institutes of Health, 1982-1986
Oncogene Study Task Group, U.S. Department of Energy, 1986

3. Editorial Boards

H. I. Adler	-	Radiation Research (Associate Editor), 1980-1987
D. Billen	-	Radiation Research (Editor-in-Chief), 1979-present
J. S. Cook	-	American Journal of Physiology (Associate Editor, 1981-present; Acting Editor, 1984-1985) Cell and General Physiology, American Physiological Society Handbook Series, 1982-present Current Topics in Membranes and Transport (Advisory Board), 1983-present News in Physiological Sciences (Editorial Board, 1985-1986) (Associate Editor, 1986-1989)
W. M. Generoso	-	Teratogenesis, Carcinogenesis, and Mutagenesis, 1979-present Mutation Research, 1985-1988
F. C. Hartman	-	BioScience, 1980-1986 Journal of Protein Chemistry, 1982-1987 Journal of Biological Chemistry, 1983-1988
K. H. Mavournin	-	Environmental Mutagenesis, 1985-present
P. Mazur	-	Cryobiology, 1967-present
A. L. Olins	-	European Journal of Cell Biology, 1982-present Molecular and Cellular Biochemistry, 1982-1985
D. E. Olins	-	Molecular and Cellular Biochemistry, 1983-1986
R. A. Popp	-	Federation of American Societies for Experimental Biology Databook, 1983-1985
R. J. Preston	-	Mutation Research, 1977-present Environmental and Experimental Botany, 1979-present Mutation Research Letters (Managing Editor), 1980-present Teratogenesis, Carcinogenesis, and Mutagenesis, 1980-present
J. D. Regan	-	Cell Biology and Toxicology, 1983-present

L. B. Russell	-	Mutation Research, 1976-present
G. A. Sega	-	Environmental Mutagenesis, 1985-
D. M. Skinner	-	Growth, 1979-1986 Gene, 1986-
R. L. Ullrich	-	Radiation Research, 1983-1989
E. S. Von Halle	-	Environmental Mutagenesis, 1979-present Mutation Research, Genetic Toxicology Testing, 1983-present Registry of Toxic Substances, 1983-present
B. L. Whitfield	-	Environmental Mutagenesis, 1985
J. S. Wassom	-	Board of Managing Editors, Mutation Research, 1981-present Risk Analysis, 1982-present
H. R. Witschi	-	Toxicology and Applied Pharmacology, 1972-1986 Toxicology, 1978-present (Associate Editor, USA) Environmental Health Perspectives, 1981-present Toxicologic Pathology, 1983-present Journal of Biochemical Toxicology, 1984-present

Awards, Honors

S. J. Kennel	-	Publication Award, Martin Marietta Energy Systems, Inc., 1986
P. Y. Lu	-	Cameron Award, National Safety Council, 1985
P. Mazur	-	Corporate Fellow, Martin Marietta Energy Systems, Inc., 1985
S. Mitra	-	Technical Achievement Award, Martin Marietta Energy Systems, Inc., 1986
R. A. Popp	-	Publication Award, Martin Marietta Energy Systems, Inc., 1986
L. B. Russell	-	Elected to Membership in The National Academy of Sciences, 1986

D. M. Skinner - National Lecturer, Sigma Xi, 1986
Association for Women in Science Award for Distinguished and Sustained Contributions to Science, 1986

H. R. Witschi - Publication Award, Martin Marietta Energy Systems, Inc., 1986

International Activities

Australia

Dr. Paul Selby continued to exchange research data and provide consultation on skeletal mutations with staff at the University of Sydney, Australia.

France

Dr. Liane B. Russell collaborated with Dr. Philip Avner of the Pasteur Institute, Paris, who is molecularly mapping the X chromosome of the mouse. A series of X-autosome translocations that arose at Oak Ridge and have been genetically characterized by Dr. Russell provide important tools for the molecular mapping of the X chromosome and have been sent to Paris. Dr. Avner has sent back to Oak Ridge cell cultures from mouse-hamster somatic cell hybrids which are being cytogenetically analyzed in banded chromosome preparations by N. L. A. Cacheiro in Dr. Russell's group.

