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BIOLOGY DIVISION
PROGRESS REPORT

For Period of October 1, 1984 - September 30, 1985

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F. C. Hartman, Molecular Genetics Section
R. J. Preston, Comparative Genetics Section
L. B. Russell, Mammalian Genetics and Reproduction Section
H. R. Witschi, Toxicology Section

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Contents

Introduction and Division Overview

R. A. GRIESEMER

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

The energy-related substances of interest are both physical and chemical. Among the physical agents, major interest is focused on the health effects of neutron and heavy ion radiations on animals with particular attention to the carcinogenic responses to low dose levels and to the relative biological effectiveness of various forms of radiation. Among the chemical agents, special emphasis is placed on problems associated with the emerging energy technologies. Since the energy-related substances to which people may be exposed tend to be complex mixtures of chemicals, the Division's activities concentrate on evaluating and understanding the toxicological interactions when mammals are exposed to multiple substances, either concurrently or successively.

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

The resources available for the Division's activities during the report period included a staff of 50 at the doctorate level, 142 support personnel, 42 predoctoral students, 10 postdoctoral students, and an average of 70 other visiting professors, students, and scientists. The Division occupies 327,000 sq. ft. of laboratory space with specialized facilities for 250,000 animals, a collection of 1,000 mutant stocks of mice, barrier facilities for the safe handling of hazardous substances, laboratories for recombinant DNA research, radiation sources, a facility for the production of large volumes of cells or microorganisms, and a library. The Division also makes extensive use of resources in other Divisions of the Oak Ridge National Laboratory including the Information Centers for mutagenesis, toxicology, and teratology and the Analytical

Chemistry Division where collaboration in research has been especially fruitful.

The Division has embarked on a long-range plan to maintain and upgrade its facilities. The laboratory buildings that house the Biology Division now are old, energy-inefficient, and in constant need of repair. A long-range plan has been developed to construct new buildings when funds can be made available and to locate them on a site that will bring together all the life science research activities at ORNL.

In 1985, the Division enjoyed its first full year as a team member with the Laboratory's new contractor, Martin Marietta Energy Systems, Inc. Several of our staff members promptly received special recognition. Drs. Peter Mazur and Ann Marchok received awards from Martin Marietta Energy Systems for their outstanding publications and Dr. Mazur received additional recognition as Author of the Year for the entire Martin Marietta Energy Systems. Two other Division scientists received awards for technical achievements during the year, Dr. Fred Hartman for his research in protein engineering and Dr. Julian Preston for his research in cytogenetics.

Technical progress during this report period is described in the following chapters but one highlight of Division activities deserves special mention. A new initiative in protein engineering (site directed mutagenesis) under the direction of Dr. Fred Hartman is well under way. The research team has succeeded in making mutants of the plant CO_2 fixation enzyme, ribulose bisphosphate carboxylase, by specific single amino acid substitutions. New methods have been developed for introducing site-specific mutations into cloned genes and for the rapid purification of enzymes.

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, 1984, through September 30, 1985. 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 current organizational structure of the Biology Division; each Section begins with an overview. It will be apparent, however, that crosscurrents 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 extramural activities of staff members, abstracts for technical meetings, and funding and personnel levels.

With this report, The Biology Division completes its 39th year of operation. We are looking ahead in anticipation of celebrating our 40th year of biomedical research next year.

Superscripts - Division Staff

[1]Loanee - Solid State Division

[2]Guest Assignment

[3]Postdoctoral Investigator

[4]Loanee - Chemistry Division

[5]Leave of absence

[6]Loanee - Maintenance Division

[7]Loanee - Finance and Materials Division

[8]Loanee - Information Division

[9]Loanee - Engineering Division

Division Staff–September 30, 1985

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Superscripts after Staff Names on Research Summaries

[1] Guest Assignment

[2] Postdoctoral Investigator

[3] Student, University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences

[4] Loanee - Chemistry Division

[5] Loanee - Solid State Division

[6] Consultant

[7] Engineering Physics and Mathematics Division

Research Activities

Molecular Genetics 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) O^6-Methylguanine-DNA alkyltransferase, responsible for the repair of promutagenic and possibly procarcinogenic O^6-alkylguanine in DNA, is tightly regulated in mammalian cells. The regulation is cell cycle-specific, and in somatic cell hybrids the repair-deficient phenotype is dominant.

(2) A 5'→3' exoribonuclease of Saccharomyces cerevisiae has been purified to near homogeneity. The enzyme has a molecular weight of 165,000 and it is localized in nuclei. Results suggest that the enzyme may be involved in the hydrolysis of the RNA primers involved in DNA replication.

(3) Incubation in a HeLa transcription extract converts a plasmid DNA containing the adenovirus type 2 major late promoter into ordered nucleoprotein complexes. Unlike naked DNA, these complexes can be transcribed

accurately without the addition of transcription specificity factors, thereby confirming that the complexes are enriched in these factors.

(4) The soluble macronuclear chromatin of Euplotes has been subfractionated by sucrose gradient ultracentrifugation to yield clear enrichment of specific genes as chromatin fragments based on immunoblotting with specific gene probes for 5S RNA, α-tubulin, and ribosomal RNA.

(5) An unusual 194 bp domain of a repeat unit of a very complex eukaryotic satellite DNA that contains a long tract comprised of three types of repetitive polypurine polypyrimidine tracts (Pu·Py) is the site of the greatest sequence divergences among the otherwise quite closely related family of molecules that comprise the satellite. An unusually long (~120 bp) segment of the domain, which has been subcloned, adopts two types of altered conformations that are driven by superhelical stress at a level typical of cellular DNAs and are stabilized by slightly acidic pH. Protonated C residues participate in the formation of such structures in vitro; methylated Cs may behave similarly in vivo. At least part of one of these altered conformations may be due to a triple stranded helical structure formed by an interaction between two homologous double helical molecules; accordingly, it offers a model for initiation of a recombinational intermediate. Pu·Py DNAs have often been found upstream from eukaryotic genes where they seem to be involved in gene regulation. Our data suggest that the changes in DNA structure associated with such tracts may also lead to the major mutations seen in the satellite.

(6) 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 10 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.

(7) Progress in the development of electron microscope tomography has included 3-D image processing on the vibrating mirror and improved specimen preparation for very thick sections. Data from semi-thin sections of transcription unit cross sections and of very thick sections used to measure transcription unit length show that DNA is less compact during transcription than when it is inactive.

Membrane biology is also considered of prime relevance to the Section, as membranes can be considered interfaces between the metabolic machinery

of the cell and its external environment. As an example of overlapping interests between the Sections, the effects of tumor promoters on membrane transport systems are being examined. The action of a phorbol ester on the uptake of neutral amino acids into cultured mammalian cells is a model system for examining early effects of the promoter. The apparent sequence of events is that the promoter intercalates into the cell-surface membrane and changes its physical properties in such a way that the inactive, cytosolic protein kinase C becomes bound to the membrane and activated. In parallel, the activity of the amino acid transporter is also redistributed, possibly by exocytosis of internal vesicles containing the transporter, and the cells' capacity for scavenging amino acids from the medium is greatly enhanced from the low level characteristic of quiescent cells toward the higher level required to sustain growth. Certain physiological diacylglycerols are also effective in initiating this cascade of events.

Another aspect of cellular-environmental interactions and membrane integrity that receives emphasis is cryobiology. Recently, the long-held view that injury to cells subjected to slow freezing is due to osmotic dehydration has been challenged. New data on erythrocytes and embryos suggest that survival is more dependent on the size of the unfrozen channels in which the cells lie than on solute concentrations. When these channels narrow, damaging rheological forces are set in motion.

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, the elucidation of enzymological consequences of suppressor mutations, 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; (2) discovery that a mutant form of tryptophan dioxygenase in D. melanogaster is inhibited by a macromolecule expressed by a suppressor locus, whereas the normal enzyme is unaffected; and (3) partial purification of a chitinase and a cysteine proteinase involved in degradation of crustacean exoskeleton.

As a logical extension of our traditional strengths in enzymology and molecular biology, a new initiative in protein engineering (site-directed mutagenesis) has evolved. This program is designed to integrate and hopefully enhance some ongoing endeavors through focusing on a common interdisciplinary theme having both fundamental and applied significance. During the past year, R. J. Mural (formerly of the Frederick Cancer Center) and T. S. Soper (formerly of Rockefeller University) joined the protein engineering effort. Current activities include mutagenesis of the active site of ribulose bisphosphate carboxylase and 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

F. C. Hartman
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R. S. Foote[1]
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T. Soper

The program in protein engineering (site-directed mutagenesis), initiated in April 1984 with partial support from the Laboratory Director's R & D Fund, has thus far been focused on specific alterations in the structural gene (rbc) of ribulosebisphosphate carboxylase (Rubisco), the CO_2-fixation enzyme ubiquitous among photosynthetic organisms and 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 (ribulose-P_2) to yield two molar equivalents of D-3-phosphoglycerate (the CO_2-fixation reaction), it also catalyzes the oxidation of ribulose-P_2 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 ribulose-P_2 carboxylase 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. As described in last year's report, the R. rubrum rbc gene, cloned in an E. coli plasmid and which expresses a fusion protein (Nargang et al., Mol. Gen. Genet. 193: 220, 1984), was engineered so that the native Rubisco is expressed in E. coli under regulatory control of lac

repressor. The rbc gene present in the plasmid as well as in the single-stranded phage vector M13 mp19 has been the target for site-directed mutagenesis induced by synthetic oligodeoxynucleotides. Although the three-dimensional conformation of Rubisco is not yet available, active-site characterization with affinity labels and comparative sequence analyses have led to the identification of several critical amino acid residues in Rubisco of R. rubrum. These include Lys-166, Lys-329 and His-44. The results of mutagenesis experiments and the preliminary characterization of mutant proteins are summarized as follows.

Synthesis of Oligodeoxynucleotides. Oligodeoxynucleotides of defined sequences for introducing mutations into the rbc gene and for use as primers for confirming the nucleotide sequences in mutants by dideoxy sequencing procedure were synthesized by the phosphoramidite procedure using a Microsyn 1450 automated DNA synthesizer (Systec Inc.). The oligonucleotides were purified by polyacrylamide gel electrophoresis and selected oligomers were further characterized by base analysis and sequencing by the Maxam-Gilbert method.

Mutagenesis with Single-Stranded DNA Template. Among the procedures for generating site-specific mutations, oligonucleotide-mediated single-stranded M13 mutagenesis is widely used. Even though the procedure is straightforward in principle, optimization of reactions in various steps is essential for high efficiency of mutagenesis. This is especially true for the rbc gene because of its unusually high (G+C) content that promotes nonspecific intra- and intermolecular H-bonding.

Following the annealing of oligonucleotide (phosphorylated at the 5' end) with the single-stranded M13 recombinant DNA, the primer-template was replicated in vitro and ligated to generate covalently closed replicative form RF I DNA that was purified by centrifugation. The transformants from the RF molecules were screened for mutants with ^{32}P-labeled mutant oligonucleotide as a hybridization probe and the suspected mutants were confirmed by direct sequencing.

"Band-Aid" Mutagenesis. A novel technique has been developed which has a significant advantage over the single-stranded template DNA-based approach. This technique, called the "band-aid" method, involves ligation of a single-stranded oligodeoxynucleotide containing the mutant sequence of the target site to protruding ends of the appropriate strand of duplex recombinant DNA of the rbc gene in the expression vector. The sequence corresponding to the oligonucleotide was removed by restriction digestion. Following transformation, the single-stranded region of the resulting circular DNA was replicated to give mutant sequences in both strands in contrast to the heterozygous sequence produced in the M13 mutagenesis system. The "band-aid" technique is apparently superior to the "cassette mutagenesis" approach in that 1) this technique requires synthesis of only one strand, 2) oligonucleotides of mixed sequence can be used for generation of simultaneous mutations and 3) the background of nonmutant clones can be significantly reduced by extensive treatment with specific restriction endonucleases that selectively degrade the residual duplex DNA

surrounding the mutation target but not the single-stranded "band-aid" that is ligated.

Engineering of Plasmid and Phage Vectors and rbc Gene. Synthetic oligodeoxynucleotides have been utilized to engineer phage and plasmid vectors for facilitating mutagenesis and expression of the rbc gene. For example, creation of a new HindIII site at nucleotide 502 allowed introduction of a 311-nucleotide-long HindIII/XmaI fragment into M13mp19 vector. The short fragment spanning the Lys-166 site can be used for generation of mutants at and around the lysine residue and for sequencing of the complete fragment in the mutant before transferring it to the expression vector. This is particularly important for in vitro synthesis of RFI DNA which is difficult because of the high (G+C) content, as mentioned earlier. A similar construct of M13 vector, containing a 340-nucleotide-long rbc fragment spanning Lys-329, has been made. The expression vector has also been engineered by removal of extraneous sites for restriction enzymes which are to be used for ligation of the mutated fragments.

The rbc gene itself was modified before using it for "band-aid" mutagenesis. The introduction of new KpnI and HindIII sites within a short segment of the rbc gene spanning Lys-166 allowed creation of a 22-nucleotide gap after digestion of the expression vector with KpnI (which leaves a 3' overhang) and HindIII (which leaves a 5' overhang).

Purification and Properties of the Rubisco Mutants. Immunoaffinity chromatography was employed to purify the wild-type and the mutant ribulosebisphosphate carboxylases. The immunoaffinity column matrix contained protein A-Sepharose to which the IgG of Rubisco was cross-linked. Bound Rubisco was eluted from the antibody column with 2.5 M $MgCl_2$ after crude extract of E. coli expressing the rbc gene was passed through the matrix. Homogeneous Rubisco was obtained by this single purification step. The mutant proteins that have been studied are ones with single amino acid replacements for Lys-166. The glycine mutant exhibits only 1% of the normal V_{max} but essentially unaltered K_m values for both CO_2 and ribulose-P_2. These observations demonstrate that Lys-166 is not involved in substrate binding but probably functions catalytically to facilitate a particular step in the complex reaction pathway. The facilitation is probably provided by the lysyl ε-amino group in its free base form rather than as a cationic acid, because the arginine mutant is devoid of catalytic activity.

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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 ribulosebisphosphate carboxylase, the plant enzyme essential for the photosynthetic assimilation of CO_2. This enzyme also possesses inherent oxygenase activity which accounts for photorespiration, a nonessential, energy-wasteful process that reduces net CO_2 fixation. 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.

In the absence of CO_2 and Mg^{2+}, ribulosebisphosphate carboxylase is devoid of both catalytic activities. The activation process entails the reaction of CO_2 with a specific lysyl ε-amino group to form a carbamate which requires Mg^{2+} for stabilization. Activator CO_2 is distinct from substrate CO_2; hence, characterization of the activator site as well as the catalytic site are pertinent to elucidation of mechanism of action and mode of regulation. Affinity labels designed by us have enabled partial mapping of the binding site for ribulosebisphosphate. Specifically, two different lysyl residues and an histidyl residue are implicated at the active site, and the high degree of sequence conservation adjacent to these residues (observed upon comparing the primary structures of the carboxylase from the

evolutionarily diverse organisms spinach and Rhodospirillum rubrum) strongly support this supposition.

To gain insight into the precise role of the active-site lysyl residues, their reactivities toward trinitrobenzenesulfonate (TNBS), a general lysyl reagent, have been examined. With both the R. rubrum and spinach enzyme, TNBS is selective for a single active-site lysyl residue, but, interestingly, with the former, Lys-166 is the target for arylation whereas with the latter Lys-334 is the target. At pH 8.0, the rates of reactions with the two enzymes are ~400-times that for the reaction with N-α-acetyllysine with TNBS. Based on the pH-dependency of inactivation of the R. rubrum enzyme by TNBS, the ε-amino group of Lys-166 exhibits a pK_a of 7.9 and an intrinsic reactivity (k_o) of 670 $M^{-1}min^{-1}$. In analogous experiments, Lys-334 of the spinach enzyme exhibits a pK_a of 9.0 and a k_o of 4500 $M^{-1}min^{-1}$. By comparison, the reaction of TNBS with N-α-acetyllysine reveals a pK_a of 10.8 and a k_o of 1250 $M^{-1}min^{-1}$. The spinach carboxylase, catalytically inactive as a consequence of selective arylation of Lys-334, still exhibits tight binding of the transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate. Therefore, Lys-334 is not required for substrate binding and may serve a role in catalysis. The unusually low pK_a of Lys-166 argues that this residue is also important to catalysis rather than substrate binding. We have recently undertaken studies with chemical cross-linking reagents designed to reveal the distance between the two active-site lysines.

We are also interested in another enzyme unique to the Calvin cycle, namely phosphoribulokinase which catalyzes the formation of ribulosebisphosphate from ribulose 5-phosphate and ATP. This enzyme is highly regulated — directly by metabolites and indirectly by the ferredoxin/thioredoxin system. In this latter light-dependent process, phosphoribulokinase is activated by reduced thioredoxin via reduction of an enzyme disulfide. With [^{14}C]bromoacetylethanolamine phosphate, we have been able to label selectively one of the sulfhydryls involved in the activation/deactivation process and also to demonstrate that this sulfhydryl is in the vicinity of the ATP binding site.

Despite the metabolic importance of phosphoribulokinase, structural studies have been hampered by the scarcity of highly purified enzyme. Thus, we have developed an efficient purification procedure utilizing affinity chromatography. Gel filtration and polyacrylamide gel electrophoresis of the purified enzyme reveal a dimeric structure of 44,000-Da subunits. Chemical cross-linking with dimethyl suberimidate confirms the presence of two subunits per molecule of native kinase, which are shown to be identical by partial NH_2-terminal sequencing. Based on amino acid analyses, each subunit contains five cysteinyl residues. The observed slow loss of activity during spontaneous oxidation in air-saturated buffer correlates with the intramolecular oxidation of two sulfhydryl groups, presumably those involved in thioredoxin-mediated regulation.

Collaborations with investigators at other institutions include X-ray diffraction studies of ribulosebisphosphate carboxylase from R. rubrum

(David Eisenberg, UCLA) and affinity labeling of fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase, the enzyme that regulates the intracellular level of fructose-2,6-bisphosphate (Kosaku Uyeda, University of Texas).

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ENZYME AND GENE REGULATION: NORMAL MECHANISMS AND RESPONSES TO TOXIC METAL IONS

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The biological consequences of exposure of humans to elevated levels of toxic substances that normally are encountered in trace amounts must be understood. In my research program the mutational and toxicological consequences have been selected for those studies and two strategies are

followed: (1) determine how the effects of certain mutations are neutralized by a natural mechanism; this is termed suppression and (2) seek the earliest events that occur when an animal is exposed to toxic metal ions and relate those to the physical-chemical properties of the metal ion.

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

Two metabolic pathways are involved: tryptophan metabolism for the vermilion mutant and pteridine biosynthesis for the purple one. In the vermilion case the synthesis of xanthommatin is eliminated and the enzyme defect is in tryptophan dioxygenase. Earlier work had demonstrated that in vermilion this enzyme activity is <2% of normal. Using the newly developed assay, based on HPLC, for kynurenine we have examined the properties of tryptophan dioxygenase from the vermilion mutant as compared to normal. The mutant enzyme was found to be subject to either inhibition or inactivation by extracts of flies that are of the $su(s)^+$ genotype whereas the normal enzyme is not. This confirms the previous finding of a similar effect on the ramiopterin synthase from purple, namely that the mutant enzyme differs from the normal in that it is inhibited by an "$su(s)^+$ substance". The next stage in this study should be to purify those enzymes and the "$su(s)^+$ substance" to determine the basis for such enzyme regulation.

A study that will provide some technically useful procedures for the study of pteridine biosynthesis has also been pursued. For this, the use of solid phase extraction procedures, using Sep-Pak C18 cartridges, has been developed for analysis of various pteridines and the pyrimidodiazepine that was discovered here.

Toxic Mechanisms. The physical-chemical properties of metal ions have been measured in many ways, but our primary goal is to determine which of these many parameters is most relevant to the biological damage metal ions cause. A related goal is to ascertain the earliest macromolecular alterations that occur after toxic metal ion is taken up by an organism. This project is an outgrowth of a seed money project and has become an interdivisional activity with two physicists (J. E. Turner and M. W. Williams, Health and Safety Research Division).

The properties of a naturally occurring cadmium chelator that we term Cadmium Binding Substance (CdBS) has been examined in some detail. The binding of cadmium *in vitro* and *in vivo* has been compared and the conditions for *in vitro* binding have been explored. The CdBS in larvae and adult Drosophila have been compared chromatographically and found to be

indistinguishable. The induction of CdBS in resistant and sensitive strains of Drosophila has been carefully evaluated and we find that the final level in both strains does not differ if they have been fed Cd^{2+} throughout their life. It remains to be seen whether the rate of induction will differ between the two strains or whether there is any structural difference of CdBS from the two strains. For the latter purpose the purification of CdBS is under way.

The effect of zinc and cadmium ion on transfer RNA is under study using RPC-5 and other physical chemical techniques. Out of this study has come the discovery of a new support material for RPC-5 that is commercially available (Voltalef beads) and this support material has been compared in some detail to the original material (Plaskon beads). We have a paper submitted to demonstrate the satisfactory chromatographic behavior of several tRNAs and of defined fragments of DNA on the Voltalef-type RPC-5 column.

The effect of divalent cations on tRNA structure has been explored through NMR, circular dichroism, ultraviolet absorption spectra, fluorescence spectra and melting behavior. A comprehensive interpretation of these studies is being written at this time to describe how the divalent cations alter tRNA structure. This model involves the ionic and non-ionic forces and interactions within the tRNA molecule and between the tRNA and the RPC-5 column.

The correlation of Alzheimer's disease with the occurrence of elevated levels of aluminum in the human brain has been reported and has been disputed in the literature. I have been acting as an advisor to the staff at The University of Tennessee Memorial Research Unit (Knoxville), who are studying Alzheimer's disease, to plan and conduct a comprehensive evaluation of the levels of aluminum and 30 other elements in the brain. This involves two laboratories in the Analytical Chemistry Division of ORNL, one for the use of inductively coupled plasma for determination of the average concentration of these elements and the other for the use of ion microprobe mass analysis for determination of the local levels of metals in each cell and group of cells. We have analyzed 12 normal and four Alzheimer's brains so far and the data are being evaluated.

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MEMBRANE DYNAMICS OF CULTURED MAMMALIAN CELLS

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The research theme of this laboratory is the regulation of membrane activities as studied in cloned mammalian cells in culture. Progress has been achieved in three areas.

1. The Effects of Amiloride and Amiloride Derivatives on Na-Dependent Hexose Transport in Cultured Renal Tubule Cells. LLC-PK_1 cells, derived from pig kidney, develop in postconfluent cultures the capacity for accumulating the nonmetabolizable glucose analog α-methylglucoside. The uptake is coupled to Na uptake, and since there is a steep electrochemical gradient for the ion high intracellular concentrations of the hexose can be achieved utilizing the energy stored in the ion gradient. This is the transport system by means of which glucose filtered at the glomerulus is retrieved in the renal proximal tubules of the intact animal. Under conditions in which the Na gradient is markedly reduced, significant uptake of hexose is still observed. To investigate whether this transport may be linked to the Na/H exchanger known to be present in these cells we have investigated the effects of amiloride and derivatives of amiloride provided to us by E. J. Cragoe of Merck, Sharp and Dohme. Amiloride is a totally synthetic loop diuretic, in clinical use, that blocks the reabsorption of Na in the distal tubules of the kidney, acting on Na,K-ATPase, Na/H and Na/Ca exchange and with lesser potency on conductive ^{Na} channels. The derivatives methylisobutyl and ethylisopropyl amiloride are more potent inhibitors of Na/H exchange, a fact that we have confirmed in LLC-PK_1 cells. We now find that these derivatives also block Na-dependent hexose

transport, but at concentrations several orders of magnitude higher than their effective concentrations on Na/H exchange. Experiments are under way to determine whether the drugs compete with Na at the cation binding site of the transporter.

2. Population Kinetics for the Development of Na-Dependent Hexose Transport in LLC-PK_1 Cells. Accumulative hexose transport is not observed in these cells during their active growth, but develops to a high degree over a period of several weeks in postconfluent, quiescent cultures. To analyze this developmental process at the cellular rather than the population level, we have derived a cell-kinetics model and the means to test it. The test, described in last year's report, requires blocking the cells' volume-regulating functions by equilibrating them with a K-free, Na gluconate medium containing 2 mM $[NH_4]_2SO_4$, which is sufficient to keep the Na,K-ATPase functioning and extruding the Na accumulated with the hexose. In this medium transport-competent cells accumulate α-methylglucoside and swell osmotically, whereas nontransporting cells are unaffected by the presence of the substrate. Transporting and nontransporting cells can thus be separated on density gradients. The model incorporates a rate constant for the recruitment of cells from the undifferentiated population onto the developmental pathway, a transition time for the traverse of the pathway, and allowance for cycling cells renewing the undifferentiated subpopulation. Two extreme cases described by the model are: (1) "Synchronous" development, in which all cells are recruited at the same time point and subsequently attain their maximum transport in parallel over a period of weeks. This form of the model predicts that on the density gradients one would observe the cells distributed in a single peak which, in the presence of α-methylglucoside, would be centered at successively lighter densities on successive days in culture as the transport capacity of the cells progressively increased. (2) The other extreme is "stochastic" development, in which the cells from the undifferentiated subpopulation are slowly recruited over a period of weeks while the development of full transport capacity occurs in a negligibly short time interval in the individual recruited cell. This form of the model predicts that two peaks would be observed on the gradients, corresponding to transporting and nontransporting subpopulations, and that with time of development one peak would become smaller as the other became larger while the center-to-center distance between the peaks, a measure of the transport capacity of the fully differentiated cell, would remain unaltered. As a first approximation this prediction corresponds to the observations.

The model can be further refined by observing the distribution of the [^{14}C]α-methylglucoside used to induce the density shift. If all of the cells in the transporting peak were fully developed, the distribution of the hexose should be symmetrically disposed under this peak. But if some of the cells in the transporting subpopulation had not yet developed their full accumulating capacity the distribution of the hexose would be skewed toward the nontransporting side, and from the model the degree of skew is a measure of the time required to traverse the developmental pathway. From these quantitative considerations we have estimated the various rate

constants in the population kinetics for the development of hexose transport in LLC-PK_1 cells. We find that: (1) after confluence is reached, cells are recruited from the undifferentiated population at a rate of 20% per day; (2) the undifferentiated population is renewed at a rate of 7% per day; (3) 5-7 days are required for the traverse of the developmental pathway; (4) once a cell achieves its maximum transport capacity, this capacity remains unaltered over subsequent periods of incubation of the culture.

The potential for separating transporting from nontransporting cells is being exploited in the biochemical analysis of the cells in the two states of development and as a preliminary screening procedure for isolating transport-deficient mutants.

3. Early Effects of TPA, Diacylglycerols, and Dialkylglycerols on Amino Acid Transport in LLC-PK_1 Cells. It is now well established that the tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate) is an activator of protein kinase C. This kinase is Ca^{++} and phosphatidylserine-dependent, and is physiologically activated by diacylglycerols; TPA substitutes for the diacylglycerols in activating the kinase. In growing LLC-PK_1 cells, the kinase is found in a membrane-rich particulate fraction and neutral amino acid transport (A system) is high. In confluent cells the kinase is found in inactive form in the soluble fraction and amino acid transport is low. TPA has little effect on transport in the growing cells, but in the confluent cells TPA treatment rapidly (minutes) reactivates transport and results in the shift of the inactive kinase from the cytosol to the membrane fraction. Diacylglycerols, especially 1,2-oleylacetylglycerol, have the same effects. A difficulty in the use of these compounds is their tendency to isomerize to the 1,3-isomers, which are inactive. The isomerization is enzymatically accelerated in whole cells. Dialkylglycerols with the same fatty acid side chains are equally effective as activators of the kinase and in reactivation of the transport system, but do not undergo the undesirable isomerization. The activation of transport appears to be the consequence of the translocation of transporters from intracellular vesicular pools to the cell surface. We are presently conducting a careful search for these pools.

Protein kinase C has been described as the "receptor" for TPA. We take a different view. Confluent cells were fractionated into kinase-deficient membranes and kinase-rich cytosol, the membrane fraction was treated with TPA, and the free TPA was removed by washing. The TPA-treated membranes were then mixed with the kinase-rich cytosol and incubated. In a few minutes the kinase was cleared from the soluble fraction and could be recovered from the membranes. The result suggests that TPA changes the physical properties of the membranes in such a way as to promote kinase binding to them. This effect is being explored in greater detail.

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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 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 mammalian 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 germ plasm 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, and there are a number of medical applications to cryobiology, especially in the transplantation of cells, tissues, and organs.

Our research during the last year has emphasized studies on the mechanisms of slow freezing injury.

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 as low as 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%), we have found that survival is affected by both the unfrozen fraction and the salt concentration in that fraction. It appears, then, that there are two main sources of injury to slowly frozen red cells. One source is rheological damage that occurs when cells become deformed at subzero temperatures as the liquid channels in which they lie become progressively narrower. Rheological damage is independent of the solute concentration in these channels and consequently independent of the degree to which the cells shrink osmotically. It is also independent of cell concentration. The second source of damage is a cell packing effect that results from cell-to-cell contacts of shrunken cells. The cell packing effect not only is dependent on the size of the liquid channels but also on the concentration of solute in those channels and the initial hematocrit.

Our experiments the past year have concentrated on mimicing the events to which cells are exposed during freezing but 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 molal glycerol in 0.15 molal NaCl and then supercooled to -5° to -8°C (supercooled in that 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 in added) some 95% survive. But if the cells are in a centrifuged pellet in the supercooled 0.5 m glycerol (0.15 m NaCl at the time the cold concentrated

solution is added) only 8% 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.

The damaging effects of cell shrinkage while the cells are pelleted decreases linearly with rise in temperature, and it disappears at 35°C.

Mouse Embryo Freezing. Three manuscripts authored by U. Schneider and P. Mazur pertaining to embryo freezing have been submitted or are in preparation. One reports the extent to which mouse and bovine embryos behave like ideal osmometers in both hyperosmotic and hypoosmotic media. Another deals with the effects of salt concentration and unfrozen fraction on embryo viability, and the third reports measurements of the permeability of bovine embryos to glycerol.

Early in the year, an interagency proposal was funded by the National Institute on Aging to establish an embryo bank of strains of mice and rats of concern to the NIA. Dr. Ulrich Schneider will carry out the work. Dr. Schneider will also collaborate with Dr. L. Russell in her Section's efforts to preserve some of their mutant mouse stocks in the form of frozen embryos.

Osmotic Response of Organized Tissues to Freezing and Simulated Freezing. Determining the osmotic response of single cells or small spherical aggregates like embryos to the hyperosmotic conditions occurring during freezing is far simpler both in theory and practice than in the case of organized tissues. Furthermore, the cells in multicellular tissues communicate with adjoining cells by specialized highly permeable regions - the gap junctions. The state of these gap junctions, i.e., whether open or closed, is in part controlled by the concentration of calcium ions, the concentration of which changes markedly during progressive freezing. The state of ions in cells and the state of the gap junctions can be determined by electrophysiological measurements of potentials between microelectrodes placed in the cell interior and electrodes in the surrounding medium. Dr. Winfried Berger, a visiting investigator from Homburg University, FRG, carried out exploratory studies on the feasibility of making these difficult measurements. The results, while not definitive, were sufficiently promising to encourage us to explore possibilities for funds for his making future visits to Oak Ridge for collaborative research with Dr. John Cook and our group.

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MOLECULAR MECHANISMS OF DNA REPAIR AND MUTAGENESIS

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A variety of genotoxic compounds are generated during energy production and utilization. These compounds, sometimes after activation *in vivo*, react with DNA and other cellular macromolecules in all organisms. The resulting alterations in DNA can lead to mutations, tumor induction and general toxic effects. However, the ultimate biological effects of chemical insult vary widely from one organism to another. These variations depend not only upon the nature of the chemical, but also on the ability of the organism to cope with the insult to its genetic material. One important parameter which determines the final consequence of DNA damage is the efficiency with which critical DNA lesions are repaired. The research objective of this laboratory is to elucidate the mechanism and regulation of repair of damages in DNA produced by simple alkylating carcinogens and mutagens as well as the mutagenic changes in DNA that are produced due to persistence of unrepaired lesions. Specifically, the current objectives of our research are (1) to determine the molecular mechanism of the regulation of repair of promutagenic and possibly procarcinogenic O^6-alkylguanine in DNA and (2) to study the specificity of O^6-alkylguanine in induction of various mutations in bacteria and mammalian cells. The results of the current experiments bear upon the first objective and are summarized as follows.

Effect of the Cell Cycle on O^6-Methylguanine-DNA Methyltransferase (MGMT) in C3H/10T1/2 Cells. A few years ago, Smith et al. (Cancer Res. 41: 1373, 1981) observed that O^6-methylguanine is not removed from DNA of mouse cells in S-phase. We decided to investigate whether this cell cycle-dependent lack of repair of the lesion is due to a selective loss of MGMT activity or results from the inaccessibility of MGMT to the lesion because of altered chromation conformation in the S-phase. We have now completed a major portion of experiments on the effects of the cell cycle on MGMT activity using parasynchronized cultures of 10T1/2 cells. The various stages of the cell cycle (i.e. cells in G1, S-phase and G2+M) were monitored by flow cytometry. Our results indicate that average MGMT levels/cell are significantly reduced as cell cultures approach S-phase and return to normal levels as cells enter G2. In confluent cells, recovery of MGMT activity in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treated cells begins about 6 h after exposure to the agent. In synchronized cultures, this recovery does not begin until the onset of G2 (ca. 24th after subculture) but is much more rapid than the recovery rate observed in confluent cultures. Preliminary experiments, in which sorted G1, S-phase or G2 populations from log phase 10T1/2 cultures were assayed for MGMT activity, show that levels of the enzyme are reduced by ~50% in S-phase cells. These results indicate that an absence of repair of O^6-methylguanine in S-phase cells may not be due solely to a lack of available MGMT. Presently, we are comparing MGMT levels in sorted populations of log phase vs synchronized cell cultures as well as the recovery of MGMT in MNNG-treated log phase vs confluent cultures. These comparisons are necessary since log phase cultures of 10T1/2 cells are more resistant to transforming and cytotoxic properties of MNNG than are synchronized cultures.

Purification and Characterization of Human O^6-Methylguanine DNA Methyltransferase (MGMT). We have purified MGMT approximately 100,000-fold from human placenta and established its molecular weight (24,000 daltons) and isoelectric point (pI 6.2). Sixty to 90% of MGMT activity is tightly associated with the chromation fraction from the homogenate of fresh placenta. The purification of the enzyme from the chromation fraction involves sonication, fractionation with polyethylene imine and ammonium sulfate, gel filtration, affinity chromatography on DNA-cellulose and chromatography on hydroxyapatite. However, difficulties in purifying sufficient quantities of MGMT for physical and biochemical characterization are formidable because of the low level (~10 μg/100g tissue) present in placenta. Preparative electrophoretic methods are being used as the final steps in purifying microgram quantities of the protein for amino acid sequencing and antibody preparation. Sequence data will be used to prepare synthetic DNA probes for cloning the MGMT structural gene from a human cell cDNA library. Immunoaffinity columns prepared from anti-MGMT antibody should simplify purification of the protein from human cells and tissues.

O^6-Methylguanine-DNA Methyltransferase in Somatic Cell Hybrids. We have assayed the methyltransferase (MGMT) activity in different somatic cell hybrids of cells proficient in repair of O^6-methylguanine (Mex^+) and those in which MGMT is undetectable. Because the hybrid cells selectively segregate chromosomes of only one parent cell (Mex^+), we hoped that we

might be able to identify the chromosome of the segregating parent that contains either the structural or regulatory sequence of MGMT. As described in the last year's report, we found that all Mex^+/Mex^- hybrids with different chromosome complement of the human Mex^+ cells, are devoid of MGMT activity. We have now shown that the hybrids of Mex^+ (human) and Mex^+ (mouse) cells have the same level of MGMT (~25,000 molecules/cell) as either parent. Thus, while there is no additive effect in the hybrid cells, we can conclude that the lack of MGMT in Mex^+/Mex^- hybrids is not due to a trivial effect of the fusion process on MGMT synthesis. We have also observed that the hybrids of Mex^- (mouse) and Mex^- (hamster) cells lack MGMT as well, suggesting that these Mex^- cells do not complement each other. The only human chromosome that was absent in all the hybrids tested was chromosome 22 and we are currently checking whether this chromosome may contain the structural (regulatory) sequence of MGMT.

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RNA PROCESSING IN YEAST

A. Stevens M. Maupin

Ribonucleases were once regarded to be solely degradative enzymes that were important to the cell for maintaining the proper balance of RNA molecules, particularly under conditions of stress. 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 the RNA molecules at both ends as well as splicing to remove introns must occur

before the final mature RNA is produced. Ribonucleases are now regarded as the key enzymes involved in the maturation processes. Ribonucleases that produce 2',3'-cyclic end groups at the 3'-termini of products were once regarded as truly degradative, but it is now known that such cyclic-ended fragments are involved in ligation reactions. The integrity of each cell is dependent on it having the proper array of mature RNA molecules. Any environmental agent that interferes with the maturation processes may be very deleterious to the cell. Studies of RNases of the lower eukaryote, Saccharomyces cerevisiae, and their possible involvement in processing of RNA continue in this laboratory.

An exoribonuclease of yeast which hydrolyzes RNA from the 5' end yielding 5'-mononucleotides has been purified to near homogeneity by poly(A)-agarose chromatography. The enzyme has a molecular weight of 165,000. Its substrate specificity has been studied using different synthetic polynucleotides. Single-stranded polyriboncleotides are hydrolyzed, but polydeoxyribonucleotides are not. The latter are inhibitory. The double-stranded molecules, poly(A)·poly(U) and poly(A)·poly(dT), are hydrolyzed at 60% and 15% of the rate of poly(A) at 37°C. The hydrolysis of these double-stranded molecules shows a temperature dependence suggesting that local melting of the molecules is required for the hydrolysis. Because of its mode and specificity of hydrolysis, the enzyme may be involved in the removal of RNA primers from the 5'-end of DNA strands during DNA replication. To test the suitability of such a substrate, Poly[^{32}P](dA)·poly(dT) with [^{3}H]poly(A) at the 5' end was made and tested as a substrate. RNases H of eukaryotes hydrolyze the poly(A) portion of such a substrate, but do not remove the last ribonucleotide linking the poly(A) to the poly(dA). The exoribonuclease hydrolyzes the poly(A) portion of this substrate, removing the final AMP residue. Assays of yeast cell fractions show that the enzyme is predominantly in the "dense crescent" fraction of yeast nuclei. This fraction contains nuclear membrane components and nucleolar structures. Antibody-localization studies are now in progress.

Studies of a pyrimidine-specific endoribonuclease of yeast in this laboratory led to the observation that RNA preparations prepared in a very diligent manner including heating at 80°C with SDS, contain detectable amounts of RNase activity with a specificity similar to that of the endoribonuclease. In an attempt to isolate small RNases that bind tightly to RNA, a peptide extraction technique was tried which is similar to that used by others for the isolation of nucleic acid-binding peptides. Low-level endoribonuclease activity is found associated with peptide fractions of yeast. Studies of the molecular weights of the active fractions by gel-filtration chromatography and membrane ultrafiltration show that one is less than 10,000 daltons and a second is less than 5000. The fractions show a specific hydrolysis of U-A and C-A bonds in RNA, as determined by end group analyses and sequencing experiments with tRNA. That the RNase could play a role in cleavage of U-A and C-A bonds which are found in mRNA precursors at the sites of polyadenylation will be examined.

