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OAK RIDGE NATIONAL LABORATORY

MARTIN MARIETTA

Biology Division Progress Report

For Period of October 1, 1983-September 30, 1984

OPERATED BY
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BIOLOGY DIVISION
PROGRESS REPORT

For Period of October 1, 1983 - September 30, 1984

Date Published: January 1985

R. A. Griesemer, Director

SECTION HEADS

G. A. Bingham, Laboratory Animal Resources
C. S. Cook, Administration and Services
R. J. M. Fry, Cancer Section
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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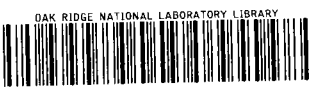

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Introduction and Division Overview

R. A. GRIESEMER

The Biology Division is the component of the Oak Ridge National Laboratory that investigates the potential adverse health effects of energy-related substances. Almost all the work of the Division is experimental and utilizes mammalian and sub-mammalian systems to obtain data for predicting and understanding hazards to human health. Work directly with the human species is limited to studies of individuals naturally or accidentally exposed to environmental agents and to investigations utilizing fluids and cells that can be obtained 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 51 at the doctorate level, 147 support personnel, 40 predoctoral students, 8 postdoctoral students, and an average of 50 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.

For the last few years, the Division has been engaged in a major effort to conserve energy and reduce operating costs related to utilities. Investments in equipment and remodeling to better control temperature and humidity along with consolidation and re-grouping to laboratory activities have resulted in the saving of over a million dollars in utility costs in 1984. The Division is engaged, too, in long-range planning to estimate the extent to which the existing buildings will still be useful in the next half century and what new buildings, if any, will be needed and when.

A notable achievement this year, in conjunction with the Chemical Technology Division, was the designation of a portion of Biology's resource as a Biotechnology Fermentation User's Facility. Thus, the Biology Division's equipment and expertise in obtaining kilogram quantities of microorganisms or mammalian cells as research materials has been made available to the scientific community. More than 30 universities and commercial companies are presently taking advantage of the opportunity to conduct large scale experiments, to obtain large quantities of research reagents, or to train their technical personnel in the techniques needed to expand from bench-scale levels to levels approaching commercial application.

An example of the Division's technology transfer, embryo freezing, has received wide commercial and scientific interest. The techniques developed here more than a decade ago to store embryos at ultralow temperatures and to revive them later in surrogate mothers have been successfully applied to food producing animals and to humans. Within the Biology Division we are continuing to apply the techniques to the freezing of mouse embryos. This is a convenient and inexpensive way to preserve valuable breeding stocks and to conserve the germ plasm of hundreds of mutant mouse stocks for future research needs.

Technical progress during this report period is described in the following sections but a few highlights of Division activities deserve special mention. In mammalian genetics, much of the data collected over nearly 40 years on experiments in mice has now been converted to automated record files making it possible to further analyze data on the thousands of mice that have been used in radiation and chemical experiments. For example, we are now able to evaluate the effects of radiation on the reduction of the number of offspring per litter by using data previously collected on 158,490 litters of mice. As another example, the results of ongoing experiments with the specific-locus mutation test (which was developed long ago in the Biology Division) can be compared with historic data on 801,406 mice. In molecular genetics, a new initiative is under way in site-directed mutagenesis in which Division scientists are attempting to use the new techniques of genetic engineering to make new and better enzymes. This promising field of research has been subsequently adopted by DOE as a new Departmental initiative. In complex mixtures research, the Division has taken the lead among national laboratories in developing a research program on toxicological interactions, using benchmark compounds among the test substances since it is not feasible to test all potentially toxic substances in biologic systems.

Of the Division's 173 publications last year, more than a third were coauthored by predoctoral and/or postdoctoral students. About 30% of the publications were coauthored by scientists at other institutions, indicating extensive collaborative interactions with universities. As indicated in the body of the report, the staff members of the Division continue to be active as officers and members of scientific societies, as advisers to federal agencies, and as members of editorial boards. Among the honors awarded the Biology Division staff members for their research was an IR-100 award for the discovery of a novel method for obtaining anerobic environments utilizing enzyme-active bacterial cell walls. The research team, headed by Dr. H. I. Adler, also received a patent for the new method.