India

Dr. Salil K. Niyogi is an Advisory Committee Member for the Indian Institute of Chemical Biology, Calcutta, India. The Committee was organized to advise the Director on the future direction of research, on holding scientific conferences to foster closer ties among scientists in India and abroad, and on other scientific matters.

Italy

Dr. Steven J. Kennel continued a collaborative research project with Dr. Ada Sacchi, Institute Regina Elena, Rome. Dr. Sacchi visited the Biology Division in February 1986, and Dr. Rita Falcioni (from the same Institute) is a visiting investigator in Dr. Kennel's laboratory. Joint work centers around tumor surface proteins on mouse and human carcinomas that function as growth factor receptors.

Japan

W. L. Carrier received a Senior Fellowship from the Japan Society for the Promotion of Science to conduct collaborative research for 2.5 months with Dr. Keiichi Nozu, Biology Department, Nara Medical University, Kashihara, Japan. He also presented seminars at several laboratories in Japan.

Korea

Dr. K. Bruce Jacobson continued his collaborative studies with Dr. John Yim, Department of Microbiology, Seoul National University, on gene suppression in Drosophila. Dr. Jacobson visited Dr. Yim's laboratory to conduct experiments and prepare manuscripts. Two papers have been published from this collaboration, which is supported by a joint research grant for the period 1984-1986 from the NSF/U.S. East Asia Cooperative Science Program.

Spain

Dr. K. Bruce Jacobson (supported by a Short-Term Research Development grant from NSF) continued to collaborate with Dr. Juan Ferré and Prof. J. L. Ménsua, Department of Genetics, University of Valencia, on the regulation of pteridine biosynthesis. A three-year grant from the Joint Spanish-North American Committee for Cooperative Science and Technology has been awarded to continue these studies in 1986-1988. Dr. Ferré visited Dr. Jacobson's laboratory in December 1985, and two papers have been published from the collaboration.

World Health Organization (WHO)

Dr. Richard A. Griesemer participated as Rapporteur in a WHO International Agency for Research on Cancer (IARC) Working Group to Revise and Update Supplement 2 to the IARC Monographs on Human Carcinogens. A Monograph entitled "An Appraisal of Long-Term Assays and Short-Term Carcinogen Screening Tests" was prepared.

Dr. Paul B. Selby served as Scientific Adviser to the U.S. Delegation, at the 35th Session of the United Nations Scientific Committee on the Effects of Atomic Radiation.

International Committees

Dr. R. J. M. Fry is a member of the International Commission on Radiological Protection.

Dr. Liane B. Russell serves on the International Commission on Standardized Genetic Nomenclature for Mice.

Special Assignment

Dr. Robert K. Fujimura was on special assignment from July 1985 to October 1986 for the Foreign Commercial Service, U.S. Department of Commerce, to assess the policies and activities related to biotechnology in Japan. He was assigned to Washington, D. C. for two months and then to the American Embassy in Tokyo. In Japan, he conferred with more than 500 people from the central and provincial governments, universities and their associated research laboratories, private industries, and national laboratories. His observations and interpretations resulted in 9 reports and 27 cables.

ABSTRACTS FOR TECHNICAL MEETINGS HELD
OCTOBER 1, 1985 — SEPTEMBER 30, 1986

Aardema, M. J., and R. J. Preston. Cytosine arabinoside (AraC) induced chromosome aberrations in an X ray sensitive mouse myeloid leukemia cell line. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Adler, Howard I., and Weldon Crow. Genetic variability in Clostridium acetobutylicum. American Society for Microbiology, Washington, D.C., March 23-28, 1986.

Adler, H. I., W. D. Crow, and R. Machanoff. Novel methods for growing anaerobes. Society for Industrial Microbiology, San Francisco, California, August 10-15, 1986.

Allison, L. J., R. J. Olson, and I.L. Munroe. ADDNET: The Acid Deposition Data Network. Tenth International CODATA Conference, Ottawa, Canada, July 14-17, 1986.

Alpen, E. L., P. Powers-Risius, E. J. Ainsworth, L. Triplett, and R. J. M. Fry. Tumor induction by heavy ions. Radiation Research Society, Las Vegas, Nevada, April 13-17, 1986.