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HELA NUCLEAR MATRIX PROTEINS: SOLUBILIZATION, SEPARATION AND DNA BINDING PROPERTIES

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S. P. Ayer[3]

After many years of research, the organization of the nucleus is only just beginning to be understood in detail. Upon removal of the membrane and most of the DNA, RNA and soluble protein from the nucleus, one is left with an insoluble structure called the nuclear matrix. It is believed to be an important structural and functional component of the nucleus. Replication, transcription, RNA processing, and steroid hormone binding are some of the functions associated with the nuclear matrix. It is believed that some of the eukaryotic DNA, arranged as supercoiled loops anchored to the nuclear matrix, may represent actively replicating and transcribing portions of the genome. It is therefore likely that there are important DNA binding proteins responsible for anchoring active genes to the nuclear matrix besides proteins that play regulatory and functional roles in transcription and replication.

The biochemistry of the nuclear matrix proteins is not well understood due to the difficulty of solubilization of these proteins without denaturation. We have tested several neutral, anionic and zwitterionic detergents, using the criteria of protein yield and DNA binding properties. We find that sodium dodecylsulfate (SDS) at low (0.06 to 0.2%) concentration is useful in solubilizing proteins with good DNA binding properties. Two detergents, CHAPS and CHAPSO (at their critical micellar concentrations), that combine the useful properties of N-alkyl sulfobetaines and bile salt anions, are also useful for solubilizing nuclear matrix proteins while retaining their DNA binding properties. Triton-X-100, a nonionic detergent, released small amounts of protein with poor DNA binding properties.

The solubilized nuclear matrix proteins were separated on DEAE-cellulose with increasing concentration of KCl. The fractions were designated FT (no salt wash), DE-100 (0.1 M KCl), DE-200 (0.2 M KCl), DE-300 (0.3 M KCl) and DE-500 (0.5 M KCl). Very little protein was present in the FT fraction. The DE-200 and DE-300 fractions each contained about twice as much protein as the DE-100 and DE-500 fractions. The protein composition of each fraction was determined by SDS-polyacrylamide gel electrophoresis. Although some fractions contained common protein bands, for the most part each fraction contained a unique set of proteins, indicating that protein separation had occurred. Each fraction was assayed

for DNA binding activity using a nitrocellulose filter binding method. All fractions contained DNA binding activity, with the most activity in the DE-200 and DE-300 fractions.

It was of interest to know which proteins were binding to DNA and to determine the specificity of binding. Protein blotting was used to screen the soluble nuclear matrix proteins as well as the fractions derived thereof. An electroblotting technique was used to transfer the protein bands from the gels to nitrocellulose paper. No DNA binding proteins were detected in the DE-100 fraction. The DE-200 fraction contained several DNA binding proteins in the molecular weight range of 55-75 kd. The DE-300 and DE-500 fractions also contained DNA binding proteins in this molecular weight range as well as some of lower molecular weights. An interesting observation was that the DE-500 fraction that had revealed little DNA binding activity by the nitrocellulose filter binding assay, contained the highest DNA binding activity by protein blotting. The reason for this discrepancy was due to the fact that this fraction contained appreciable amounts of nucleic acid that interfered with the nitrocellulose DNA binding assay.

In order to determine the specificity of binding, we used DNA from the following sources: SV40 virus, HeLa cell, pBR322 and pFLBH (a plasmid containing the adenovirus major late promoter). The results showed little specificity. Only when an 850 base pair restriction fragment containing the SV40 regulatory sequences was compared with the rest of the SV40 genome, was any specificity apparent. The 850 base pair fragment bound only slightly to the 55 kilodalton protein in the DE-200 fraction and to the 30 kilodalton protein in the DE-300 fraction, both of which bound strongly to the remainder of the SV40 genome. No clear-cut differences were seen when SV40 DNA, HeLa cell DNA, pBR322 (a bacterial plasmid) or the cloned adenovirus promoter were used as probes. It is possible that the probes used were too general to detect specific DNA binding. The use of synthetic oligonucleotides containing specific sequences of interest may prove to be more useful for detecting proteins that bind to specific sequences.

The results would lend support to the model that the nuclear matrix, composed predominantly of proteins, serves as anchorage points for the replication and transcription machinery and the DNA template is reeled through the anchorage points. Therefore, the participating proteins should display a general rather than a specific affinity for DNA.

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MODIFICATION OF STRUCTURE AND FUNCTION OF PROTEINS INVOLVED IN PHAGE T5 DNA REPLICATION

R. K. Fujimura B. C. Roop

Recent advances in recombinant DNA technology and protein engineering have made it feasible to modify the structure and function of highly complex proteins. DNA polymerase has multiple functions and often exists in a complex with other proteins. DNA replication occurs with high specificity and even a slight alteration in physiological conditions affects rates and functions of the process. In vitro systems that mimic the situation in vivo are being developed in increasing numbers. Such systems are ideal to study the effects of external agents such as by-products of energy production and to analyze the ultimate sites of action of mutagens in DNA.

We have characterized T5 DNA polymerase extensively, and have shown that it is associated with an exonuclease that serves in editing the replicated strand of DNA. Both replication and editing have been shown to be processive.

We have isolated the segments of T5 DNA of varying length that encode the DNA polymerase gene. The physical locus of the gene is located 58.3% to 61.3% from the left end of the DNA (1). It includes the loci for conditional lethal mutants of the gene (left to right) D7, D8, am1, ts5E, ts53, am6, and D9. The gene has been cloned as reported subsequently.

Another protein that has been purified to homogeneity and characterized by us is a DNA binding protein, gpD5, which has a dual role in DNA replication and control of transcription. It is the most abundant DNA binding protein synthesized in T5 phage-infected cells. It binds cooperatively to double stranded DNA with no apparent preferred site on DNA. It will be cloned eventually.

The following is a description of the progress since the last report.

The Direction of Transcription of the DNA Polymerase Gene. We have identified by immunobinding assay the polypeptides synthesized as the result of amber mutations in the DNA polymerase gene of bacteriophage T5 (2). Comparison of the sizes of such polypeptides revealed the order of mutagenic loci of these mutations and the direction of transcription of the gene. The extracts of cells infected with wild type T5 and with five amber

mutants of the polymerase gene (D7, D8, D9, am1 and am6) were prepared and the proteins resolved by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis. After transfer of the proteins to a nitrocellulose sheet, a radioimmunolabeling technique was used to identify the T5 DNA polymerase and its amber mutant polypeptides. Based upon the relative sizes of the polypeptides, the direction of transcription of the T5 DNA polymerase gene was determined to proceed in the order D7, D8, am1, D9 and am6. The molecular weights of DNA polymerase polypeptides coded by D8, am1, D9, am6 and T5 were 23,000, 45,000, 75,000, 83,000 and 96,000, respectively. The D7-coded polypeptide was not detectable. These data suggest that the carboxyl terminal region of the enzyme is essential for the polymerase function.

Cloning of the DNA Polymerase Gene. We have attempted to clone DNA segments of varying length that may contain the DNA polymerase gene. The following segments were shown to have the gene by the Southern technique and a marker rescue method.

EBp11 × mp18 (pRF3)	:	a 1.5 kbp piece containing D9 locus.
EBtsp11 × mp18 (pRF12)	:	a 1.5 kbp piece containing ts53 locus.
EBtsp11 × pUC9 (pRF122)	:	EBtsP11 cloned into pUC9.
CEp4 × mp18 (pRF45)	:	a 1.8 kbp segment from A15 (obtained from John Davison, Brussels, Belgium) and contains D8 and D9 loci.

In addition to the above, a segment adjacent to EBp11 at the right side was cloned and shown not to contain the D9 locus. Presumably it is outside of the polymerase gene. Attempts to clone the segment to the left of D8 have been unsuccessful. Prefixes to fragment numbers in the above clones stand for restriction enzymes used to cut out the segment. The clones pRF3 and pRF45 are being sequenced in collaboration with Chin-Yih Ou (Louisiana State University) with oligonucleotide primers synthesized by Robert Foote of this Division.

The expression of the cloned segments is being studied in collaboration with John Davison of the International Institute of Cellular and Molecular Pathology, Brussels, Belgium.

A culture of E. coli containing the plasmid pRF122 was infected with the amber mutant D9. When the phage produced in such a culture was assayed, a larger fraction of temperature-sensitive plaques was obtained. Since pRF122 should contain the ts53 locus, the result is as expected, and is additional proof that the plasmid contains the polymerase gene.

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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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A Complex G+C-Rich Satellite DNA: A Model System for the Definition of "Hotspots" for Mutation. The secondary and tertiary structures of repeat units of three variants of a very complex G+C-rich eukaryotic satellite DNA have been characterized by digestion with S1, mung bean, Bal 31, and P1 nucleases, enzymes that are sensitive to DNA conformation. The variants studied were RU (2089 bp), close to the average size of repeat units recovered from digests of cellular satellite with any of the restriction enzymes that cut at a single site in a repeat unit; TRU (1674 bp), truncated by the presence of an EcoRI site ~300 bp from the 3' end; and EXT (2639 bp), extended by the presence of ~600 bp more DNA than the average size repeat unit. Analyses revealed that the three most divergent domains in RU, TRU, and EXT are sensitive to S1 nuclease under physiological levels of torsional stress. These three domains contain tracts of G·C embedded in similarly-biased regions of repetitive polypurines·polypyrimidines (Pu·Py) that have abrupt interruptions in bias adjacent to the tracts. Because of the high incidence of mutations associated with these domains, the abnormal DNA structures detected by the nucleases appear to be involved in cellular genetic recombination.

In order to understand the more precise nature of these unusual DNA structures, one domain of exceptionally high divergence was subcloned from the RU variant. The repetitive Pu·Py tracts present in the domain may be represented by ("CCT")$_{27}$CC(CCT)$_{15}$CTTAAC$_3$TC$_{22}$(CGCAC)$_5$("CGCAC")$_2$ (quotes = variations on sequence). We find that a region of ~120 bp centered on the TTAA sequence exhibits sensitivity to S1, mung bean, and P1 nucleases, and that mild negative superhelical densities ($-\sigma$ = 0.02–0.03) and slightly acidic conditions (pH 4.5 ~ 6) are required to stabilize the altered conformations. Studies with higher NaCl concentrations identify two structural types within the domain, differing in their stability to 50–300 mM NaCl.

High resolution S1 mapping of this subclone shows that only the pyrimidine strand is nicked to the right of TTAA in the sequence above, and only the purine strand (not shown) is nicked to the left of TTAA; the point where Pu·Py bias is interrupted corresponds to a region of highly distorted

structure where nuclease sensitivity exchanges between strands. This correlates with the fact that the three S1-sensitive divergent domains of RU, TRU, and EXT have interruptions in Pu·Py bias and might form analogous but unique structures in these domains, which could in turn be recognized as sites for mutations (recombination) in vivo.

Previous data indicated that every copy of the G+C-rich satellite may be different. To test this hypothesis we cloned an EcoRI digest of cellular satellite into the plasmid vector pUC9; 1000 satellite clones were recovered. Using restriction enzymes, we have screened 34 pUC9 clones as well as all 126 pBR322 clones in a smaller satellite library. Every clone analyzed to date contains at least one band in one restriction digest that distinguishes it from all others. This indicates that no one sequence is present in more than 16 copies. In order to show, at the 95% confidence level, that all 16,000 related sequences are unique in detail, a total of 310 clones must be analyzed.

In EcoRI digests of labeled cellular satellite, two discrete populations of 360 and 320 bp fragments are observed in overexposed autoradiograms. Restriction mapping and sequencing of a number of these fragments indicate that they are the 3' ends of truncated satellite repeat units (such as TRU, described above). Certain structural changes are associated with a segment rich in alternating purines and pyrimidines known to adopt a Z conformation, suggesting that specific unusual secondary structures are the loci of high mutation frequency.

Degradative Enzymes with Activities Correlated with the Crustacean Molt Cycle. Calcium-dependent proteinase activity (CDP) has been characterized in land crab claw muscle and in lobster claw and abdominal muscles. The CDPs are cytoplasmic cysteine proteinases that degrade myofibrillar proteins during proecdysial muscle atrophy. In vitro studies of crab claw muscle showed that the CDP constitutes a major catabolic pathway; the contribution of lysosomal processes was negligible. Four CDP activities in lobster claw and abdominal muscles have been resolved by high-performance liquid chromatography. These enzymes, which are not generated by autolytic or other degradative processes, differed from each other in molecular weight, net charge, and sensitivities to calcium and inhibitors. Our data are the first to show more than two forms of CDPs in any tissue, vertebrate or invertebrate.

In members of seven families of brachyuran crabs, apolysis, the separation of the epidermis from the exoskeleton in premolt crustaceans, was induced by chilling (forced apolysis). Inhibitor studies of extracts prepared from integumentary tissue from crabs which had undergone forced apolysis indicated the presence of two types of proteinases: a carboxyl proteinase, cathepsin D, and several alkaline cysteine proteinases (ACPs). Forced apolysis could not be induced in two families of astacuran crustaceans represented by crayfish and lobsters. Extracts of astacuran integument yielded cathepsin D activity, but not ACP activity. ACPs hydrolyzed proteins extracted from the innermost layer of the crab exoskeleton, the layer that is degraded during the premolt period. They

also displayed other characteristics that correlated with the physiological conditions of apolysis. The presence of more than one enzyme was confirmed since two peaks of ACP activity were seen following chromatography of crab extracts on several types of HPLC columns. Estimates of ACP sizes ranged from 53-67 kD by gel filtration and 61 kD by SDS PAGE. The data suggest that ACPs play a critical role in physiologic apolysis.

Heterogeneity of Myofibrillar Proteins. Multiple variants of myofibrillar proteins have been analyzed by SDS-polyacrylamide gel electrophoresis. In lobster, a total of two isoforms of paramyosin, three of troponin-T, five of troponin-I, three of troponin-C, three of myosin alpha light chain, and two of myosin beta light chain occurred in six muscles of the claw and abdomen. In lobster and land crab only paramyosin showed a variant pattern consistent with fiber type: slow-contracting fibers contained a variant (105 kD) smaller than the major variant in fast-contracting fibers (110 kD). The expression of more than one variant of a myofibrillar protein in a single fiber forms unique assemblages by which subgroups can be discriminated within the broader categories of fast and slow fibers.

Observations on the Crustacean Molt Cycle. Mud crabs, Rithropanopeus harrisii, were induced to molt within two weeks following removal of six walking legs; the mean time to ecdysis for control crabs was 40 days. This brings to 15 the number of crustaceans that have been induced to molt by leg autotomy. Some specimens were parasitized by a castrating barnacle, Loxothylacus panopaei. No crab parasitized by an adult barnacle has molted in the laboratory including those which had autotomized six legs.

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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. In

order to capitalize upon these advantages we have spent considerable time with the following projects: (1) large-scale cultivation and harvesting of protozoa, especially Oxytricha and Euplotes; (2) lysis of cells and isolation of macronuclei, with minimum nucleolytic and proteolytic degradation; (3) preparation of macronuclear DNA fragments, gel electrophoresis and blot-hybridization with specific gene probes; (4) preparation of soluble chromatin, with characterization of nucleosome repeat lengths and companion biophysical parameters (i.e., thermal stability and circular dichroic spectra); (5) analysis of various nuclear histones and non-histones; (6) development of unique cytochemical reactions for the Replication Band; (7) isolation and characterization of the chromatin properties of RB; and (8) ultrastructural analysis of the patterns of replicating DNA in the RB. Current experiments are focusing upon: (1) gel electrophoretic analysis of acid-extractable macronuclear proteins from Euplotes; (2) preparation and characterization of monoclonal antibodies directed against macronuclear chromatin and replication band chromatin, and (3) preparative fractionation of soluble macronuclear chromatin "gene-size" fragments. During the past year we succeeded in enriching for specific gene-chromatin fragments by differential sedimentation in sucrose gradients as assayed by specific gene probes.

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. Current experiments are focused upon: (1) reconstruction of thick sections (i.e., 1-3 μm thick) rather than the semi-thick sections used earlier (i.e., 0.1-0.25 μm); (2) analysis of sections treated with a DNA-specific electron-dense stain; and (3) visualization of the reconstructed transcription structures on a user-interactive 3-D display system for editing the data and creating models in real time. During the past year we completed reconstructions of six entire BR gene loops in thick sections at low resolution (i.e., > 300 Å), yet detailed enough for us to calculate the length of the transcription units in situ, and to estimate the DNA compaction ratio during transcription.

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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 $P2_1$ 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 10 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 10 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 are also under way on the histone octamer. This series of studies will provide very important information regarding the size and shape of the histone octamer in solution, and should help distinguish between two alternative structures for the histone core in chromatin (5).

3. National Center for Small-Angle Scattering Research. The facilities of the National Center for Small-Angle Scattering Research have been used by seven groups from external laboratories and two groups from within ORNL. Several of the external groups have made more than one trip to the facility. The major part of sample preparation and data analysis was 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 crystallographic structural analyses is to elucidate and understand the structure of compounds in terms of their functions. Several years ago, we initiated a project to analyze the structure of antischistosomal drugs, hycanthone and its analogs. Our results revealed that the thioxanthene ring system of this group of drugs is invariably roughly planar and that the terminal nitrogen atom of the side chain is responsible for the formation of various intermolecular hydrogen bonds, thus verifying the hypothesis made for this family of drugs regarding the drug-DNA interaction.

Recently a new type of antischistosomal drug, 4-methyl-5-(2-pyrazinyl)-3H-1,2-dithiole-3-thione (oltipraz) has been developed. This is a slow-acting drug which functions by reducing the glutathione stores of the worms (Bueding et al., Res. Commun. Chem. Pathol. Pharmacol. 37: 293-303, 1982). Although some dithiolethione analogous to oltipraz do not show antischistosomal activity, they are of great interest because of their chemoprotective and antimutagenic activities. Following the X-ray structural report of oltipraz (C. H. Wei, Acta Crystallogr. C39: 1079-1082, 1983), we have completed structural determinations of five such dithiolethiones as part of our health-related program to provide information regarding structure-function relationships. These five drugs are: 3H-1,2-dithiole-3-thione, 4-methyl-5-(2-pyrazinyl)-3H-1,2-dithiole-3-one, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione, and 4- and 5-phenyl-3H-1,2-dithiole-3-thiones.

In addition, X-ray diffraction data for four different small molecules of biological interest have recently been collected and await further analyses. All drugs related to oltipraz were furnished by Professor Ernest Bueding of the Johns Hopkins University, and X-ray data were collected by the use of the diffractometer of Drs. G. M. Brown and W. R. Busing of the Chemistry Division of ORNL.

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Comparative Genetics Section

SECTION OVERVIEW - R. J. Preston

The Comparative and Cellular Genetics Section has been reduced in number of scientists over the past year and now consists of four research groups. The majority of the research is now concerned with genetic studies in mammals or in mammalian in vitro cell systems, with less emphasis on comparative genetics as such. However, there is also a research effort in prokaryotic genetics, that has provided information that is complementary to the mammalian studies.

The emphasis of the mammalian genetics research is to attempt to understand the mechanisms of induction of mutations and chromosome alterations and the involvement of DNA repair systems in these processes. The information that has been and will be obtained can be utilized in the assessment of the potential induction of adverse health effects, both genetic and somatic, in humans as the result of occupational or environmental exposure to chemical and physical agents. A few specific studies will be described to exemplify our approaches.

The research of the Mammalian Biochemical Genetics group has been directed towards determining the control of expression of globin genes during development, and it has been shown that regulation is at the level of transcription. In addition, mice with a spontaneous beta-thalassemia mutation have about three times as much transcribed beta-minor RNA as normal mice. This beta-thalassemia mutation provides an excellent mouse model for human beta-thalessemia.

The Mammalian Cytogenetics Group has been studying the involvement of a specific chromosome alteration in the development of neutron-induced myelogenous leukemia in mice. At the same time they have utilized cultured somatic cell systems to determine the mechanisms by which chromosome aberrations are induced by radiation and chemicals, and how specific chromosome aberrations could be induced. It is apparent that there are sensitive or fragile regions in the genome, and that these can result in non-random induction of chromosome aberrations.

It is very clear that mutations and/or chromosome aberrations can be produced as a consequence of misrepair of DNA damage, and also that the frequency of both end points can be influenced by the fidelity and efficiency of repair processes. The DNA Repair Group has utilized a range of somatic cells, including human fibroblasts, to better understand excision repair processes, and to more accurately measure the kinetics of excision repair. Of particular interest is the production and repair of a UV-induced photosensitive lesion. Normal cells repair these lesions in a few hours, but cells from a xeroderma pigmentosum type A complementation

group become more photosensitive over the same time period. XP-variant cells show no change in photosensitivity up to 24 h. The variant cells are excision repair proficient, and it might be that their UV-sensitivity is a consequence of failure to repair lesions other than pyrimidine dimers.

The Genetics of Anaerobic Organisms Group has been studying the process of oxygen sensitivity and the genes involved. Such studies will be particularly important in providing an understanding of the possible involvement of oxygen radicals in the induction of mutations and lethality. There has also been considerable effort in studying the genetics and physiology of the anaerobic bacterium, Clostridium acetobutylicum because of its potential utility for the production of, for example, acetone, butanol and ethanol.

There are interactions within the Section and with other Sections that have allowed us to consider a broader approach to genetics research. The studies reported in this Progress Report are most encouraging and provide us with considerable optimism over the next year.

COMPARATIVE MUTAGENESIS

J. L. Epler	S. W. Perdue
F. W. Larimer	R. L. Schenley
C. E. Nix	S. I. Simms
L. C. Waters	E. R. Wilkerson
A. A. Hardigree	R. D. Wilkerson

The major goals of the Comparative Mutagenesis group are (1) to provide, through a battery of short-term tests, an evaluation of the genetic effects of energy-related compounds, and (2) to obtain basic information necessary for evaluating test results and for estimating possible genetic risks to man. This estimation of potential long-range health effects requires both a broad data base and an understanding of the biological response. Such factors as metabolism, repair, genetic constitution, solubility, and method of exposure must be explored experimentally. The approach includes (a) the use of a battery of short-term assays including bacterial, yeast, and Drosophila systems; (b) bio-directed chemical characterization of actual process materials and wastes; (c) examination of the effects of biological factors on the response; (d) identification of basic biochemical mechanisms of mutagenesis in eukaryotic systems; and (e) development of a data base on the identified hazardous chemicals. The benefits of such an approach are (a) more confident estimates of risk from energy-related materials, (b) the correlation of biochemical data with biological end points, and (c) the comparison of tests with mammalian cells and intact animals. These comparative studies serve as a connecting link between short-term bioassays in simple systems and mammalian tests.

I. Energy-Related Studies (J. L. Epler, in collaboration with the Analytical Chemistry Division)

The Comparative Mutagenesis Group has continued research with energy-related materials during this year. Funded studies (Fossil Energy) included (1) consideration of alternate methods of treatment of fuels in order to mitigate potential health effects, and (2) investigation of benchmark materials and/or compounds.

Studies at the Oak Ridge National Laboratory and at Pacific Northwest Laboratory on hydrogenated coal liquids have shown that hydrotreatment significantly reduces the toxicological properties of coal liquefaction products. The cost of treatment is significant, however, thereby possibly making alternative or supplemental upgrading processes economically attractive.

Although reactions with pure compounds are useful for identifying reduction products, the presence of a complex mixture such as a coal liquid for the sample matrix can drastically alter the product distribution from hydrogenation. Chemical studies compared the yield of hydrogenated products for reductions conducted with and without vacuum gas oil present. Whereas the octahydrobenzo(a)pyrene is the major product in the reduction of benzo(a)pyrene in a pure solvent, the addition of a vacuum gas oil considerably alters the reduction, and the main product identified is the dihydro-derivative. The effect this can have on the potential tumorigenicity of the product is suggested by the mutagenic activities measured for the different reduction products of benzo(a)pyrene, using strain TA-98 with S-9 activation: benzo(a)pyrene, 58 rev/μg; 4,5-dihydrobenzo(a)pyrene, 22 rev/μg; 7,8,9,10-tetrahydrobenzo(a)pyrene, 3.8 rev/μg; and 4,5,7,8,9,10,11,12-octahydrobenzo(a)pyrene, 0.2 rev/μg. A greater degree of hydrogenation results in a less mutagenic product, with mutagenicity being almost completely eliminated at the octahydro level of reduction.

Additional studies considered alternative upgrading treatments and compared mutagenicity, tumorigenicity and benzo(a)pyrene measurements to gauge reduction in toxicity. Thermal (distillation) separation, acid/base washing, precipitation, solvent extraction, and adsorption were evaluated. With respect to mutagenicity as measured by Salmonella histidine reversion (Ames assay), distillation was an effective means of segregating mutagens (and tumorigens) from coal liquids. Mutagenicity test data indicated that acid treatment, deasphalting, pentane/furfural partitioning, and clay percolation reduced mutagenicity. Much of the reduction can be traced to the removal of highly mutagenic aromatic amines. Thus, the reduction in toxicity as measured by mutagenicity was not paralleled by a reduction in tumorigenicity which is mainly caused by polycyclic aromatic hydrocarbons.

II. Regulation of Cytochrome P-450 Expression in Drosophila Melanogaster (L. C. Waters and C. E. Nix)

We previously reported that the cytochrome P-450 in Drosophila is resolved into only two protein bands by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The larger protein band (MW = 59.3 kDa) is present in all strains examined; the smaller (MW = 55.8 kDa) in only a few. The structural genes, or the regulatory gene(s) required for expression, for the 55.8 kDa proteins are located on chromosome II and map at or near a major locus for insecticide resistance. Trans-acting regulatory genes are required for the maximum expression of the 55.8 kDa proteins and are located on chromosome III. Strains containing the 55.8 kDa proteins are significantly more resistant to phenylurea than are those with only the 59.3 kDa proteins. Since others have concluded that a major component of resistance to insecticides is P-450 associated, we believe that it is the 55.8 kDa proteins that we have genetically and biochemically characterized that confer resistance in this organism.

Our major progress in the past year was in the purification of Drosophila cytochrome P-450. The 59.3 kDa proteins were purified to homogeneity as judged by SDS-PAGE from either the sensitive Oregon-R strain or the resistant Hikone-R strain by successive chromatography on octylamino-sepharose, DEAE cellulose and hydroxyapatite. The specific activities of these preparations range from 6.9 to 8.6 nmol/mg. Preparations enriched markedly for the 55.8 kDa proteins (SA = 4.6 - 7.4) have been obtained from Hikone-R microsomes. These preparations contain a significant amount of the 59.3 kDa proteins (~20-30%), but are apparently free of other proteins, again as judged by SDS-PAGE. Preparations of the 59.3 kDa proteins are suitable for use in making either polyclonal or monoclonal antibodies. Preparations of the 55.8 kDa proteins can be used to generate monoclonal antibodies specific for them to the exclusion of those for the 59.3 kDa proteins by the use of selective screening techniques.

Our goal is to use the system, the purified P-450's, specific antibodies and finally P-450-specific DNA clones to investigate the molecular mechanisms that control P-450 expression, especially as they relate to insecticide resistance. From a practical point, we anticipate that antibodies to the 55.8 kDa protein will be useful as probes for resistance-related P-450's in other insects of more economic concern.

III. Isolation and Partial Characterization of the REV1 Gene of Saccharomyces Cerevisiae (F. W. Larimer)

The REV1 gene is required for normal induction of base-pair substitutions and frame-shift alterations by UV or chemical mutagens. To characterize the role of the REV1 gene product in mutagenesis, we have cloned REV1 from a cosmid library of yeast genomic DNA. A cosmid derived from the URA3-CEN4 plasmid YCp50 was used to construct a total genomic DNA library

from a Sau3AI partial digest. A rev1-1 lys1-1 ura3-52 host was transformed to Ura+ with the cosmid library. 440 transformants were screened for UV mutability to Lys+. Two Rev+ transformants yielded cosmids with 30 and 39 kbp inserts, which contained a 15 kbp overlap. An 8 kbp BglII fragment internal to the overlapping segment (subcloned into YCp50) conferred Rev+ activity indistinguishable from wild-type. Further subcloning using specific restriction sites and Bal31 partial digests indicate that the REV1 gene exceeds 3 kbp in length. Subclones from a Bal31 digest beginning 5' to REV1 display mutability intermediate to REV1 and rev1-1. Sequencing of REV1 and characterization of REV1 mRNA is in progress.

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MAMMALIAN CYTOGENETICS GROUP

R. J. Preston	G. J. Hook
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C. B. Bast[3]	H. S. Payne
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T. Ho	R. A. Winegar[3]

The Mammalian Cytogenetics Group has continued its various approaches to understanding the mechanisms of induction of chromosome aberrations by radiation and chemical agents, the role of DNA repair kinetics in this process, and the application of the information obtained to studying differences in sensitivity of different species or cell types to the induction of chromosome aberrations. In addition, the results obtained have been used to determine the role of chromosomal alterations in increasing genetic risk from environmental or occupational exposure. We have now expanded the program to include much more emphasis on the potential risk of non-random chromosome aberrations in the induction or development of tumors, and on possible progressive chromosomal changes during the development of tumors.

1. The Relationship Between Neutron-Induced Chromosome Aberrations in Bone Marrow and Their Role in the Development of Myelogenous Leukemia. We have continued this collaborative study (with R. L. Ullrich, Biology Division) to measure the frequency and distribution of neutron-induced chromosome aberrations in bone marrow cells of RFM mice, and to determine if specific chromosome aberrations can be observed in neutron-induced myelogenous luekemias. The intention is to determine the role of chromosome aberrations in the induction or development of leukemia, to provide a method for estimating leukemia risk from measurement of chromosome aberrations in bone marrow cells, and also to determine if the Relative Biological Effectiveness (RBE) for chromosome aberration induction in bone marrow cells by neutrons compared to X rays is similar to that obtained for the induction of leukemias.

We have analyzed the chromosome aberrations induced by neutrons in bone marrow cells in two ways. Firstly, we have developed a technique that enables us to determine for each cell analyzed its stage in the cell cycle at the time of irradiation. The method is based upon the staining patterns observed following bromodeoxyuridine (BrdU) incorporation. It is important to be able to establish the chromosome aberration dose response curve for cells in the different stages of the cell cycle for a cycling cell population such as the bone marrow. The second method of analysis is performed with banded chromosome preparations to enable us to determine the distribution of neutron-induced chromosome aberrations.

The major conclusions from this part of the study are that linear dose response curves are obtained over the range of 10-40 rads for all aberration types, in all cell cycle stages. An approximate RBE value for fission neurons, compared to X rays, for chromosome deletions (for example) in the bone marrow is 8. This value is considerably higher than that of

2.5 for the induction of myelogenous leukemias, and emphasizes the fact that it is not readily possible to use RBE values for total chromosome aberrations to predict the RBE for tumors. In addition, on the basis of some 500 banded karyotypes, it appears that chromosome-type reciprocal translocations and deletions are distributed randomly as regards involvement of particular chromosomes and major regions of each chromosome.

We have also analyzed spleen and bone marrow chromosomes from mice confirmed as leukemic, and have observed that an interstitial or terminal deletion of chromosome 2 is present in each of the 10 cases to date. Other aberrations were observed in a particular animal, but were not observed in other cases. This observation is interesting and important, since deletions of chromosome 2 have been observed in X ray and spontaneous leukemias. The increases in leukemia following irradiation are quite possibly a consequence, in part, of the increased probability of inducing a deletion of chromosome 2. This study is being continued and expanded.

2. The Sensitivity of a Mouse Leukemic Cell Line and Normal Bone Marrow Cells to the Induction of Chromosome Aberrations by X Rays. It was described in our previous Annual Progress Report that the mouse leukemic cell line (ML-1) was more sensitive than normal bone marrow cells to the induction of chromosome aberrations by X rays when cells were treated in the G_1 stage of the cell cycle. In addition, chromatid-type aberrations were observed in ML-1 cells only, and these would have to be formed in the S-phase, although the DNA damage was induced in G_1 cells. The differences in sensitivity and the presence of chromatid-type aberrations in the ML-1 cells can be explained, in part, by a lack of radiation-induced delay of progression of ML-1 cells into the DNA synthesis phase; normal bone marrow cells do show a delayed entry into S. The delay allows cells to repair DNA damage before replication, so in the ML-1 cells unrepaired DNA damage remains at the time of replication, and this is converted into chromatid aberrations. The increased sensitivity to the induction of chromosome-type aberrations in ML-1 cells appears to involve some altered DNA repair capacity, and this has been studied in an additional series of experiments, utilizing cytosine arabinoside (ara-C) an inhibitor of the resynthesis step of DNA excision repair.

The frequency of X-ray-induced chromosome aberrations in G_1 ML-1 mouse myeloid leukemia cells and normal mouse bone marrow cells increased with post irradiation incubation with ara-C but the frequency of aberrations in the leukemic cells increased with quite a different time response compared to the normal cells. Irradiated normal mouse bone marrow cells had a rapid increase in the frequency of chromosome exchanges and deletions with increasing ara-C incubation time, for example, and increase was observed with 0.5 h ara-C incubation. In contrast, the ML-1 cells did not have a significant increase in aberrations until 1-2 h post irradiation incubation with ara-C. These results suggest the ML-1 cells, per unit time, initially undergo less repair of the X-ray-induced DNA damage that can be converted into chromosome aberrations. We previously showed that the ML-1 cells have a higher frequency of X-ray-induced chromosome aberrations compared to normal cells and the results presented here indicate that a slower rate of

repair resynthesis is contributing to the increased sensitivity of the ML-1 cells.

We have recently shown that ML-1 cells are also more sensitive than normal bone marrow cells to the induction of chromatid-type aberrations by X rays in the G_2 stage of the cell cycle. This suggests that a slowing of the resynthesis step of excision repair in ML-1 cells also occurs in G_2. It was also found that when ML-1 cells were treated in G_2 with araC there was an enormous increase in chromatid deletions, some twentyfold greater than that seen in normal bone marrow cells. Some preliminary experiments suggest that perhaps the DNA of the leukemic cells has an inherent fragility that is particularly apparent in the presence of ara-C. There is also a much higher frequency of deletions in ML-1 cells treated with X rays plus ara-C than in bone marrow cells.

3. Background Frequency of Chromosome Aberrations and Sister Chromatid Exchanges in Human Lymphocytes. This study is a collaborative effort with Dr. Michael Bender of Brookhaven National Laboratory and has now been under way for about 4 years. The initial study population from BNL contained approximately 400 persons, who were partially selected in order to have a stratification on the basis of age groupings, sex, and race. The second part of the study was designed to include persons in younger and older age groups than represented by the BNL population. A standard protocol was established through preliminary experiments and has been utilized throughout the main study. The frequencies of chromosome aberrations and sister chromatid exchanges have been analyzed in some 500 samples, but a complete analysis of the data will not be performed until all the proposed samples have been analyzed.

4. Studies of the Induction of Random vs Non-Random Chromosome Aberrations by Radiation, Chemical Agents, and Restriction Endonuclease. Our initial studies on the distribution of induced chromosome aberrations made use of hybrid cells between Chinese hamster and mouse genomes. Mouse genomes contain high proportions of repetitive sequences, particularly in the centromeric regions, whereas Chinese hamster genomes contain only very low amounts. This hybrid cell system allows us to simply determine random vs non-random aberration induction by observing whether chromosome aberrations are restricted to interactions between hamster/hamster (H/H) and mouse/mouse (M/M) chromosomes or whether hamster/mouse (H/M) chromosomes can be involved in exchanges and whether the relative frequencies are proportional or not to the numbers of hamster and mouse chromosomes in the different hybrid cell lines. In addition, the localization of aberrations to specific chromosome regions can be analyzed, as well as the involvement of highly repetitive regions, since these are present almost exclusively in the mouse genome.

Our initial studies indicated that chromatid exchanges were distributed randomly among the H/H, M/M and H/M genomes following irradiation in G_2, early S-phase or late S-phase. On the other hand, cells treated with mitomycin C contained aberrations, as expected, only when cells were in the S phase at the time of treatment. There was also a very non-random

distribution such that M/M combinations were in great excess, with a localization of break points in the centromere region. There were a few exchanges involving hamster chromosomes but only in H/H combinations, never as H/M. It appears that the distribution of mitomycin C induced exchanges is the result of interactions between coincidentally replicating regions that are normally close together spatially at the time of replication, as would be the case for the late replicating, highly repetitive centromeric regions of mouse chromosomes.

Our current approach is to attempt to produce DNA lesions that are specific for DNA sequence and genome localization and determine their involvement in the production of chromosome aberrations. The best way to do this is by restriction endonucleases. A variety of techniques have been used to permeabilize cells in order to facilitate incorporation of restriction endonucleases, and we have been able to induce significant frequencies of chromosome aberrations. In order to develop the system we have to date used enzymes that will cut in the genome: Alu 3, Cfo 3, Msp 3, and BamHl. Future plans include the use of enzymes that will produce fewer cuts with a more specific genome distribution, and also to study the interaction of endonuclease lesions with lesions produced by radiation and or chemical agents to try to better understand the mechanism of induction of chromosome aberrations, and, in particular, on the production of non-random aberrations.

5. Gene Mapping in Baboon Species. This is a project that has been continued in the Mammalian Cytogenetics Group, following the departure of Dr. Peter Lalley, the graduate student adviser originally involved.

In order to extrapolate from data obtained with laboratory animals to man, it is frequently of importance to know the relationships between chromosomal locations of particular genes under study and the preservation of linkage groups between species. The study being conducted here involves the assignments of genes in baboon species. The genus Papio (family Cercopithecidae) consists of the five species of baboons whose karyotypes are identical. To determine whether the gene maps of these species are also identical and to discover which linkage groups have been conserved between man and these more distantly related primates, somatic cell hybrids were formed between rodent cells and cells from baboons of two species of Papio, P. papio and P. hamadryas. Thirty-three mouse × P. papio and fifteen Chinese hamster × P. hamadryas primary hybrid clones, all segregating baboon chromosomes, were analyzed for the expression of 32 gene markers. We have discovered the assignment of one new gene, identified at least one new independently segregating marker in P. papio and described 15 linkage groups in P. hamadryas. The gene coding for mitochondrial malate dehydrogenase (MDH2) segregates concordantly with the gene for soluble superoxide dismutases (SOD1) and can be assigned to P. papio chromosome 3 (PPA3). This supports the previously described homology between human chromosomes 7 and 21 (HSA7 and 21) and PPA3. The gene coding for aminoacyclase 1 (ACY1) and peptidase B (PEPB) segregate independently of each other and of the other markers examined in P. papio and, therefore, represent two separate linkage groups. However, a linkage of PEPB with

LDHB (both on HSA12) and an assignment of PEPB to PPA11 cannot be excluded, as LDHB cannot be examined in this set of hybrids. A total of 27 genes have been assigned to 15 syntenic groups in P. hamadryas. Where comparisons can be made, the linkage maps of the two Papio species correspond. Three syntenic groups which have not been examined in P. papio are found to be conserved in P. hamadryas and man.