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, 1983, through September 30, 1984. At the end of each summary is a list of publications covering the same period (published or accepted for publication). For convenience, the summaries are assembled under Sections in accordance with the current organizational structure of the Biology Division; each Section begins with an overview. It will be apparent, however, that 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 programs, research conferences, and international interactions, as well as extramural activities of staff members, abstracts for technical meetings, and funding and personnel levels.

Division Staff– September 30, 1984

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[3]Postdoctoral Investigator

[4]Chemistry Division

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[6]Consultant

[7]Student, Southern Colleges University Union

Research Activities

Molecular and Cellular 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 birth defects may 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, replication and transcription of DNA as well as their regulation, enzymology of repair of DNA damaged by chemicals or radiation, and molecular genetics. During the past year, notable findings in these general areas of molecular biology include the following:

(1) The newly developed method, Electron Microscope Tomography, has been extended to 1 μm thick sections in which entire transcription units are reconstructed in three dimensions. A vibrating mirror has been introduced to permit rapid user interactive visualization of a three dimensional structure.

(2) 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 15 Å. The path and properties of the super-helical DNA and the location of the histone domains can be clearly seen. These features will provide important new information about the function and dynamic mechanisms of chromatin.

(3) Three cloned variants of a complex G+C-rich (63%) satellite DNA have some domains as long as ~590 base pairs that have been highly conserved; other domains are divergent. Specific nuclease sensitivities in the latter domains show that unusual secondary or tertiary structures are present at the "hotspot" sites of divergence.

(4) Two proteins, covalently linked by alkali-resistant bonds to 5' termini of Kilham rat virus replicative form DNA, have been identified. These two polypeptides of 90 and 40 Kda appear to be related to each other and are not of viral origin.

(5) Ordered nucleoprotein complexes, formed by incubation of a plasmid DNA containing the adenovirus 2 major late promoter in a HeLa transcription extract, were accurately transcribed with an RNA polymerase II-containing chromatographic fraction. Naked template DNA was utilized for specific synthesis only upon addition of another chromatographic fraction containing protein specificity factors. Thus, the nucleoprotein complexes contain the essential specificity factors.

(6) The genetic locus (or loci) that confers resistance to dietary cadmium ion has been identified on the X-chromosome of D. melanogaster.

Membrane biology is also considered of prime relevance to the Section, as membranes can be considered interfaces between the intracellular metabolic machinery and the external environment. In many cases, membranes are effective barriers to noxious chemicals and as such preclude the necessity of genetic repair pathways. In other cases, membrane components are rendered inactive by combination with exogenous agents and repair mechanisms must then be invoked. As an example of overlapping interests among Sections, the effects of tumor promoters on transport proteins located in the cell membrane are being examined. Amino acid uptake by cultured mammalian cells provides a functional assay for the activity of the relevant membrane-bound transport protein. The so-called "A-system" transporter is activated by tumor promoters (TPA), an effect now shown to be mediated by the Ca^{++}- and phospholipid-dependent protein kinase C. The activation of the transporter is paralleled (or preceded) by the movement of the kinase from the cytosol of differentiated cells to the membrane compartment of TPA-treated cells.

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. Some of these studies relate to toxicology in that acute toxic poisoning frequently reflects the specific interaction of a chemical with a key metabolic enzyme; e.g. the inactivation of acetylcholine esterase by nerve gases. Recent advances include (1) partial mapping of the ATP-binding site of phosphoribulokinase, a light-regulated enzyme of the Calvin cycle, (2) identification of an 8-β-D-glucoside of xanthurenic acid as a metabolite that accumulates in the "cardinal" mutant of D. melanogaster, 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 been initiated. This program will integrate and hopefully enhance some ongoing endeavors through focusing on a common interdisciplinary theme having both fundamental and applied significance. We envision the addition of two junior-level professionals to this program very shortly.