Bast, Cheryl Barbati, and R. Julian Preston. Induction of chromosome-type aberrations by chemicals in G_0-treated human lymphocytes: A possible method of increasing the sensitivity of the lymphocyte assay. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Cadilla, C. L., A. E. Roberson, J. Harp, A. L. Olins, and D. E. Olins. Subfractionation of soluble macronuclear chromatin and enrichment of specific genes as chromatin from Euplotes eurystomus. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Carrier, William L. DNA damage and repair. Japanese Radiation Research Society, Nara, Japan, October 25, 1985.

Carrier, W. L., A. A. Francis, and J. D. Regan. DNA damage and repair in mammalian cells. Japanese Society of Radiation Research, Nara, Japan, October 15-18, 1985.

Carrier, W. L., and J. D. Regan. Excision of UV-light induced pyrimidine dimers from mammalian cells. American Society for Photobiology, Los Angeles, California, June 22-26, 1986.

Chang, L. Y., F. E. Myer, and W. K. Yang. On the defective MuLV-related sequences: The involvement of short direct repeat in the generation of deletions. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 20-25, 1986.

Cook, John S., Carolyn Shaffer, and Edward J. Cragoe, Jr. Inhibition of NA+-dependent uptake of hexose and amino acids in LLC-PK1 cells by amiloride and amiloride-derivatives. Federation of American Societies for Experimental Biology, St. Louis, Missouri, April 13-18, 1986.

Crow, W., M. V. Adler, Mary Jean Bryant, and H. I. Adler. A new anaerobic method for clinical microbiology. Southern Association for Clinical Microbiology, Knoxville, Tennessee, September 10-12, 1986.

Dunn, W. E., R. S. Foote, P. A. Lalley, R. E. Hand, Jr., and S. Mitra. Regulation of repair of O^6-methylguanine in DNA of mammalian cells. International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis, Lawrence, Kansas, October 6-10, 1985.

Evans, R. R., R. M. Furkes, and W. K. Yang. Partial characterization of viral proteins from murine Type C retrovirus. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Ferré, Juan, Wolfgang Pfleiderer, and K. Bruce Jacobson. Synthesis, purification and properties of a new class of pteridine derivatives. 8th International Symposium on Pteridines and Folic Acid Derivatives, Chemical, Biological and Clinical Aspects, Montreal, Canada, June 15-20, 1986.

Fowler, R. F., L. A. Stringfellow, and D. M. Skinner. Characterization of a Z-DNA structure associated with sequence divergence in a complex satellite. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Fry, R. J. M. Radiation carcinogenesis in skin. Workshop on Radiation Damage to Skin: Fundamental and Practical Aspects, Paris, France, October 9-11, 1985.

Fry, R. J. M. Radiation effects in space. Committee on Space Research (COSPAR), Toulouse, France, June 30-July 12, 1986.

Fry, R. J. M., J. Gray, L. Triplett, and S. Ogle. Radiation effects on Langerhans cells. Radiation Research Society, Las Vegas, Nevada, April 13-17, 1986.

Furkes, R., R. R. Evans, J. G. Levin, and W. K. Yang. Identification of endonucleases in gross passage a strain Murine leukemia virus. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 20-25, 1986.

Furkes, R. M., R. R. Evans, and W. K. Yang. Purification and characterization of an endonuclease from gross passage A strain murine leukemia virus. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Goad, M. E. P., A. F. Tryka, and H. Witschi. Morphometric analysis of acute lung injury induced by bleomycin and hyperoxia. American Thoracic Society, Kansas City, Missouri, May 11-14, 1986.

Griesemer, R. A. Interspecies quantitative extrapolation. 14th International Cancer Congress, Budapest, Hungary, August 21-27, 1986.

Griesemer, R. A. In vivo/in vitro assays for respiratory carcinogenicity. Conference on "Short-Term Test Method Development and Deployment," Nikko, Japan, May 27-29, 1986.

Griesemer, R. A. Short-term in vivo assays for carcinogenicity. Conference on "Short-Term Test Method Development and Deployment," Nikko, Japan, May 27-29, 1986.

Hartman, Fred C., and Eva H. Lee. Active-site characterization of ribulose bisphosphate carboxylase-oxygenase. Royal Society, Discussion Meeting on Ribulose Bisphosphate Carboxylase-Oxygenase, London, England, December 4-5, 1985.