6. Method for Increasing the Sensitivity of the Human Lymphocyte Assay. Human lymphocytes exposed to environmental agents in vivo are in the G_0, or noncycling G_1 stage of the cell cycle, and require mitogenic stimulation to re-enter the cell cycle, so that chromosome aberrations can be analyzed at metaphase. Following a chemical exposure to a clastogenic agent, in almost all cases, chromosome aberrations are induced during the DNA replication phase. Thus, for human lymphocytes repair of DNA damage can occur in G_0 cells, prior to sampling and mitogenic stimulation, and during the long G_1 phase of the first in vitro cell cycle. This considerably reduces the sensitivity of the lymphocyte assay for detecting chromosome aberrations.

In a series of experiments conducted over the past several years, we have shown that ara-C can inhibit the excision repair of DNA damage induced by radiation and chemical agents, and that on reversing the inhibition with deoxycytidine, the gaps accumulated at repair regions can interact to produce chromosome aberrations. If lymphocytes are treated in G_1 with 4NQO or MMS, no chromosome aberrations are produced. However, if the G_1 cells are incubated with ara-C after chemical treatment, chromosome aberrations can be produced, and they are of the chromosome-type that were normally induced by radiation in G_1 treated cells, but not by chemical agents.

Thus, it seemed that if human lymphocytes were treated in G_0 (prior to mitogenic stimulation) with chemical agents, stimulated, and incubated with ara-C, the repair of damage that would normally reduce the sensitivity to aberration induction could be accumulated and converted into chromosome aberrations on reversing the inhibition. In this way, advantage could be taken of the feature of the assay that is normally a major disadvantage namely repair in G_1. In addition, the types of aberrations produced (dicentrics and deletions) can be scored very precisely, and they occur at much lower background frequencies than chromatid-type aberrations, giving the assay a greater sensitivity for detecting human chemical exposures.

Preliminary experiments with 4NQO and N-AAAF, have shown that the chemicals alone do not induce aberrations following G_0 treatment, but when incubated for 12 h with ara-C, significant frequencies of dicentrics and deletions were observed. It is intended to expand this study to include chemicals to which humans are exposed environmentally or occupationally, and to include experiments performed for different times between treatment and mitogenic stimulation, since following human exposures, samples are not or cannot be taken immediately.

7. Changes in Karyotype with Tumor Progression, and the Possible Role of Specific Chromosome Alterations. We have recently initiated studies

with R. J. M. Fry of the Biology Division to determine if specific chromosome changes are associated with early stages in tumor production and whether additional progressive changes in karyotype occur that are dependent on the agent inducing the tumor. The method involves the induction of sarcomas by the implantation of small plastic discs into mice. Sarcomas develop on the plastic disc, such that the discs can be removed at different times after implantation, and the associated cells cultured and karyotyped. In addition, the animals can be irradiated or treated with chemical carcinogens, and the cells associated with the plastic disc can again be analyzed to determine early and progressive karyotypic changes.

To date we have analyzed mainly well-developed sarcomas, and have shown that there are wide variations in chromosome number between sarcomas, with much less variation within a particular sarcoma. In addition, two tumors contained double minutes that are indicative of gene amplification, and we intend to determine the amplified region. The cell cap that initially forms around the plastic disc, prior to any apparent pre-neoplastic changes, consists of cells with the diploid number of chromosomes.

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THE REPAIR OF HUMAN DNA

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It is essential that the vital information contained in DNA remain intact. However, there are numerous environmental agents capable of damaging DNA including UV and ionizing radiation and chemical carcinogens, which can compromise the information encoded in the DNA and lead to mutation, carcinogenesis and cell death. Thus it is not surprising that most organisms have evolved molecular mechanisms for the detection, removal, and repair of DNA lesions. These biochemical repair processes are responsible for maintaining the fidelity of the DNA and are therefore critical in the prevention of mutations and cellular transformation to the malignant state. Our studies focus on the process of DNA excision repair and its relationship to human carcinogenesis. Both repair proficient normal human cells and cells derived from patients with the hereditary, cancer-prone disease, xeroderma pigmentosum (XP), are used in these studies. 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).

Model DNA Lesions: Pyrimidine Dimers. Much of our understanding and many of our models of DNA repair have been derived from studies of UV light damage to cells. The major process by which cells repair many kinds of DNA damage is termed excision repair. It is a multienzymatic process by which a damaged portion of DNA is removed and the genetic fidelity restored. Ionizing radiation and chemical damage cause various types of damage to DNA. UV light causes a specific type of damage to DNA, mainly dimer

formation between adjacent pyrimidines. Studies have shown that dimers produce their effects by altering both transcription and translation. Above certain critical doses, this block is irreversible and must therefore lead to cell death. The number and kinds of UV-photoproducts formed in DNA are related to the base ratio, the wavelength and the total exposure. Although thymine-containing dimers are the most numerous at all wavelengths, cytosine-containing dimers become increasingly important at the wavelengths contained in sunlight. Pyrimidine dimers, formed in [^{3}H]thymidine labeled cells, are stable to acid hydrolysis, easily separated by paper chromatography, and can be monitored by liquid scintillation counting. These properties permit the study of the production and fate of DNA damage (dimers) in mammalian cells. Human cells are proficient in the removal of UV-light-induced pyrimidine dimers. Normal human cells have the capacity to remove and repair about one million dimers over a 24 h period. During this process, which may take several days, all forms of dimers are removed. At least in human cells, cytosine-containing dimers are removed faster than thymine-containing dimers. This may account for the two components of excision rate observed in human cells. It is hoped that continued detailed analyses of the repair of DNA damage and further clarification of the correlation between biochemical events and changes in cell behavior will lead to an understanding of the mechanisms of tumor induction.

Repair of a UV-Induced Photosensitive Lesion in the DNA of Normal and Xeroderma Pigmentosum Human Cells. Exposure of human cells to UV light results primarily in the formation of pyrimidine dimers in the DNA. However, other lesions are known to occur and could be of great importance due to the carcinogenic nature of sunlight. We have evidence of such a non-dimer lesion produced in the DNA of human cells. The lesion is produced by exposure of cells to either 254 nm UV light or by simulated sunlight (filtered FS-40 fluorescent lights). Its presence makes the DNA sensitive to irradiation with 313 nm light which causes strand breaks to occur. These breaks can be analyzed by alkaline sucrose sedimentation techniques and are proportional to the UV (265 nm or FS-40 light) dose. These photosensitive lesions in the DNA caused by a 20 J/m^2 dose of UV (254 nm) light disappear from normal cells in 3 to 5 h. In excision deficient XP type A cells, the photosensitivity doubles during this time period. We think this indicates processing of the original lesion to an excisable intermediate which, in this case, cannot be excised and accumulates. XP-variant cells are excision proficient and no completely satisfactory explanation for the severe clinical symptoms has been available. These cells, however, do not change the photosensitive nature of this lesion up to 24 h after UV irradiation. The processing of the lesion seen in XP type A cells evidently is lacking in XP-variant cells, thus blocking its repair at a different step.

The photolysis kinetics are used in the bromodeoxyuridine photolysis excision repair assay for calculation of the patch size. A curvilinear relationship between photolysis and 313 nm light dose is indicative of a patch size greater than about 50 nucleotides. If these kinetics are applied to the photolysis of the photosensitive lesion, a similar

curvilinear relationship is found including 313 nm light saturation. These results are consistent with the lesions not occurring at random in the DNA, but being formed in clusters over at least 50 bases. This could result in underestimating the actual number of lesions as well as their occurring in "hot spots" where their genetic effect could be very great if they are not readily repaired.

Certain Lower Vertebrate Cells are Extremely Useful Tools in Molecular Repair Studies. Skin cells isolated from the rattlesnake Crotalus horribus have two efficient means of repairing UV damage to their DNA, excision repair and photoreactivation. After a dose of 30 J/m^2 of UV, these cells removed 60% of the pyrimidine dimers initially present via the excision pathway in a period of 24 h. We have studied the process of photoreactivation (PR) in these cells using various wavelengths of near UV and visible light and have determined the optimum wavelengths and times of exposure for PR. We were able to monomerize about 75% of the thymine dimers with 1.9×10^4 J/m^2 of 365 nm light delivered over an 18-minute period. This degree of photoreversal was also observed at the higher wavelength of 435 nm in which case we could remove 75% of the dimers after 15 minutes with the previously mentioned dose of light. Since the dose of UV given to these cells was 80 J/m^2, the degree of PR is equivalent to the photoreversal of approximately 6 million pyrimidine dimers (equivalent to 56 J/m^2 of UV). We also determined survival in these cells prior to and following photoreactivation. After a UV dose of 20 J/m^2, survival as measured by colony counts could be increased from 51% to 98% by PR. Similarily, 8 minutes exposure to light delivered from a sunlamp resulted in 58% survival whereas, after PR, the survival increased to 86%. These data indicate that UV-induced pyrimidine dimers in DNA are directly involved in cell killing. We have also examined excision repair in these cells using the bromodeoxyuridine photolysis assay following exposure to photoreactivating light. Since PR removes a majority of the pyrimidine dimers, any repair observed is indicative of repair of non-dimer damage. The significant result of these findings is that, for the first time, we have a vertebrate cell line with both repair systems intact and highly efficient. With the ability to remove pyrimidine dimers from DNA using PR, we now have the capacity to undertake in-depth studies into the repair of non-dimer damage to DNA and to examine the role this damage plays in the regulation of DNA repair, survival and mutagenesis.

Measuring Initial Rates of DNA Repair in Human Cells. Through implementation of the cytosine arabinosine (ara-C) repair arrest assay, we have been able to make more precise measurements of initial rates of DNA repair in human fibroblasts following UV irradiation. Ara-C, when converted to ara-CTP, inhibits the resynthesis step of DNA excision repair, producing an accumulation of DNA breaks as monitored by velocity sedimentation in alkaline sucrose gradients. The accumulation of DNA breaks followed first-order kinetics and was analyzed via Michaelis-Menten calculations. Using a Lineweaver-Burke plot of UV dose vs the number of DNA breaks produced per hour, the apparent K_m and V_{max} for the repair rates were calculated as 16.7 pyrimidine dimers/10^8 daltons of DNA and 0.045 repair/10^8 daltons/minute. Based on the initial number of UV-induced

pyrimidine dimers in the DNA and the apparent K_m and V_{max} the rate constants for the excision repair process were calculated. Using these rate constants, the time needed for the removal of 50% of the pyrimidine dimers induced by 254 nm UV at fluences of 5 J/m^2 and 20 J/m^2 was calculated to be 7.6 h and 17.3 h, respectively. These values are in good agreement with our other direct measurements of pyrimidine dimer excision which are made by radiochromatography. The reversal by deoxycytidine of the accumulated DNA strand breaks due to the presence of ara-C was found to be a non-competitive inhibition. The results of these studies confirm our kinetic model of excision repair.

Assaying Carcinogen-Induced DNA "Bulky" Lesions Using a Bacterial Repair Enzyme Complex. Recent studies by others have shown that the endonuclease complex coded for by the uvrA, uvrB, and uvrC genes of Escherichia coli (UVR ABC excision nuclease) can incise DNA containing a variety of "bulky-type" lesions, such as those resulting from UV light or benzo(a)pyrene diol epoxide (B(a)PDE). Using partially purified UVR ABC excision nuclease, we have quantitated the number of endonuclease sensitive sites (ESS) in purified DNA isolated from human fibroblasts treated with UV light or B(a)PDE. The number of ESS/10^8 daltons of DNA were calculated from the number average molecular weight of the DNA as determined by sedimentation in alkaline sucrose gradients. The number of endonuclease sites increased linearly with increasing doses of either UV light or B(a)PDE. The UVR ABC excision nuclease was able to incise a majority of the B(a)PDE-DNA adducts. XP fibroblasts, complementation group A, (XP12BE) had 20-25% more ESS at each dose than the BPDE-treated normal (HSBP) cells. Cells treated with 4 μM B(a)PDE and allowed 12 h of incubation to perform excision repair showed removal of 60% of the initial number of ESS from HSBP DNA and 40% of the ESS from XP-A DNA. Beyond 12 h, XP12BE cells lost no additional ESS while HSBP cells continued to lose ESS, although at a slower rate, until at 48 h only 22% of the initial ESS remained. In cells treated with 10 J/m^2 of UV light, the UVR ABC excision nuclease detected 60% of the sites recognized by the pyrimidine dimer specific Micrococcus luteus glycosylase/apyrimidinic endonuclease. The results demonstrate the potential use of the UVR ABC excision nuclease in a quantitive assay for determining the number of carcinogen-induced lesions in human DNA.

Benzo(a)pyrene Damage to Human DNA: Quantitation of the Number of Sites Undergoing Repair. Benzo(a)pyrene is an ubiquitous polyaromatic hydrocarbon. Its active intermediates, the benzo(a)pyrene diolepoxide [B(a)PDE] diastereomers, are carcinogenic. We wished to determine the number of sites undergoing DNA excision repair in human fibroblasts following treatment with the highly carcinogenic anti- and less carcinogenic syn- diastereomers of B(a)PDE. The levels of DNA repair were assayed by several different techniques: bromodeoxyuridine photolysis; ara-C induced accumulation of DNA incisions, nucleoid sedimentation and a newly developed endonuclease sensitive site assay using E. coli uvr ABC excision nuclease. During excision repair of B(a)PDE-DNA adducts, DNA incisions occur in normal cells and to a much lesser extent in repair deficient XP (XP12BE) cells. When compared on a per adduct basis, syn-B(a)PDE produced approximately twice as many repairing sites than

anti-B(a)PDE in normal cells. Few repairing regions were detectable in XP12BE cells.

The kinetics of repair in normal human diploid fibroblasts (HSBP) for both syn- and anti-B(a)PDE adducts were biphasic, having fast and slow repair components. The repair defective XP12BE cells exhibited only a fast repair component, losing 30-40% of the B(a)PDE-DNA adducts in the first 12 h following treatment, with no apparent B(a)PDE-DNA adduct removal at later times. The slow component for anti-B(a)PDE repair in HSBP cells extrapolated to 58% of the initial number of DNA adducts. The time needed for 50% removal of this component was 28.2 h, while the slow component of syn-B(a)PDE repair extrapolated to 69% of the initial number of DNA adducts. The time needed for 50% removal of this component was 17.2 h. These small differences in the kinetics of repair for anti- and syn-B(a)PDE are significant, but cannot solely explain the marked differences in the biological activities of these two compounds. Experiments correlating the number of adducts removed with the number of repair sites indicated a more rapid loss of adducts than sites undergoing repair. This may reflect a two step repair process for a specific class of DNA adducts, such as the removal of an alkylated base by the action of a DNA glycolase, followed by an apurinic/apyrimidinic endonuclease incision.

Clearly, cells from normal individuals possess the ability to repair a variety of damage to DNA. Numerous studies indicate that defects in DNA repair may increase an individual's susceptibility to cancer. It is hoped that continued studies of the exact structural changes produced in the DNA by environmental insults and the correlation of specific DNA changes with particular cellular events, such as DNA repair, will lead to a better understanding of cell-killing, mutagenesis and carcinogenesis.

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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.

We reported last year on the use of an ethylnitrosourea-induced hemoglobin mutation to show that the expression of the two adult beta-globin genes changed from primarily the beta-minor to beta-major type during gestation and that modulation toward relatively higher beta-minor globin synthesis was observed under conditions of hematopoietic stress. RNA was isolated from the reticulocytes of mice at different stages of development and from mice of several genotypes. The RNA was immobilized on nitrocellulose paper and subjected to dot-blot analysis with beta-minor and beta-major specific probes to determine whether shifts in beta-globin expression during development and in genetically anemic mice were controlled by transcriptional or translational processes. The results showed that the quantity of beta-minor RNA in reticulocytes at 14.5 and 17.5 days of gestation was nearly the same as in induced reticulocytes of adult mice. In contrast, the level of beta-major RNA in reticulocytes at 14.5 days of gestation was 0.23 times and at 17.5 days of gestation was 0.66 times the amount found in reticulocytes of adult mice. These results correlate well with earlier observations that the beta-minor globin gene approaches its normal adult level of expression by 14.5 days of gestation, whereas the beta-major globin gene expression increases between 14.5 days of gestation and 6 days postnatally. Thus, the differential expression of the globin genes during development is regulated at the level of transcription. Expression of the beta-minor globin gene in reticulocytes of adult normal mice is not maximal, however, because reticulocytes of beta-thalassemic mice contain about three times as much beta-minor specific RNA as do reticulocytes of normal mice. This increase in the quantity of transcribed beta-minor RNA probably accounts for the more than expected quantity of beta-minor globin synthesized in beta- thalassemic mice.

The spontaneous beta-thalassemia mutation was found among (C57BL/6 × DBA/2)F_1 progeny in a mutation experiment. The mutation is now being

placed on two genetically inbred backgrounds to make better use of this mouse model of human beta-thalassemia both to analyze the progression of the anemia and to study other side effects of this mutation. A C57BL/6-$\underline{Hbb}^{th}$ congenic strain is at the N7 generation; bone marrow, spleen and fetal liver transplantation experiments are being done to investigate the nature of the perturbed hematopoiesis in these mice. A DBA/2-$\underline{Hbb}^{th}$ congenic strain is at the N4 generation. Mice from this congenic strain will be treated with Friend leukemia virus to induce an erythroleukemia cell line for the purpose of testing various approaches for introducing and regulating beta-globin genes that have been integrated from exogeneous sources. Such studies in the mouse are necessary before gene therapy is attempted in human thalassemia patients. In the course of developing these congenic lines, we have noticed that the WBC counts are significantly higher when this mutation is placed on the DBA/2 background. The genetic basis for this is under investigation. This study and other studies using hematological data have been facilitated recently by the installation of an Ortho ELT-8/ws Hematology Analyzer that has been calibrated for mouse blood. The instrument collects data from 100 microliters of whole blood to record 15 hematological parameters within one minute.

Tissues of age- and sex-matched beta-thalassemia and control mice are being examined. Beta-thalassemic mice have large quantities of stainable iron in liver parenchymal cells, splenic and bone marrow macrophages, and proximal tubule cells of the kidney; lesser but significantly more than normal quantities of stainable iron are also present in the islets of Langerhans, adrenal glands and myocardium of beta-thalassemic mice. Frozen tissues have also been sent to Dr. David Van Wyck (University of Arizona, Tucson) for quantitative measurements of tissue iron. The results show that the spleen, liver and kidney have 4 to 10 times the normal amount of tissue iron. Although iron overloading in beta-thalassemic mice is more severe than in alpha-thalassemic mice, it is not as severe as in beta-thalassemic humans.

We reported last year that SEC/R1 mice are particularly sensitive to inhaled ethylene oxide (EtO). When these mice are exposed to EtO their urine becomes bloody and histological sections show that tubular necrosis occurs in their kidneys. The germinal tissue of SEC/R1 males are also uniquely affected. Exposures to EtO that do not affect the fertility of C57BL/6 males cause SEC/R1 males to become sterile for 4 weeks or longer and histological sections show pronounced tubular degeneration in the testes. (SEC/R1 × C57BL/6)F_1 mice are resistant to EtO like their C57BL/6 parent, suggesting that sensitivity to EtO is a recessive trait. In efforts to uncover the genetic basis for EtO sensitivity, we found that 34 of 70 backcross mice were sensitive to EtO like their SEC/R1 parent. This suggests that sensitivity to EtO is controlled by a single pair of recessive genes. C57BL/6 and SEC/R1 mice differ at 37 visible and biochemical markers on 12 autosomes. Sensitivity to EtO among the backcross mice is not linked with 4 visible and about a dozen biochemical markers analyzed to date. We have recently found that SEC/R1 mice are also more sensitive than C57BL/6 mice to ethylene glycol, a hydrolysis product of EtO. Once the genetic and biochemical bases for the differential

sensitivity of mice to EtO are known, it will be possible to determine whether homologous factors cause humans to be more sensitive to EtO and its derivatives.

Because humans working near gas sterilizers are likely to be exposed to high concentrations of EtO for short periods, we have examined the effects of 1500 ppm of EtO exposure to C57BL/6 and SEC/R1 mice for two 10-minute periods a day for 1 to 4 days. The hematological effects were quantitated on an Ortho ELT-8/ws Hematology Analyzer. When blood was analyzed within 2 h after the last exposure, a significant depression in lymphocyte and granulocyte numbers was observed in both strains. Blood from these mice was analyzed 6 days later to assess the ability of the bone marrow to replace the cellular populations lost through EtO exposure and removal of blood for the first analysis. The ability of SEC/R1 mice to rebound (replace their WBC loss) within 6 days was not affected by exposure to EtO, but the WBC levels did not return to normal within 6 days in EtO exposed C57BL/6 mice. Many diseases are accompanied by disturbed ratios of subsets of hematopoietic cells; thus cellular perturbations for prolonged periods of time may not be inconsequential.

The congenic strain C57BL/10.F-H-2 (B10.F) mice survive half as long as its inbred partner strain C57B1/10 (B10) mice. The accelerated aging of B10.F mice is not associated with any detectable tumors or other pathological diseases. These mice have a common genetic background but differ genetically at the major histocompatability locus (H-2) and express significantly different levels of IgA immunoglobin. Lifespan data on F_2 and backcross progeny from reciprocal F_1 hybrids of B10.F and B10 mice showed that both lifespan and IgA levels were influenced by the strain of the maternal parent. Moreover, the IgA levels could be modulated by foster nursing of neonates, by blastocyst transfer into foreign mothers, and by neonatal injection of histocompatible spleen cells. These results are consistent with the possible transfer of allotype specific immune suppressor cells or vertical transfer of a viral particle. In collaboration with J. A. Otten, Biology Division, we found that an endogenous ecotropic virus is actively shed in B10.F but not in B10 mice. Transfer of a cell-free splenic filtrate established that the long-term suppression of IgA levels in neonatally injected offspring was not cell mediated. Analyses of DNA of liver, spleen and lymph node showed that DNA from the shed virus had reintegrated into the DNA of some lymphoid cells of B10.F but not of B10 mice. These observations suggest an etiological role for the ecotropic virus in regulation of IgA levels and life shortening. Viruses shed by the B10.F mother and passed on to her offspring reintegrate in lymphoid cells resulting in a deficient IgA humoral response, which, in some way, compromises long-term survival.

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GENETICS OF ANAEROBIC ORGANISMS

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"Degeneration" in Clostridium Acetobutylicum. The bacterium, Clostridium acetobutylicum, is a spore-forming anaerobe that produces acetone, butanol, ethanol, acetic acid, butyric acid, hydrogen and carbon dioxide from a wide variety of materials containing starch or sugar. At one time, this organism was used commercially for the production of acetone and butanol in large quantities. One of several reasons that led to the abandonment of this process was the observation that the organism rapidly "degenerated" to a non-sporulating, low solvent producing variant.

For many years the genetic concepts that would allow the design of experiments which might explain "degeneration" were not available. The phenomenon is not readily explainable in terms of simple mutational events and has some characteristics which suggest it could be a model for differentiation in higher organisms. Today, the appropriate concepts and several new techniques for handling oxygen-sensitive organisms are available. Therefore, we are attempting to determine the mechanism of "degeneration" because this may lead to the exposure of an unusual genetic mechanism in this bacterium and other organisms. Such a study may also reveal methods for the stabilization of Clostridium acetobutylicum in the spore-forming, solvent producing phase. The organism would then become more attractive as a catalyst for the production of solvents and fuels from waste materials and renewable resources.

During the past year, we have developed a microscopic technique for the observation of detailed morphological features of bacterial colonies. The technique is based on the use of oblique lighting and selective staining reagents. When the technique is applied to colonies of Clostridium acetobutylicum grown from individual spores plated on the surface of agar, we observe that each and every colony contains large regions or sectors that are obvious only when the oblique lighting and appropriate stains are applied. These roughly triangular sectors do not extend to the midpoint of the colony but seem to arise after the original plated spore has germinated and produced a large number (circa 10^5) cells. Every colony contains such sectors and there are, on the average, 6 to 8 sectors/colony. Several kinds of sectors can be distinguished in each colony. They differ in texture, stainability and density. If cells picked from the sectors are examined microscopically, it becomes obvious that they have properties distinct from those associated with the cells in the central, unsectored region of the colony. Cells from the central region are relatively wide, short rods and almost all contain well developed, highly refractile spores. Cells from some of the sector types are long and relatively thin (circa 1 μ × 6 μ) and spores are rare but definitely present. Completely asporogenic long, thin, motile cells are found in only one of the several types of sectors. If liquid cultures are grown from sectors that contain a few sporulating cells, it can be observed that they are usually relatively poor solvent producers and poor spore producers. If colonies are grown on agar surfaces from the poor spore producers, they also sector and these latter sectors contain the completely asporogenic thin, rod-shaped organisms that are usually extremely poor solvent producers.

This cascade of high frequency sectoring always moves from the spore-forming, high solvent producing type towards the "degenerate" asporogenic, low solvent producers. We have not observed any tendency for spontaneous reversion from either the intermediate or extremely "degenerate" types back towards the original cell type.

The high frequency sectoring phenomenon is observed with several laboratory strains of Clostridium acetobutylicum and 12 recent soil isolates of spore-forming, solvent producing anaerobes. It can be observed on synthetic media as well as complex organic media. It is, therefore, not a phenomenon restricted to one strain or one set of conditions. We believe it is a reflection of the basic events that underlie the "degeneration" phenomenon. Because the sectors are initiated at a particular stage in colony development, and because the frequency of sectoring is so high, we do not believe that the phenomenon can be explained in terms of random mutational events. The observation that sectoring always results, through a series of intermediate types, in the production of a relatively stable, asporogenic cell line with no tendency towards reversion also argues against certain kinds of mutational events. Our current working hypothesis is that, at the time the initial group of cells in a colony begin to segregate their DNA for incorporation into spores, some daughter cells are produced that inherit limited genetic information. These defective cells, produced by unequal segregation of DNA, are viable but lack the information

necessary for sporulation, solvent production and possibly other properties. Because they cannot sporulate, or sporulate only poorly, they continue to divide and give rise to the "degenerate" sectors which grow out from the perimeter of the central colony. For this hypothesis to be compatible with all our observations, we must postulate that the original, spore-forming, high solvent producing cells have multiple copies of many genes. This would help explain the appearance of semi stable intermediate types of sectors as well as other observations we have made. If the hypothesis is correct, one expectation is that the most degenerate types of cells would have less DNA than the original cell type. Preliminary data obtained in collaboration with the Biology Division's Cytometrics group indicates that this is the case. Experiments are under way with colleagues at the University of Florida to determine if the genome of Cl. acetobutylicum is organized as a single circular chromosome as in E. coli or consists of several distinct structures possibly including large "mini chromosomes." Other experiments are being conducted in collaboration with colleagues at The University of Tennessee. These hybridization and DNA melting experiments should establish if the copy number of genes in the original strain is, in fact, higher than that in the "degenerate" types.

The Effect of Oxygen on DNA of Clostridium Acetobutylicum. We previously reported that if actively growing anaerobic cells of Cl. acetobutylicum are exposed to air, the apparent average molecular weight increases from about 80×10^6 to 300×10^6. This process is complete after about 60 seconds of exposure. The lower molecular weight DNA reappears rapidly (circa 30 minutes) when the cells are regrown under anaerobic conditions. We have now observed that the apparent average molecular weight of the DNA is not constant throughout the different stages of growth. The apparent average molecular weight decreases as the cells go from lag to log phase. Density determination by alkaline cesium chloride gradients and length determination by examination of electron micrographs suggest that exposure to oxygen increases the length of the DNA.

We have now examined several strains of Clostridium acetobutylicum and other anaerobic and faculatitive organisms to see if they exhibit this same phenomenon. Twenty-two different strains of Clostridium acetobutylicum were examined and all showed an increase in the apparent average molecular weight after exposure to air. A very strict anaerobe, Clostridium thermoacetocum, was examined and showed a decrease in molecular weight after exposure whereas a less strict Clostridium butyricum showed only a slight shift in molecular weight after exposure. We will attempt to correlate these effects of oxygen on DNA with lethal and mutagenic effects in these organisms.

Genes Controlling Sensitivity to Oxygen. Eighteen mutants of Escherichia coli K-12 A81157 have been isolated after exposure of the parental strains to ultraviolet (2650A) light. These mutants are unable to form colonies on agar surfaces in the presence of oxygen. They do form colonies if incubated anaerobically. The mutant cell lines retain all of the amino acid requirements and antibiotic resistance characteristics of the parental strain. Survival studies indicate that, for these mutants,

oxygen is bacteriostatic rather than bacteriocidal. All the mutant cell lines are F-(recipients in genetic crosses) and they are being crossed with a recently obtained collection of oxygen tolerant Hfr (donor) strains of E. coli.

These crosses should reveal the map location of genetic loci that control oxygen sensitivity. To date, one mutation has been assigned to the region between 88 and 91.5 minutes. A known gene controlling heme biosynthesis is in this region and could affect oxygen sensitivity because of the presence of this structure in enzymes such as catalase and the cytochromes. Genetic and biochemical characterization of the remaining mutants should help us understand why some organisms thrive in oxygen while others are inhibited.

The Repair of Potentially Lethal Damage Caused by the Indirect Effects of X-Irradiation in Escherichia Coli. In bacteria and mammalian cells the indirect effects of water radiolysis products, most likely OH radicals, are responsible for a major portion of cell lethality and DNA strand breaks. In bacteria, a DNA polymerase I-dependent system repairs most of the single strand breaks in DNA induced by the indirect action of X rays. In this study with E. coli an investigation was made of the role of DNA polymerase I in preventing cell death caused by potentially lethal damage resulting from the indirect effects of X rays.

The radiosensitivity of an Escherichia coli mutant deficient in DNA polymerase I was measured in the presence of OH radical scavengers. The extreme X-ray sensitivity of the mutant could be abolished by OH radical scavengers if a sufficiently high level of radioprotector was present. There was a direct correlation between the OH radical scavenging activity of the chemicals tested (NO_2^-, n-butanol, glycerol t-amyl alcohol, and t-butanol) and their protective ability. The data suggest that the indirect actions of X rays (primarily OH radicals) result in major damage to the bacterial DNA which in large part consists of potentially lethal lesions. This potentially lethal damage is repaired through an enzymatic pathway requiring DNA polymerase I. In the mutant lacking DNA polymerase I, these potentially lethal lesions are expressed as cell lethality.

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Mammalian Genetics and Reproduction Section

SECTION OVERVIEW - LIANE B. RUSSELL

The work of the Mammalian Genetics and Reproduction Section has continued to concern itself almost exclusively with mouse germ-line mutagenesis and genetics. The major themes of the Section's research have been germ-line mutagenesis (biological factors affecting yields of various classes of mutational damage), basic genetics and cytogenetics, developmental genetics and the study of phenotypes, the interaction of chemical agents with germ-cell DNA, and gametogenesis (normal and abnormal). With the retirement during the year of E. F. Oakberg, the last-named area of research, which is inextricably tied to all the others, is in danger of being neglected in the future unless the staff gap can be filled. Altogether, the permanent doctoral staff of the Section, already small, shrank by 33% (from 6 to 4). Because of the unusually energetic involvement of the remaining investigators, and a small number of collaborative studies with other laboratories, the Section has remained remarkably productive. It is, nevertheless, particularly heartening that we have been able to hire a mammalian molecular geneticist (E. M. Rinchik, who will join us 1/1/86) and are being permitted to recruit other scientists to help exploit the unique opportunities for progress in basic areas of mammalian genetics.

The research conducted during the past year has addressed — at least to some extent — all the major components of the information needed to understand the mechanisms for, and assess the risk from the induction of heritable mutational damage. In addition, it has addressed some basic genetic and cytogenetic questions, using as tools the altered genes and chromosomes produced in the course of more routine mutagenesis experiments. Interfaces of this type are an important aspect of the Section's research and are often of reciprocal benefit; e.g., if detailed knowledge of the molecular structure of a specific chromosomal region can be achieved, this will, in turn, elucidate the manner of action of certain mutagenic agents. Even mundane mutagenesis testing of selected chemicals can be conducted in such a way as to contribute to method development or to an understanding of the basic properties of the biological material.

Approaches to the understanding of heritable mutational damage include (1) detection of such damage, (2) quantitation — specifically, exploration of factors affecting yield, (3) investigation of organismic expression, and (4) analysis of the quality of the damage at the chromosome, gene, or molecular level.

(1) We have in the past developed most of the methods currently in use for the detection of the major classes of genetic damage. This year, our major effort in method development has been as follows:

- Monitoring DNA breakage induced in spermiogenic stages by measuring DNA strand breaks in sperm via alkaline-elution techniques. For some chemicals, the correspondence in yield to that of transmitted clastogenic damage was shown to be good.

- Development of a genetic marker scheme for detecting autosomal trisomy directly, instead of by complementation, and exploration of a trisomy prescreen. Funding has been obtained to validate these methods.

Using methods developed earlier, we have attempted to determine whether a number of chemicals are mutagenic in the mammalian germline. Although testing is not a major objective of this Section, such work serves to enlarge the data base for comparisons with other mutagenesis systems. Chemicals investigated this year in specific-locus tests were urethane, 6-mercaptopurine, ethylene dibromide, acrylamide, and platinol. A survey of 11 "environmental" chemicals previously demonstrated to be positive in Drosophila or mammalian somatic cells showed that all 11 were mutagenically negative in spermatogonial stem cells. In conjunction with other evidence, this led to the conclusion that such cells have an effective repair capability. One widely used chemical, acrylamide, was found to be highly clastogenic in certain postspermatogonial stages.

(2) Factors affecting quantitative yield of heritable damage include sex, germ-cell stage, and genotype. These may act via differences in cell selection or in repair capability. A large number of experiments during the past year addressed biological as well as physical factors affecting yields of various genetic endpoints following exposure to various agents.

- Following injection of plutonium (which is deposited near the spermatogonial stem cells), the yield of heritable translocations increases with time after exposure; but, surprisingly, the yield from this α-particle irradiation is more comparable to that from X rays than that from neutrons.

- The specific-locus mutation yield from X-ray dose repetition is additive when doses are separated by monthly intervals.

- Dose fractionation greatly decreases the yield of specific-locus mutations from ENU-exposed spermatogonial stem cells. Mutation rate after 20×5 mg/kg is even lower than after 10×10 mg/kg, which in turn is almost an order of magnitude lower than at 1×100 mg/kg. Additional evidence from DNA interaction studies indicates that repair is responsible for the differences. Additivity of the 100/mg response (4 weekly doses) yields a mutation frequency 36 times that reported for the most effective chemical mutagen previously known for stem-spermatogonia. The prediction that our discovery of the mutagenic effectiveness of this chemical would be useful in the production of mouse models of human genetic diseases has now been realized in both the USA and the UK.

- A marked dose-rate effect has been found for clastogenic effects produced by ethylene oxide — the first effect of dose protraction demonstrated for a chemical mutagen. The finding is of major regulatory importance.

- A strain difference was found in the yield of translocations induced by X rays in spermatogonial stem cells. At least part of the difference can be accounted for by a difference in the proportion of unbalanced sperm descending from aberrant meiocytes.

- While strain differences in response to dibromochloropropane appear minor, mice and rats differ with respect to the chemical's interaction with germ-cell DNA, as measured by unscheduled DNA synthesis.

- In-depth studies with the supermutagen ENU show a major effect of sex (oocytes very resistant), and no major effect of age of males. Combined radiation-ENU exposures indicate that ENU may be most effective in the S phase of spermatogonial divisions.

- Elucidation of the basic properties of normal germ cells and of cytotoxic effects in germ cells provides information necessary to the interpretation of heritable genetic damage. Experiments this year addressed gonocyte and stem-cell properties in young mice (days 1-36). They also investigated the important question of whether spermatogonial stem cells of adults consist of more than one population with respect to sensitivity to environmental agents.

(3) The organismic expression of mutational damage must be explored in order to be able to translate information on mutation yield into projections of human risk. In addition, investigations in this area can provide essential data for studies on gene expression and developmental genetics. We expect an increasingly fruitful interface between this field of investigation and that dealing with the structure of normal and mutated chromosomal regions (see 4, below). Highlights of last year's findings dealing with organismic expression were as follows.

- No excess skeletal anomalies were detected in offspring of Plutonium-exposed males. With the sample size used, this outcome was expected from specific-locus mutation frequencies unless the spectrum of mutations had been different from that after X-ray exposure.

- Dominant skeletal mutations on a congenic background markedly interact with respect to the expressed syndrome. Both synergisms and antagonisms are found. Synergistic interactions of unlinked dominants may be the cause of some human disorders that are presently thought to have recessive inheritance.

- An extensive computer analysis involving almost 160,000 litters indicates, among other things, that if men were exposed to 1R of low-LET low-dose-rate radiations, the number of deaths between conception and late childhood caused by induced dominant mutations would be about 19 out of every million individuals who would have reached that age in the absence of radiation. An approximately equal number of offspring would be afflicted by viable disorders.

- A neurological disorder has been found to be totally associated with heterozygosity for a balanced reciprocal translocation. Total absence of anatomical findings indicates a biochemical basis for the defect.

(4) Investigation of the quality of genetic damage at the chromosome, gene, or molecular level not only contributes information on the mechanisms of mutation induction but can greatly add to our knowledge of the basic structure and behavior of the genetic material. Contributions to both of these areas were made in the past year.

- The use of radioactively labeled chemical mutagens has been expanded from the measurement of total germ cell alkylation to the study of specific adducts. Non-linear increases in N7-hydroxyethylguanine were found following EtO inhalation exposure, indicating saturation of other nucleophilic sites.

- Additional evidence has been obtained bearing on the hypothesis that the formation of unstable N-7 alkylguanine products in the DNA of post-meiotic germ cells is associated with the production of heritable translocations, while that of stable alkylation products leads primarily to dominant lethals.

- The qualitative scope of specific-locus test data has been greatly enlarged by genetic and molecular analyses of mutations at certain of the loci, allowing a grouping of mutations into (a) multilocus deletions, (b) small (possibly single-locus) deletions, and (c) intragenic lesions. Using these criteria, it was shown that the spectrum of mutations induced in stem cells by ENU are qualitatively indistinguishable from the control spectrum, while radiation causes larger lesions, especially so in certain germ-cell stages.

- Dissection of the d-se region on Chromosome 9 was accomplished by an extension of genetic analyses and by a detailed molecular analysis that utilized DNA probes from both sides of the proviral integration site within the d locus. Molecular substantiation was obtained for the deletional nature of many of the mutations, and the physical (DNA) map was found to be generally concordant with the genetic map. Two of the probes were able to distinguish between members of a single complementation group.

Basic studies involving chromosome aberrations have provided information on gene action and chromosome behavior.

- A new X-autosome translocation, discovered in the course of a specific-locus experiment, will be of great use in the study of X inactivation, since it appears that the proximal portion of the X chromosome — from centromere to translocation breakpoint — is unable to inactivate contiguous autosomal material, and may itself turn out not to be subject to X inactivation when separated from the site of control of primary X inactivation.

- An inversion involving a major portion of the X chromosome was utilized in electron-microscope studies of the synaptonemal complex to provide additional evidence supporting the hypothesis that X-Y pairing is non-homologous.

Future directions. Currently, certain government agencies, including DOE, are interested in the possibility of detecting heritable or potentially heritable DNA damage directly in human beings. Mouse genetics will play a major role in this quest. For example, method development will be greatly aided by the ability to increase the mouse mutation rate to 200-fold the control rate (as can be done with certain ENU regimens). Among potentially fruitful future approaches will be some that involve analysis of germ-cell DNA. We have already made progress toward this through our work on detection of single-strand breaks by alkaline elution of sperm DNA.