Unfortunately, further contraction of the doctoral-level staff has taken place. Dr. Warren E. Masker, well known in the field of mutagenesis and DNA repair, moved his program to Temple University. After a distinguished 30-year career in the Biology Division, Dr. Elliot Volkin retired. Among his many achievements was the discovery of messenger RNA.

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

MOLECULAR MUTAGENESIS AND PROTEIN ENGINEERING PROGRAM

F. C. Hartman
F. W. Larimer
R. Machanoff
S. Mitra
R. S. Foote[2]
R. K. Fujimura
S. K. Niyogi

With partial support from the Laboratory Director's discretionary funds, a new program in protein engineering (site-directed mutagenesis) was initiated in April, 1984.

Protein engineering utilizes recombinant DNA technology to systematically alter protein structure by changing the nucleotide sequence of the corresponding gene. This provides a powerful tool for the elucidation of

enzyme mechanisms and, potentially, for altering the properties of enzymes in predictable and desirable fashions. Initial efforts are focused on the CO_2-fixation enzyme (ribulosebisphosphate carboxylase), ubiquitous to photosynthetic organisms and essential for the net conversion of atmospheric CO_2 into carbohydrates. The enzyme is pertinent to production of energy from biomass and to questions concerning the global CO_2 issue (i.e., the "greenhouse effect"). The enzyme is bifunctional; in addition to catalyzing the carboxylation of ribulosebisphosphate to form 3-phosphoglycerate (the initial step in the photosynthetic assimilation of CO_2), it also catalyzes the oxygenation of ribulosebisphosphate to form phosphoglycolate and 3-phosphoglycerate (the initial step in photorespiration). The latter physiologically nonessential reaction reduces yields of many plants by 50%. Thus, manipulation of the enzyme's relative specificity for CO_2 and O_2 is of fundamental importance to agricultural productivity.

In contrast to ribulosebisphosphate carboxylase from most organisms, which is comprised of eight, 52,000-Da catalytic subunits encoded by a chloroplast gene and eight, 12,000-Da subunits of ill-defined function encoded by a nuclear gene, the enzyme from Rhodospirillum rubrum (a purple, nonsulfur photosynthetic bacterium) is a dimer of 52,000-Da subunits and hence a more attractive choice for cloning experiments in which expression of wild-type enzyme in terms of structural integrity and catalytic properties is crucial. Because we have already determined the total primary structure of the R. rubrum carboxylase and mapped its catalytic site with affinity labels and because others have cloned and sequenced its gene (Nargang, F., et al., Mol. Gen. Genet. 193: 220-224, 1984), the enzyme is a prime candidate for protein engineering.

Polyclonal antibody to homogeneous ribulosebisphosphate carboxylase from R. rubrum was raised in rabbits injected intraperitoneally and intramuscularly. This antibody has been used to quantitate the enzyme by the immuno-dot-blot method and to screen colonies of bacteria in which the carboxylase gene is expressed from a plasmid. The antibody will be useful for detecting bacteria harboring in vitro mutagenized plasmids that code for inactive but immunologically cross-reacting carboxylase.

We are using automated solid phase oligodeoxynucleotide synthesis to prepare DNA sequences encoding specific amino acid changes in ribulosebisphosphate carboxylase. Several oligomers, 15-24 nucleotides in length, have been prepared by the recently developed phosphoramidite method, which requires only 10 minutes for the addition of each base at a coupling efficiency of >98%. Synthetic oligonucleotides require purification to remove molecules resulting from incomplete synthesis, depurination and other side reactions. We have simplified the purification procedure by using conditions of high temperature and pH to cleave molecules containing damaged sites, followed by isolation of the full-length oligonucleotide by gel electrophoresis. Oligonucleotide sequences are then confirmed by the Maxam-Gilbert DNA sequencing method.