Hartman, Fred C., Richard J. Mural, Sankar Mitra, Thomas S. Soper, Salil K. Niyogi, Robert S. Foote, Richard Machanoff, Eva H. Lee, and Frank W. Larimer. Site-directed mutagenesis of lysine-166 of ribulose bisphosphate carboxylase/oxygenase from Rhodospirillum rubrum. American Society of Biological Chemists, Washington, D.C., June 8-12, 1986.

Holmes, Roger S., Raymond A. Popp, and John L. VandeBerg. Genetics of ocular NAD^+-dependent alcohol dehydrogenase and aldehyde dehydrogenase in the mouse - evidence for genetic identity with stomach isozymes and localization of Ahd-4 on chromosome 11. 3rd Congress of the International Society for Biomedical Research on Alcoholism, Helsinki, Finland, June 8-13, 1986.

Hotchkiss, J. A., and S. J. Kennel. A monoclonal antibody specific for fully mature type I pneumocytes in the BALB/c mouse. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Jacobson, K. Bruce, and J. Ferré. Use of C18 silica cartridges to purify and characterize pteridines and pterin derivatives. 8th International Symposium on Pteridines and Folic Acid Derivatives, Chemical, Biological, and Clinical Aspects, Montreal, Canada, June 15-20, 1986.

Jamison, C. Scott, and Howard I. Adler. Mutations in Escherichia coli affecting sensitivity to oxygen. American Society for Microbiology, Washington, D.C., March 23-28, 1986.

Kao, J., and J. W. Hall. Cutaneous metabolism in skin absorption of topically applied chemicals. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Kennel, S. J., L. S. Foote, and R. L. Ullrich. Cell surface phenotype of mouse mammary epithelial cells correlated with tumorigenic potential. American Association for Cancer Research, Los Angeles, California, May 7-11, 1986.

Kuemmerle, N. B., L.-Y. Chang, C. K. Koh, L. R. Boone, and W. K. Yang. Solitary LTRs of the MuLV type in chromosomal DNA of laboratory mouse strains. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 20-25, 1986.

Larimer, F. W., R. Machanoff, R. J. Mural, T. S. Soper, S. K. Niyogi, S. Mitra, R. S. Foote, and F. C. Hartman. Mutations probing an implicated active-site residue of ribulose bisposphate carboxylase. American Society for Microbiology, Washington, D. C., March 23-28, 1986.

Lee, Eva H., and Fred C. Hartman. Distance between two active-site lysines of ribulosebisphosphate carboxylase/oxygenase. American Society of Biological Chemists, Washington, D.C., June 8-12, 1986.

Lee, K.-L., K. R. Isham, A. Johnson, and F. T. Kenney. Insulin enhances transcription of the tyrosine aminotransferase gene in rat liver. American Society of Biological Chemists, Washington, D.C., June 8-12, 1986.

Lewis, B. A., W. D. Dawson, and J. S. Cook. Effects of TPA on subcellular localization of protein kinase C and lipid bilayer structure. Biophysical Society, San Francisco, California, February 9-13, 1986.

Lindenschmidt, R. C., and H. P. Witschi. Inhibition of lung tumor formation by hyperoxia. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Lock, Simon. Growth and fetal viability in mice exposed to high concentrations of ethylene oxide (EtO). Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Lu, P. Y. Toxicology information and environmental protection. The Association of Chinese Scholars in Southeastern United States, Atlanta, Georgia, June 14-15, 1986.

Lu, P. Y., and J. T. Ensminger. Preparation of material safety data sheets. Hazardous Material Spills Conference, St. Louis, Missouri, May 5-8, 1986.

Lu, P. Y., J. T. Ensminger, and S. M. Hubner. Generation of material safety data sheets. The Tenth International CODATA Conference, Ottawa, Canada, July 14-17, 1986.

Machanoff, R., F. W. Larimer, R. J. Mural, S. K. Niyogi, R. S. Foote, T. S. Soper, S. Mitra, and F. C. Hartman. Protein engineering of ribulose bisphosphate carboxylase. Society for Industrial Microbiology, San Francisco, California, August 10-15, 1986.