Assuming that methods for use in human populations are eventually developed, it is likely that they will provide plus-minus data only. Mouse experiments will continue to be needed to determine the effects of variables on mutation yield. Even more important, the experimental data are essential for exploring the link between DNA damage and organismic effects, especially so since human DNA-damage detection methods are likely to be weighted toward the 90-99% of the genome that is not expressed. Finally, to evaluate any approaches that rely on somatic → germ-cell extrapolations (the so-called "parallelogram" approaches), mouse experiments must explore such factors as differences between loci and effects of cell type on intrinsic sensitivity, repairability, and cell selection.

In addition to mutagenesis-oriented investigations, the basic work on gene structure and expression shows much promise of progress through the wedding of classical genetic and molecular techniques in the exploration of the invaluable genetic material that has been and continues to be generated in mutagenesis experiments.

DNA DAMAGE IN MAMMALIAN SPERM ASSAYED BY ALKALINE ELUTION

G. A. Sega E. E. Generoso

Alkaline elution, a procedure used to measure DNA strand breaks in cells, has been adapted to monitor DNA breakage in spermiogenic stages of the mouse following mutagen treatment. In each experiment, one group of male mice have their germ-cell DNA pre-labeled with ^{3}H-thymidine and are subsequently exposed to the test mutagen. A second group of males have their germ-cell DNA pre-labeled with ^{14}C-thymidine and serve as controls.

At daily intervals through 3 or 4 weeks following mutagen treatment, spermatozoa from the vasa deferentia of treated and control animals are placed together on a polycarbonate filter and lysed. DNA is eluted through the filter overnight, using an alkaline buffer. Any small pieces of single-stranded DNA resulting from breakage by the mutagen will pass rapidly through the filter, while normal-sized DNA will take considerably longer to pass through. The amounts of treated and control DNA eluted are then determined using liquid scintillation counting techniques.

Studies carried out with injected ethylene oxide (EtO) have 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 is induced by injected EtO. A more relevant exposure route for EtO in humans is by inhalation, and we have recently been able to demonstrate breakage of mouse germ-cell DNA when EtO is administered by that route. Work is under way to determine the minimum inhalation exposure to EtO at which we can still detect breakage of germ-cell DNA in the mouse. Work is also planned to determine how the level of DNA breakage in the germ cells is affected by variations in the time over which a fixed EtO inhalation exposure is given. Information on these points is important in establishing guidelines for permissible human exposure levels to potentially genotoxic chemicals in the environment.

Not all mutagens studied with our alkaline elution technique have been demonstrated to induce DNA breakage in mouse sperm cells. A notable member of this class of mutagens is X rays. No increased elution of DNA from sperm can be observed after exposures as high as 1200 R to various spermatogenic cell types, even though genetic and cytogenetic evidence has shown X rays to be very effective in causing chromosome rearrangement and breakage in meiotic and postmeiotic germ-cell stages of male mice. Furthermore, numerous 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, we are currently working on methods to purify earlier germ-cell stages directly from the testis. These stages include late spermatids, early spermatids, and pachytene spermatocytes. It may be possible to demonstrate DNA breakage in these earlier germ-cell stages

shortly after X-ray exposure, rather than waiting until they have advanced to the mature spermatozoa stages found in the vasa deferentia.

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EXPERIMENTAL APPROACHES FOR THE DETECTION OF CHROMOSOMAL MALSEGREGATION OCCURRING IN THE GERMLINE OF MAMMALS

L. B. Russell K. F. Stelzner

A critical survey of existing methods for the detection of heritable aneuploidy led to a proposal for new methods. We analyzed test systems that involve direct chromosomal examination, and those relying on genetic means for the detection of malsegregation. Among the former are: observations involving the first meiotic prophase, metaphase-2 counts, analysis of postmeiotic germ cells, examination of pronuclear chromosomes at first cleavage, and cytogenetic analysis of embryos. Of these, the study of pronuclear chromosomes was judged to be the most universally informative and reliable, provided tertiary trisomy can be ruled out.

Genetic methods may be grouped into those based on complementation (use of tester stocks with frequent spontaneous malsegregation) and those in which aneuploidy is directly detected. With complementation methods, only a fraction of the products of aneuploid gametes is detectable. Direct methods do not suffer from this problem; however, if observation of animals is to be made postnatally (as is the case with complementation methods), only sex-chromosome aneuploidy can reliably be detected, since almost all autosomal aneuploids in the mouse are now known to die prenatally.

We have therefore devised a genetic marker scheme for detecting autosomal aneuploidy in late fetal stages. The scheme involves mice carrying two closely-linked recessive markers in repulsion, mated to a tester stock that is homozygous for both markers. We have recently received funding from the EPA Genetics Toxicology Division to develop and validate this method.

We have also explored trisomy prescreens that may be combinable with other mutagenesis assays. Most mouse trisomics are now known to die during the second half of pregnancy, in contrast to most "dominant lethal" carriers, which die around the time of implantation. Work previously reported for ethyl alcohol by Generoso's group (Washington, W. J., et al., Mutat. Res. 147: 205-210, 1985) confirms the feasibility of detecting such late deaths; and a small 6-mercaptopurine experiment carried out by us this

year provided similar, though not yet statistically significant, data. As a result, we have proposed the scoring of late fetal deaths as a trisomy prescreen. The postmidterm death prescreen will be validated under the EPA contract mentioned above.

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SPECIFIC-LOCUS EXPERIMENTS AND RELATED STUDIES WITH SIX CHEMICALS

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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. This year, we completed experiments on one chemical (urethane), almost completed studies on two (6-mercaptopurine and ethylene dibromide), and initiated studies on two more (acrylamide and platinol). In addition, follow-up study was done on a chemical found to be negative last year (DBCP). The work is being carried out under contract with the National Toxicology Program.

1. Urethane. Urethane is a rodent carcinogen that has given widespread, though erratic, evidence of mutagenicity and carcinogenicity. 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. Such an experiment was begun last year and completed this year, using the maximum tolerated dose of 1750 mg/kg. No mutations were observed among 13,315 offspring derived from treated spermatogonial stem cells. This result is negative by Gene-Tox criteria. The minimum multiple of the control rate that is ruled out (at the 5% probability level) is 3.2. Among 19,513 offspring derived from treated poststem-cell stages there was one with a possible mutation, which, however, died before verification by allelism tests. Regardless of whether or not this animal is counted as a mutant (minimum multiples of the control rate ruled out are 3.5 or 1.9,

respectively), the result is negative by Gene-Tox criteria. The results of the specific-locus test thus do not support the claim by Nomura that urethane causes heritable genetic lesions resulting in F_1 cancers.

Both the productivity data (lower-than-average litter number for conceptions made in the 8th week post treatment) and detailed histological analysis of the testis (possible slight effect on A-paired and A-aligned differentiating spermatogonia) provide suggestive evidence that urethane reaches germ cells.

2. 6-Mercaptopurine (6MP). With the collection of over 65,000 offspring, we almost completed a specific-locus study on the base analog 6-Mercaptopurine (6MP) administered at 150 mg/kg. The experimental design involved weekly matings with fresh females to sample the poststem-cell period, except that during weeks 5 and 6, females were replaced in the middle of the week as well. This was done to increase sample sizes for days 32-38 post-treatment, a period implicated by earlier work as possibly vulnerable to mutagenic damage. Altogether, 10 replicate experiments were run. A clear drop in litter size found for matings made during the 32- to 38-day interval confirms earlier findings of dominant-lethal effects (Generoso et al., Mutat. Res. 28: 437-447, 1975; Oakberg, Mutat. Res. 94: 165-178, 1982). Productivity data, ancillary to the specific-locus test, confirm Oakberg's finding that there is no stem-cell killing.

No mutants were found among 33,213 offspring derived from exposed poststem-cell stages. This result rules out (at the 5% probability level) a multiple of 0.7 times the historical control rate. For the 32-38 day interval alone, the multiple ruled out by the data (0 mutations in 5214 offspring) is 9.8. Among 31,040 offspring derived from exposed spermatogonial stem cells there were 3 mutations. The experimental rate is not significantly different from the spontaneous rate, and rules out a multiple of 3.7 times the historical control. The result is thus negative by Gene-Tox criteria.

3. Ethylene Dibromide (EDB). Specific-locus data collection was completed for offspring derived from EDB-exposed spermatogonial stem cells (160 or 167 mg/kg). Small exploratory groups exposed for up to 18 weekly injections of 160 mg/kg EDB (weighted average exposure accumulated = 1152 mg/kg) continue to produce at a low rate. The spermatogonial stem-cell results — 0 mutations in 26,242 offspring — rule out (at the 5% probability level) a multiple of 1.2 of the historical control rate. If the small repeated-exposure groups are added, the multiple of historical control ruled out is 1.0; i.e., the doubling dose for EDB is presumably larger than ca 160 mg/kg. No mutations were found among 2370 offspring derived from exposed poststem-cell stages.

Productivity data give no evidence for spermatogonial cell killing, dominant-lethal effects, or morbidity of treated males. Males remain fertile even after 18 weekly injections of 160 mg EDB/kg (total exposure, 2880 mg/kg). The evidence that EDB does reach male germs comes from an

experiment of Sega's showing adduct formation. The interacting EDB is removed less rapidly from the testis than is ENU.

4. Acrylamide (AA). Acrylamide, the monomer used in the production of polyacrylamides, 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, and clearcut dominant-lethal effects have recently been found at our laboratory.

Toxicity studies indicate that 125 mg/kg, given in a single injection, is the maximum tolerated dose. However, dose fractionation increases tolerance. Specific-locus studies on AA were initiated in offspring of males injected on 5 successive days with 50 mg/kg AA. Productivity data provide no evidence for the killing of differentiating spermatogonia. No mutations have been observed to date (9385 offspring). The multiple of the historical-control rate ruled out by the spermatogonial stem-cell data is 5.0 (at the 5% probability level). Data collection for this germ-cell stage is continuing. We are also initiating experiments to determine mutation rates in poststem-cell stages.

5. Platinol (PLA). Cis-diaminedichloroplatinum (II) abbreviated platinol (PLA), is a chemotherapeutic agent used for the treatment of testicular and other cancers. On the basis of preliminary toxicity studies, male (101 × C3H)F_1 mice were injected i.p. with 10 mg/kg PLA. To date, all offspring have been derived from exposed spermatogonial stem cells.

Productivity data obtained incidental to the specific-locus experiment give no indication of stem-cell killing for the stages sampled. However, other tests, involving exposed poststem-cell stages, clearly point to major cytotoxicity of PLA in differentiating spermatogonia, and possibly during meiotic divisions as well (proving, incidentally, that PLA reaches male germ cells). The specific-locus data are not yet very extensive: no mutations have been observed among 3198 classified offspring. This result rules out only a very large multiple of the control rate, namely 16.5 (at the 5% probability level).

6. 1,2-Dibromo-3-chloropropane (DBCP). Last year, we found that DBCP produced no fertility disturbance, germ-cell cytotoxicity, dominant lethals, or specific-locus mutations. The treated (101 × C3H)F_1 mice were found to be Ah responsive, but other findings make it questionable whether biotransformation of DBCP to reactive intermediates is accomplished via the Ah-receptor system. This year, in order to explore the negative DBCP results with (101 × C3H)F_1 mice, we tested 8 different mouse strains and SD rats for germ-cell UDS response (see p. 81). All mouse strains gave a negative or, at best, marginal UDS response, while the rats responded more strongly than the mice, and in a dose-related manner. The results are consistent with the species difference in dominant-lethal tests.

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POSITIVE GENETIC HAZARD PREDICTIONS FROM SHORT-TERM TESTS HAVE PROVED FALSE FOR RESULTS IN MAMMALIAN SPERMATOGONIA WITH ALL ENVIRONMENTAL CHEMICALS SO FAR TESTED

W. L. Russell[6]

The major portion of genetic risk in human males exposed to environmental mutagens is assumed to occur from mutations induced and accumulated in the stem-cell spermatogonia. Specific-locus mutation tests in mouse stem-cell spermatogonia have now been performed on a total of 11 environmental chemicals, i.e., chemicals encountered in the everyday environment or workplace. None has given a mutation frequency higher than the control, and the mutation frequency for all 11 combined (12 mutations in 298,502 offspring) is actually less than the historical control, though not, of course, significantly lower. Yet all 11 chemicals are positive in the Drosophila sex-linked lethal test, and all of the 10 tested in mammalian somatic cells proved positive. Absence of mutation induction in mouse spermatogonia cannot be attributed to: (a) small sample size (the samples are large), (b) failure of the chemicals to reach the testis (10 of them are known to reach the testis in active form), (c) insensitivity of the test (with X rays, the test detects approximately ten times as many mutations as does a comparable Drosophila test; and with ethylnitrosourea, it detects almost one order of magnitude more mutations than are obtained with X rays). It is concluded that mammalian stem-cell spermatogonia have an effective repair capability. This is supported by the dose-response and dose-fractionation results with ethylnitrosourea. Therefore, positive genetic hazard predictions from short-term tests on chemicals may frequently give false warning of what to expect in mammalian spermatogonia, the cells considered to be of major concern for genetic risk in human males.

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ACRYLAMIDE INDUCES DOMINANT LETHALS IN MALE MICE

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Acrylamide (AA) is a vinyl monomer widely used in the paper industry, as a flocculant in water treatment, and in research laboratories for preparing polyacrylamide gels for electrophoresis. AA is presently used extensively in laboratories engaged in DNA sequencing. The 1974 U.S. production of AA was approximately 70 million pounds, and at that time NIOSH estimated 20,000 workers were potentially exposed.

AA is highly soluble in water and may be absorbed through the skin. Through the conjugated ethylenic group, AA can undergo addition reactions with nucelophilic sites and thus may be expected to react with DNA. AA has been shown to be rapidly distributed in test animals and is found bound to proteins, particularly hemoglobin (TSCA Chemical Assessment Series, EPA-560/11-80-016, 1980). The neurotoxicity of AA has been the primary basis for human health concerns.

AA is structurally similar to two other industrially important vinyl monomers, acrylonitrile (ACN) and vinyl chloride (VC), both of which are mutagens and rodent carcinogens. Because of the evidence for genetic toxicity of structurally related monomers, the expectation that AA binds with DNA, and the widespread opportunities for human exposure, a study was begun to determine the genetic effects of AA on mammalian germ cells. We are here reporting the results of the dominant-lethal study in male mice.

Acrylamide monomer (CAS #79-06-1) of purity > 99% was provided by the National Toxicology Program Chemical repository, Radian Corporation, Austin, Texas. Acrylamide solutions were prepared in Hank's balanced salt solution (HBSS) and injected intraperitoneally in a maximum volume of 1.0 ml. Control mice were given 0.8 ml of HBSS. A preliminary 30-day acute toxicity study for single doses was performed in order to determine the maximum tolerated dose.

Three dominant-lethal experiments were conducted. In the first experiment, 60 (C3H × 101)F_1 male mice, about 12 weeks old, were randomized into two equal groups. One group received a single dose of 125 mg/kg acrylamide and the other group was used as control. Each male was caged

with two T-stock females (about 12 weeks old) early in the morning following the day of injection. Females were checked for vaginal plugs each morning for 46 days following treatment. Mated females were replaced by fresh ones. Uterine analysis was performed 12-15 days after observation of the vaginal plug. In the second experiment, the same conditions as in the first were followed with the exception that the females were (SEC × C57BL)F_1 hybrids and matings were limited to the period 6-10 days after treatment, a period that samples sperm treated as late spermatids or epididymal sperm. In the third experiment, 120 males from the same stock were randomized equally into experimental and control groups. Each mouse in the experimental group was given a 50 mg/kg dose daily for 5 days; controls were administered HBSS on the same schedule. Each male was caged with one T-stock and one (C3H × 101)F_1 female early in the morning following the last day of treatment. Females were examined for vaginal plugs each morning for 14 days following the last day of treatment.

All three experiments showed unequivocably that AA induces dominant lethals in late spermatids and early spermatozoa (exclusively). It is noteworthy that the stages sensitive to AA are the same ones that are sensitive to EMS, MMS or MNU. It also appears that the yield of AA-induced dominant lethals was higher when treated males were mated to T-stock females than when they were mated to (C3H × 101)F_1 females. These results once again demonstrate that the strain of female mice used in dominant-lethal studies may play an important role in the outcome of the test.

The present results indicate that AA induces chromosomal damage in certain male germ cells. Thus, AA is another example of an environmental chemical that is a mutagen in mammalian germ cells. In 1980, the EPA concluded that the neurotoxicity of AA was sufficient reason to control human exposure and that no further health-effects testing would be required. However, because of its structural similarities to known mutagenic and carcinogenic vinyl monomers, and in view of the present results indicating that AA induces chromosomal damage in mouse germ cells, it is possible that AA poses a mutagenic risk. A re-evaluation of the potential health hazards associated with human exposure to AA may be in order. A study to determine if AA is also capable of inducing heritable translocations is underway.

This study was conducted in collaboration with Dr. M. D. Shelby of the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

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^{239}PLUTONIUM-INDUCED HERITABLE TRANSLOCATIONS IN MALE MICE

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^{239}Pu-citrate deposited in the testis is retained for a long time. Because it is localized in the interstitial tissue outside the basement membrane of seminiferous tubules, the spermatogonial stem cells receive a relatively higher dose than the testis as a whole — estimated to be about 2 to 4 times in mice. This study was conducted in order to determine the frequency of transmitted reciprocal translocations per rad of exposure to the alpha emitter.

The present heritable translocation study was an adjunct to the specific-locus study by W. L. Russell. Male (101 × C3H)F_1 mice were injected via the tail vein with 0.25 μCi/mouse of monomeric ^{239}Pu citrate (equivalent to 10 μCi/kg body weight). Testicular dosimetry was performed by Drs. A. Lindenbaum and J. Russell of Argonne National Laboratory. Treated males were mated to T-stock females. Male progeny conceived at two intervals following treatment, approximately 6 and 12 months, were tested for translocations. These groups of progeny were collected from two series of experiments. Series 1 was started in December of 1976 and Series 2 in March of 1980. Male progeny were screened for presence of translocations using the procedure used routinely in our laboratory. They were fertility tested and those found sterile or partially sterile were studied cytogenetically.

Results of Series 1 seemed to indicate an increase in the frequency of heritable translocations produced with time postinjection. Series-1 data, however, were not sufficient to establish an effect of interval because the difference between the frequencies at 12 months (6/1560) and at 6 months (2/987) was not significant ($P = 0.342$, Fisher's exact test). Therefore, a larger replicate was initiated through Series 2. Like Series 1, Series 2 showed a time-related increase in the frequency of translocation carriers. However, this time, the two frequencies are significantly different: 6/2633 at 6 months, and 14/2505 at 12 months ($P = 0.046$). The combined Series (1 and 2) also indicate a significant difference between the early and later groups, with frequencies of 8/3620 and 20/4065, respectively ($P = 0.036$).

The dosimetry data for the two series appear to be in good agreement. Moreover, the dose rate was reasonably stable over a long period (last sampling, 354 days posttreatment). The estimated mean dose rate to the whole testis is 1.53 rads per week. Thus, considering that the spermatogonial stem cell received a dose about two to four times higher than that for the testis as a whole, the dose-rate estimate for the spermatogonial stem cell is 3.06-6.12 rads per week. Subtracting 6 weeks (the minimum time for post-stem cell development up to appearance in the ejaculate) from the total exposure time of the germ cells gives the number of weeks the stem cells were exposed. Thus, the dose to the stem cell can be estimated by multiplying this adjusted number of weeks by 3.06 - 6.12 rads.

In estimating the rate of induction per rad of exposure, the spontaneous frequency must be subtracted from the observed frequency. Unfortunately we do noc have sufficient data at the present time for the particular cross [T-stock ♀ × (101 × C3H)F_1 ♂] used in the present study. On the assumption that genotype of females has a negligible effect on the spontaneous occurrence of translocations, we may use for our calculations the spontaneous frequency of 3 translocations in 8095 tested progeny (3.7×10^{-4}) obtained in our laboratory for the cross (SEC × C57BL)F_1 ♀ × (101 × C3H)F_1 or (C3H × 101)F_1 ♂. Using this frequency and the factor 2-4 (see above), and assuming that the poststem-cell exposure was of negligible consequence, we estimate the rate of induction of heritable translocations in spermatogonial stem cells to be $1.45\text{-}2.91 \times 10^{-5}$ per rad per gamete. It is interesting that unlike neutrons, which are much more effective than acute X rays, the alpha particles from ^{239}Pu have an effectiveness that is not markedly different from that we determined for acute X rays (3.89×10^{-5}).

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1. Generoso, W. M., K. T. Cain, N. L. A. Cacheiro, and C. V. Cornett. ^{239}Plutonium-induced heritable translocations in male mice. Mutat. Res., in press.

EFFECT OF X-RAY DOSE REPETITION ON MUTATION FREQUENCY IN MOUSE SPERMATOGONIA

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Male mice exposed at monthly intervals to 4 doses of 500 R of X rays (total dose 2000 R) were bred by W. M. Generoso to measure translocation frequency and then turned over to us for a standard specific-locus mutation test. A total of 13 mutations was obtained in the 3,040 offspring derived from irradiated stem-cell spermatogonia. An estimate of expected mutation frequency from a single dose of 500 R can be obtained by interpolation of our results obtained earlier at 300 and 600 R. Four times this interpolated value gives an expected number of 9.5 mutations in 3,040 offspring. Thus, the observed frequency of 13 mutations indicates that the effect of dose repetition is not less than would be expected on the basis of additivity. A dose regimen of this kind could, therefore, be used whenever high radiation-induced mutation rates are required.

The results also demonstrated an important feature of the design of such experiments. The 13 mutations included 3 clusters: one each of 4, 3 and 2 mutants. Each cluster probably arose from a group of germ cells descending from a single spermatogonial mutation. Thus, the 13 mutations represented only 7 independent mutational events. Such clusters occur when the number of spermatogonial stem cells surviving a high dose of radiation

is small, and when the number of offspring obtained per treated male is relatively large. In this experiment, the number of offspring per male, for the 41 males mated, ranged from 10 to 177, with a mean of 74. The clusters of 2 and 4 both occurred to the same male who had 176 offspring; and a male with 83 offspring had the cluster of 3. Future experiments with high doses should, therefore, be designed to limit the number of offspring obtained per male (increasing the number of treated males if necessary), thereby minimizing the chance of clusters. Clusters demonstrate redundancy in the data and greatly complicate statistical tests of significance.

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, W. L., 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 is the continuation of an experiment in which 20 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 to date are 14 mutations in 40,335 offspring. The induced mutation rate (experimental minus control) is now down to 55% 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 9.5% of that obtained in experiments using a single 100 mg/kg exposure.

ENU Exposure of Preirradiated Males. This experiment, which was completed this year, was designed to test the hypothesis that ENU is most effective in the S phase of cell division. E. F. Oakberg (personal communication) has found evidence that the proportion of stem-cell spermatogonia in S phase is somewhat increased above normal 3 days after an X-ray dose of 150 R, and 5 days after a dose of 300 R. Therefore, we have injected 100 mg/kg of ENU at these intervals after these doses of X rays. With the 150 R X-ray exposure, 13 specific locus mutations were obtained in

3,890 offspring. This is identical with the number of mutations expected on the basis of additivity (using earlier results obtained separately for radiation-induced, ENU-induced, and spontaneous mutation rates). In the 300 R X-ray experiment, with the 5-day interval before ENU treatment, 19 mutations have been scored in 3,613 offspring. Only 13.1 mutations would have been expected on the basis of additivity. Although the data are limited, they provide some support for the hypothesis that ENU is most effective in S phase.

Test of Effect of Age of Males on ENU-Induced Mutation Frequency. This experiment was prompted by earlier results indicating that ENU-induced mutation frequency was lower in males injected at 19 weeks of age than in males aged 12 weeks at injection. In the current experiment, two groups of males, aged 8 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. To date, 41 mutations have been obtained in 18,956 offspring from males treated at the younger age, and 38 mutations in 13,683 offspring from the males that were older at treatment. The mutaton rates are not significantly different at the 5% probability level.

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. To date, no specific-locus mutations have been obtained in offspring conceived during the first six weeks of mating after injection. This includes 1,046 offspring from matings started at the beginning of the third week after injection, thereby increasing the sample size from oocytes exposed in an intermediate stage of maturation. Conceptions occurring later than six weeks after injection produced 11,923 offspring and one mutation. The total mutation frequency of 1 mutation in 17,114 offspring is not significantly greater than the historical control (spontaneous) mutation frequency. The results continue to confirm the conclusion that oocytes are extremely resistant to mutation induction, even to a chemical that is highly effective in spermatogonia. On the basis of results reported earlier, the number of mutations that would be expected among 17,114 offspring if stem-cell spermatogonia were exposed to 50 mg/kg of ENU is 12.7. The one mutation obtained rrom oocyte exposure is significantly below this calculated value ($P = 0.00028$).

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

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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 do so, 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. These studies (which are by far the most extensive, to date, for heritable translocation induction 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 in 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 × C3H)F_1 males, each of which was caged individually with an unexposed (SEC × 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. So far, close to 18,000 male offspring have been produced.

ETHYLENE OXIDE DOSE AND DOSE-RATE EFFECTS IN THE MOUSE DOMINANT-LETHAL TEST

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Ethylene oxide (EtO) is an indispensable agent for sterilizing many types of heat-labile hospital and laboratory equipment. It is during the sterilization operation that most human exposures to EtO takes place. Exposure of workers does not usually occur over many hours of more or less constant low concentrations, but generally from short bursts of relatively high concentrations. Because of lack of data on the relative effectiveness of short-term vs long-term exposures, OSHA decided early in 1985 not to impose any limit for short-term exposure beyond that which is already covered in the long-term exposure limit (1 ppm averaged over an 8 h period). This decision stimulated considerable controversy.

G. A. Sega has determined by use of the labeled chemical that inhaled EtO reaches the testes of mice (see elsewhere in this volume). Our earlier studies have demonstrated that inhaled EtO is effective in inducing both dominant-lethal mutations and heritable translocations in certain male postmeiotic germ-cell stages. In addition, these studies indicated that induced dominant-lethal mutations, which are primarily the result of chromosomal aberrations induced in parental germ cells, can be used as an endpoint in quantitating effects of various EtO exposure conditions. We therefore conducted a study designed to determine the effects of varying dose and dose-rate on the dominant-lethal response in male mice.

For each experiment, 96 (C3H × 101)F_1 males (about 12 weeks old) were randomized into equal experimental and control groups. Control mice were not sham-exposed. In both dose-response and dose-rate experiments, males were exposed daily for four consecutive days. In the dose-response study, males were exposed to either 300, 400, or 500 ppm EtO in air for 6 h each day, for a total of 7,200, 9,600, or 12,000 ppm.h, respectively. In the dose-rate study, males were exposed to a daily total of 1800 ppm.h (overall total, 7200 ppm.h) but at concentrations of either 300 ppm (for 6 h), 600 ppm (for 3 h) or 1200 ppm (for 1.5 h). (SEC × C57BL)F_1 females, 10-12 weeks old, were used in matings to determine dominant-lethal response. In the dose-response study, matings were examined for 12 days after the last exposure in the 500 ppm group in order to determine the germ-cell sensitivity pattern; they were examined for only 8 days in the 400 and 300 ppm groups. In the dose-rate study, matings took place from the fifth to the eighth day following the last exposure. This mating period was chosen because it generally gave the highest level of effect in the earlier experiments.

The dose-response curve based on matings made 4.5 - 7.5 days after the last exposure clearly indicates nonlinearity. The 300 ppm concentration induced only a marginal dominant-lethal response (4%), while clear-cut dose-dependent responses were observed for 400 and 500 ppm concentrations (27% and 61% dominant lethals, respectively).

In the dose-rate study, exposure of mice to 300 ppm for 6 h per day (total of 1800 ppm.h/day) again induced a low level of dominant-lethal mutations (11%). When the same total exposure was given in only 3 h at 600 ppm or in 1.5 h at 1200 ppm, markedly higher dominant-lethal responses were observed (32% and 64%, respectively) indicating a large dose-rate effect. These results mean that, even though the total dose is the same, short-term exposure is more effective than long-term exposure in producing genetic damage.

Because of high cost and low resolving power, carcinogenesis and teratogenesis studies are usually not conducted with the explicit objective of determining the effect of variations in exposure conditions. In mutagenesis studies, a major (order-of-magnitude) effect of dose fractionation has been found on the yield of gene mutations from ethylnitrosourea-exposed spermatogonial stem cells (Russell et al., Proc. Natl. Acad. Sci. USA 79: 3592-3593, 1982). To our knowledge, the EtO experiments here reported provide the first evidence for chemical dose protraction effects on clastogenic damage induced in mammalian germ cells.

This study was conducted in collaboration with Dr. M. D. Shelby of the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

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DIFFERENCE IN THE RESPONSE OF TWO HYBRID STOCKS OF MICE TO X-RAY INDUCTION OF CHROMOSOME ABERRATIONS IN SPERMATOGONIAL STEM CELLS

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Reciprocal translocations are certainly the most extensively studied endpoint of radiation-induced chromosomal damage to mammalian spermatogonial stem cells. In mice, most of these studies involve quantitation of multivalent chromosome association in meiocytes of irradiated males. Meiocytes with reciprocal translocations are expected to give rise to unbalanced and balanced segregants, hence to conceptuses that die early in development or are viable (with either normal chromosomes or the balanced translocation). However, the pathway between irradiation of the chromosomes of spermatogonial stem cells and the transmission and expression of the resulting aberrations is complex and little understood.

Factors that could affect the yield of transmissible aberrations include repair, spermatogonial cloning and repopulation of the seminiferous epithelium, and selective disadvantage during spermatogenesis. Direct studies on the relationship between these factors and transmissible aberrations have been scarce because of the limited opportunity for such studies. At least a partial understanding of the factors involved may come from the results summarized in this report, which show a clear-cut difference between two F_1 hybrid stocks in the yield of lethals arising from reciprocal translocations induced by X rays in spermatogonial stem cells.

The study was conducted in two series (A and B). Experimental males were exposed to four 500-R doses, four weeks apart, delivered at approximately 89 R/min. In series A, the males used were (101 × C3H)F_1 hybrids. They were about 12 weeks old at the time they received the first dose. At intervals 203-212 and 258-265 days after the last dose, irradiated and control males were mated to virgin (SEC × C57Bl)F_1 females, about 12 weeks old. In series B, two types of males, (C3H × 101)F_1 and (SEC × C57BL)F_1, were exposed simultaneously to the same irradiation regimen as that used in Series A. They were mated to virgin (C3H × C57BL)F_1 and (SEC × 101)F_1 females (about 12 weeks old), at intervals 153-159 and 278-289 days, respectively, after the last dose. On day 156 after the last dose, 6 males of each type were killed and their testes processed individually using an air-dry technique. Slides were coded and 25 diakinesis/metaphase-I spermatocytes were scored from each testis.

Series-A experiments were conducted in order to measure the incidence of embryonic mortality resulting from exposure of spermatogonial stem cells of (101 × C3H)F_1 males to 500 R × 4. Clear-cut increases in embryonic lethality in the irradiated group were observed in each of the two mating intervals (25% in each case). Death occurred either shortly before or shortly after implantation.

Series-B experiments were conducted in order to study the relationship between induced embryonic lethality and induced reciprocal translocations scored cytologically in meiocytes of exposed males and to determine if this relationship was the same for the two F_1 hybrids used. Data for both mating intervals clearly show that the embryonic lethalities induced by irradiation of (C3H × 101)F_1 males are similar, qualitatively and quantitatively, to those for the reciprocal hybrid used in Series A. In contrast to the marked effects on the incidence of embryonic lethality observed for these two reciprocal hybrids, only a marginal effect (7% embryonic lethality) was observed when (SEC × C57BL)F_1 males were irradiated. Even in the latter case, however, the frequencies of dead implantations in the experimental groups were significantly higher than those in the control groups ($P < 0.001$ for both mating intervals studied). Significant reductions were also observed in the numbers of living implants per female.

Results of the cytological study parallel those on embryonic lethality. The number of translocations per cell for the (C3H × 101)F_1 is significantly higher than that for (SEC × C57BL)F_1 (0.57 vs 0.33, $P < 0.005$). However, the difference in the frequencies of embryonic lethality may not be totally attributable to processes occurring prior to metaphase I. The difference in the cytological frequencies of reciprocal translocations appears to be smaller than that observed for embryonic lethality. Following the assumptions used by Ford et al. (Cytogenetics 8: 447-470, 1969), we may estimate the incidence of embryonic lethality from the cytological data – i.e., meiocytes with one reciprocal translocation are expected to produce balanced normal, balanced translocated, and unbalanced sperm in a 1:1:2 ratio. Thus, based on the cytological data, expected frequencies of embryonic lethality are 25.1% and 15.4% for (C3H × 101)F_1 and (SEC × C57BL)F_1, respectively. The observed frequency of embryonic lethality for the (C3H × 101)F_1 matches the expected frequency well. On the other hand, the observed frequency for (SEC × C57BL)F_1 (7%) is only about half of that expected ($P < 0.01$ for significant difference).

Thus, the cytological and embryonic-lethality data, together, suggest not only that fewer aberrations were present in the meiocytes of (SEC × C57BL)F_1 males, but that the proportion of unbalanced sperm descending from aberrant meiocytes is lower in (SEC × C57BL)F_1 than in (C3H × 101)F_1 males. The differential transmission of unbalanced chromosome constitutions may be attributed to one of two possible phenomena: selection during the postmeiotic stage against translation-carrying gametes (Ford et al., Cytogenetics 8: 447-470, 1969), or metaphase-I delay (Crocker, Mutat. Res. 103: 339-343, 1982). It should be noted, however, that accurate quantitation of the frequency of embryonic mortality expected on the basis of spermatocyte cytological data, as well as accurate comparison of the expected with the observed frequency of embryonic lethality, require many more data than those available for the present report. The relationship between the cytological frequency of reciprocal translocations in exposed males and embryonic lethality from the resulting unbalanced segregants is under further investigation in these two types of males. Prior to the present finding, evidence for the influence of genotype in radiation induction of chromosomal aberrations in spermatogonial stem cells has been equivocal.

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DNA REFAIR STUDIES IN MAMMALIAN GERM CELLS

G. A. Sega M. Ellahuene[1]
W. M. Generoso

1,2-Dibromo-3-chloropropane (DBCP), a potent nematocide used extensively in agriculture, has been linked to an increased incidence of male sterility among workers in processing plants. The chemical appears to be metabolized more rapidly in the rat than in the mouse. In the mouse, DBCP does not cause germ-cell cytotoxic effects (Oakberg and Cummings, Environ. Mutagen. 6: 621-625, 1984), dominant lethals (Generoso et al., Mutat. Res. 156: 103-108, 1985), or specific-locus mutations (Russell et al., Mutat. Res., submitted). In the rat, on the other hand, dominant lethals are induced by DBCP in postmeiotic stages, especially in early spermatid stages.

To provide more information on possible species differences, DBCP was studied for its ability to induce unscheduled DNA synthesis (UDS) in the germ cells of male mice and rats. Two exposure levels of the chemical were given to each of eight different strains of mice and to SD rats. The DBCP was dissolved in corn oil and administered to the animals by i.p. injection. A testicular injection of [^{3}H]dThd was given either at the same time as the DBCP injection or at various times after injection of the chemical. Spermatozoa were recovered from the caudal epididymides 16 days after exposure of the mice, and both 24 and 31 days posttreatment in the rats. The timing of sperm recovery was such that postmeiotic germ-cell stages were being sampled in both species.

With all of the mouse strains tested, there was either no UDS response or, at best, a marginal response. (A positive response would have to be at least 2-3 times the control values.) In the rat studies there was a greater UDS response of the germ cells than was observed with the mice. The UDS response of the rat germ cells appeared to be dose-related. It was also observed that it took 2 h after the DBCP treatment before the UDS response of the rat germ cells was at a maximum, suggesting that it takes some time for the DBCP and/or its metabolites to produce DNA damage in the germ cells.

Our negative to marginally positive results with DBCP in the mouse suggest that this chemical interacts relatively less with the germ-cell DNA of the mouse than of the rat. Such observations are important in learning how best to evaluate the potential hazard to human germ cells from chemicals in the environment.

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THE SPERMATOGONIAL STEM CELL POPULATION OF THE MOUSE FROM BIRTH TO OLD AGE

E. F. Oakberg C. C. Cummings

We have previously demonstrated that gonocytes of male mice initiate DNA synthesis on the day of birth, and that cells with morphology characteristic of the A_s spermatogonia appear at 4 days of age. Stem cells in juvenile animals divide more rapidly than do those of the adult, with cell-cycle properties characteristic of the adult appearing by 21 days of age (Oakberg, Proc. Vth Workshop on Development and Function of the Reproductive Organs, July 6-9, 1981, Excerpta Medica pp. 149-152).

In our study of young animals, we had, however, not used ^{3}H-TdR labeling followed by radiation, as we had in the adult. Accordingly, mice aged 1, 5, 11, 15, 19, 21, or 36 days were given 1 μCi ^{3}H-TdR/gm body weight. Three groups of mice were used at each age. One group was killed 1 h after ^{3}H-TdR. Groups 2 and 3 were given 300 R 24 h after ^{3}H-TdR, and killed either 5 days or 207 h after irradiation. Autoradiographs were prepared of paraffin sections of the testis and are being scored to determine the frequency and radiation resistance of the long-cycling spermatogonial stem cells in young mice. Collection of data is almost complete, but results have not yet been summarized.

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DIVISION OF SPERMATOGONIAL STEM CELLS AFTER BOTH SINGLE AND FRACTIONATED RADIATION EXPOSURE

E. F. Oakberg C. C. Cummings

Experiments are being conducted to attempt to answer the following questions: (1) What is the effect of radiation on the mitotic activity of spermatogonial stem cells surviving either a single exposure to 300 R of X rays or a 600 R exposure given in two equal fractions 24 h apart? (2) Is there evidence for populations of different radiation sensitivity other than those attributable to stage of the mitotic cycle of the radiation-resistant survivors?

Hybrid male (101 × C3Hf)F_1 mice, 12 weeks old, were randomized into 4 groups. Group 1 received 25 μCi ^{3}H-TdR intraperitoneally and was killed 1 h later to assess number of stem cells in DNA synthesis. Group 2 was given 300 R, followed by 25 μCi ^{3}H-TdR 23, 41, 47, 71, 95, 119, 167, and

215 h later, and killed 1 h after ^{3}H-TdR injection. Group 3 was exposed to 300 R, given 25 μCi ^{3}H-TdR 24, 42, 48, 72, 96, 120, 168, and 216 h later, then exposed to a second 300 R dose 24 h after ^{3}H-TdR injection. Mice in this group were killed either 120 or 207 h after the second irradiation. Group 4 was given 25 μCi ^{3}H-TdR, exposed to 300 R 24 h later, and killed either 120 or 207 h after the X-ray exposure.

One testis was used to prepare tubule whole mounts; the contralateral testis was used to prepare autoradiographs. The whole mounts were used to assess cell survival, to identify the cell types in division for the different treatments, and to determine the mitotic activity of surviving stem cells. Autoradiographs were scored to determine the frequency of labeled cells at the different times and treatments in order to assess the radiation sensitivity of the dividing cell populations.