Mann, Reinhold C., Betty K. Mansfield, and James K. Selkirk. Parametric versus non-parametric gel image analysis. National Electrophoresis Society, Gaithersburg, Maryland, March 26-28, 1986.

Mansfield, Betty K., Reinhold C. Mann, and James K. Selkirk. 2,5-Diphenyloxazole (PPO) in dimethyl sulfoxide (DMSO) regeneration for re-use in fluorography. National Electrophoresis Society, Gaithersburg, Maryland, March 26-28, 1986.

Marchok, A. C., and L. M. Arnseth. Quantitation of the effects of fractionated doses of benzo(a)pyrene (BaP) on tumor initiation sites using an open-ended rat tracheal implant-cell culture model. 77th Annual Meeting of the American Association for Cancer Research, Los Angeles, California, May 7-10, 1986.

Martin, F. M. Health effects associated with exposure to methyl methacrylate. Society for Toxicology, Southeastern Regional Chapter, Athens, Georgia, September 4-5, 1986.

Mazur, Peter. Do embryos respond as ideal osmometers during slow freezing? Society for Cryobiology, Augusta, Georgia, June 17-20, 1986.

Mazur, Peter, and Kenneth W. Cole. Responses of packed and suspended human red cells to hyperosmotic glycerol-NaCl solution at subzero temperatures in the absence of ice-formation. Society for Cryobiology, Augusta, Georgia, June 17-20, 1986.

Mitra, Sankar, and Robert S. Foote. Site-directed mutagenesis of DNA-O^6-methylguanine methyltransferase of *E. coli*. American Society of Biological Chemists, Washington, D.C., June 8-12, 1986.

Mural, R. J., and R. S. Foote. "Bandaid" mutagenesis: A novel technique for oligonucleotide-directed site-specific mutagenesis. Sixth Annual Congress for Recombinant DNA Research, Baltimore, Maryland, January 26-29, 1986.

Mykles, D. L., and D. M. Skinner. A comparative study of crustacean Ca^{2+}-dependent proteinases that degrade myofibrillar proteins. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Niemann, S. L., and R. A. Popp. Sensitivity of mouse strains to ethylene oxide is due to a single genetic trait. Genetics Society of America, Champaign, Illinois, June 3-5, 1986.

Niemann, S. L., A. F. Tryka, and R. A. Popp. Ethylene oxide induces germ and somatic cell damage in strain SEC mice. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Niemann, S. L., C. J. Wawrzyniak, and R. A. Popp. Fetal liver stem cell concentration is decreased in beta-thalassemic embryos during development. 14th Molecular and Biochemical Genetics Workshop, Bar Harbor, Maine, August 26-30, 1986.

Niyogi, Salil K., Robert S. Foote, Richard J. Mural, Frank W. Larimer, Sankar Mitra, Thomas S. Soper, Richard Machanoff, and Fred C. Hartman. Site-directed mutagenesis of histidine-291 of ribulosebisphosphate carboxylase/oxygenase from Rhodospirillum rubrum. American Society of Biological Chemists, Washington, D.C., June 8-12, 1986.

O'Brien, J. J., and D. M. Skinner. A proteinase that degrades crustacean exoskeleton. American Society of Zoologists, Baltimore, Maryland, December 28-31, 1985.

O'Brien, J. J., and D. M. Skinner. Inhibition of limb regeneration and ecdysis by the parasitic barnacle, Loxothylacus panopei in the mud crab, Rithropanopeus harrisii. American Society of Zoologists, Baltimore, Maryland, December 28-31, 1985.

O'Bryan, T., R. H. Ross, and P. Y. Lu. Chemical scoring for hazard and exposure assessment. Society of Toxicology, New Orleans, Louisiana, March 3-6, 1986.

Oen, C. J. The world of scientific information. Association for Women in Science, East Tennessee Chapter, March 17, 1986.

Olins, Ada L. Electron microscope tomography: 3-D reconstruction of asymmetric structures. Electron Microscopy Society of America, Albuquerque, New Mexico, August 10-15, 1986.

Owen, P. T. An overview of decommissioning and decontamination technical information support activities funded by the U.S. Department of Energy's remedial action programs. Health Physics Society's 19th Midyear Topical Symposium, February 2-6, 1986.

Pfuderer, H. A. Information resource management: the "in" thing in government. American Society of Information Science, Chicago, Illinois, September 28-October 2, 1986.

Popp, D., S. Lock, and R. A. Popp. Effect of high dose, short term ethylene oxide (EO) exposure in mice. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Popp, D. M., J. Otten, and R. A. Popp. Viral influence on basal serum IgA levels. 14th Molecular and Biochemical Genetics Workshop, Bar Harbor, Maine, August 26-30, 1986.

Popp, R. A., S. D'Surney, and C. J. Wawrzyniak. Changes in α- and β-globin gene expression during development of the mouse. Fifth Hemoglobin Switching Conference, Airlie, Virginia, September 30-October 1, 1986.

Popp, R. A., S. L. Niemann, and S. Lock. Ethylene oxide induces dominant lethals in C57BL and sterility in SEC male mice. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Porter, Michael A., and Fred C. Hartman. The thioredoxin binding site of ribulose 5-phosphate (Ru5P) kinase overlaps the catalytic site. VII International Congress on Photosynthesis, Providence, Rhode Island, August 10-15, 1986.

Reed, W. M., and H. I. Adler. Application of bacterial membranes for the growth of thermophilic, anaerobic bacteria. Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tennessee, May 13-16, 1986.

Roseberry, L. M. Database administration in a matrix organization: Playing the game and coaching the team. American Society for Information Science, 15th Midyear Meeting, Portland, Oregon, May 1986.

Ross, R. H. Chemical scoring for hazard and exposure assessment. Society of Risk Analysis, East Tennessee Chapter, Oak Ridge, July 15, 1986.

Ross, R. H. Clearinghouse for indoor air quality: The role of the Oak Ridge National Laboratory. The Second Annual Conference Indoor Air Quality, Washington, D.C., September 18-19, 1986.

Ross, R. H., M. G. Ryon, and M. L. Daugherty. Teratology, immunotoxicology and inhalation toxicology. American Society for Pharmaceutical and Experimental Therapeutics/Society for Toxicology, Baltimore, Maryland, August 17-21, 1986.

Roy, Dipak, and Sankar Mitra. Degradation of chlorinated aromatic compounds by dehalogenases of two new pseudomonas species. Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, Tennessee, May 13-16, 1986.

Russell, Liane B. Qualitative analysis of radiation- and ENU-induced specific-locus mutations. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Russell, W. L., P. R. Hunsicker, and J. W. Bangham. High resistance of mouse oocytes to mutation induction by ethylnitrosourea. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Selkirk, James K. The role of metabolism in chemical carcinogenesis. Southeastern Cancer Research Association, Durham, North Carolina, October 2-4, 1985.

Siracusa, Linda D., Liane B. Russell, Eva M. Eicher, Neal G. Copeland, and Nancy A. Jenkins. Molecular genetic approach to the study of the agouti locus of mouse chromosome 2. International Workshop on the Molecular Genetics of the Mouse, Warth (Zurich), Switzerland, June 15-20, 1986.

Stringfellow, L. A., R. F. Fowler, and D. M. Skinner. Major deletions from a complex G+C-rich satellite are associated with a domain of Z-DNA. American Society for Cell Biology, Atlanta, Georgia, November 17-22, 1985.

Turner, J. E., H. A. Wright, R. N. Hamm, and K. Bruce Jacobson. A model for calculating fragmentation of linear polymers irradiated in water. Radiation Research Society, Las Vegas, Nevada, April 13-17, 1986.

Ullrich, R. L. Time-dose relationships in radiation carcinogenesis. Radiation Research Society, Las Vegas, Nevada, April 13-17, 1986.

Wasson, J. S., P. Y. Lu, and J. T. Ensminger. Quality assurance and searchable toxicology data bases for risk analysis. Society for Risk Analysis, Alexandria, Virginia, October 7-9, 1985.

Waters, Larry C., and Carroll E. Nix. Drosophila cytochrome P-450: Partial purification of insecticide resistance-related forms. UCLA Symposia on Molecular Entomology, Steamboat Springs, Colorado, March 30-April 6, 1986.