Scoring of slides is almost completed.

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NO EVIDENCE FOUND FOR INDUCTION OF DOMINANT SKELETAL MUTATIONS BY PLUTONIUM

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

Earlier experiments of W. L. Russell showed that plutonium induces specific-locus mutations in stem-cell spermatogonia. The frequency observed was much lower than expected, but several lines of evidence suggested that the mutations might be qualitatively more severe than those induced by low-LET radiation. There was concern that the amount of induced overall dominant damage might be considerably higher than that predicted from the specific-locus mutation frequency.

In order to provide evidence that could be used in making a direct estimate of genetic risk from plutonium, a skeletal non-breeding-test (NBT) experiment was carried out. Male mice of the inbred strain 102 (formerly strain 101) were injected intravenously with 10 μCi of monomeric ^{239}Pu-citrate per kg body weight, the mean gametic exposure being 58 rads. According to usual procedures, these males were mated with females of the C3H inbred strain, and the skeletons of offspring conceived 9 weeks or more after injection were examined. After correcting for preexisting spontaneous mutations, the frequencies of presumed dominant skeletal mutations are 0.0018 mutation/gamete (6/3353) in the experimental group and

0.0020 mutation/gamete (4/1987) in the concurrent control. The lack of evidence for induction of dominant skeletal mutations in a sample of this size is in line with the specific-locus mutation frequency found earlier. It was expected that induction of dominant skeletal mutations could be demonstrated only if the level of dominant damage was much higher than that predicted from the specific-locus mutation frequency. From the level of risk that can be ruled out from the observed frequency of dominant skeletal mutations, it appears that the usual quality factor of 20 that is applied for alpha-particle effects probably overestimates genetic risk by at least a factor of 2 and possibly by much more.

Three preexisting mutations were found in this experiment. Two with low penetrance occurred repeatedly in both the experimental and control groups, and a third, with complete penetrance, occurred exclusively in one litter in the control. None of these mutations was present in our earlier work, using the same strains, in Germany. We are presently making a detailed computer-assisted study of the pedigrees of both inbred strains in the hopes of learning which strain carried each preexisting mutation. The skeletons of most of the inbred parents of the mice with preexisting mutations will also be studied in our effort to solve this puzzle. This analysis could lead to a much greater understanding of the reasons for surprisingly high levels of heterogeneity sometimes found in inbred strains. Future skeletal experiments will incorporate a modification that will make it much simpler to distinguish preexisting mutations from new ones, either spontaneous or induced.

FURTHER STUDY OF THE SYNERGISTIC INTERACTIONS OF TWO RADIATION-INDUCED DOMINANT SKELETAL MUTATIONS

P. B. Selby
T. W. McKinley, Jr.
S. L. Niemann[3]
G. D. Raymer

Last year we reported several interesting interactions between two radiation-induced dominant skeletal mutations that are provisionally identified as Ccd (Cleidocranial dysplasia) and Dsh (Short digits). These mutations have now been made congenic on the C57BL/10 genetic background so that the interactions can be studied in the absence of other types of genetic variability. The syndromes of both mutations on the C57BL/10 background are quite similar to those on the original mixed genetic background, with the exception that the mice with the Ccd mutation have a large gap in the orbit of the eye on the C57BL/10 background. We have never seen such an effect in any other mice.

Double heterozygotes were constructed to determine whether these mutations might together cause malformations that neither one can cause alone. We have earlier shown that the mutations are homozygous lethal and unlinked. Each causes several skeletal anomalies in heterozygotes. Penetrance is complete for some anomalies and incomplete for others.

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. Double heterozygotes were found to have a few interactions that were far more extensive than expected on the hypothesis of additivity of effects. Thus, the size of the hole between the frontals far exceeds that expected on the basis of additivity. Whereas the interparietal bone is of the same length in Dsh mice as in non-mutant mice, animals, with mutation Ccd have this bone reduced to 74% normal length. In the double heterozygote, this bone is only 77% as long as it is in mice with mutation Ccd alone. These differences are statistically significant. Interactions are not always in the direction of enhancement. E.g., mutation Ccd entirely suppresses the ability of Dsh to cause large boney plates on many of the ventral ribs.

Although the earlier study on mixed genetic background gave no suggestion that double heterozygotes have decreased survival, only 9 double heterozygotes were found on the C57BL/10 background when 21 were expected. This represents a statistically significant reduction in survival to young adulthood and yet another synergistic interaction.

Synergistic interactions of unlinked dominant mutations may be the cause of some human disorders that are presently thought to have autosomal recessive inheritance. If such interactions turn out to be common, they will have to be taken into account in genetic risk estimation for chemicals and radiation and in human genetic counseling.

FIRST-GENERATION LITTER-SIZE REDUCTION FOLLOWING IRRADIATION OF SPERMATOGONIAL STEM CELLS IN MICE

P. B. Selby W. L. Russell[6]

As part of our ongoing effort to measure organismic effects of heritable genetic alterations, we completed a computer analysis of the litter-size-reduction (LSR) results from 14 earlier specific-locus experiments involving 158,490 F_1 litters. Litter sizes at about three weeks after birth were compared between experimental and control groups. In order to reduce variability, comparisons were made only with concurrent controls and only between groups of litters having mothers of approximately the same age. From these results we reached the following conclusions.

The results at the dose rate of 90 R/min show a humped dose-response curve, with the LSR being significantly lower after 1000 R than it is after 300 R or 600 R. The response per R is also significantly lower at 600 R than it is at 300 R. There is a pronounced dose-rate effect, the LSR being much less at dose rates of 0.009 and 0.001 R/min than of 90 R/min. The dose-rate reduction factor for LSR is about 6.7. The weighted average of the LSR per R, based on the 9 low-dose-rate experiments, was 0.00194% ± 0.00076% (SE).

These data fill a gap in our knowledge about the amount of radiation-induced dominant damage that results in death between conception and weaning age in mice. Application of the results to human beings suggests that if men were exposed to 1 R of low-LET low-dose-rate radiation, the total number of deaths between conception and late childhood caused by induced dominant mutations would be about 19 out of every million individuals that would have reached that age in the absence of irradiation. These data, together with other evidence, suggest that many of these 19 deaths probably occur long before birth and, in fact, before pregnancy is recognized in human beings. To give a total estimate of induced dominant damage, the total number of induced deaths can be added to the earlier estimate (based on dominant skeletal mutations) of an approximately equal number of viable disorders in all body systems.

The data show that very little, if any, of the observed litter-size reduction is caused by reduced sperm count. Thus, it seems clear that it results from induced dominant lethality from all causes, including segmental aneuploidy (i.e., unbalanced segregants from reciprocal translocations), gene mutations, small deficiencies, monosomy, trisomy, and so on.

The data permit the following additional conclusions. (1) Translocations are the major cause of LSR after large acute exposures. (2) Under chronic irradiation conditions, roughly one-half of the LSR results from translocations. (3) Only an extremely small proportion of serious radiation-induced genetic disorders among live-born humans would be expected to result from segmental aneuploidy. (4) Death between birth and the age of skeletal examination does not lead to a serious underestimation of genetic risk when it is based on skeletal results obtained from examination of young adult mice.

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A CHEMICALLY INDUCED BALANCED RECIPROCAL TRANSLOCATION IN MICE WITH NEUROLOGICAL DEFECT

W. M. Generoso
N. L. A. Cacheiro[1]
K. T. Cain
C. V. Cornett

It has generally been believed that most transmitted balanced, reciprocal translocations cause no ill-effects in heterozygous carriers, except for reduced chance to reproduce (death of aneusomic offspring). In mouse and man, however, there is an increasing number of examples of apparently balanced reciprocal translocations with adverse physical effects on the carrier. In the course of a mutagenesis experiment, we found, among offspring of triethylenemelamine-treated males, a semi-sterile male translocation heterozygote which exhibited obvious neurological symptoms and an inability to swim in a normal fashion. Both the phenotype and the semisterility were found to be transmitted, and a well-producing stock has been established, permitting extensive genetic and pathological studies.

The most striking part of the phenotype, which is already evident on day 1 and well expressed by day 6, is the inability to swim. The mutant swims in random tight circles, often on its side, and eventually dives, continuing the circular swimming underwater. The mutation has therefore been named diver. There is no worsening or improvement in the condition with age, and very little heterogeneity among affected animals. Four generations of backcrosses to the SEC strain have not changed the behavior. Divers do not exhibit seizures, tremors, abnormal postures, compromised coordination, difficulty with fine movements, or visual or auditory defects. Pain and position sensation appear intact.

Although adult diver mice are clearly smaller than their normal littermates, no dysmorphic features were seen on external examination, and the gross and histologic features of the internal organs were unremarkable. Extensive anatomical studies at the gross, histological, and transmission- and scanning-electron-microscopical levels failed to detect any abnormalities of the brain, muscle, spinal cord, otoconia and other inner-ear structures of diver mice at all ages. Furthermore, no neuropathological defects were noted in large numbers of 12-18 day p.c. fetuses from matings expected to produce heterozygotes and homozygotes.

A total of 621 male and female progeny from reciprocal matings between diver and normal animals were included in the analysis of association between the translocation and the neurological symptoms. Of these, 144 males and 142 females were divers and 174 males and 161 females were normal. All male diver progeny were fertility tested: 134 were semisterile and 10 were sterile. On the other hand, 170 of the 174 normal males had normal fertility and four were sterile. Thus, there is complete association between semisterility and the abnormal behavior. Crosses between diver animals produced 75 divers and 54 normal offspring of which 11 and 15, respectively, were fertility tested. Again there was complete association between the diver phenotype and semisterility. Homozygotes appear to die prenatally. On the average, semisterile diver males produced

57.6% (± 5.8) dead implants (unbalanced segregants) when mated to at least 3 (C3H × C57BL)F_1 females each, while normal males produced 3.75% dead implants (± 3.3). The chromosomes involved in this reciprocal exchange have been identified as 2 and 14.

As far as is known, diver is the only neurological mutant in the mouse that resulted from a reciprocal nonhomologous chromosome exchange. The absence of anatomic defects either in the ear or in the central nervous system suggests that the translocation-associated defect may have a biochemical basis. One possible mechanism is that the induction of a rearrangement by the chemical mutagen may concommitantly have damaged genetic material at the rearrangement points and that this particular genetic material is essential for normal neurological functioning. Alternatively, the DNA may still be intact but producing an abnormal product as a result of its changed position.

A more practical aspect of the findings is that the translocation-associated neurological mutant will be useful as an animal model for similar neurological conditions in human beings. The findings also raise the question whether certain human genetic disorders that have been assumed to be the result of single-gene mutations may not, in fact, be associated with rearrangements. Since certain chemical and physical agents can induce translocations with higher frequencies than they do gene mutations, the organismic effects of translocations may cause a reassessment of risk estimation.

This study was conducted in collaboration with Drs. J. C. Rutledge and G. Wright of the University of Texas, Medical Branch, Dallas, Texas.

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CHEMICAL DOSIMETRY STUDIES IN MAMMALIAN GERM CELLS

G. A. Sega P. A. Brimer

Chemical dosimetry experiments using radioactively labeled chemical mutagens have provided an excellent method for learning more about the molecular events going on within germ cells after mutagen treatment. The procedure is extremely sensitive and can be used to measure binding of chemical agents to germ cells and germ-cell DNA at exposure levels that are orders of magnitude lower than those necessary to produce a statistically significant genetic effect. As our chemical dosimetry data are combined with genetic and cytogenetic data for the same chemicals, much will be learned about the relationship between the extent of chemical damage in the

germ cells and the amount of genetic damage expected at realistic human exposure levels. Since human beings are exposed to many energy-related compounds by inhalation, we are also using this route of exposure in some of our chemical dosimetry studies.

Experiments using tritium-labeled ethylene oxide ([^{3}H]EtO) are currently being conducted in order to look for particular DNA lesions in the mouse testis. The lesions being studied are N7- and O^6-hydroxyethylguanine. (101 × C3H)F_1 males were given i.p. injections of [^{3}H]EtO of 16 or 160 mg/kg, and testicular DNA was recovered from the treated animals from 90 min. to 6 days after the exposures. The results showed that while there was a 10-fold difference between the two EtO exposure levels, the total alkylation of testicular DNA was from 20 to 40 times greater at the higher EtO exposure. In a similar manner, the amount of N7-hydroxyethylguanine formed in the testis increased by 40- to 50-fold in going from the low to the high EtO exposure. The amount of O^6-hydroxyethylguanine formed in the testis appears to be quite low, and it was not possible accurately to measure this lesion in the testis DNA. The non-linear increase in the number of testis DNA lesions produced with increasing EtO exposure suggests that there may be saturation of other nucleophilic sites within the animal as the exposure is increased, or else that repair mechanisms designed to remove DNA alkylation damage are impaired as the EtO exposure is increased.

A semidynamic inhalation chamber has been used to expose mice to [^{3}H]EtO gas. In each experiment, 12 males were placed in the inhalation chamber and exposed for 1 h to the [^{3}H]EtO. Exposures have ranged from 3 to 188 ppm.h. There appears to be a generally linear increase in alkylation of testis DNA with the EtO inhalation exposures up to about 80 ppm.h. However, the amount of alkylation of testis DNA after exposure to 188 ppm.h of EtO is considerably lower than predicted from a linear extrapolation of the lower exposure data. The reason for the discrepancy between the results of the i.p. injections and the inhalation exposures to EtO is not presently known.

Besides looking at chemical binding to the total DNA recovered from the testis we are very much interested in looking at chemical binding levels in specific germ-cell stages within the testis. We are continuing to improve upon elutriation techniques to isolate late spermatids, early spermatids, and pachytene spermatocytes from the mouse testis, and will shortly begin to look at binding of [^{3}H]EtO in these separate germ-cell stages.

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MECHANISMS FOR INDUCTION OF CHROMOSOME ABERRATIONS IN MALE GERM CELLS

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, and chromosome 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 alkylguanine 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.

In an attempt to provide additional evidence bearing on this hypothesis, we compared the progenies of males mated 6.5 to 9.5 days after MMS treatment (early spermatozoa to late spermatids sampled) with those of males mated 16-18 h posttreatment (mature sperm sampled) with respect to relative incidence of dominant-lethal mutations and heritable translocations. We assumed that there should be marked differences between these two groups in the number of hydrolysis-generated apurinic sites present at the time of mating and pronuclear DNA synthesis. Thus, if the hypothesis is correct, the groups should differ in the relative frequencies of dominant-lethal mutations and heritable translocations because of the degree of transformation of premutational lesions into some intermediate form.

The induction of heritable translocations at the two mating intervals was compared using MMS doses that produce about 50% dominant-lethal mutations, namely, 40 mg/kg for late spermatid/early-spermatozoa stages, and 120 mg/kg for mature sperm. The frequencies of heritable translocations at the two mating intervals are 15.2% (70/460) and 10.2% (26/254), respectively. These results indicate a higher rate of induction of heritable translocations relative to dominant-lethal mutations in the late spermatid/early-spermatozoa stages than in the mature sperm, which is consistent with our hypothesis. However, the difference between the two intervals is smaller than we anticipated. Since the short mating interval is 16-18 h long, it is possible that substantial numbers of apurinic sites had already been produced during this period and the period prior to sperm entry. We are currently conducting a similar comparative study in which progeny of males mated 6.5-9.5 days after MMS treatment will be compared to those of males mated only about two hours posttreatment.

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CONTRIBUTIONS FROM QUALITATIVE ANALYSES OF SPECIFIC-LOCUS MUTATIONS TO THE SCOPE OF MUTAGENESIS DATA

L. B. Russell C. S. Montgomery

Molecular corroboration is now available for what had been surmised from genetic analyses, namely, that many radiation-induced mutations are deletions of genetic material (see preceding abstract for d-se region; and 1984 Progress Report, ORNL-6119, p. 93, for p region). In genetic tests,

we have shown that combinations of certain lethal deletions that overlap at c (albino locus) give complementation for lethality but not for the visible effect, albinism (Russell, Genetics 100: 427-453, 1982). We therefore conclude that c is a non-vital locus, i.e., its total absence from both chromosomes does not affect viability. The same conclusion may be drawn for se (Russell, Mutat. Res. 11: 107-123, 1971). Thus, any c- or se-locus mutation that affects viability in homozygous condition, or any d mutation that is homozygous lethal (rather than opisthotonic) may now be concluded — without further testing — to be a deletion. Such diagnoses enlarge the qualitative scope of specific-locus test data; previously, only d-se "double" mutants could be assumed to be deletions.

The inverse assumption, namely that viable mutations that have null expression for these markers are intralocus lesions, is not legitimate because it is at least theoretically possible that other non-vital genes with, so far, undetected phenotypes may flank the loci in question. On the other hand, if such viable nulls are multi-locus deletions, they are very probably shorter than those that are homozygous lethal. They could also be single-locus deletions or even intragenic changes of various sorts. A third class of mutations, the so-called "intermediate alleles" at c, se, and d, may be assumed to be intragenic lesions on the basis of the evidence (see above) that overlapping deletions produce the null phenotype.

Mutations involving c, d, and/or se, derived in recent experiments with ethylnitrosourea and in older experiments with various radiations, were analyzed for phenotype and viability and then grouped on the basis of the above criteria into (a) multilocus deletions, (b) small (possibly single-locus) deletions, and (c) intragenic lesions. The number of independent radiation-induced mutations analyzed was 174 and 90 for the d-se and c regions, respectively, for the total of all exposed germ-cell stages. All ENU mutations analyzed to date had been induced in spermatogonial stemcells; 67 and 28 were available for the d-se and c regions, respectively. There were 28 and 17 control mutations.

Multi-locus deletions were found to constitute the majority of mutations when postspermatogonial stages or oocytes had been irradiated. Their frequency was higher after neutron than after low-LET irradiations, regardless of what germ-cell stage was exposed. Spontaneous mutations had only a very low incidence of multi-locus deletions (4-6%) and a relatively high incidence of intra-locus mutations. Of all irradiated groups, low-LET-exposed spermatogonia yielded distributions most closely approaching those in controls; however, even these distributions were shifted in the direction of the larger lesions. By contrast, spermatogonia exposed to ENU yield distributions that are not distinguishable from controls. If anything, the spectrum is shifted in the direction of smaller lesions. Among the 95 mutations, none was clearly identifiable as a multi-locus deletion, and 26.3% were intragenic lesions.

Among the several conclusions that may be based on the qualitative analysis, an important one is that radiation-induced mutations differ

qualitatively from spontaneous ones, throwing some doubt on the doubling-dose approach for risk estimation. The results also make it clear that the spectrum of types of mutations detected by the specific-locus test varies strongly with the mutagen and with germ-cell stage exposed. The qualitative analysis allows conclusions about relative potential harmfulness of different types of exposures that may yield equal mutation rates. It also provides genetic material that is leading to molecular characterization of the normal markers and of mutations involving them (see preceding abstract). Once that is accomplished, the high efficiency of the SLT for mutation detection will be combinable with detailed qualitative analysis.

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GENETIC AND MOLECULAR ANALYSIS OF THE DILUTE-SHORT-EAR (*d-se*) REGION OF THE MOUSE

L. B. Russell C. S. Montgomery

Among the markers used in standard specific-locus experiments, two are very closely linked: dilute (*d*) and short-ear (*se*), 0.16% recombination. Mutations to *d*, *se*, or *d-se* have fallen into several distinct classes on the basis of their phenotype and survival in homozygotes. We have earlier demonstrated the genetic complexity of the *d-se* region by extensive complementation analyses involving over 800 pairwise combinations of individual mutations (Russell, L. B., Mutat. Res. 11:107-123, 1971). A recent extension of this genetic analysis has made it possible to postulate 10 functional units (including 5 prenatal-lethal factors) and to subdivide the mutations into 26 complementation groups, most of which fit a linear arrangement.

Molecular analysis of the *d-se* region was initiated following the discovery that the original *d* mutation carried in common inbred strains of mice is associated with the site of integration of an ecotropic murine leukemia provirus, *Emv-3* (Jenkins *et al.*, Nature 293: 370-374, 1981). In collaborative studies with Drs. E. M. Rinchik, N. G. Copeland, and N. A. Jenkins (working in the Department of Microbiology and Molecular Genetics of the University of Cincinnati College of Medicine), we have isolated a series of unique-sequence cellular DNA probes from regions both 5' and 3' to the proviral integration site within the *d* locus. These probes have been used for deletion-mapping experiments with a large number of radiation-induced *d-se* mutations.

All d-se mutants (involving both marker loci) and many, but not all, d^{pl} mutants (prenatally lethal dilutes) were found to delete sequences homologous to a hybridization probe, p0.3, that is located approximately 3 kb distal to the 3' LTR of the Emv-3 provirus. These and other d-se region mutations have been assumed to be deletions since they were defective in more than one functional unit (Russell, Mutat. Res. 11: 107-123, 1971). The new molecular data now substantiate this hypothesis.

One of the d^{pl} deletion mutants, Aa2, yielded a restriction fragment that hybridized to the p0.3 probe but was much shorter than normal. Cloning of this deletion breakpoint fusion fragment and subsequent analysis confirmed the hypothesis that the deletion extends in the 5' direction past the Emv-3 integration site. A probe, p94.1, was derived from the 5' end of the cloned deletion breakpoint fusion fragment, and two additional probes were derived by different pathways. The p0.3 probe was instrumental in orienting the physical map of the d-se region with respect to the genetic complementation map. The two maps are, in general, concordant. Both p0.3 and p94.1 were able to distinguish among members of a single complementation group, as well as among members of closely related complementation groups. Additional cloned probes, which more precisely define the extent of the deletions, will ultimately allow the determination of the smallest segment of DNA that must be deleted to produce a specific mutant phenotype.

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T(X;7)18Rl, A NEW X-7 TRANSLOCATION, SHEDS LIGHT ON THE SPREAD OF X INACTIVATION

L. B. Russell J. W. Bangham

Ever since they were first discovered (Russell and Bangham, Genetics 44: 532, 1959), mouse X-autosome translocations [T(X;A)s] have provided highly useful material for interpretations of X inactivation and is spread to autosomal genes (Russell, Progress and Topics in Cytogenetics 3A: 205-250, 1983). In the course of a quarter of a century, only 17 mouse T(X;A)s have been reported. The noncontiguous X, divided in different places by different T(X;A)s, can be utilized to test hypotheses concerning X inactivation by examining activity in the X and autosomal segments. Particularly useful for comparative purposes are series of T(X;A)s that involve the same autosome but with different breakpoints. Of the 15

T(X;A)s recently reviewed (Russell, Progress and Topics in Cytogenetics 3A: 205-250, 1983), six were T(X;7)s. We have now discovered and investigated a seventh such translocation, T(X;7)18R1 (abbreviated R18), which is of particular interest because one autosomal segment appears not be subject to inactivation.

R18 was found in a specific-locus experiment, among offspring derived from an ENU-exposed spermatogonial stem cell. Since, however, T(X;A)s in general (including R18) are known to be unable to pass through male meiotic divisions, it is likely that the translocation arose spontaneously in a postmeiotic, or even early post-fertilization stage. An R18 stock has been established, but is yielding genetic information only slowly, since R18 males are sterile, and the lifetime production of R18 daughters per R18 female is only 1.7.

R18 resembles other T(X;A)s in the following general features: some autosomal loci 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 normal in all respects, except for their total sterility.

The Chromosome-7 breakpoint of R18 has been shown, genetically, to lie between c (albinism) and p (pink-eyed dilution) and very close to the former. While the autosomal p-locus is clearly subject to X inactivation, the c-locus is not. In this respect, R18 is like R2, which has similar, though not identical breakpoints. The c locus is under the influence of X inactivation in all other T(X;7)'s. For T(X;A)s in general, all informative genes that have been investigated in the autosomal and/or X-chromosomal portion of the longer translocation product are subject to inactivation. This is true even of genes as far as 25 cM from the breakpoint (Russell, Progress and Topics in Cytogenetics 3A: 205-250, 1983). For the reciprocal translocation products, on the other hand, no critical information has been available, except in the case of R2, where at least three autosomal loci have been shown not to be subject to inactivation. R18 now provides another case. This would indicate that the proximal portion of the X, from centromere to R18 breakpoint, is unable to inactivate contiguous autosomal material, and that this segment may itself not be subject to X inactivation when separated from the site of control of primary X inactivation.

SUPPORT FOR NONHOMOLOGY OF X-Y CHROMOSOME SYNAPSIS FROM SYNAPTONEMAL COMPLEX ANALYSIS OF MALES CARRYING AN X-CHROMOSOME INVERSION

T. Ashley[1] C. S. Montgomery

Although it has long been supposed that synapsis between the distal portions of the mammalian X and Y chromosomes is homologous, this view has recently been challenged (Ashley, T., Hum. Genet. 67: 372-377, 1984) based

on the following findings: (a) the synapsed region is longer than would be expected from the correlation between genetic and cytological maps, and (b) molecular data indicate that several DNA sequences common to the X and Y do not lie within the pairing region, while some sequences unique to the X or Y do.

This year, we have obtained evidence from an extensive sex-chromosome rearrangement that supports the hypothesis of non-homology of X-Y synapsis. The rearrangement is In(X)1H, a very long paracentric inversion that involves at least 85% of the X chromosome, with breaks in A1 and F3 or F4 (Evans and Phillips, Nature 256: 40-41, 1975). Synaptonemal complexes from pachytene spermatocytes of male translocation heterozygotes were microspread, silver stained, and studied in electronmicrographs.

If synapsis were homologous, it should lead to one of the following configurations: (a) pairing involving only the most distal 5% of the X, i.e., progressing no farther than the distal X breakpoint; or (b) pairing of the Y with the long inverted interstitial segment of the X, an arrangement that would leave centromeres facing in opposite directions and all four end segments unpaired. The latter configuration was not found; this and additional reasons make it highly unlikely that homologous synapsis of the long inverted region occurs. In almost all of the nuclei scored (96%) synapsis involved more than 5% of the X; in half of these nuclei, in fact, it involved over 20%. This means that a large portion of the inverted X segment must be paired as if it were noninverted.

In the autosomes, nonhomologous synapsis can be observed when certain rearrangements are present, but only subsequent to a stage of homologous synapsis (early pachytene). The finding that, in the case of normal X and Y chromosomes, maximum synapsis occurs in early pachytene was previously taken as an argument for homology of the X-Y synapsis. Because of this, we sampled pachytene nuclei also from juvenile inversion males, aged 13 to 14 days, in which most spermatocytes are in early pachytene. The configurations found were similar to those in adult males — >50% of the nuclei exhibited X-Y synapsis involving >20% of the X, i.e., nonhomologous synapsis. The finding that nonhomologous synapsis of the X and Y in these inversion males occurs prior to nonhomologous synapsis of the autosomes is consistent with our hypothesis that early-pachytene synapsis of the normal X and Y is also nonhomologous.

Toxicology Section

SECTION OVERVIEW - H. R. WITSCHI

Complex mixtures are an area of concern to DOE. Exposure potential to complex mixtures is practically limitless. Yet for all practical purposes only limited information is available on how to predict, on a rationale basis, the toxicology of complex mixtures.

Some work done in the Toxicology Section during the past year has begun to develop a generic approach on how to evaluate and to predict untoward health effects produced by acute or chronic exposure to complex mixtures. In principle, exposure may occur via three major routes: the skin surface, the respiratory tract and the gastrointestinal tract. It is conventional to consider the stratum corneum, the outermost layer of the skin, to be a passive and inert barrier through which chemicals pass essentially through a diffusional process. This view has by now been proven wrong. We developed new methods which allow us to measure in both a qualitative and a quantitative way how skin is involved in the metabolism of foreign compounds. This made available some novel information on how different mammalian species, including subhuman primates and man, handle exogenous and endogenous biologically active compounds.

Once the metabolic capabilities of skin were recognized it was only a small step to ask the question whether such events might play a role in skin carcinogenesis. It was found that a known and potent skin carcinogen, benzo(a)pyrene, forms adduct with the DNA of skin cells. Adduct formation is thought to be a critical step in carcinogenesis. The newly developed techniques of measuring skin DNA adducts now enable us to quantitate the "biologically-effective" dose for the carcinogenic action of benzo(a)pyrene in the skin. The method has the potential to measure actual levels of exposure of the population at large to skin carcinogens.

Complex mixtures may, by themselves, influence both skin carcinogenesis and the transport of foreign compounds across the skin. Preliminary data have been acquired which seem to indicate that a highly carcinogenic coal liquefaction product is not quite as effective in allowing an inert paraffin to cross the skin barrier than is a more innocuous and not carcinogenic coal derivative. This points to the possibility, to explore in future toxicokinetic studies, that the ultimate toxicity of selected marker compounds may be modulated and influenced by their carrier media.

The capability to measure adducts of benzo(a)pyrene with macromolecules begins to be exploited in molecular dosimetry. Benzo(a)pyrene metabolites bind covalently to hemoglobin. Future studies will work out quantitative relationships between adduct formation and intake of

benzo(a)pyrene into the body, thus giving us the tool to arrive at realistic and quantitative estimates of exposure.

A second possible portal of entry is the respiratory tract. It cannot be expected, given the physicochemical properties of most aerosols originating from complex mixtures, that all inhaled material will penetrate across the air-blood barrier. Rather it must be expected that complex mixtures may produce pulmonary diseases such as fibrosis or neoplasia.

Progression of neoplasia in the respiratory tract has been characterized in studies with the flow-through tracheal implant (FTTI) model. A very early marker of carcinogen-induced alterations in tracheal cells is a lack of need for pyruvates. A biochemical explanation for this observation has been found: carcinogen-altered cells and tumor cells have higher levels of particulate-bound NADP-dependent malic enzyme activity and are capable of catalyzing pyruvate from malate. The FTTI model has also been used successfully to study interactions between benzo(a)pyrene and formaldehyde. The validity of the model for mechanistic studies and to characterize the pathobiology of interactions which are commonly thought to be associated with exposure to complex mixtures has thus been documented.

The response of the alveolar zone to chemical insult has been examined with an analysis of cell turnover in normal and damaged lungs. It was once thought that acute lung injury was followed by a sterotyped pattern of recovery, involving first epithelial cell proliferation and later endothelial and interstitial cell growth. A series of studies done with toxic inhalants $CdCl_2$ or $BeSO_4$ or with a systemic lung toxic agent such as bleomycin, cyclophosphamide or busulfan have shown that patterns of recovery appear to be more specific. Agents which produce acute pulmonary lesions usually produce a marked and early epithelial response. Chemicals known to produce a more slowly and developing lesion elicit a rather delayed epithelial proliferation. An interesting note was added in a study in which we examined the recovery from acute oxygen toxicity in mice, rats, hamsters and marmosets. Patterns of cell proliferation suggest that mouse and marmoset most resemble man in recovery from acute oxygen toxicity. This reopens the question as to which species is the most appropriate for studies in inhalation toxicology to produce data relevant to man.

During these studies it was also discovered that oxygen prevented the development of chemically-induced lung tumors in mice and in rats. Whether this observation can be exploited in the treatment of cancer metastasis to the lung is presently under investigation.

The last portal of entry for complex mixtures, the gastrointestinal tract, has received less attention than the other sites. We found that the common food additive, the phenolic antioxidant butylated hydroxytoluene, enhances the development of chemically-induced tumors in the gastrointestinal tract of mice and rats, provided exposure to the antioxidant begins after exposure to the carcinogen has been completed.

These are some of the highlights of work accomplished in the Toxicology Section during the last year. Many more observations have been made and several questions related to the pathobiology of both individual compounds and of complex mixtures have been answered. Most of the work has been published in the open literature. One reason why our work progressed as well as it did was a continuing collaboration with colleagues from other Biology Division Sections, other ORNL Divisions (particularly the Analytical Chemistry Division), and institutions such as The University of Tennessee and others across the country. To all of them go our thanks for continuous support and collaboration.

SYSTEMIC TOXICOLOGY

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Mechanisms of acute and chronic lung injury and the toxicokinetics of complex mixtures were the main points of attention during the past year. The ongoing toxicity testing program was continued to study the acute effects of selected chemicals.

The lung may become damaged by both airborne or bloodborne agents. Acute and chronic damage can be recognized with biochemical techniques that include measuring lavage enzyme levels or quantitating the presence of structural macromolecules such as collagen. However, biochemical measurements alone do not distinguish various pathological lesions, and histology is needed to provide additional important information and to evaluate tissue renewal. When the methods of studying lung collagen are applied to different toxic agents, different patterns of hydroxyproline accumulation and cellular kinetics are revealed. It is speculated that the development of fibrotic lung disease may be at least partially determined by the initial lesion and that quantitative analyses of cell kinetic patterns help us to understand and to predict the nature and evolution of disease processes.

This is best exemplified in a study in which we correlated cell kinetics with the development of a chronic pulmonary lesion produced by a toxic inhalant. Male BALB/c mice were exposed to aerosols of 4.9 μg of Cd/liter for 1 h while controls were exposed to water aerosol. Immediately after, half of each group was placed in 80% O_2 for 6 days, while the rest were left in room air. End points used to assess lung injury were measurement of hydroxyproline, [^{14}C]thymidine incorporation into DNA, histopathology and lung cell labeling indices (LI) determined by autoradiography. A 1-h exposure to $CdCl_2$ aerosols caused marked cell proliferation in the lung with the peak of cell labeling occurring at day 5. In animals exposed to both $CdCl_2$ + 80% O_2, the cell labeling peak

was delayed until day 9. Cell differentiation studies showed a delay in the peak of type II epithelial cells and endothelial cell division when $CdCl_2$ exposure was followed by the 80% O_2 treatment. On day 15 most of the labeled cells were identified as interstitial cells in both treated groups. Bronchiolar cell labeling was suppressed at the early time period in the $CdCl_2$ + O_2 group. During the following weeks and months, the histologically visible lung lesions tended to resolve in animals exposed to $CdCl_2$ or $CdCl_2$ and 80% O_2, whereas total pulmonary hydroxyproline remained at all times (3, 6, and 12 months) significantly higher in Cd-treated animals when compared to controls. It was concluded that acute lung injury by a toxic inhalant can be amplified if there is an initial delay in pulmonary cell proliferation following an acute insult.

With the same approach we studied the acute response to lung injury produced by another metal aerosol. Rats and mice were exposed in a nose-only chamber to an aerosol of beryllium sulfate ($BeSO_2$) and sacrificed thereafter over a time period of 21 days. Lung cell kinetics were studied with autoradiography and the labeled cells were differentiated as to type. In rats, the LI showed a peak response on day 8 following exposure, while the LI of mice showed a maximum response at day 5. In rats, the proliferative response involved type II alveolar epithelial cells, interstitial and capillary endothelial cells. In mice, the response was mainly found in the alveolar macrophage population and the interstitial and endothelial cells. In general, the response to injury was more pronounced in rats, presumably because mice accumulated less beryllium in their lungs.

Detailed cell kinetic studies also proved useful in an experiment designed to test the hypothesis that in mouse lung, enhancement of tumor development could occur independently of diffuse alveolar cell hyperplasia. Male A/J mice were given 1000 mg/kg of urethan or 10 mg/kg of 3-methylcholanthrene (MCA). Alveolar cells were labeled through continuous infusion of [^{3}H]thymidine for 6 weeks after administration of the carcinogen. Urethan produced a significant hyperplasia of the type II alveolar cell population, whereas MCA had no such effect. Five repeated injections of 300 mg/kg of butylated hydroxytoluene (BHT), a procedure known to enhance lung tumor development, produced cell hyperplasia only during the first 2 weeks; later the mice became resistant to the action of BHT. In animals treated with piperonyl butoxide prior to BHT, cell proliferation was abolished. BHT still had a small but significant enhancing effect on tumor development. However, this effect was dwarfed by the observation that piperonyl butoxide alone greatly inhibited tumor development. In a later experiment we circumvented this problem with a different approach. Animals were given a series of intraperitoneal BHT injections until they became tolerant to BHT. They were then treated with MCA. Subsequent BHT injections did not elicit pulmonary toxicity and cell proliferation was suppressed in all lung cell populations. BHT nevetheless greatly enhanced the development of lung tumors induced by 3-methylcholanthrene (MCA). It was concluded that overall cell hyperplasia is not a necessary prerequisite for enhancement of lung tumor formation by BHT and that a different mechanism of action should be sought.

Bleomycin is a valuable anticancer drug in man. It is also notorious for producing lung damage. In most experimental studies bleomycin is given intratracheally, whereas man is exposed to the drug via the bloodstream. This called for a comparative study in which we examined in detail the differences in the response to lung injury between intratracheally instilled (IT) bleomycin and intravenous (IV) administration. Mice were treated with either 4 U/kg IT or 100 U IV bleomycin and killed at intervals up to 21 days after treatment. Cell proliferation, histopathology, lung lavage and hydroxyproline content were examined. There was a biphasic response in the cell proliferation in the IT treated mice, while the IV treated mice showed a single delayed peak in proliferation. The histopathologic features of interstitial pneumonitis, elevation in lung lavage enzyme activities and lung hydroxyproline content were qualitatively similar between the two routes of administration, although the IT mice response was always greater in magnitude. Differences exist between the lung reaction to these two routes of administration, but these differences reflect nonspecific inflammatory response and magnitude of initial injury. We concluded that the response to bleomycin-induced acute lung injury is basically similar, whether the injurious agent is administered IT or IV.

Bleomycin was also used in rats to study the development of a progressive pulmonary lesion. Animals were exposed to 70% oxygen for 72 h following an intratracheal instillation of bleomycin (0.2 U/kg body weight). Animals were killed 15, 30, 60 and 90 days after treatment for hydroxyproline, cell kinetics and histopathologic analysis. A 16% increase in hydroxyproline over controls was seen 15 days after treatment which was manifested by the proliferative phase of diffuse alveolar damage and an increase in cell labeling by tritiated thymidine. Thirty days after treatment hydroxyproline remained elevated while lung injury appeared to be healing with a residual focal interstitial pneumonitis and a drop in cell labeling. Between 60 and 90 days, there was an additional significant increase in hydroxyproline to 44% over controls. Diffuse interstitial pneumonitis with fibrosis was observed. Cell labeling remained constant between 60 and 90 days. It was concluded that the treatment of rats with bleomycin and hyperoxia results in a slowly progressive pulmonary fibrosis. The increase in hydroxyproline in the chronic phase was not accompanied by an increase in cell proliferation, and therefore may have resulted from an increase in cellular production of hydroxyproline rather than an increased number of cells producing collagen.

In previous experiments we documented that the antioxidant BHT would enhance tumor development in mouse lung. The observation appeared to be somewhat at odds with a current concept which says that oxygen-free radicals are instrumental in tumor promotion, whereas antioxidants might be expected to have anti-promoting properties. Exposure of animals to hyperoxia most likely increases the flux of oxygen-free radicals in the lung. This was used to test the hypothesis that continuous hyperoxia would enhance the development of lung tumors in mice. In strain A/J mice treated with a single dose of 1000 mg/kg of urethan and exposed to 70% O_2 for 16 weeks, an average of 5 tumors per lung developed, whereas in animals kept in air, an average of 20 tumors per lung was found. When the animals

were returned to air after oxygen exposure it was found that a difference of 15 tumors per lung between the two groups persisted up to one year later, indicating that O_2 was tumoricidal. The shortest duration of O_2 exposure to be effective was 4 weeks and delay of O_2 exposure up to 12 weeks after urethan still was effective in reducing the number of developing tumors. Histopathology showed that continued exposure to 70% O_2 produced some hyperplasia of the bronchiolar epithelium and only very discrete changes in the pulmonary parenchyma. Analysis of cell proliferation patterns with a continuous [^{3}H]thymidine labeling technique showed a persistent high cell labeling in the bronchiolar epithelium and a temporary increase in alveolar wall cell labeling. Chronic hyperoxia failed to alter the activities of pulmonary superoxide dismutase or glucose-6-phosphatase dehydrogenase. Ornithine decarboxylase, on the other hand, was increased as long as the animals remained exposed to oxygen. It was concluded that hyperoxia kills developing tumor cells in mouse lung and that the data are not consistent with the free-radical theory of tumor promotion.