Wawrzyniak, C. J., and R. A. Popp. Expression of the two adult β-globin genes in mouse yolk sac and fetal liver erythrocytes. 14th Molecular and Biochemical Genetics Workshop, Bar Harbor, Maine, August 26-30, 1986.

Wei, Chin Hsuan. The crystal structure of 4-methyl-5-(2-vinylenepyrazinyl)-3H-1,2-dithiole-3-thione, $C_{10}H_8N_2S_3$. American Crystallographic Association, Hamilton, Ontario, Canada, June 22-27, 1986.

Winegar, R. A., and R. J. Preston. The induction of chromosome aberrations by restriction endonucleases - frequencies from specific lesions and chromosome distribution. Environmental Mutagen Society, Baltimore, Maryland, April 9-13, 1986.

Witschi, Hanspeter. Complex organic mixtures and toxicological interactions. National American Chemical Society Fall Meeting, Anaheim, California, September 7-12, 1986.

Witschi, Hanspeter. Complex organic mixtures and toxicological interactions. Symposium on "Health and Environmental Effects of Complex Organic Mixtures," American Chemical Society, Anaheim, California, September 7-12, 1986. (Expanded version)

Witschi, H. P. Interactions: The problem and its biology. American Statistical Association, Chicago, Illinois, August 18-21, 1986.

Witschi, H. P., and R. C. Lindenschmidt. Modification of gastrointestinal tumor development by dietary butylated hydroxytoluene. Society of Toxicology, New Orleans, Louisiana, March 3-7, 1986.

Yang, W. K., C. Y. Ou, L. Y. Chang, F. E. Myer, D. M. Yang, and L. E. Roberson. Expression of three distinct families of related endogenous retroviral gene elements in normal mouse tissues. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 7-11, 1986.

FINANCIAL SUMMARY AND PERSONNEL DISTRIBUTION

Biology Division*

FY 1986

Funding Source	Funding in thousands	Percent of total budget	Person-years
Department of Energy	12,128	78.2	110.2
National Cancer Institute	1,495	9.6	16.0
National Institute of Environmental Health Sciences	1,105	7.1	16.9
Environmental Protection Agency	227	1.5	2.4
Miscellaneous	176	1.1	1.4
National Institute of Allergy and Infectious Diseases	116	0.7	1.6
Army	62	0.4	0.6
Department of Agriculture	58	0.4	0.1
National Institute of General Medical Sciences	49	0.3	
National Institute on Aging	48	0.3	0.5
National Heart, Lung and Blood Institute	23	0.1	0.2
National Institute of Child Health and Human Development	23	0.1	0.3
TOTAL	15,510		150.2

*Does not include Information Research and Analysis Section.

FINANCIAL SUMMARY AND PERSONNEL DISTRIBUTION

Information Research and Analysis Section

FY 1986

Funding Source	Funding in thousands	Percent of total budget	Person-years
Environmental Protection Agency	1,354	29.7	15.2
Department of Energy	1,133	24.9	8.1
National Institute of Environmental Health Sciences	749	16.4	10.0
Miscellaneous	414	9.1	3.5
National Library of Medicine	396	8.7	4.6
Navy	262	5.7	2.9
Army	249	5.5	3.2
TOTAL	4,557		47.5

FINANCIAL SUMMARY AND PERSONNEL DISTRIBUTION

Total Biology Division

FY 1986

Funding Source	Funding in thousands	Percent of total budget	Person-years
Department of Energy	13,261	66.1	118.3
National Cancer Institute	1,495	7.5	16.0
National Institute of Environmental Health Sciences	1,854	9.2	26.9
Environmental Protection Agency	1,581	7.9	17.6
Miscellaneous	590	2.9	4.9
National Library of Medicine	396	2.0	4.6
Army	311	1.5	3.8
Navy	262	1.3	2.9
National Institute of Allergy and Infectious Diseases	116	0.6	1.6
Department of Agriculture	58	0.3	0.1
National Institute of General Medical Sciences	49	0.2	
National Institute on Aging	48	0.2	0.5
National Heart, Lung and Blood Institute	23	0.1	0.2
National Institute of Child Health and Human Development	23	0.1	0.3
TOTAL	20,067		197.7

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