Experiments were initiated to establish whether BHT would enhance tumor development not only in mouse lung, but possibly also in mouse liver in the GI tract of mice or of rats. BALB/c mice were treated with six sc injections of 1,2-dimethylhydrazine (DMH), 20 mg/kg, and then fed a diet containing 0.5% or 0.05% BHT. Ten months later, colon tumor incidence in control animals was 10%. In the animals given 0.5% BHT it was 32% ($p < 0.05$). In mice fed 0.05% BHT, colon tumor incidence was 0%. BHT did not influence GI tract tumor incidence in mice treated with three intrarectal instillations (4.5 mg total) of N-nitro-n-methylurea. F344 rats were given two sc injections of DMH, 40 mg/kg. They were fed diets containing 0.5% BHT, 0.1% BHT, or 0.5% butylated hydroxyanisole (BHA). Seven months later it was found that 0.1% BHT had significantly increased the incidence of GI tumors at all sites (incidence: controls 50%; 0.5% BHT 66%; 0.1% BHT 78%; BHA 70%). BHT did not influence the development of colon tumors, but in animals fed 0.5% or 0.1% BHT, significantly more tumors were found in the small intestine than found in animals fed the control diet or 0.5% BHA. BHT may enhance GI-tract tumor development, provided exposure to BHT begins after exposure to the carcinogen.

A major effort was begun in studying the toxicokinetics of complex mixtures. We chose, for an initial study, two complex mixtures having widely different biological properties: fuel oil blend No. 931, which produces within one year a 100% incidence of skin tumors in C3H mice, and the same fuel oil blend following low severity hydrotreatment (to 2650 ppm N); this mixture has, within the first year, no carcinogenic activity. To both mixtures was added a sample of ^{14}C-labeled dotriacontane, a paraffin with 32 carbons.

Male BALB/c mice were shaved on the back and 50 µl of the spiked sample (4.5×10^6 dpm ^{14}C) was painted onto the skin. The animals were kept in metabolism cages for collection of feces and urine. One, 6 and 24 h later three animals per group were killed for determination of radioactivity (liquid scintillation counting) in blood and various internal

organs. Radioactivity in blood rose steadily over the entire 24-h period; in the animals exposed to the non-carcinogenic mixture, diffusion of the marker substance into the blood appeared to occur at a faster rate than in the animals painted with the carcinogenic oil. Accumulation of label in internal organs followed a similar pattern: at 6 h after exposure to the mixture, more of the marked compound originating from the noncarcinogenic mixture (compared to the marker compound originating from the carcinogenic fuel oil blend) was found in fat, testes, brain, kidney, liver and gastrointestinal tract. After 24 h, however, the differences between radioactivity measured in the various organs were statistically no longer significant, with the exception of the kidney. At this time, the total amount of the administered doses in individual organs was found to be: liver 11-12% of total dose; kidneys 0.2-0.4; brain 0.1% and testes 0.01-0.02%.

Initially, the same amount of radioactivity was administered in each of the two mixtures. If uptake and elimination kinetics of the marker compound were identical for the two mixtures, then the ratios of tissue specific activities should have stayed close to unity at all time points. This was not the case. Six hours after exposure the ratio of dpm/g tissue for the noncarcinogenic vs the carcinogenic mixture was around 6.6 in the testes, around 5.0 in liver and kidney, and 2.5 to 3.0 in blood, GI tract and ear (a measure of skin not directly exposed) whereas it remained close to the theoretical value of 1.0 in the exposed skin. In the feces, the ratio was 9.3 and in urine 2.1. After 24 h, the ratios in all tissues examined had fallen below 2.0, including urine and feces.

The marker substance used in the present experiment was a largely inert long-chain paraffin. The pharmacodynamic behavior was largely determined by the complex mixture in which it was dissolved. Within the first 6 h, the hydrotreated mixture provided for a much faster and better absorption of the marker from its site of application. This was accompanied by a higher accumulation of the marker in kidney, liver and GI tract and a considerably larger excretion, predominantly through the feces. The salient feature thus is that after application of the carcinogenic mixture more material appears to be staying at the site of application. Whether this is related to the carcinogenic process remains to be established.

If dpm/g tissue in several organs are averaged, it can be calculated that following one single exposure up to 80% of the applied doses are taken up within the body. In a conventional mouse skin painting study, where 3 × 100 µl of mixture are applied over a two year period, this could mean that the final concentration of the mixture in the animal would be 1 kg/g of body weight, obviously an impossibility. The equilibrium kinetics must be different from this calculation and it will be important, in future studies, to understand better the pharmacokinetics of dermally applied complex mixtures, particularly after repeated exposure.

Finally we examined several chemicals in acute toxicity studies. Under contract with Sandia National Laboratories and for the Toxic

Materials Advisory Committee, we have conducted one or all of the following tests on chemicals of industrial importance: (a) oral LD_{50} in mice, (b) dermal toxicity (screen) in rats, (c) eye irritation in rabbits, (d) skin irritation in rabbits, (e) delayed-type allergic hypersensitivity in guinea pigs. The chemicals tested were: 2-(5-cyanotetrazolato-pentaaminecobalt (III) perchlorate, 1,4-[bis(1-hydroxycyclopentyl)-butadiyne], 2-ethyl-4-methylimidazole, silicon carbide, silicon nitride, titanium carbide, boron nitride, titanium diboride, osmocene, dirhenium decacarbonyl, iridium acetylacetonate, mesitylene tungsten tricarbonyl, platinum acetylacetonate, and 1-4-bis(phenylethynyl)benzene. Except for platinum acetylacetonate and dihrenium decacarbonyl, none of these chemicals would appear to constitute a health hazard. Based on oral LD_{50} values in mice, these two chemicals would be considered very toxic.

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TUMORIGENICITY TESTS OF SYNFUELS

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Programs designed to evaluate energy technologies involving production of liquid fuels from coal must include studies of the potential health hazards of process substances and the final products, e.g. gasoline, fuel oil. Part of the Biology Division's mission is to provide a data base for potential health hazards of these materials. The principle focus this year has been a chronic dermatotoxic study of a blend of coal-derived oils and its hydrogenated derivatives. This study was begun in 1983 and involves testing of the blend and four of its derivatives: (a) the H-coal blend consisting of a mixture of light and heavy oils, (b) a low severity hydrotreated preparation of the blend, (c) a high severity hydrotreated preparation of the blend, (d) a home heating oil derived from the high severity hydrotreated oil, and (e) a naphtha reformate (gasoline) derived from the high severity hydrotreated oil. A petroluem-derived naphtha (gasoline) and home heating oil were included in the test for comparison purposes. Each test substance and control material i.e., acetone (vehicle), benzo(a)pyrene (positive control) was applied to the shaved backs of C3H mice (25 males and 25 females) 3 times a week. The dose levels were 100, 50, and 25% (w/v) and the application volume was 50 µL. The initial design called for an 18-month period of testing, but was

changed to a life-time study. As mice died or developed skin tumors, they were necropsied, and the tissues were preserved for histological evaluation.

Results of the study showed that the H-coal blend is a potent skin tumorigen and that hydrotreatment of the blend significantly reduces its tumorigenicity. High severity hydrotreatment, however, is no more effective than low severity hydrotreatment of the blend in reducing its skin tumorigenicity. The coal-derived naphtha reformate (gasoline) is essentially non-tumorigenic for skin in contrast with petroleum-derived naphtha which resulted in a low but possibly significant tumor incidence. The coal-derived home heating oil appears to be detectably tumorigenic and is quite probably more potent than its petroleum-derived counterpart.

A histopathologic evaluation showed that about half of the skin tumors were papillomas and half were carcinomas. Gross necropsy observations indicated that the percutaneously applied H-coal liquids tested did not increase the incidence of tumors in tissues other than skin.

Another protocol is being used in an attempt to determine whether a shorter test period involving fewer mice can be used to screen materials for their potential as skin tumorigens. Five male and 5 female mice constitute a treatment group to which a test material is applied to the skin twice a week for 52 weeks. Using this protocol, we have completed testing of 19 preparations derived from H-coal liquids that have been upgraded by physical or chemical means other than hydrotreatment, e.g. distillation, acid treatment, pentane deasphalting, oxidation. Although data analysis is incomplete for this test protocol, it appears that none of the upgrading procedures are as effective as hydrogenation in reducing tumorigenicity of H-coal liquids.

Skin tumorigenicity testing is carried out in conjunction with members of ORNL's Analytical Chemistry Division who provided all test materials.

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 hazardous 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. 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 our first series of experiments we determined the number of altered cell populations (SPC) induced in tracheas exposed to beeswax pellets containing 200 μg of the very potent carcinogen, DMBA. Tracheas were exposed to the 200 μg pellets for 2 wks (135 μg released), 4 wks (165 μg released) or the pellets left in (all released). From these exposures 1.8, 5.0, 7.2 SPC/trachea, respectively, were isolated at 2 months after the start of exposure. At 6 and 9 months, the number of SPC induced by the 2-wk DMBA-exposure decreased to 1.3/trachea while the numbers of SPC remained the same for the two longer exposed groups. As reported earlier, the 2-wk-DMBA exposed group was also distinguished by a slower average growth rate in primary culture and less subculturable primary cultures. 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. We found a close correlation between an increase in severity of the lesions and the acquisition 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. This first major study with DMBA, spawned two new lines of investigation with DMBA discussed below.

a) Initiation and promotion of carcinogenesis with DMBA and 12-O-tetradecanoylphorbol-13-acetated (TPA). 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. We have been carrying out a study in which tracheal implants were first exposed for 2 wks to 200 μg DMBA-beeswax pellets. The tracheas were then made into FTTI and exposed 2×/wk to 7 μg TPA-gelatin pellets up to 25 wks. Control FTTI were exposed to the DMBA followed by gelatin pellets or to beeswax pellets followed by TPA. In this study we again saw a decrease in SPC/trachea, this time from 2.50 to 1.43, in tracheas exposed to the carcinogen for 2 wks. Tracheas exposed to DMBA + TPA had 4.0 SPC/trachea. This indicates that the TPA not only promoted those cells which were not permanently altered by the carcinogen, but may have promoted non-detectable (by our culture conditions) initiated cells as well. TPA alone induced no SPC. Markers for the progression of neoplasia (anchorage-independent growth, etc.) in the subcultured SPC are now being studied to determine whether the later stages of carcinogenesis are promoted as well.

b) Induction of carcinogenesis with split doses of DMBA. With the advent of the FTTI, we are now able to expose rat tracheas to repetitive low doses of carcinogen, thereby reducing acute toxicity. A project was started during the past year to study the carcinogenicity of DMBA given in this manner. To begin this study, the acute morphological responses of the FTTI to twice weekly exposures of different amounts of DMBA were examined. Doses of 10, 20 and 40 μg DMBA given 2×/wk for 2, 4, 8 wks and the same total doses given over different lengths of time were compared. At each time point, 4 FTTI from each exposure group were taken for histopathologic assessment. At the lowest amount of DMBA [10 μg given 2×/wk for 2 wks (total dose 50 μg)] 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 2×/wk, 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 have been started to quantitate the induction and progression of carcinogenesis. FTTI were exposed to 20 μg DMBA pellets 2×/wk for 2.25 months (total dose 360 μg). After an additional month without carcinogen, SPC are now being determined. Preliminary data indicate that a very high number of SPC which will test tumorigenic will be isolated. We are now exposing FTTI to 5 μg DMBA, 2×/wk for 2.5 months (total dose 90 μg), and will determine SPC and progression of neoplasia. If a large number of SPC are obtained, a lower dose of DMBA will be tested. 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.

2. In-Vivo-In Vitro Study of Carcinogenesis Induced by 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 2×/wk 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, 0.67 SPC/trachea was obtained. The number increased to 0.92/trachea if the 1440 μg B(a)P was spread over 2.25 months (80 μg, 2×/wk). Increasing the exposure to 4.5 months (40 μg, 2×/wk) induced 2.0 SPC/trachea. This is clear evidence that long-term low doses of a carcinogen are more tumorigenic, and the condition is closer to the human situation.

We questioned whether the B(a)P had initiated carcinogenesis in some cells which had not reached the stage detected by our selection procedures. Therefore, we established primary cultures from FTTI exposed for 4.5 months to the 40 μg B(a)P pellets, but instead of starting the selective media at 14 days of culture, we changed to the selection media at 63 days. With this regimen we obtained 5.25 SPC/trachea, indicating that some cells progressed during culture in complete medium. With these more sensitive procedures, we should be able to study agents with low tumor potency.

3. Initiation-Promotion and Co-Carcinogenesis with B(a)P and Formaldehyde (HCHO). Recently we began a major project 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 (0.1% 2×/wk, 30 wks) given after exposure for 1 month to 800 μg B(a)P-beeswax pellets induced severe lesions in the tracheas which were

detected in histological sections as well as in the cytopathology of the cells which exfoliated into the tracheal lumens. No dysplastic lesions beyond mild atypia were seen in the tracheas exposed to B(a)P or HCHO alone. This promotion effect of HCHO will be studied in additional experiments in which carcinogen-altered cell populations (SPC) will be quantitated as well as the progression to tumor development in vivo followed. Experiments have also been started to study the co-carcinogenic effects of B(a)P and HCHO. FTTI are being exposed to HCHO followed by B(a)P and the reverse. The acute morphological changes are now being assessed. Since HCHO is known to induce DNA-protein crosslinks as well as DNA strand breaks, these will also be assessed using alkaline elution techniques.

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, 3, 12 and 24 wks after inserting the pellets. We have found that 62% of the radioactivity from the lumens has been taken up by the tissue at 3 h and 91% of the activity is gone from the lumens by 12 h. In the future we will determine the effect of pre-exposure to B(a)P and to HCHO on the utilization of B(a)P by the FTTI.

4. Biochemical Markers for Stages in the Progression of Neoplasia. Since we found that the ability of tracheal cells to survive in pyruvate-deprived medium is a very early marker of carcinogen-induced alterations, we have put considerable effort toward elucidating the cellular mechanisms underlying the requirement of pyruvate by normal tracheal cells in culture and the lack of this need by the carcinogen-altered cells. Two key earlier findings were: (1) normal tracheal cells actually metabolize [^{14}C]pyruvate for macromolecular synthesis and lactic acid production at 3-4 times higher levels than carcinogen-altered cells; (2) carcinogen-altered cells and tumor cells have markedly higher levels of particulate-bound $NADP^{+}$-dependent malic enzyme activity. This enzyme catalyzes the formation of pyruvate from malate. Since malic enzyme is involved in lipid metabolism, we recently began looking at the utilization of [^{14}C]acetate and of [^{14}C]palmitate for energy production by normal primary cell cultures and SPC. So far, we have not detected differences between the normal and carcinogen-altered cells. We have also looked at the utilization of [^{14}C]alanine and [^{14}C]glutamine, two sources of pyruvate in cells. Uptake for macromolecular synthesis and for energy production was similar for both of the metabolites in the normal cells and SPC.

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SKIN TOXICOLOGY

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The focus of the Skin Toxicology Research Program is to characterize the various parameters that can influence and modulate the interactions between mammalian skin and the toxic and carcinogenic chemicals of concern from various energy technologies. Studies are directed toward developing a better understanding, at both the molecular and cellular levels, of the mechanisms involved in chemical toxicity and carcinogenicity in mammalian skin. Toxicokinetics of skin penetration, cutaneous metabolism and disposition, biochemical dosimetry, and interactions of reactive intermediates with tissue macromolecules are important determinants in the response of the skin to topical exposure of xenobiotics. Thus knowledge of factors which may modulate these processes, and hence the tissue response in different species, is an important prerequisite in the risk and safety evaluation of chemicals. From the results of such studies it should be

possible to establish a more direct and quantitative basis for extrapolating animal data to human risk and permit a more rational approach to the identification and elimination of hazardous exposures.

In the past few years the research activities of our group have centered around two overlapping but independent areas, namely: (1) cutaneous metabolism and toxicokinetics of skin absorption, and (2) dosimetry of polycyclic hydrocarbons (PAH) in skin and carcinogenesis. Both in vivo and in vitro techniques are employed in developing mechanistic and functional approaches to address problems. Progress made during this report period is summarized below.

1. Cutaneous Metabolism and Toxicokinetics of Skin Absorption. The major barrier to skin absorption is generally considered to be the stratum corneum. This outermost layer of the skin is composed essentially of dead cells containing keratin; consequently, the skin has frequently been thought of as a passive, inert barrier covering the body, and percutaneous absorption of chemicals was thought to be essentially a diffusional process. This has resulted in the belief that nonviable skin samples which have been stored frozen may be used as acceptable tissue preparations in a multitude of in vitro permeability studies. In general, the consideration of the skin as merely a diffusional barrier persists, despite the fact that the skin is an organ system active in many metabolic functions, including those involved in the metabolism of xenobiotics. These metabolic processes in the skin could play an important role in the percutaneous fate of topically applied substances. However, to what extent cutaneous biochemical processes may contribute to the physiological disposition and toxicity of chemicals following topical exposure remains to be investigated.

Using the organ culture systems developed in our laboratory, we have previously demonstrated that in mouse skin maintained in organ culture, the in vitro permeation and cutaneous metabolic fate of topically-applied benzo(a)pyrene [B(a)P] was significantly influenced by the biochemical viability of the cultured tissue. The results strongly implicated cutaneous metabolism to be an important factor for consideration in the percutaneous fate of surface-applied chemicals. In a recently completed series of studies we have examined the in vitro penetration and percutaneous fate of topically applied B(a)P and testosterone in the skin from six mammalian species maintained as short-term organ cultures.

Skin samples from mouse (C3H), rat, rabbit, guinea pig, marmoset and man were examined; the results from these studies showed that, in all species, metabolic viability was a major factor involved in the in vitro skin permeation of these surface-applied chemicals. Permeation was dependent upon diffusion, but was also accompanied by extensive cutaneous "first pass" metabolism. For B(a)P, negligible amounts of unmetabolized parent compound was translocated across the skin, and essentially only metabolites were found in the receptor medium with water soluble metabolite constituting the major components. For testosterone, on the other hand, both parent compounds and a full spectrum of metabolites were found in the

receptor fluid from viable skin preparations. Extensive species variations in the metabolite profiles in the skin and in the receptor medium from viable tissues were observed; however, in previously frozen nonviable skin preparations, essentially only unchanged parent compounds were detected in the receptor fluid. Permeation of both compounds was highest in mouse skin, but no other correlations between species, e.g. in the thickness of the cultured skin and the extent of penetration as assessed in terms of the percentage of the applied radioactivity recovered in the receptor fluid, 24 h after topical application, was apparent. For B(a)P, the order of extent of penetration was mouse > marmoset and human > rat and rabbit >> guinea pig. Permeation of B(a)P in guinea pig skin was essentially negligible. For testosterone, the order was mouse > rabbit and human > rat > guinea pig and marmoset.

In a comparative study using skin samples from hairless and haired mice, no apparent strain differences in the in vitro permeation of topical testosterone were observed, but the overall permeation of B(a)P in skin samples from hairless strains (HRS, SKH) was 3- to 4-fold lower than in skin samples from normal haired mice (C3H, DBA/2, C57BL/6 and BALB/c). Moreover, in similar studies with an in-house strain of mice which carried three phenotypic variants due to their hair densities, the results showed that the overall permeation of B(a)P in the variant with a full coat of hair was 6-fold higher than the hairless variant. Permeation in the intermediate variant was 2-fold higher than the hairless animals. These results strongly implicated the possible involvement of the skin appendages in the percutaneous absorption and cutaneous metabolism of topical B(a)P.

Significant sex and age differences were observed in studies with topical B(a)P in C3H mouse skin in organ culture. Overall permeation in female skin was approximately 2-fold greater than in male skin; and in the males, permeation was highest in the skin preparations from geriatric animals (90 weeks old) and lowest in immatured animals (4 weeks old). Also, as illustrated by the 2- to 3-fold increase in the in vitro permeation of B(a)P in mouse skin pretreated in vivo with enzyme inducers and the significant reduction in permeation following the addition of a metabolic inhibitor (KCN) to the culture medium, modulation of the metabolic stratum of mouse skin can greatly influence the percutaneous absorption of some topically applied chemicals. For B(a)P this increased permeation in response to in vivo pretreatment with inducers of mixed function monooxygenase activities also showed the expected strain differences. Following pretreatment with 3-methylcholanthrene, increased permeation was observed in the skin from the "inducible" C57BL/6 mice, while no changes were observed in the skin from the "non inducible" DBA/2 mice. However, with prior in vivo exposure to the potent inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin, increased permeation of B(a)P was observed in skin preparations from both mice strains.

In contrast to what we found with B(a)P and testosterone, studies with benzene showed that percutaneous absorption of this compound was not influenced by viability and metabolic status of the tissue. In all species studied which included mouse, guinea pig, rabbit and man, in vitro

permeation of topically applied benzene was not accompanied by any detectable biotransformation and appeared to be influenced solely by diffusional determinants. There were significant species differences which appeared to be inversely related to the thickness of the cultured skin. Mouse skin, the thinnest, was most permeable to benzene, being at least 10-fold higher than human breast skin. Permeability of benzene in guinea pig and rabbit skin was about twice that of human skin.

Recently we have also examined the percutaneous fate of three closely related estrogens. The initial results supported our contention that in addition to diffusional determinants, percutaneous absorption of certain chemicals is closely associated with their cutaneous metabolism. Following the topical application of esterone to mouse skin in organ culture, permeation was accompanied by extensive bioconversion to estradiol. For the equivalent amount of estradiol, permeation through mouse skin in vitro was approximately 2-fold higher than esterone and there was only a small amount of biotransformation of estradiol to esterone. For estriol, the product of esterone and estradiol metabolism, the extent of permeation through mouse skin was low, being only about 20% of that of esterone. Moreover, evidence for the cutaneous biotransformation of estriol during percutaneous translocation through mouse skin was not apparent.

Taken together, the results of these investigations showed that, in all species studied, the translocation of surface-applied chemicals through the skin was accompanied by varying degrees of cutaneous "first pass" metabolism. This demonstrated that, in addition to tissue viability, the metabolic status and capabilities of the skin are important determining factors in modulating skin absorption. This is contrary to the currently accepted view that diffusion through the stratum corneum dictates percutaneous absorption and determines the systemic bioavailability of topically applied chemicals. Our results support the concept that both diffusional and metabolic processes are intimately involved in percutaneous absorption of chemicals. The relative importance of these processes is a function of the physiochemical properties of the compounds and the ability of the epidermal cells to metabolize the compound in question. Furthermore, it is evident that meaningful in vitro studies of the cutaneous fate of topically applied chemicals should include not only a measure of the diffusion of the compound through the skin, but also an assessment of the cutaneous biotransformation of the applied compound.

In summary, skin permeability is a complex phenomenon influenced by a variety of biochemical and physiochemical factors. In addition to being a drug metabolizing organ, the skin is also a target organ for chemical toxicity and a primary portal for entry for xenobiotics. Understanding the interrelationship of these functional properties of the skin is therefore an important aspect in elucidating the mechanism of chemically induced skin lesions. Studies to date have demonstrated that our in vitro approach using skin in organ culture offers a useful model to further advance our knowledge of the disease process in skin brought about by exposure to toxic chemicals.

2. Dosimetry of PAH Skin Carcinogenesis. Evaluation of exposure of the general population to environmental chemicals is hampered by the lack of quantitative exposure data for individuals. Estimates of levels of compounds of interest in the environment are often the only data available.

We are interested in devising techniques which will allow us to detect and quantitate exposure to chemical carcinogens and which eventually can be used in risk analysis with humans. Our recent research with HPLC/fluorescence analysis has demonstrated that we can detect, identify, and quantitate the binding of the model PAH, B(a)P, with the DNA of mouse skin. The technique allows detection of femtomole amounts of B(a)P associated with DNA using conventional instrumentation.

It is known that topically applied carcinogens, such as PAHs, are rapidly absorbed, metabolized, and excreted by the mouse. In the cells of the skin at the site of application and where subsequent tumor formation occurs, metabolites of PAHs are converted to electrophilic agents that react with nucleophilic centers in nucleic acids and proteins. A good correlation has been demonstrated between the amount of B(a)P bound to DNA of mouse skin and its carcinogenic activity. In fact, the ultimate carcinogenic form is thought to be the anti diol epoxide of B(a)P, and it is possible that quantitation of adduct formation of this compoud with DNA can be used as a measure of the "biologically-effective" dose for B(a)P carcinogenicity.

The cellular metabolism of B(a)P, a prototype of PAHs, to the chemically reactive diolepoxides B(a)PDE I and B(a)PDE II [often referred to, respectively, as anti-B(a)PDE and syn-B(a)PDE] results in the formation of covalent B(a)PDE-adducts with DNA which may be the initiating events responsible for the observed carcinogenic properties of B(a)P. However, in those instances in which the sources of B(a)P are substances found in the environment, it is not possible to use radiometric detection because the B(a)P is unlabeled. Recently, immunoassays and post-derivatizational procedures for B(a)P-DNA adducts have been introduced; nevertheless, only meager estimates exist of the burden in human tissue of B(a)P derivatives.

Fluorescence/HPLC assay. Previous fluorescence studies of the intact B(a)PDE complex have utilized photon counting, or low temperature fluorimetry or a combination of these methods to detect as little as one molecule of B(a)PDE bound per 10^5 DNA nucleotides. However, in order to analyze the minute amounts of B(a)P derivatives bound to DNA of animals exposed to PAHs in the environment, more sensitive detection methods are necessary. Other workers have pointed out that the interaction of the bases in DNA with covalently bound B(a)PDE strongly quenches the fluorescence of the pyrene moiety. On the other hand, when the pyrene moiety is in the form of the free tetrol, the fluorescence quantum yield in the deoxygenated solution is similar to that of pyrene itself. In addition, tetrols are easily separated by HPLC and this method establishes the stereochemical origin of the diolepoxide involved in adduct formation. These results suggest the use of HPLC coupled with fluorescence detection

to analyze the pyrenyl content of B(a)PDE-DNA following acid hydrolysis to release the tetrols.

Our initial investigations have involved the development of a fluorescence/HPLC assay for the quantitation of B(a)PDE-DNA adducts. The data from these studies have established that with conventional instrumentation, a lower limit of about 5 pg of B(a)PDE-DNA adducts as tetrols is detectable. This is equivalent to approximately one molecule of B(a)PDE bound per 10^7 DNA nucleotides in 100 µg of DNA.

Animal studies. Since the metabolism of carcinogens may be influenced by the interference and/or interactions of all compounds present, we measured the covalent interaction of the carcinogen with cellular DNA in the target tissue. The rationale for this approach is based on the hypotheses that (i) adduct formation at the site of tumor formation represents the critical step in carcinogenesis, and, (ii) the adduct concentration in the tissue represents the "biologically effective" dose of the carcinogen. Thus, the fluorescence/HPLC technique appeared to be well suited toward an anlysis of adduct formation by the model carcinogen, B(a)P.

Analysis of DNA isolated from mouse skin after topical application of B(a)P demonstrated that adduct formation occurred via the metabolic activation of the carcinogen to the diolepoxides which is similar to results of those who used radiolabeled B(a)P.

The sensitivity of our method is such that parallel studies could be performed with B(a)PDE-DNA isolated from mouse skin maintained in organ culture where B(a)P-exposure approached 1 µg/5 cm^2 of skin.

In subsequent experiments we were able to demonstrate that (i) ellagic acid, a plant phenolic compound known to exhibit an inhibitory effect on B(a)P-mutagenicity and cytotoxicity, interfered specifically with the covalent binding of anti-B(a)PDE with cellular DNA; (ii) prior treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin, a known inducer of microsomal aryl hydrocarbon hydroxylase and glucuronyl transferase activities, caused differences in the degree of covalent binding of B(a)PDE to DNA of mouse skin, most probably as a consequence of an induced change in the pathway of cellular metabolism of B(a)P; and (iii) B(a)PDE-DNA adducts persist in skin cells long after their disappearance by DNA turnover would predict. In the first week after topical application of a single dose of B(a)P, the t1/2 of B(a)PDE-DNA adducts and of DNA were approximately 5 days. At 30 days post treatment, less than 15% of these adducts are detectable in the skin; however, their t1/2 has increased to 30 days.

B(a)PDE-hemoglobin adduct formation. The observation that exposure of animals of different species to B(a)P results in covalent binding of B(a)P metabolites to DNA and protein of most tissue prompted us to examine the concomitant adduct formation in tissue other than the skin. We found that mild acid hydrolysis of hemoglobin preparations from erythrocytes of

mice, previously exposed topically to B(a)P, released tetrols which were detectable by fluorescence/HPLC analysis, and it was concluded that prior to acid hydrolysis the tetrols were covalently attached to the hemoglobin as a result of the metabolic conversion of the applied carcinogen to the chemically reactive anti-B(a)PDE. Furthermore, there was a dose response relationship between the amount of B(a)P applied to the skin of the mouse and occurrence, 24 h later, of B(a)P adducts to hemoglobin, while the adduct, once formed, disappeared with a t1/2 of 6 days. The amount of anti-B(a)PDE bound to hemoglobin and DNA of the skin at the site of exposure, at various doses of B(a)P, appeared to be qualitatively similar.

The administration of B(a)P topically to pregnant mice during days 13-17 of gestation results in the adductive modification of the hemoglobin in the mother and progeny. Concomitant adductive modification of the DNA of the skin with B(a)P in the progeny was not observed and was probably due to the small amount of carcinogen applied to the mother. These data indicate that B(a)P administered externally to the mother passed across the placental membrane, either as B(a)P or some metabolite, and was present in the fetal tissue as anti-B(a)PDE, the carcinogenic form of B(a)P, before binding to hemoglobin.

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Cancer Section

SECTION OVERVIEW - R. J. M. FRY

The Cancer Section is concerned with both the fundamental changes involved in carcinogenesis at the molecular and cellular level and the responses of the whole animal to carcinogenic agents, in particular, radiation. The spectrum of studies is broad — in fact, a breadth only possible because of the interactions and collaboration with other Sections. This has become increasingly so with the decrease in the number of the staff members in the Section.

The events leading to cancer after exposure to agents that range from radiation to various chemicals are very different, at least up to some point. The question remains whether or not there is a common final pathway in the carcinogenesis process independent of the causative agent. One of the attractions of the oncogene hypothesis is that the carcinogenicity, common to so many diverse agents, could be explained in a unified theory. It is often said that cancer is many diseases — is it? — and if so in what ways?

Gene expression and its control lie at the very heart of the behavior of cells whether it be differentiation, normal growth, or neoplastic growth. The gene products differ qualitatively and quantitatively during normal cell and cancerous cell development. In the case of cancer the change in gene expression may be due to structural change in the gene, or a change in its normal spatial relationships with other genes, that may suppress or promote its functional activity, or to changes in the levels of hormones affecting the expression of the particular genes. It is becoming increasingly clear that although the structural changes, or the gene-gene relationships, associated with initial events in cancer induction are essential, they may not be the determining factor in all tissues. The mechanisms of expression or the suppression of the initial events is equally or more complex than the induction of the initial events. The way in which age, the strongest determinant of cancer, increases the probability of cancer must be related largely to the factors that influence expression.

The Section's research concerns normal gene function, its control, and the ways in which perturbation of DNA may lead to changes in the normal gene expression. Damage to DNA as a consequence of exposure to ionizing radiation, ultraviolet radiation, and chemical agents can lead to both changes in the position of genes and/or subtle changes in the genes themselves.

It is perhaps worth noting that the more we understand about cancer induction the more intriguing become the insights into the defenses against

cancer. The defenses stretch from the molecular level, such as the genes influencing integration of genomic sequences, to factors at the tissue and systemic level by which the development of tumors is suppressed. For example, at the gene level, the Fv-1 gene has been studied here for quite some time by W. K. Yang, R. W. Tennant, and their colleagues. This gene influences movement and integration of murine leukemia virus genomes. The importance of suppressor genes and their inactivation by carcinogenic agents will surely be studied more intensely in the future. Similarly, new work on the host factors, such as cell-cell interactions, and even the somewhat neglected immune system, are justified by the findings here and at other laboratories. As with many other aspects of the study of mechanisms of carcinogenesis, radiation provides a most worthy tool.

The reports of the individual groups give the details of their recent work and its implications. Some of the highlights are:

In the studies of the murine leukemic virus (MuLV) in the mouse germ line it has been shown that copies of the proviral sequences are dispersed in different chromosomal sites and are different in nucleotide structure from that of MuLV.

Retro-transposons is the name coined to describe genes with a retrovirus-like structure and that may move to other sites in the genome. A molecular clone named pIS-23 that contains two LTR-IS and structural gene sequences has been isolated. It has been found that about 100 copies of these, or homologous sequences, are generally found in the chromosomes of the laboratory mouse. The evidence, including sex and mouse strain dependency, indicates that a new family of retro-transposable gene elements has been found.

The previous work on the Fv-1 gene has helped greatly in the development of a new and major effort to investigate genetic factors that may control the expression of altered oncogenes. Clones of NIH 3T3 cells have been isolated that are resistant to transformation by Ha-ras and the resistance has been transferred to other cells by DNA transfection. These findings promise some very interesting work in the future.

In the studies of 'Regulation of Gene Expression' a gene, provisionally called gene 33, has been isolated and further characterized. The gene responds to hormonal inducers in a similar manner to the gene related to tyrosine aminotransferase (TAT) but hybridization distinguishes the genes. The information gained from the sequencing of the newly identified gene opens up ways of investigating the mechanisms of regulation of the gene. The messenger RNA of gene 33 is on the nonmembrane-bound polysomes but the protein product awaits precise definition.

Progress has also been made in this group's long-term interest, namely, hormonal regulation using TAT as the model. Work in the last year or so has made it clear that insulin accelerates transcription of the TAT gene; the contention of several groups that induction of this enzyme by insulin is controlled at the translational level was incorrect. It was

shown that the control of transcription by insulin was also true for gene 33.

A new approach to the study of liver differentiation is the use of mutant mice from the Division's Mouse Genetics Group that have c locus deletions. The importance of a trans acting regulatory gene for the activation of transcription of certain genes has been confirmed in studies of these mice. Initial results suggest that 5-azacytidine induced demethylation may partially obviate the need for the regulatory gene. This opens up the study of the role of selective gene demethylation in the action of the trans acting gene.

In the last couple of years S. J. Kennel's group has been tackling the challenging task of developing monoclonal antibodies to dioxins. Since very low levels of dioxins are toxic, exquisitely sensitive assays are required and monoclonal antibodies could be the answer. However, specificity, the immunologist's touchstone, has been hard to come by and requires further research.

The cytometrics group continues to make itself increasingly indispensable. R. C. Mann's contributions to the development of techniques for the quantitative two-dimensional gel electrophoresis with J. K. Selkirk have advanced the handling and analysis of the vast amount of data that can be obtained from such gels. The work has brought closer the goal of an error free (or nearly so) and "intelligent" computer technique of analysis. Legionella bacteria are widespread and vary in virulence. Interestingly, flow cytometry can detect a pattern associated with virulence.

Flow cytometrics has helped members of other Sections not only sort their cells but sort out their research problems, for example, the detection of a differential in X ray-induced cell cycle delay between normal and leukemic cells. In other experiments the cell-cycle stage dependency of repair of O^6-methylguanine has been established.

The saga of benzo(a)pyrene [B(a)P] metabolism continues to reveal interesting facets of the cell's metabolism of toxic compounds. The type of metabolism that in turn depends on the metabolic pathway determines the consequences of exposure. New studies have shown that human mammary tumor cells metabolize B(a)P very actively but glucuronide conjugation, in contrast to other cells, such as hamster embryo fibroblasts, is of minor consequence. The role of glutathione conjugation seems to predominate in these human cells.

It has been shown that the induction of sister chromatid exchanges as a result of exposure to B(a)P appears to be correlated with production of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. This singular result underlines the usefulness of variant cell lines in correlating metabolic pathways, their products and biological effects.

Neutron studies continue to be a major function of the radiation carcinogenesis program. Many of the data from recent neutron studies,

carried out by J. B. Storer and R. L. Ullrich, have been used in the judgement decision by the International Commission for Radiation Protection to change the quality factor for neutrons used in setting protection standards. We have not heard the last of this question and the result of our current work will continue to influence the decisions about the standards.

The *in vivo in vitro* technique using mammary and tracheal cells have been used to complement *in vivo* studies and to answer questions that can more easily, and perhaps can only be answered with *in vitro* systems. Results suggest that low dose rate neutron irradiation may be more effective than single doses because of a promotion-like effect of protraction.

The importance of host factors to the outcome of initiation has been alluded to earlier. A good demonstration of the importance of cell-cell interactions has been demonstrated by M. Terzaghi-Howe using tracheal epithelial cells. Normal cells have a marked inhibitory effect on the development of both preneoplastic and neoplastic cells to become frank tumors. It appears that the effect may be mediated by release of an inhibitor from normal cells. Surprisingly, the inhibitor appears to be the transforming growth factor type-β!

Studies of the ability of various agents to promote, or more correctly, inhibit suppression of X ray-induced initiation of epidermal cells, have shown the remarkable capability of the skin to suppress expression of potentially cancerous cells. Protracted treatment with ultraviolet radiation, 8-MOP and UVA (PUVA), and TPA appear to act synergistically with prior exposures to X rays. It has been shown that such protraction can have diverse effects. For example, both the Langerhans cells, part of the immune system, and glucocorticoid receptors, important in the hormonal influence on epidermal cell growth, are decreased.

MOLECULAR GENETICS OF CARCINOGENESIS

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To understand the molecular mechanisms of carcinogenesis, we have continued to perform our experimental research with the same working hypothesis that activation of specific transposable gene elements in the chromosomal DNA, caused by environmental carcinogenic insults to the cell,

may lead to gene rearrangement and eventually, neoplastic phenotypic expression involving altered oncogenes. Emphasis is also placed on cellular genetic factors that control the insertional process of transposable gene elements (e.g. Fv-1 gene) and that prevent altered oncogenes from causing neoplastic transformation (e.g. ras-specific "anti-cancer gene").

Characterization of Murine Leukemia Virus-Related Proviral Genes in the Mouse Germ Line. There are 20 to 50 copies of murine leukemia virus (MuLV-)-related sequences dispersely located in various chromosomal sites in the genome of laboratory mice. We have studied these gene sequences by molecular cloning and nucleotide sequencing approaches. Our results have clearly indicated that these sequences contain novel nucleotide structure features that are distinctly different from murine leukemia virus genomes, as briefly summarized in the following:

(a) All molecular clones of MuLV-related sequences isolated from chromosomal DNA of the mouse contain a distinct 170 to 200 nucleotide segment in the mid-U3 region of their long-terminal repeats (LTR) (Ou et al., Nucleic Acids Res. 11: 5603-5620, 1983). This sequence segment is absent in all known horizontally infectious murine leukemia virus genomes but is also found in another type of LTR, called LTR-IS, that exists in vast numbers (1000 to 2000 copies) in germ lines of laboratory strain mice.

(b) Nucleotide sequences of the region that corresponds to the site of tRNA primer binding for a functional retrovirus were determined in six MuLV-related sequence clones from mouse chromosomal DNA. The 3'-terminal 18-nucleotide sequence of a major glutamine isoacceptor tRNA was found to match well with the putative primer binding site: 18 of 18 in four clones, 17 of 18 in one clone and 16 of 18 in one clone. This implies that most of these endogenous proviral sequences of the mouse genome, if replicated as retroviruses, will be different from ecotropic murine leukemia viruses and other infectious mammalian type C retroviruses in using glutamine tRNA, rather than proline tRNA, as a primer for minus DNA synthesis in the reverse transcription process.

(c) We have molecularly cloned a HindIII chromosomal DNA fragment that harbors an apparently intact MuLV-related provirus from the RFM/un strain mouse. Nucleotide sequencing analysis revealed that the 5' and 3' LTRs were of identical nucleotide sequences except three base changes in the U3 "enhancer" area. The tRNA primer binding site showed perfect sequence complementarity to the 3' 18 nucleotides of glutamine tRNA. The putative plus DNA strand primer site in this proviral clone was a polypurine stretch, not distinctly different from those found in murine leukemia viruses. With reference to the nucleotide sequences elucidated for the region corresponding to the 5' portion of env gene of an infectious retrovirus, this proviral clone is closely related to the mink cell cytopathic focus-forming viruses, or MCF-type murine leukemia viruses, but not to the xenotropic-type virus. Thus, this work provides definite

experimental evidence for the proviral nature of MuLV-related sequences in the mouse chromosome.

(d) With the subcloning of specific sequences that distinguish the endogenous MuLV-related provirus from ecotropic or xenotropic MuLV proviruses, it is possible to perform RNA gel blot analyses to examine the expression of these endogenous retroviral genes in various mouse organs. Expression of these MuLV-related proviruses was found to be highest in the liver in two mouse strains that we studied. In this aspect, this class of endogenous retroviral genes appears to be also different from ecotropic or xenotropic murine leukemia proviruses that are found to express mainly in the lymphoid organs.

(e) Previous molecular cloning studies have often obtained MuLV-related proviral genomes with apparent sequence deletions in the proviral structure gene regions. This was observed in two of the six molecular clones we randomly selected for restriction mapping analysis; both of them showed sequence deletion within a BglII/BglII DNA fragment which includes 3' portion of gag gene and 5' portion of the pol gene. Employing specific sequence probe derived from this gag-pol junction region, we found in gel blot analysis of mouse chromosomal DNA that MuLV-related proviral genomes with the sequence deletion represent only a minor population while most of the endogenous MuLV-related proviruses are apparently intact. This indicates that recombinant DNA procedures, or bacterial hosts used for molecular cloning, tend to preferentially select for the isolation of defective proviral genome. We have also determined the nucleotide sequences at the site of sequence deletion in these two molecular clones and found that sequences deleted in the defective provirus are bounded by 7 base-pairs direct repeats in the intact provirus. This finding suggests that sequence deletions in the proviral structure gene region are not random events and may take place either by homologous recombination involving 7 base-pair repeats in the integrated proviral DNA within the mouse genome or by "jumping" of reverse transcriptase from one repeat to another during the process of proviral DNA formation prior to integration into the mouse genome.

A Novel Family of Retro-Transposons in the Mouse Genome. In a search for retrovirus-like structure genes that may be associated with the distinct type of LTR (called LTR-IS) in the mouse genome, a molecular clone, designated pIS23, was isolated. The mouse chromosomal DNA insert of pIS23 contained wo LTR-IS separated in a 5.6 kbp sequence distance. The 5.6 kbp sequence in pIS23 clone was found to represent a retroviral genome structure on the basis of the following findings: (a) nucleotide sequences of the two LTR-IS were nearly identical; (b) immediately linked to the 5' side of the 5' LTR-IS and to the 3' side of the 3' LTR-IS are CATT/CAT, suggesting the presence of short direct repeats; (c) at the putative tRNA primer binding site on the immediate 3' side of the 5' LTR-IS, the 18 nucleotide sequence is a perfect complementary match to the 3' portion of proline tRNA, while at the putative plus DNA strand primer site is a polypurine tract as expected; (d) at the corresponding regions located between the LTR-IS, nucleotide sequences are present which show significant

degrees of apparent homology to the gag-pol gene sequences of the Moloney murine leukemia virus genome, although there are apparent sequence deletions, particularly in the putative env gene region. We have employed a molecular probe derived from the putative gag-pol gene region of pIS23 and found that laboratory mice possess about 100 copies of this and homologous sequences dispersely in various chromosomal DNA sites. Two of these sequences are found in the DNA of male mice but not in the DNA of female mice, suggesting their location in the Y chromosome. Unique sequences located on both sides of the proviral structure in pIS23 were used to prepare molecular probes to characterize the integration site in the genome of the BALB/c mouse, the original source of pIS23, and five other laboratory mouse strains. It was found that the IS23 provirus integration is present only in the genome of BALB/c and A strain mice, but not in the same genomic site of the other 4 laboratory mouse strains. These results indicate that the proviral structure found in the pIS23 clone represents a novel family of the retro-transposable gene elements in the mouse genome.

Studies on "Anti-Cancer Genes" or Dominant Genetic Factors for Inhibition of Neoplastic Phenotype Expression of Oncogenes. Although we have still maintained a low level of research on the Fv-1 gene and other host genetic factors that affect the movement of murine leukemia virus genomes, a major new research effort has been initiated to investigate the possible presence of cellular genetic factors that affect the neoplastic phenotype expression caused by altered oncogenes. This involves especially two research projects. In the first research project, we have isolated different cell clones from our stock of NIH3T3 cell line, which was subcultured only a few times following receipt from Dr. Todaro in the early 1970's and tested for their susceptibility to neoplastic transformation of ras oncogenes by DNA transfection. While all 11 NIH3T3 cell clones were comparable in competence for DNA transfection, they differed considerably in showing transforming foci after DNA transfection with human bladder carcinoma oncogene, pT24 Ha-ras clone. Tests by animal inoculation indicated that two clones appeared to be resistant to neoplastic transformation by pT24 Ha-ras. Genetic cell hybrids between these two cell clones and a Ki-ras-transformed NIH3T3 cell clone, DT cells, mostly showed flat cell morphology, suggesting the genetic dominance of the ras resistant factor in these two NIH3T3 cell clones. The resistant property of these two cell clones appeared to be unstable and decreased considerably after several serial subcultivations in vitro. The second research project is a collaborative effort with Dr. Robert Bassin of NCI to investigate the molecular basis of dominant resistance to ras oncogene in his flat revertant cell clones that were isolated from mutagenized ras-transformed NIH3T3 cells. We have prepared DNA from these dominantly resistant revertant cells and found that the resistance property may be transferred by DNA transfection. This activity was lost following digestion of the DNA preparation with most restriction endonucleases except two, suggesting that the resistance property is residing in a DNA entity. We are currently trying to isolate this DNA entity by combining the DNA transfection and molecular cloning approaches.

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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 to bring about 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. Significant progress has been made in a number of experimental approaches.

Gene Isolation and Analysis. From a library of cloned cDNAs, synthesized via size-selected mRNAs from livers of hydrocortisone-treated rats, we have isolated six discrete cDNAs cognate to mRNAs whose expression is sensitive to modulation by glucocorticoids. Further testing for sensitivity to control by insulin and cyclic AMP revealed that one of these, cloned in plasmid number 33, hybridized to a 3.4 kb mRNA that is 5- to 6-fold increased by treatment with both insulin and cAMP as well as by hydrocortisone. Transcriptional runoff assays in isolated nuclei have demonstrated that the rate of transcription is increased by each of the hormonal inducers, and to an extent commensurate with increased mRNA levels. Southern blotting of rat genomic DNA after cutting with a variety of restriction enzymes yielded single bands when probed with pc-33, indicating that the gene is present as a single copy in the haploid rat genome. This gene is also regulated during hepatocyte differentiation, being expressed at very low levels during rat development until activated just before and at birth. These findings of a single gene, subject to multihormonal as well as developmental regulation, have prompted us to isolate and characterize the gene, which we have provisionally termed "gene 33".

Screening of two libraries of cloned rat genomic DNA, using pc-33 as probe, has yielded a clone carrying a 14.5 kbp insert of rat DNA of which 13.9 kbp comprise the entirety of gene 33. Several restriction-cut fragments were subcloned in plasmids, and one of these, a 2 kbp fragment shown to contain the 5' terminus as well as ca. 1 kbp of the 5' flanking region, was selected for sequencing. The transcriptional start site was identified by S1 nuclease protection after hybridization to the mRNA (purified by hybrid selection); transcription is initiated at a G preceded by a C, as is common to eucaryotic genes. A total of 999 bp was sequenced, 519 bp in the mRNA coding region and 480 bp of the 5' flanking region. A TATA box was found 31 bp upstream and a CAAT box 89 bp upstream from the start site; these are also common to eucaryotic genes. Four regions were detected with significant homology to concensus sequences for glucocorticoid receptor binding. Two of these are overlapping, found at -433 and -423 bp in the sense strand; they are 75% and 69% homologous, respectively, to the known site of the chicken lysozyme gene. A third putative site begins at -268 in the antisense strand, and the fourth is at -241; these are 56% and 69% homologous, respectively, to known sites for receptor binding. We found no sequences homologous to published sites implicated in control by cAMP. As yet there are no published data suggestive of specific regions in DNA involved in regulation of gene expression by insulin. The entire region sequenced is unusually rich in GC bp, 70% compared to the 41% of total rat DNA. Also the dinucleotide sequence CG, a potential site for methylation and thereby for control of expression, is overrepresented. Between positions -1 and -180 the CG sequence occurs 27 times, nearly three times as often as the expected random occurrence. These data will be of great value in future probes of the mechanisms of hormonal and developmental regulation of expression of this gene.

In the mRNA coding region a protein synthesis initiation codon was found at +161; this is followed by an open reading frame (less a putative intron from +198 to +498, bounded by typical splice junctions) which continues to the 3' end of the sequenced DNA. From these data a 19 amino acid sequence beginning at the N-terminus of the gene 33 protein product could be derived. Computer search of the polypeptide sequences stored in the Protein Identification Resource data base showed no significant homology with known proteins. Extensive searching of three nucleic acid data bases revealed no major homologies of any of the stored sequences to either the mRNA coding region or the 5' flanking region of gene 33. From these data we believe that this gene has not been characterized before. This is in accord with our inability to match other known properties of the gene 33 protein to known proteins, for example its subunit size (53 kDa) or its highly unusual capacity to respond to the three hormonal regulators cited. In these properties as well as in the kinetics of response to hormonal inducers in adult liver it is virtually identical to tyrosine aminotransferase, but other physiological aspects of expression of these genes differ and by hybridizations we have shown that they are not related.

Gene 33 mRNA is found on nonmembrane-bound polysomes in the liver and is, then, presumably translated into an intracellular protein. Attempts were made to further characterize the protein synthesized in reticulocyte

lysates supplemented with the mRNA purified by hybrid selection. The 53 kDa protein under denaturing conditions has a pI of 6.65 and does not migrate on nondenaturing gels. When examined on nondenaturing sucrose gradients greater than 90% of the translation product sedimented as a 53 kDa species, suggesting a monomeric protein. However that the reticulocyte lysate lacks components necessary for oligomerization cannot be excluded. We are continuing in attempts to define the protein product of this gene and especially its physiological significance.

Hormonal Regulation. In earlier studies on the mechanisms by which the various hormonal agents induce tyrosine aminotransferase (TAT), we reached the tentative conclusion that induction by insulin reflects control at the translational level. This was based, necessarily, on measurements of only the protein gene product and on indirect indicators such as the kinetics of induction, effects of inhibitors, and the consequences of sequential treatment with the various hormonal inducers. With the means now in hand for a definitive analysis (cloned cDNA specific for TAT, courtesy of Dr. Günther Schütz, German Cancer Research Center, Heidelberg) we reexamined this question, assessing the levels of TAT mRNA by hybridization, then directly measuring the rate of transcription of the gene in nuclear transcriptional runoff analyses. The results are unequivocal, establishing that transcription is enhanced about 4-fold after insulin treatment of adrenalectomized rats, an effect entirely sufficient to account for similar increases in the mRNA and the enzyme. The effect of insulin treatment on transcription is very fast; the rate is significantly increased within a few minutes of an intraperitoneal injection. The rapidity of this effect of insulin — much faster than that of glucocorticoids — may account for the discrepancy between our earlier conclusions and these definitive results.

We have established then that insulin enhances transcription of both TAT and gene 33 in rat liver. Others have analyzed the effect of insulin on phosphoenolpyruvate carboxykinase (PEPCK) in rat liver; in this instance transcription of the gene is rapidly repressed. In all three cases it is known that the effect of insulin is mediated by reaction with its membrane-bound receptor, but there is no information as yet on how this interaction results in control of expression of specific genes, sometimes positive and in one case, at least, negative. We anticipate that definition of the mechanisms involved will be an intriguing aspect of future research.

We have recently completed a project designed to determine the spectrum of responsiveness in fetal liver of genes known to be responsive to hormonal agents in the adult. Five cloned cDNAs were used in these analyses, two specific for known genes (TAT and PEPCK, the latter courtesy of Dr. Richard Hanson, Case Western Reserve University), and three of unknown specificity (termed 33, 35, and 38). All of these hybridize to glucocorticoid-inducible mRNAs in adult liver, some are responsive to insulin, others to cAMP, and two (TAT and gene 33) are inducible by all three of these agents. The results of analyses of the respective mRNAs (dot and northern blot hybridizations) after treatment of fetuses in utero with these hormones can be summarized as: all genes responsive to cAMP in

the adult are similarly responsive in fetal liver; both insulin and glucocorticoids enhance expression of gene 33, but do not affect either TAT or PEPCK significantly; insulin, but not glucocorticoids, has marginal effects on expression of genes 35 and 38. Two aspects of these data must be noted: (i) the significance of marginal responses is dubious, owing to the very low levels of these mRNAs and consequent difficulties in quantitating them; (ii) where responses are considered truly significant (e.g., effects of cAMP on several genes, or of glucocorticoids and insulin on gene 33) the "fold" increase in mRNAs is comparable to that in the adult, but this is from a much lower basal level. This is entirely consistent with the emerging realization that hormones act as "enhancers" of gene expression but not as "activators"; the limited expression of these genes in fetal liver prior to their developmental activation can be accelerated to an extent by hormones, but the full response is determined by promoter activity. With this realization we can conclude that all components of induction by cAMP are functional in fetal liver, and the cAMP-responsive genes tested are all capable of response at this stage. Both insulin and glucocorticoids, however, are effective in fetal liver only on certain genes. As the capacity for these hormones to effect induction is clearly present, the differential response appears to lie at the level of the specific gene. This implies that some structural modification conferring responsiveness to insulin or glucocorticoids, perhaps demethylation, occurs at different times during development for specific genes.

The capacity of TAT to respond to glucocorticoids is known to occur at birth, when expression of this gene is activated to the adult level. We asked if responsiveness to insulin is similarly timed, but found that expression is not enhanced until 48 to 72 h post partum. To evaluate the mechanistic significance of this and the other observations from this project, all essentially descriptive at this point, will require much further experimentation defining gene structures and their modifications during development.

Tissue-Specific Expression. In earlier reports we have described analyses of the tissue specificity of expression of TAT and gene 33. TAT was found to be truly liver-specific, while gene 33 is expressed in all tissues examined but at various levels; expression of this gene is highest in liver, very significant in kidney, and low but definitely detectable in lung, testis, and heart. TAT and gene 33 are hormonally regulated in identical fashion in the liver and it was therefore of interest to determine their responses in other tissues. Expression of TAT was not influenced in tissues other than liver; thus it is truly silent in nonhepatic tissues. Hydrocortisone induced gene 33 in each of the tissues, but either insulin or cAMP were effective only in liver and kidney. Whether the latter two agents are delivered to the tissues that did not respond in effective quantities after an intraperitoneal injection is a problem that has not been resolved. That a similar injection of the steroid is effective in all tissues would argue against this interpretation.

Differentiation. Our recent work in this area has focused on deletion mutants of the c locus in mice from the Division's mouse genetics facility. In certain of these mutant strains differentiation of the liver is impaired, the impairments including failure of expression of liver enzymes such as TAT to become activated in the normal fashion at birth. Since earlier measurements were made entirely at the level of protein gene products we began by assessing mRNA levels in newborns of genes for which cloned cDNA probes are available. Synthesis of albumin and α-fetoprotein has been reported to be severely reduced in homozygous mutants (albino); we found these mRNAs to be at the same or higher levels than in heterozygotes or homozygous normal litter mates. Apparently the defective synthesis of these secreted proteins relates to abnormalities in the endoplasmic reticulum and not to expression of the genes. An interesting corollary is the implication that mRNAs normally translated while membrane-bound are poorly translated if membranes are defective. TAT-specific mRNA was not increased after birth in albinos while it was in heterozygous or normal litter mates, in accord with earlier enzyme measurements. As we have established that developmental activation of this gene reflects activation of transcription, the earlier conclusion, that the c locus region contains a trans acting regulatory gene whose product is required for activation of transcription of certain genes in the liver, is confirmed. We also measured PEPCK mRNA; this gene is developmentally activated at birth like TAT but the enzyme was reported to be at normal levels in newborn albino mutants. Contrary to these reports we found the mRNA levels to be reduced in homozygous mutants, although not as severely as TAT. Developmental activation of gene 33, on the other hand, was not influenced by the mutation. For most of these analyses we used cRNA probes, prepared by cloning cDNAs into the SP6 transcription vector. This provides much greater sensitivity to these hybridization assays using heterologous (i.e., rat DNA) probes.

In earlier work we have treated rat fetuses in utero with the DNA hypomethylating agent, 5-azacytidine, and found it to have profound effects on differentiation of the liver. Morphological and biochemical changes indicate a drug-induced advancement in time of events normally occurring only after birth, including activation to full expression of genes such as TAT, PEPCK, and gene 33. It was therefore of interest to ask if this agent might overcome the faulty activation of gene expression in mutants lacking the trans acting regulatory gene. Azacytidine was administered to mouse fetuses in the uterus of heterozygous dams, two days prior to their expected time of delivery. Unlike comparable experiments with rats, these mice do not tolerate the required surgery and/or drug treatment well, and in only one did the treated fetuses survive long enough to complete the experiment. But the data from this experiment are clear and very promising, as expression of the TAT gene was clearly activated by the drug in homozygous mutants as well as in heterozygotes and homozygous normals. A number of attempts to repeat this, by treatment of fetuses in utero, those delivered early by caesarian section, or newborns delivered normally, have not succeeded. We are continuing these attempts to confirm the preliminary indication that artificial demethylation bypasses the requirement for the regulatory gene, an indication that may implicate

selective gene demethylation in the action of the putative trans acting product of that gene. We are also currently cloning the mouse TAT gene to aid in future analyses of these phenomena.

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MONOCLONAL ANTIBODIES FOR DIAGNOSIS AND THERAPY

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Tumor Antigen Characterization. Cell surface proteins mediate interaction between cells and their environment. Unique tumor cell surface proteins are being identified and quantified in several tumor systems to address the following questions: (1) How are genes for cell surface proteins regulated during cell transformation? (2) What properties make these proteins good targets for antibody dependent tumor cell destruction in vivo? (3) Can sensitive immunoassay of these proteins provide a means to quantify transformation frequencies?

A tumor surface protein of 180,000 M_r (TSP-180) has been identified on cells of several lung 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.

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. M_r 180 kd is phosphorylated. Differential labelling experiments indicate that the larger forms (M_r 180 kd and M_r 160 kd) are external while a large membrane bound but internal pool of smaller molecules (M_r 140-150 kd, M_r 135 kd and M_r 115 kd) represents the majority of cell associated forms detected by the two-site assay. Finally, the distribution of the different forms appears to vary with the stage of cell malignancy as mammary cell cultures progress from a preneoplastic to neoplastic state.

Tumor Therapy with MoAb. Syngeneic monoclonal antibodies (MoAb) to Moloney sarcoma cells (MSC) were produced by fusion of spleen cells from MSC regressor mice to myeloma SP2/0. MoAb 244-19A, a mouse IgG_{2b}, bound to MSC cells and did not bind to 12 other murine sarcomas, a carcinoma (Line 1), a fibroblast (A31) or a fibroblast infected with C-type virus (A31-Moloney leukemia virus). Radiolabelled MoAb 244-19A was cleared rapidly from circulation of tumor bearing animals, but circulated normally in normal animals indicating that the epitope reacting with this MoAb is not present in normal animals and may be specific to the MSC tumor. In contrast, MoAb 271-1A bound to the MSC and Ha2 sarcoma and Line 1 carcinoma as well as to the normal and infected fibroblast cultures. Antibodies were tested for therapeutic effect using three schedules of antibody injection. Intraperitoneal injection of ascites fluid containing 244-19A MoAb given on days -1, 0, and +1 relative to tumor cell injection, increased life span significantly over that of control animals given injections (P3, immunoglobulin G, or MoAb 271-1A) and produced seven of 19, one of five, and one of five long-term survivors in three separate experiments. Antibody given to animals with established tumors (4 days after implantation) also prolonged life span significantly and produced three long-term survivors of nine treated animals. Antibody given to animals with very large tumor burdens (10 days after implantation) did not prolong life span significantly.

Doses up to 1-2 mg per animal increase chances of survival and cure. In some experiments 100% of the treated groups are cured. Splitting the total dose of antibody into several injections is a more effective treatment than giving one single injection. Finally, the therapy is effective in nu/nu mice lacking T cells, BALB/c mice which are depleted of complement activity, mice depleted of macrophage activity, and mice compromised by 400 or 800 rads of X ray. These studies indicate that the antibody probably works through a mechanism which involves the host immune system for an antibody dependent cell mediated cytotoxicity function, but the host cell which mediates this function remains poorly defined.

Since this MoAb appears to be unique in tumor specificity, it was important to determine if such antibody specificity is common during rejection phases of the tumors. About 40 MoAb producing hybridomas were isolated from fusion of spleen cells of MSC regressor animals with SP2/0 myeloma. Most of these antibodies bind to fibroblasts as well as to tumor cells and seem to lack any functional relationship to the regression phenomenon.

Monoclonal Antibody for Lung Toxicology. Rat MoAb 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. Preliminary evidence indicates that one MoAb monitors mature Type I cells in mouse lung sections. This antibody is being used to assess cell death and turnover as a function of time after different toxic insults.

Five other MoAb appear to be useful for flow microflorimeter studies. If these MoAb's define different cell types, monitoring of cell kinetics will be greatly simplified, and mechanisms of development of fibrosis and emphysema will be more easily studied.

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

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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 Division.

During the past year the Cytometrics Group became involved in work in the area of high resolution digital image analysis by computer. A general purpose digital image processing system and a high resolution image scanner were added to the existing computer facility in the Division. In collaboration with the Chemical Carcinogenesis Group a system of programs has been developed that enables us to perform quantitative two-dimensional gel electrophoresis.

Computer-Assisted Quantitative Two-Dimensional Gel Electrophoresis. Mammalian cells contain thousands of proteins, each representing a specific function for maintaining the cell's biochemical, genetic, or structural homeostasis. When a physical process or a chemical insult occurs, enzymatic systems are stimulated to repair the damage and/or metabolize xenobiotics to less toxic forms for excretion. Two-dimensional gel electrophoresis is uniquely sensitive in its ability to separate these proteins in the first dimension by isoelectric focusing as a function of net charge, and in the second dimension as a function of molecular weight. In order to fully comprehend the transformation of a normal cell to a diseased cell (e.g. cancer, mutation) it is critical to determine the cascade of events that accompany this transformation process. Analysis of the voluminous information inherent in a two-dimensional gel requires localization, identification and quantitation of each protein. It is also necessary to make facile comparisons between multiple gels that reflect protein patterns from various disease states or after carcinogen or mutagen treatment, in order to demonstrate subtle changes that occur in the progression to the diseased state. The images to be analyzed are generated on X-ray film by radiolabeled proteins in the electrophoresis gels. We are dealing with one spectral band, i.e. images with grayvalues between 0 (black) and 255 (white), which correspond to optical densities (0-3 OD). The system under development accomplishes the following main tasks: (1) the detection of protein spots, (2) quantification of spots, i.e. intensity distribution and to a certain extent spot shape, and (3) the statistical comparison of several images in order to assess changes in the protein patterns. We have been performing experiments involving computer simulations to assess the performance of the analysis algorithms. For isolated spots we found relative errors in the radioactivity estimates between 10% and 20%. An increase in computational effort can be expected to lower these error margins, and work is under way to achieve this. Preliminary data on spot detection error rates were obtained by comparing the computer results to the analyses by two independent human observers based on a sample of 20 image sections. An average false negative rate (i.e. spots missed by computer) of ~8%, and false positive rate of ~4% could be observed. Detection errors mainly occurred with very faint spots and in crowded areas of the gel where many spots sometimes badly overlapped. Work is under way to improve these error rates by following a different conceptual approach to gel image analysis that allows us to incorporate a priori knowledge obtained from the analysis of multiple gels in a particular experiment under consideration.

Studies Involving Flow Cytometry and Cell Sorting. We had previously shown that Legionella, the bacterium that causes a sometimes fatal pneumonia, also known as Legionnaire's disease, can be detected in environmental water samples using flow cytometry. Preliminary results indicated that it might also be possible to distinguish between Legionella populations of high and low virulence on the basis of their FCM data patterns. With assistance from the ORNL Exploratory Studies Program three sets of experiments were performed: serial animal passage test of virulence with simultaneous FCM analysis, analysis of Legionella at different temperatures, and analysis of clinical isolates. Our results

show that high-virulence bacteria populations do indeed consistently exhibit an FCM data pattern that we previously had associated with high virulence (pattern B). However, not all Legionella populations showing this data pattern are virulent when tested in guinea pigs. Thus, FCM pattern B is a necessary but not sufficient condition for virulence. Further work on this problem is under way.

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 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 O^6-methylguanine repair in C3H10T1/2 cells by measuring the enzyme O^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.

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METABOLIC ACTIVATION AND CARCINOGEN METABOLISM

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The study of polyaromatic hydrocarbons and their role in carcinogenesis has grown in logarithmic proportions over the last decade. The growth has largely been due to more sensitive methods of analysis, better synthetic schemes and a whole series of bacterial and mammalian assays for toxicity, mutagenicity, and carcinogenicity. It is generally assumed that the parent molecules of environmentally prevalent chemical carcinogens are structurally stable and relatively inactive metabolically. Therefore, it is apparent that chemical carcinogens require activation within the body in order to exert their carcinogenic potential. Our laboratory has therefore been attempting to probe more deeply into the cellular processing of the carcinogen as it passes through the steps of activation and on toward detoxification. We have been focusing more directly on those particular biochemical steps that are most critical in determining whether an individual cell will undergo malignant transformation or continue on its normal phenotypic state by successfully detoxifying and excreting the carcinogen. Activation of the carcinogen is accomplished by the microsomal monooxygenases that transform the parent molecule into its highly reactive electrophilic intermediate that can interact with cellular nucleophiles such as RNA, DNA, and protein. We have chosen benzo(a)pyrene [B(a)P] as the prototype chemical carcinogen. It is ubiquitous in the environment and created by the incomplete pyrolysis of carbonacious material. Over the last decade the majority of metabolic studies with polycyclic aromatic carcinogens has centered around the organic solvent soluble derivatives, such as dihydrodiols, phenols, and quinones. However, it is becoming increasingly clear that both susceptible and resistant cells to malignant transformation form the identical pattern of organic solvent soluble metabolites. It is now important to probe the water soluble fraction containing various glucuronides, sulfates, and glutathione conjugates, in order to understand the metabolic role these detoxification steps play in directing the reactive carcinogen away from critical cellular sites for malignant transformation. It is probable that the conjugation reaction within the biochemical process helps determine susceptibility and resistance of a given cell toward transformation.

High-Pressure Liquid Chromatography of Benzo(a)pyrene Glucuronide, Sulfate, and Glutathione Conjugates. While high-pressure liquid chromatography (HPLC) has made significant progress in understanding organic solvent-solvent metabolites, conjugation of water-soluble products,

which are of high molecular weight and contain numerous functional groups, have been more difficult to separate using current HPLC technology. However, we have found by adding acid in the solvent regimen, we have been successful in separating B(a)P glucuronides from glutathiones and have begun studies to understand the possible role of this conjugation step in detoxifying reactive electrophiles of polycyclic hydrocarbons. The relative selectivity of various oxygenated B(a)P intermediates may be an important factor in determining how much reactive electrophile will be available for interaction with critical target sites in the cell for malignant transformation. Incubation of B(a)P with hamster embryonic fibroblasts for 24 h resulted in metabolism of ~88% of the initial B(a)P. Organic soluble metabolites consisted of tetrols, triols, dihydrodiols, quinones, and phenols, comprising about 16% of the total metabolites. Water-soluble metabolites were subjected to enzymatic hydrolysis by β-glucuronidase treatment and the resulting freed metabolites were extracted into ethyl acetate. 9-Hydroxy and 3-hydroxy phenols were the predominant products released, accounting for ~38% of the total metabolites. Quinones and tetrols were also liberated by β-glucuronidase treatment. Addition of sulfatase to culture media after organic extraction failed to release any detectable levels of B(a)P metabolites. The remaining 31% of water-soluble B(a)P metabolites were resistant to β-glucuronidase and sulfatase treatment. HPLC analyses of these water-soluble metabolites were performed using a water:methanol (0.075% trifluoroacetic acid) gradient (0.40%/10 min:40-70%/30 min) and showed most of the remaining radioactivity eluting as a complex of peaks at low UV absorbance ($A_{254}<0.005$). These peaks eluted in the area of the chromatogram with the BaP-4,5-GSH standard, inferring that these metabolites are a family of glutathione conjugates.

Based on the following results, similar studies were undertaken using human mammary tumor T47D cells. This cell line is the most active metabolizer for B(a)P with a turnover rate of greater than 95% at 1 g/ml after 24 h incubation. As with the hamster embryonic fibroblasts B(a)P metabolites were predominantly tetrols, triols, dihydrodiols, quinones, and phenols. Glucuronide conjugation was minor in human cells. Many water-soluble metabolites could be extracted with butanol or removed from the culture medium protein with methanol:water, and also indicated these products to be a family of glutathione conjugates.

Sister Chromatid Exchange Studies in Variant Mouse Hepatoma Cells. Two variant mouse hepatoma cell lines had been separated from the parent cell line HEPA-1C1C7 by fluorescence activated cell sorting. Our earlier metabolic studies showed that the variant TAOC1BP^{r}C1 was more active in the metabolism of B(a)P than the variant BP^{r}C1. In an extension of these studies the relationship between the metabolic capabilities of these two cell variants and the induction of sister chromatid exchanges by B(a)P was investigated. It was observed that the TAOC1BP^{r}C1 yielded a significant dose-dependent increase in the induction of sister chromatid exchange by B(a)P, whereas BP^{r}C1 did not show a response significantly greater than control. The metabolic results indicated that the induction of sister chromatid exchange in TAOC1BP^{r}C1 was due to the production of

7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene by this variant. This metabolite did not appear to be produced by BP^{r}Cl. Furthermore, TAOClBP^{r}Cl required only 40 nM of B(a)P to produce a twofold increase in sister chromatid exchange frequency. This concentration is considerably lower than that required to elicit a similar response in other reported cell lines. This may be the first use of a mouse hepatoma cell line for determining the relationship of metabolic capability to the induction of sister chromatid exchange.

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RADIATION CARCINOGENESIS

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M. Terzaghi-Howe
B. E. Allen
N. D. Bowles
M. C. Jernigan
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C. K. McKeown
S. P. Ogle
L. C. Satterfield
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Research in the ionizing radiation carcinogenesis program emphasizes studies on neutron carcinogenesis, time-dose relationships, the role of host factors in radiation carcinogenesis, and the dynamics of the carcinogenic process after exposure to radiation and chemicals. These problems are being approached on several different levels using in vivo as well as in vitro and in vivo/in vitro methods. Common to all these studies is an emphasis on the examination of mechanisms and the establishment of general principles which may allow a better understanding of the risks to humans from radiation exposure. Data from these studies also are used to develop more direct 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. The radiation exposures which are being performed at the Health Physics Research Reactor will be complete by the end of 1985.

A program to study myeloid leukemogenesis following neutron irradiation has also been under way for the past 3 years. These studies are designed to examine the neutron dose response, as well as the possible role of specific chromosome aberrations in myeloid leukemogenesis after neutron exposures in collaboration with R. J. Preston (Biology Division). Data obtained thus far indicate that the dose response for the induction of myeloid leukemia is linear over the 0–80 rad neutron dose range. A comparison of these data with previous data for γ-ray induction of myeloid leukemia indicates an RBE of ~2.5.

We have previously demonstrated that protraction of neutron exposures enhances the mammary tumorigenic effect of neutrons. We postulated that this effect could be a result of either enhanced transformation rates or a

result of influences on tumor expression similar to tumor promotion. To differentiate between these possibilities we have examined the sequential cellular changes which occur during in vivo tumor development following neutron irradiation and determine the effects of neutron dose rate on that sequence of events. This approach separated the initial transformational effects of neutron irradiation from those effects which influenced tumor expression. Female BALB/c mice were irradiated with fission-spectrum neutrons at dose rates of 1 rad/min or 1 rad/day. Twenty four hours or 16 weeks after irradiation, mammary cells were obtained by enzymatic dissociation. Mammary outgrowths were derived by injection of 10^4 cells into gland-free fat pads of 3-week-old female BALB/c mice. The frequency of ductal dysplasias in outgrowths from cells irradiated at high or low dose rates were similar. Persistence of dysplasias differed markedly. Few of the dysplasias in outgrowths derived from cells irradiated at the high dose rate persisted, while a large fraction of the dysplasias in outgrowths derived from cells irradiated at low dose rate persisted. When cells remained in situ for 16 weeks prior to dissociation, a higher frequency of persistent altered cells was also observed in outgrowths derived from cells irradiated at low neutron dose rates. These data suggest that low dose rate neutron exposures enhance the probability of progression of carcinogen altered cells rather than increase the numbers of initiated cells.

Time-Dose Relationships. Interpretation of differences in the effects on tumorigenesis of different rates of exposure may be confounded by their effects on factors influencing tumor expression rather than the induction of initial events. To develop reliable estimates of radiation risk and to determine the general applicability of the principles derived from experimental studies, more information on time-dose relationships for the induction of solid tumors and the basis for these relationships is required. Further information on the underlying mechanisms for these time-dose relationships is also essential. A program designed to examine time-dose relationships for radiation carcinogenesis has been undertaken to develop needed information. The objectives of these studies are to: (1) examine time-dose relationships for the induction of lung adenocarcinomas and mammary adenocarcinomas in BALB/c mice after gamma ray irradiation, including the influence of dose rate and fractionation; (2) determine whether dose rate effects are a result of repair or recovery from initial carcinogenic events or due to changes in mechanisms related to tumor expression; and (3) examine the persistence of latent carcinogenic effects. Data obtained relevant to objective one indicate that the biophysical model based upon the theory of dual radiation action adequately predicted the effects observed following both protracted and fractionated exposures. When exposures are fractionated the response is dependent upon the dose/fraction. If the dose/fraction is a dose which lies on the predominantly linear portion of the dose response, then the response should be dose-rate independent and the incidence similar to that after low dose-rate exposure. If the dose/fraction is in a region where the quadratic portion of the response makes a significant contribution to the dose response, then the incidence is no longer dose-rate independent and is predicted to be higher than that observed following a low dose-rate

exposure. Studies related to objectives two and three are being conducted using the mammary cell dissociation system.

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

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

Effect of Cell Interactions on Neoplastic Development. We have evaluated (1) the influence of intracellular 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 (Gray et al., In Vitro 19: 559-570, 1983) or in vivo in denuded tracheal grafts (Terzaghi et al., J. Natl. Cancer Inst. 65: 1039-1048, 1980). Following coculture in vivo or in vitro the relative proportions of diploid (normal) and aneuploid (carcinogen-altered) cells are determined. Mixed populations are harvested enzymatically, stained with propidium iodide, and DNA distribution profiles generated by means of flow cytometry.

Normal cells exert a strong inhibitory effect on both preneoplastic and neoplastic cells both in vivo and in vitro. In vivo inhibition is observed even when a small fraction of normal cells is inoculated with preneoplastic or neoplastic cells. This suggests that direct cell contact is not of primary importance. In cell culture neoplastic and preneoplastic RTEC are inhibited when cocultured with normal RTEC. This effect is at least partially mediated by the release of an inhibitor into the culture medium of an inhibitor by normal RTEC. This inhibitor has been identified as transforming growth factor type -β. It is not clear whether inhibition of carcinogen-altered cell in vivo by normal RTEC is also mediated by TGF-β.

Malignancy of Cells Transformed by Radiation and Chemical Carcinogens. In order to determine whether type III transformed 20 T1/2 cells initiated by chemical carcinogens, low LET radiation (x rays) or high-LET radiation (neutrons) systematically differ in malignancy the following experiments are being carried out. Type III foci isolated following exposure to various carcinogens are injected into syngeneic, C3H mice at doses of 10^3 to 10^6 cells per site. Tumors are scored up to 6 mos after cell inoculations. Differences between type III foci in terms of the number of cells required to yield a tumor were determined. To date no systematic differences between various type III foci have been observed. However, many foci are not yielding tumors even after inoculation of 10^6 cells. Thus, we are now trying C3H nude mice as test animals. To date no conclusions can be reached as to whether or not the initiating agent affects the "malignancy" of resulting type III foci.

Effect of Low and High LET Radiation on the Growth of Rat Tracheal Epithelial Cell in Culture. Previous studies indicated that low-LET radiation did not enhance the growth of rat RTEC as did chemical carcinogens. It was thus of interest to determine whether high-LET radiation could affect the growth potential of tracheal epithelial cells in culture. These experiments have been temporarily delayed until such time as we can develop a serum-free medium for rat tracheal epithelial cells. Attempts to date have been hindered due to overgrowth of fibroblasts. We hope to control this problem by dropping serum levels to <0.5%.

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) The basis of strain-dependent differences in susceptibility, in particular, the role of hormonal growth factors such as glucocorticoids. (3) 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). (4) 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 previously shown that protracted exposures to UVR enhanced the expression of skin cancer initiated by PUVA and also by urethane. There has long been a question of whether ionizing radiation and other agents also interact in the induction of skin cancer. Some thirty years ago Shubik et al. (Nature 171: 934-935, 1953) obtained equivocal results about the promotion of beta-irradiation-induced skin cancer with croton oil.

The question of whether or not UVR enhances X ray-induced skin cancer is of importance clinically and in estimating risks. For example, space workers will be exposed to appreciable skin doses of ionizing radiation while in space and to UVR on the earthly sojourns. The evidence is accumulating that exposure to sunlight increased the risk about eightfold of skin cancer in patients treated for tinea capitis with X rays (Shore et al., Radiat. Res. 100: 192-204, 1984).

Mice exposed to soft X rays in a fractionation regimen developed no carcinomas or sarcomas, and only 6% of benign tumors when the total exposure was 4000R. When the various fractionation regimens were followed by 24 weeks of treatment with TPA, cancers occurred. Exposure to 2000R and 4000R, plus TPA, resulted in an incidence of 36% and 82% carcinomas, respectively. When the 16 X ray exposures (4000R) were followed by 24 weeks of exposures to low doses of UVR, 60% of the mice developed carcinomas. Initial results suggest that exposures to small doses of PUVA also enhance the expression of X ray initiated cells. These findings illustrate the importance of interactions in skin carcinogenesis. The question of interest is whether or not the mechanisms by which such diverse agents as TPA, UVR, and PUVA increased the expression of initiated cells. It is not known whether quite different mechanisms are involved with the different agents or if a common mechanism is involved, for example, immune suppression.

The role of the skin in immunologic responses is now known to be complex and even to involve keratinocytes. We have chosen to investigate Langerhans cells and the effects of X rays, UVR, PUVA and TPA. The effect of TPA appears to be associated with the epithelial hyperplasia that TPA induces. When hyperplasia follows TPA treatment the number of Langerhans cells also increases. This is consistent with the apparent correlation, that we have noted, between the number of keratinocytes per unit area and the number of Langerhans cells. Exposures to X rays, UVR and PUVA reduce the number of Langerhans cells that can be demonstrated in a dose-dependent manner. However, the kinetics of recovery are quite different after exposure to X rays from that after UVR with the apparent reduction in Langerhans cells lasting considerably longer after X rays. X-irradiation reduces DTH whereas UVR has little effect. It is not clear whether the X ray-induced effect on the Langerhans cells is related to the change in cellular immunity indicated by the reduction in DTH. It seems likely from our results that the target in the Langerhans cells is different for X rays and UVR.

Another factor that influences the susceptibility for skin cancer is the hormonal effect on epidermal cell growth. Our approach to this question has been to assay glucocorticoid receptors in strains that show a marked difference in susceptibility to the induction of skin cancer and after exposure to UVR or UVR and TPA.

Previous experiments demonstrated that the concentration of glucocorticoid (GC) receptor in skin is significantly reduced in SENCAR mice treated topically with TPA or other hyperplasiogenic agents. Treatment protocols revealed that after a single application, the reduction in GC receptor correlated with hyperplasiogenicity, whereas, after repetitive applications, the reduction correlated with tumor promoting activity. In order to determine if GC receptors may be involved in the mechanism by which ultraviolet radiation (UVR) induces skin cancer in mice or if susceptibility to UVR-induced skin cancer is influenced by changes in GC receptor content, SKH- and HRS-hairless mice were exposed to 1250 J/m^2 UVR 3 times a week for varying lengths of time (3-54 exposures). The

animals were killed 24, 48 and 72 h after exposure; GC receptor content in the skin was determined. The results showed that in SKH-hairless mice, susceptible to UVR-induced skin cancer, the reduction in the concentration of GC receptor was greater than in the less susceptible HRS-hairless mice, but only after 54 exposures. The magnitude of the reduction was less than that observed in SENCAR mice after TPA treatment and may reflect the longer latency and a less marked hyperplasia in hairless mice.

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11. Sinclair, W. K., and R. J. M. Fry. General discussion on assessment of radiation risks for space flight. Adv. Space Res. 4(10): 287-288, 1984.
12. Storer, J. B. Carcinogenic effects on humans: An overview. In: Radiation Carcinogenesis, ed. by A. C. Upton. Elsevier/North-Holland Biomedical Press, Amsterdam, in press.

13. Terzaghi-Howe, M., and C. McKeown. Inhibition of carcinogen-altered rat tracheal epithelial cells by normal epithelial cell-conditioned-medium. Cancer Res., in press.
14. Totter, J. R., H. I. Adler, and J. B. Storer. The relationship of cancer mortality to life span and food supply rate. Nucl. Sci. Eng. 90: 459-466, 1985.
15. Ullrich, R. L. The rate of progression of radiation-transformed mammary epithelial cells is enhanced after low dose rate neutron irradiation. Radiat. Res., in press.
16. Weinberg, A. M., and J. B. Storer. Ambiguous carcinogens and their regulation. Risk Analysis 5(2): 151-156, 1985.

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. 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 9 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 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 40 students working toward the Ph.D. degree and 1 in the Master's program. As of October 1985, 104 students have been awarded the Ph.D. degree.

The fall quarter of 1985 marks the beginning of the School's twentieth year. W. Edgar Barnett is the Director. The School also has three full-time 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 "shared" faculty.

The students form a very active group of investigators in training, and their names appear on a number of manuscripts each year. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division.

Master of Science Degree Program in Biotechnology

A new Master of Science degree program in biotechnology has been started in conjunction with the University of Tennessee. The first year's class consists of 7 students who carry out part of their laboratory training in the fermentation plant of the Biology Division. Staff members of the Biology Division and other Divisions have been involved in setting up the program and are actively teaching in it.

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 13 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 1985

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. 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, 1984 through September 30, 1985.

Speaker	Affiliation	Subject
Brain, J.	Harvard School of Public Health Boston, Massachusetts	Biomagnetometry: A new tool for pulmonary cell biology
Brinster, Ralph L.	School of Veterinary Medicine University of Pennsylvania Philadelphia, Pennsylvania	Introduction of genes into animals
Burma, D. P.	Banaras Hindu University Varanasi, UP, India	Translocations in protein synthesis
Ehling, Udo H.	Institüt für Genetik Neuherberg, FRG	Induction of gene mutations in mice: The multiple end point approach
Elliott, Rosemary	Roswell Park Memorial Institute Buffalo, New York	Analysis of three gene families in the mouse
Ethier, S. P.	Michigan Cancer Foundation Detroit, Michigan	The proliferative life-span of normal and carcinogen-treated rat mammary epithelial cells *in vitro*
Gallahan, Daniel	University of Maryland College Park, Maryland and National Cancer Institute Bethesda, Maryland	Identification and characterization of a new mmTV proviral insertion site (*int*-3) in mammary tumors of *Mus musculus* wild mouse populations

Godleski, John	Brigham & Womens Hospital Boston, Massachusetts	Surface antigens on lung macrophages
Hampsey, D. Michael	University of Rochester Rochester, New York	Dispensable and indispensable residue of cytochrome *c*: A genetic approach
Ihle, James N.	Frederick Cancer Research Center Frederick, Maryland	Interleukin-3 regulation of growth of normal and neoplastic hematopoietic cells
Klyosov, A. A.	A. N. Bach Institute of Biochemistry USSR Academy of Sciences Moscow, USSR	Enzyme engineering in the USSR
Lehrach, Hans	European Molecular Biology Laboratory Heidelberg, FRG	Molecular approaches to developmental mutations: The *T-t* complex of the mouse
Ma, Din-Pow	University of Oklahoma Norman, Oklahoma	Sequence and gene organization of Xenopus mitochondrial DNA
Margaretten, Nadine C.	Utah State University Logan, Utah	The effect of anti-epileptic drugs on the immune system
Rinchik, E.	University of Cincinnati Cincinnati, Ohio	Molecular structure of the dilute-shortear (*d-se*) complex in the mouse
Rosenberg, Michael P.	University of Michigan Medical School Ann Arbor, Michigan	Developmental regulation of human globin genes in transgenic mice
Searle, Anthony G.	MRC Radiobiology Unit Harwell, England	Translocation intercrosses and the trouble with parthenogenesis

Schull, W. J.	University of Texas Houston, Texas	Radiation effects on the development of the central nervous system
Sherr, Charles	St. Jude Children's Hospital Memphis, Tennessee	The fms oncogene
Soper, Thomas S.	Rockefeller University New York, New York	Enzyme-activated inhibitors of D-amino acid transaminase
Stack, Steven	Colorado State University Fort Collins, Colorado	Synapsis, recombination nodules, and crossing-over
Traiger, G. J.	University of Kansas Lawrence, Kansas	Effects of substituents on the toxicity of thiobenzamide
van Houton, B.	University of North Carolina Chapel Hill, North Carolina	Reconstitution of E. coli nucleotide excision repair in vitro
Wiley, H. Steven	University of Utah College of Medicine Salt Lake City, Utah	Receptor dynamics and the responses of cells to hormones

Extramural Activities

1. Officer of Society

D. Billen	- Executive Committee, Radiation Research Society, 1979-present
J. Y. Kao	- Secretary-Treasurer, Southeastern Regional Chapter of the Society of Toxicology, 1985-1988
P. Mazur	- Board of Governors, Society for Cryobiology, 1981-1987
A. L. Olins	- Councilor, American Society for Cell Biology, 1984-1986
R. J. Preston	- Councilor, Environmental Mutagen Society, 1983-present
L. B. Russell	- President, Environmental Mutagen Society, 1984-1985
D. M. Skinner	- Chairperson, Section G, Biological Sciences, American Association for the Advancement of Science, 1985
H. R. Witschi	- Vice-President, Society of Toxicology, Inhalation Specialty Section, 1985

2. Society Committees

H. I. Adler	- Education and Training Committee, Radiation Research Society, 1980-1986
D. Billen	- Finance Committee, Radiation Research Society, 1979-present
G. A. Bingham	- Forum Review Committee, American College of Laboratory Animal Medicine, 1984-present

J. S. Cook - Publications Committee, Society of General Physiologists, 1982-1984
Publications Committee, Federation of American Societies for Experimental Biology, 1982-1985
Membrane Biophysics Subgroup (Chairman), Biophysical Society, 1984
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. Mazur - Publications Committee (Chairman), Society for Cryobiology, 1974-present

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

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

L. B. Russell - Executive Committee, Environmental Mutagen Society (Chairman, 1984-1985; member 1985)

P. B. Selby - Committee on Critical Issues: Accreditation/Certification/Standardization, Environmental Mutagen Society, 1984-present

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

3. Advisory Committees

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

D. A. Billen - Research Manpower Training Committee (ad hoc member), National Cancer Institute, 1980-present

J. S. Cook - Special Study Section, National Institutes of Health, 1981, 1982 (Chairman), 1984
Member of the Corporation, Mount Desert Island Biological Laboratory
Peer Review Group on Bioelectromagnetics, Office of Naval Research, 1983-1984
Member, Special Review Committees (2), National Institutes of Health, 1984
Advisory Committee, Department of Medicine, Baylor College of Medicine, 1984-1986

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
Advisory Committee, Radiation Effects Research Foundation, National Academy of Sciences, 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

R. A. Griesemer - Committee on Toxicology, National Research Council, 1983-1986
Ad hoc Panel on Chemical Carcinogenesis Testing and Evaluation, National Toxicology Program, 1982-1985
Third Task Force for Research Planning for the National Institute for Environmental Health Sciences, 1983-1985
Chemical Selection Committee, Department of Energy, 1984-1985
Subchairman for Animal Studies, Committee for the Evaluation of the Carcinogenicity of Cyclamates, National Research Council, 1984-1985

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
Scientific Advisory Board, U.S. Environmental Protection Agency; Chairman, Environmental Health Committee of the Science Advisory Board, 1984-present
Chronic Hazard Advisory Panel on the Use of DEHP in Consumer Products, U.S. Consumer Product Safety Commission, 1985
Health and Environmental Research Advisory Committee (HERAC), Department of Energy; Chairman, Subcommittee on Ionizing Radiation, 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

F. T. Kenney - Advisory Committee for Personnel in Research, American Cancer Society, 1978-1984
Ad hoc member, Postdoctoral Fellowship Committee, National Institutes of Health, 1985
Adviser, National Cancer Institute Outstanding Investigator Grants Program, 1984-present

S. Mitra - Ad hoc member, National Cancer Institute Site Visit Committees, 1984-present

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

D. E. Olins - Research Council, University of Tennessee, 1981-present
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
Committee on Population Monitoring, World Health Organization, 1983-present
Committee on Aneuploidy (Chairman), Environmental Protection Agency, 1984
Talent Pool for Committee on Radiation Research and Policy Coordination, 1985-

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, 1981-1987
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-present
Genetic Risk Assessment Guidelines Review Group, Science Advisory Board, U.S. Environmental Protection Agency, 1984-present

W. L. Russell - Scientific Adviser to U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation

P. B. Selby - Task Group XI of National Council on Radiation Protection Committee 57, Genetic Risk from Internal Emitters, 1984-present

J. K. Selkirk - Biochemistry Study Section, American Cancer Society, 1983-1988
Metabolic Pathology Advisory Committee, National Institutes of Health, 1984

D. M. Skinner - Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-present
Member, Peer Review Committee, Visiting Professorships for Women, National Science Foundation, 1985

A. L. Stevens - Postdoctoral Fellowship Advisory Committee, National Institutes of Health, 1984

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-

M. Terzaghi - Chemical Pathology Study Section, National Institutes of Health, 1982-1985

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

H. R. Witschi - Scientific Review Panel for Health Research, Office of Research and Development, Environmental Protection Agency, 1980-present
Toxicology Study Section, National Institutes of Health, 1980-1984
Special Study Section, National Heart, Lung, and Blood Institute, National Institutes of Health, 1984
Toxicology Data Bank Peer Review Committee, Division of Research Grants/National Library of Medicine, National Institutes of Health, 1984-1985

Complex Mixtures Committee, Commission of Life Sciences, BOTEHS, National Research Council, 1985-1986

W. K. Yang - Experimental Virology Study Section, National Institutes of Health, 1982-1986
Special Review Committee, Cancer Center Support Grant Program, National Institutes of Health, 1983-present

4. 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, 1982-1984
Current Topics in Membranes and Transport (Advisory Board), 1983-present

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

P. Mazur - Cryobiology, 1967-present
Revue Francaise de Transfusion et Immunohematologie, 1979-present

A. L. Olins - European Journal of Cell Biology, 1982-1986
Molecular and Cellular Biochemistry, 1982-1985

D. E. Olins - Molecular and Cellular Biochemistry, 1983-present

R. A. Popp - Federation of American Societies for Experimental Biology Databook, 1983-

R. J. Preston - 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-

J. K. Selkirk - Cancer Research, 1982-1985
Carcinogenesis, 1980-present

D. M. Skinner - Growth, 1979-1986

R. L. Ullrich - Radiation Research, 1983-1986

H. R. Witschi - Toxicology and Applied Pharmacology, 1972-present
Toxicology, 1978-present
Environmental Health Perspectives, 1981-present
Toxicologic Pathology, 1983-present
Journal of Biochemical Toxicology, 1984-present

5. Awards, Honors

F. C. Hartman - Technical Achievement Award, Martin Marietta Energy Systems, Inc., 1985

K. B. Jacobson - National Science Foundation U.S./East Asia Cooperative Science Grant, 1984-1986

A. C. Marchok - Publication Award, Martin Marietta Energy Systems, Inc., 1985

P. Mazur - Jefferson Cup Award, Martin Marietta Corporation, 1985
Author of the Year Award, Martin Marietta Energy Systems, Inc., 1985

R. J. Preston - Technical Achievement Award, Martin Marietta Energy Systems, Inc., 1985

D. M. Skinner - National Lecturer, Sigma Xi, 1985, 1987

International Activities

Australia and England

Dr. Paul Selby continued to send mutant stocks of mice, exchange research data, and provide consultation on skeletal mutations with staff at the University of Sydney, Australia. He also provided consultation and sent mouse strains to staff at the Medical Research Council, Harwell, England, to investigate genetic changes in mouse strains.

Bulgaria

Dr. Peter Mazur lectured at a summer school course in cryobiology at Toulbuhin, Bulgaria. The course, organized and chaired by Dr. Tsvetan Tsvetkov, Director of the Central Problem Laboratory for Freeze-Drying and Cryobiology in Sofia, was designed for young European scientists.

Chile and Antartica

Dr. James K. Selkirk continued his collaboration with staff at the University of Chile at Santiago on a study of carcinogen metabolism in species in the Antarctic region.

The People's Republic of China

Dr. Wen K. Yang and Den-Mei Hsu Yang participated in the China Medical Seminar Tour, which was organized by the American Center for Chinese Medical Sciences and the Chinese Embassy and was hosted by the Chinese Medical Association and the All-China Taiwanese Association. Ten U.S. citizen couples of Chinese heritage, all in medical professions, were invited to participate in this tour to visit 12 medical colleges or hospitals in eight major Chinese cities for the purpose of giving advice for future advances of medical science practices and research in China.

Federal Republic of Germany

Dr. K. Bruce Jacobson conducted collaborative research on structures of pteridines with Prof. Wolfgang Pfleiderer, Department of Chemistry, University of Konstanz. He also collaborated with Prof. Inmgard Ziegler, Department of Cell Chemistry, Gesellschaft fur Strahlen - und Umweltforschung, Munich, on the stimulation of T-cell lymphocytes by a pyrimidodiazepine that was discovered at ORNL.

Two German scientists have been conducting research in the laboratory of Dr. Peter Mazur. Dr. Ulrich Schneider, School of Veterinary Medicine, Hanover, was sponsored by the German Research Council for two years to pursue research on the permeability of embryos to freezing-protective substances. He returned to Germany in December, 1985. Dr. Winfred K. Berger, Professor at the Physiologisches Institut, Universitat des Saarlandes, worked with Dr. Mazur for six months on cryopreservation of electrically coupled tissues.

During a visit to Germany, Dr. Sankar Mitra initiated collaborative research with Prof. Wolfgang Pfleiderer, University of Konstanz, on promutagenic behavior of O^6-alkylguanine and O^4-alkylthymine incorporated into plasmid DNA via synthetic oligodeoxynucleotides.

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 has established a collaborative research project with Dr. Ada Sacchi, Institute Regina Elena, Rome, on tumor surface proteins of murine lung carcinomas. Monoclonal antibodies and immunochemical techniques developed at ORNL have been used by Dr. Sacchi to detect differences among Lewis lung carcinoma cell lines of high or low metastatic potential. This collaboration resulted in a publication in Cancer Treatment Reports and other manuscripts are in preparation.

Japan

Japan indicated its interest in Biology Division scientists by inviting Dr. James D. Regan for a three-month stay in the Biology Division of the National Institute for Radiological Sciences at Chiba. The Japan Ministry of Science awarded Dr. Regan a Research Award for Foreign Specialists to study and teach on the subject of DNA repair and carcinogenesis.

W. L. Carrier received a Senior Fellowship from the Japan Society for the Promotion of Science. In collaboration with Dr. Keiichi Nozu, Biology Department, Nara Medical School, Kashihara, he conducted experiments on ultraviolet damage to DNA in Japan.

Korea

Dr. K. Bruce Jacobson is continuing his collaborative studies with Dr. John Yim, Department of Microbiology, Seoul National University, on gene suppression in Drosophila. Dr. Yim will visit Dr. Jacobson's laboratory in January 1986. This collaboration is supported by a joint research grant for the period 1984-1986 from the NSF/U.S. Asia Cooperative Science Program.

Spain

Dr. K. Bruce Jacobson (supported by a Short Term Research Development grant from NSF) visited the Department of Genetics at the University of Valencia, to collaborate with Dr. Juan Ferre and Prof. J. L. Mensua 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.

World Health Organization (WHO)

Drs. Walderico M. Generoso and Gary W. Sega participated in the International Program on Chemical Safety Collaborative Study, sponsored by WHO and the Government of France. WHO will publish in book form individual reports of investigators and the various evaluations and summaries of the collaborative study.

Dr. Richard A. Griesemer served on the WHO International Agency for Research on Cancer (IARC) Working Group to review and evaluate the evidence for the carcinogenicity of smoking tobacco. The IARC review will assist nations in understanding the urgency for taking steps to reduce the cancer risks from tobacco smoking. The results of the review will be published as Volume 38 of the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.

Dr. William L. Russell served as Scientific Adviser to the U.S. Delegation, at the 34th 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.

ABSTRACTS FOR TECHNICAL MEETINGS HELD
OCTOBER 1, 1984 — SEPTEMBER 30, 1985

Aardema, M. J., and R. J. Preston. The mechanism of induction of chromosome aberrations in mouse bone marrow and myeloid leukemia cells. Environmental Mutagen Society, Las Vegas, Nevada, February 25-March 1, 1985.

Adams, L. M., and R. L. Ullrich. Epidermal growth factor and 12-O-tetradecanoyl phorbol-13-acetate ellicit a differential proliferative response in mammary epithelial cell lines from normal, carcinogen treated and tumor bearing mice. American Association for Cancer Research, Houston, Texas, May 22-25, 1985.

Allen, R. L., S. J. Kennel, and D. E. Olins. Characterization of the replication band in Euplotes eurystomus macronuclei with monoclonal antibodies. The American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Bender, M. A, R. J. Preston, R. C. Leonard, and M. D. Shelby. Chromosomal aberration and sister chromatid exchange frequencies in lymphocytes from a large random sample of normal, healthy people. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Cadilla, C. L., J. Harp, A. L. Olins, and D. E. Olins. Biophysical studies of soluble macronuclear chromatin from the Hypotricha Euplotes eurystomus. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Carrier, W. L., W. H. Lee, and J. D. Regan. Photoreactivation and excision repair in snake cells. American Society for Photobiology, New Orleans, Louisiana, June 24-27, 1985.

Charp, P. A., W. L. Carrier, and J. D. Regan. Photoreactivation of pyrimidine dimers in rattlesnake cells (Crotalus horribus). American Society for Photobiology, New Orleans, Louisiana, June 24-27, 1985.

Charp, P. A., and J. D. Regan. DNA repair inhibition by anticalmodulin agents. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Cook, John S., W. David Dawson, and Ellen R. Weiss. Development of Na^+-dependent hexose transport in cultured renal epithelial cells (LLC-PK_1). Conference on "Membrane Transport Driven by Ion Gradients," New York Academy of Sciences, New York, New York, October 2-4, 1984.

Crow, Weldon, Richard Machanoff, and Howard Adler. A gene dose effect on production of anaerobiosis by Escherichia coli membrane fragments. American Society for Microbiology, Las Vegas, Nevada, March 3-8, 1985.

Cullen, W. C., R. G. Helman, and J. Y. Kao. Stereologic analysis of skin in dermatotoxicity studies. Society of Toxicology, San Diego, California, March 18-22, 1985.

Dawson, W. David, and John S. Cook. Protein kinase C, A-system amino acid transport, and exocytosis in LLC-PK_1 cells. Federation of American Societies for Experimental Biology, Anaheim, California, April 21-26, 1985.

Dawson, W. D., and J. S. Cook. Protein kinase C, TPA, and A-system amino acid transport during development of LLC-PK_1 cells. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Dunn, W. C., R. S. Foote, and S. Mitra. Regulation of repair of O^6-methylguanine, a promutagenic base, in mammalian cells. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Fishman, J. C., and J. S. Cook. Recycling pathway of HeLa cell surface sialoglycoproteins. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Flanagan, J. M., and K. B. Jacobson. Alteration of tRNA structure in solution by zinc ions. Fourth Conversation in the Discipline-Biomolecular Stereodynamics, Albany, New York, June 4-8, 1985.

Fowler, R. F., and D. M. Skinner. Z-DNA: A site of sequence divergence. Federation of American Societies for Experimental Biology, Anaheim, California, April 21-26, 1985.

Francis, Andrew A., and James D. Regan. Repair of a UV induced photosensitive lesion in the DNA of human cells. American Society for Photobiology, New Orleans, Louisiana, June 24-27, 1985.

Fry, R. J. M. Mechanisms of carcinogenesis and dose-response models. American Statistical Association Conference on Radiation and Health, Berkeley Springs, West Virginia, July 7-12, 1985.

Fry, R. J. M. Report on neutron carcinogenesis workshop. Workshop on Radiobiological Effectiveness of Neutrons, Washington, D.C., April 9, 1985.

Fry, R. J. M., and D. S. Nachtwey. Radiation effects and space travel. Health Physics Society, Chicago, Illinois, May 26-31, 1985.

Fry, R. J. M., S. Ogle, and L. Triplett. Experimental induction of skin cancer with X-rays. Radiation Research Society, Los Angeles, California, May 5-9, 1985.

Fry, R. J. M., P. Powers-Risius, E. L. Alpen, E. J. Ainsworth, and R. L. Ullrich. High LET-radiation carcinogenesis. Symposium on Heavy Charged Particles in Research and Medicine, Berkeley, California, May 1-3, 1985.

Generoso, W. M. Relationship between alkylation sites and induction of dominant lethals and heritable translocations in mice. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Godfrey, G., R. C. Lindenschmidt, L. E. Sendelbach, and H. P. Witschi. Analysis of cell kinetics and bronchoalveolar lavage fluid after bleomycin. Society of Toxicology, San Diego, California, March 18-22, 1985.

Goldberg, S. Z., P. W. Kantoff, D. Kuebbing, D. R. Trauber, S. Lewis, R. Popp, and W. F. Anderson. Molcular characterization of β-thalassemic mice. 26th Annual Meeting of the American Society of Hematology, Miami Beach, Florida, December 1-4, 1984.

Grell, R. F. Meiotic processes and chromosome segregation in Drosophila. Symposium on Aneuploidy, Washington, D.C., March 25-29, 1985.

Hartman, Fred C., Sylvia Milanez, and Eva H. Lee. Ionization constants of two active-site lysyl ε-amino groups of ribulosebisphosphate carboxylase/oxygenase. Symposium of American Protein Chemists, San Diego, California, September 30-October 3, 1985.

Helman, H. G., J. W. Hall, and J. Y. Kao. In vivo and in vitro toxic responses of mouse skin. Society of Toxicology, San Diego, California, March 18-22, 1985.

Hook, Graham J., Stephen E. Holt, M. W. Williams, and K. Bruce Jacobson. Cadmium toxicity in Drosophila: Identification and characterization of cadmium-binding proteins. Southeastern Regional Chapter of the Society of Toxicology, Knoxville, Tennessee, December 14-15, 1984.

Hotchkiss, J. A., and S. J. Kennel. A monoclonal antibody which monitors destruction and repair of respiratory epithelium in butylated hydroxytoluene treated mice. Southeastern Regional Chapter, Society of Toxicology, Chattanooga, Tennessee, September 13-14, 1985.

Jacobson, K. Bruce, M. W. Williams, N. T. Christie, and S. E. Holt. Cadmium toxicity in Drosophila: Genetic and physiological parameters. Meeting on Heavy Metals in the Environment, Athens, Greece, September 10-13, 1985.

Jacobson, K. Bruce, M. W. Williams, L. J. Richter, S. E. Holt, G. J. Hook, S. M. Knoop, F. V. Sloop, and J. B. Faust. Cadmium resistance in Drosophila: A cadmium binding substance that is smaller than metallothionein. Second International Meeting on Metallothionein, Zurich, Switzerland, August 21-24, 1985.

Jamasbi, R. J., E. H. Perkins, R. E. Hand, Jr., and R. C. Mann. Stability of DNA content of mouse squamous cell carcinomas during in vitro cultivation. American Association for Cancer Research, Houston, Texas, May 22-25, 1985.

Johnson, A. C., K.-L. Lee, and F. T. Kenney. Hormonal control of developmentally regulated genes in fetal rat liver. Federation of American Societies for Experimental Biology, Anaheim, California, April 21-26, 1985.

Kao, John Y. Skin absorption and metabolism of topically-applied chemicals: In vitro studies with mammalian skin in organ culture. Southeastern Regional Chapter, Society of Toxicology, Chattanooga, Tennessee, September 13-14, 1985.

Kao, John Y. Skin metabolism in percutaneous absorption: New approaches and concepts. Complex Mixture Meeting, Argonne, Illinois, June 17-18, 1985.

Kennel, S. J., L. J. Foote, and K. M. Flynn. Quantitation of tumor antigen on murine lung carcinoma. Fourth Annual Congress for Hybridoma Research, San Francisco, California, February 3-7, 1985.

Kennel, S. J., and P. K. Lankford. Factors affecting passive monoclonal antibody therapy of Moloney sarcoma in BALB/c mice. American Association for Cancer Research, Houston, Texas, May 22-25, 1985.

Kennel, Stephen J., and Stephen H. Safe. Monoclonal antibodies to chlorinated dibenzo-p-dioxins. Southeastern Regional Chapter, Society of Toxicology, Chattanooga, Tennessee, September 13-14, 1985.

Lalley, P. A., D. Nakamura, R. Popp, and E. Eicher. Comparison of the arginosuccinate synthetase gene family in mouse and man. Gene Mapping Meeting, Helsinki, Finland, August 5-9, 1985.

Larimer, Frank W., and Alice A. Hardigree. Isolation and partial characterization of the REV1 gene of Saccharomyces cerevisiae. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, August 13-18, 1985.

Larimer, Frank W., Richard Machanoff, Robert S. Foote, Sankar Mitra, Thomas S. Soper, Robert K. Fujimura, and Fred C. Hartman. Site-directed mutagenesis of the active-site of ribulosebisphosphate/carboxylase. American Chemical Society, Chicago, Illinois, September 8-13, 1985.

Lindenschmidt, R. C., and H. P. Witschi. Hyperoxia and lung tumor development. Society of Toxicology, San Diego, California, March 18-22, 1985.

Lindenschmidt, R. C., and H. P. Witschi. In vivo alterations in collagen production via cyclic nucleotides. Southeastern Regional Chapter of the Society of Toxicology, Knoxville, Tennessee, December 14-15, 1984.

Lock, S., R. E. Hand, Jr., and F. Stenglein. Species comparison of bone marrow perturbations in mice and rats exposed to ethylene oxide. Society of Toxicology, San Diego, California, March 18-22, 1985.

Mann, Reinhold C., Betty K. Mansfield, and James K. Selkirk. Automated analysis of digital images generated by two-dimensional gel electrophoresis. International Congress of Applied Pattern Recognition II, Amsterdam, The Netherlands, June 19-21, 1985.

Marchok, Ann C. Biological effects of chemicals in the open-ended tracheal implant model: Applications to complex mixtures. Complex Mixture Meeting, Argonne, Illinois, June 17-18, 1985.

Marchok, A. C., M. Shiba, and A. J. P. Klein-Szanto. Multiple exposure to carcinogens and promoters in an open-ended, flow-through tracheal implant (FTTI) model. Federation of American Societies for Experimental Biology, Anaheim, California, April 21-26, 1985.

Mazur, Peter. Debate on mechanisms of freezing injury. Society for Cryobiology, Madison, Wisconsin, June 18-21, 1985.

Mazur, Peter. Freezing of living cells. Second National Summer School on Cryobiology and Freeze-Drying, Toulbuchin, Bulgaria, August 1-10, 1985.

Merrick, B. A., and J. K. Selkirk. Distribution of organic soluble, sulfate, glucuronide and glutathione conjugates of benzo(a)pyrene (BaP) metabolites in human mammary tumor T 47D cells. American Association for Cancer Research, Houston, Texas, May 22-25, 1985.

Merrick, B. A., and J. K. Selkirk. Separation of glucuronide, sulfate and glutathione conjugates of benzo(a)pyrene by HPLC. Ninth International Symposium on Polynuclear Aromatic Hydrocarbons, Columbus, Ohio, October 30-November 1, 1984.

Moore, K. L., and P. A. Lalley. New gene assignments and linkage groups in baboon (Papio) species. Eighth Human Gene Mapping Workshop (HGM8), Helsinki, Finland, August 4-10, 1985.

Nakamura, D., R. Popp, P. Lalley, and E. Eicher. The arginosuccinate synthetase gene family in the mouse. Biochemical Genetics Workshop, Park City, Utah, January 28-31, 1985.

Niemann, S. L., S. E. Lewis, and R. A. Popp. Genetic control of differential sensitivity to ethylene oxide of two mouse strains. Genetics Society of America, Boston, Massachusetts, August 11-15, 1985.

O'Brien, J. J., D. L. Mykles, and D. M. Skinner. Enzymatic activities associated with apolysis in anecdysial crustaceans. American Society of Zoologists, Denver, Colorado, December 27-30, 1984.

Popp, D. M., S. Lock, and R. A. Popp. The use of fetal tissue to demonstrate the cytostatic effects of ethylene oxide (EO). Society of Toxicology, San Diego, California, March 18-22, 1985.

Popp, D. M., J. A. Otten, and R. A. Popp. Reduced serum IgA levels and lifespan. Southeastern Immunology Conference, Stone Mountain, Georgia, November 1-2, 1984.

Popp, Diana M., and R. A. Popp. Hematology and pathology of murine thalassemias. 9th Annual Meeting of the Tennessee Society for Histotechnology, Gatlinburg, Tennessee, April 17-19, 1985.

Popp, Raymond A. Synopsis of the scientific activities of Raymond A. Popp. Symposium in Honor of Dr. Clement L. Markert's Retirement, Westcliffe, Colorado, July 28-31, 1985.

Popp, R. A., F. Stenglein, and S. Lock. Strain SEC mice are sensitive to ethylene oxide. Society of Toxicology, San Diego, California, March 18-22, 1985.

Porter, Michael A., and Fred C. Hartman. An active-site peptide from spinach ribulose-5-phosphate (Ru5P) kinase. Federation of American Societies for Experimental Biology, Anaheim, California, April 21-26, 1985.

Porter, Michael A., and Fred C. Hartman. Purification and properties of spinach ribulose-5-phosphate (Ru5P) kinase. American Society of Plant Physiologists and Canadian Society of Plant Physiologists, Providence, Rhode Island, June 23-28, 1985.

Preston, R. J., M. A Bender, and M. D. Shelby. Analysis of chromosome aberrations and sister chromatid exchanges in human lymphocytes - population monitoring studies. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Rinchik, E. M., L. B. Russell, N. G. Copeland, and N. A. Jenkins. Molecular analysis of the dilute-short ear (d-se) complex in the mouse. Biochemical Genetics Meeting, Park City, Utah, January 19-23, 1985.

Rothrock, R., and F. T. Kenney. Changes in DNA methylation during hepatic differentiation. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Russell, Liane B. Heritable effects of chemicals in mammalian germ cells: Relation to results from other test systems. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Russell, W. L. For some chemicals, genetic risks based on tests other than germ-cell mutagenicity in the whole mammal may be exaggerated. Environmental Mutagen Society, Las Vegas, Nevada, February 25-March 1, 1985.

Russell, W. L. Positive genetic hazard predictions from short-term tests have proved false for results in mammalian spermatogonia with all environmental chemicals so far tested. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-28, 1985.

Selby, P. B., T. W. McKinley, Jr., and G. D. Raymer. Dominant skeletal mutation study shows that the quality factor commonly used for alpha irradiation probably leads to a sizeable overestimation of genetic risk in males. Genetics Society of America, Boston, Massachusetts, August 11-15, 1985.

Selby, P. B., S. L. Niemann, T. W. McKinley, Jr., and G. D. Raymer. Synergistic interactions of two radiation-induced dominant skeletal mutations. Environmental Mutagen Society, Las Vegas, Nevada, February 25-March 1, 1985.

Selkirk, J. K., and B. Alex Merrick. Analysis of benzo(a)pyrene water soluble conjugates in human and rodent cells. Fourth International Conference on Environmental Mutagens, Stockholm, Sweden, June 24-25, 1985.

Sendelbach, L. E., H. P. Witschi, and A. F. Tryka. Acute beryllium toxicity in rats and mice. Society of Toxicology, San Diego, California, March 18-22, 1985.

Sendelbach, L. E., and R. C. Lindenschmidt. The affect of cyclosporin on pulmonary fibrosis induced by bleomycin, butylated hydroxytoluene and beryllium sulfate. Southeastern Regional Chapter of the Society of Toxicology, Knoxville, Tennessee, December 14-15, 1984.

Shugart, Lee. Molecular dosimetry: Technical development, skin and transplacental exposure. Complex Mixture Meeting, Argonne, Illinois, June 17-18, 1985.

Sozer, A. C., H. I. Adler, C. T. Hadden, and A. A. Francis. An effect of oxygen on the DNA of Clostridium acetobutylicum. American Society for Microbiology, Las Vegas, Nevada, March 3-8, 1985.

Tindal, M. H., K. L. Lee, and F. T. Kenney. Cloning of a hormone-inducible gene. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Tryka, A. Francine, and Hanspeter Witschi. Patterns of cell proliferation during recovery from oxygen injury. United States-Canadian Division of the International Academy of Pathology, Toronto, Canada, March 11-15, 1985.

Tyndall, Richard, Russell Hand, Jr., Reinhold Mann, Cynthia Evans, and Rebecca Jernigan. Application of flow cytometry in the analysis of environmental Legionella populations. Symposium on Legionella in the Environment: Enumeration, Distribution and Interrelationships, Miami, Florida, November 1, 1984.

Ullrich, R. L. Experimental data and dose-response models. American Statistical Association Conference on Radiation and Health, Berkeley Springs, West Virginia, July 7-12, 1985.

Ullrich, R. L. The rate of progression of radiation transformed cells is enhanced after low dose rate neutron irradiation. Radiation Research Society, Los Angeles, California, May 5-9, 1985.

Waters, L. C., and J. Kao. Studies on the cytochrome P-450 system in mouse skin. Southeastern Regional Chapter of the Society of Toxicology, Knoxville, Tennessee, December 14-15, 1984.

Wawrzyniak, C. J., and R. A. Popp. Quantitation of the $\underline{Hbb}^{8m}$ haplotype products during mouse development. Fourth Hemoglobin Switching Conference, Airlie, Virginia, September 30-October 3, 1984.

Wawrzyniak, C. J., and R. A. Popp. Transcriptional control of altered hemoglobin expression in anemic mice. Genetics Society of America, Boston, Massachusetts, August 11-15, 1985.

Wei, C. H. Crystal structures of two structural isomers of phenyl-3$\underline{H}$-1,2-dithiole-3-thione, $C_6H_5.C_3HS_3$. American Crystallographic Association, Stanford, California, August 18-23, 1985.

Weiss, E. R., and J. S. Cook. Separation of hexose-transporting from non-transporting LLC-PK_1 cells. American Society for Cell Biology, Kansas City, Missouri, November 12-16, 1984.

Winegar, Richard A., and R. Julian Preston. The induction of random vs. nonrandom chromatid exchanges by X-rays and mitomycin C. Radiation Research Society, Los Angeles, California, May 5-9, 1985.

Witschi, H. P., and R. C. Lindenschmidt. Cell kinetics in acute lung injury produced by antineoplastic agents. Society of Toxicology, San Diego, California, March 18-22, 1985.

Witschi, H. P., and R. C. Lindenschmidt. Modulation of lung and colon tumor development by butylated hydroxytoluene. Second International Conference on Modulation and Mediation of Cancer by Vitamins and Micronutrients, Tucson, Arizona, February 10-13, 1985.

Witschi, H. P., and L. H. Smith. Toxicokinetics of complex mixtures. Complex Mixture Meeting, Argonne, Illinois, June 17-18, 1985.

Financial Summary and Personnel Distribution

FY 1985

Funding Source	Funding in thousands	% of total budget	Person-years
Department of Energy	13,227	78.8	142.5
National Cancer Institute	1,504	9.0	17.7
National Institute of Environmental Health Sciences	1,164	6.9	15.8
National Institute of Child Health and Human Development	256	1.5	2.7
Miscellaneous	246	1.5	1.0
Environmental Protection Agency	229	1.4	2.5
Department of Agriculture	42	0.2	0.2
National Institute of Allergy and Infectious Diseases	126	0.7	1.5
TOTAL	16,794		183.9

AUTHOR INDEX

ORNL-6248
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