The lac/carboxylase hybrid gene from plasmid pBR36 (Nargang, F., et al., Mol. Gen. Genet. 193: 220-224, 1984) has been resectioned by the

attachment of oligonucleotide linkers at position -38. This removes all lac coding sequences and 12 base pairs of the 5' non-coding leader of carboxylase. Similar resections have been constructed at position -10. These resections were used to construct expression vectors with both the lac and trp-lac promoters. Using such vectors, carboxylase can be produced at a level of 1 mg/l of cells in E. coli. The results suggest that optimum expression utilizes the native R. rubrum ribosome binding site at positions -18 to -9. We are currently confirming that our modified plasmid indeed codes for the native R. rubrum enzyme, rather than the lac/carboxylase fusion protein.

The trimmed carboxylase gene (resectioned at position -38) has been used to construct M13 hybrid phage that is being used as a source of single-stranded DNA templates for oligonucleotide-mediated mutagenesis. Control studies of complement strand synthesis have been completed and initial mutation experiments are in progress.

STUDIES OF DNA-PROTEIN INTERACTION DURING TRANSCRIPTION OF DNA TUMOR VIRUSES

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One of the consequences of energy production is the increase in deleterious agents that could interact with the genetic material, thereby leading to possible mutagenesis and carcinogenesis. Fundamental studies involving model systems are necessary to elucidate the underlying mechanisms of such processes. Understanding the control of transcription in eukaryotic systems is of fundamental importance. We are interested in elucidating the molecular mechanisms of transcription in two animal viral systems, namely, simian virus (SV) 40 and adenovirus 2. We are particularly interested in elucidating the biochemical roles of protein factors in the key process of correct chain initiation. The protein factors are being classified on the basis of their interaction with the viral DNA or RNA polymerase II or both.

Transcription Studies. Understanding the mechanism of chain initiation by eukaryotic RNA polymerase II has been greatly aided by the development of crude cell-free systems in which correct initiation can be achieved by the use of promoter-containing viral or cloned DNA templates. Recent studies from our laboratory have shown that the template DNA (either SV40 or adenovirus) is converted into ordered nucleoprotein complexes (NPCs) that structurally resemble eukaryotic DNA but lack histones. The complexes are formed by the interaction of nonhistone proteins with DNA. Ordered NPCs formed with a plasmid (pFLBH) DNA containing the adenovirus major late promoter is able to serve as template for accurate transcription in vitro. Use of preformed NPCs lessened the stringent extract to template ratios needed when transcribing naked DNA and alleviated the bulk DNA dependence

of the in vitro transcription system. In fact, use of NPCs eliminated the requirement for poly(dI-dC) in transcription reactions performed at template concentrations that were too low to yield a detectable product from naked DNA. These results show that the NPCs can serve as templates under conditions where DNA cannot, thereby suggesting that the NPCs are the true templates for transcription and are enriched in essential transcription components.

Recently, we have purified the HeLa transcription extract on phosphocellulose into four chromatographically distinct fractions (designated A, B, C, and D), essentially following the procedures of Matsui et al. (J. Biol. Chem. 255: 11992-11996, 1980) and Samuels et al. (J. Biol. Chem. 257: 14419-14427, 1982). Fraction C contains the HeLa RNA polymerase II, two additional transcription components and poly(ADP-ribose) polymerase. Fractions A and D contain specificity components and stimulatory factors for RNA polymerase II transcription. It has been shown by Samuels et al. that accurate synthesis requires fractions A, C and D and that optimal synthesis also requires the addition of exogenous purified RNA polymerase II. Fraction B, containing most of the nuclease activity of the crude extract, is not needed for specific transcription.

In recent studies we have found that with "naked" pFLBH DNA (a plasmid DNA containing the adenovirus major late promoter) as template, reconstitution of specific RNA synthesis is dependent upon the addition of fractions A and C. The dependence on fraction D for specific RNA synthesis is not found, although D serves to enhance the specific signal. The addition of exogenous purified RNA polymerase II is also not necessary. The reasons for these differences are not quite clear and further studies are underway.

NPC-pFLBH - formed by incubation of pFLBH DNA with a HeLa transcription extract - was tested with the purified fractions to investigate the notion that the NPCs represent authentic transcriptional intermediates and are enriched in essential transcription factors. We find that only fraction C, which contains the RNA polymerase II activity, is needed to accurately transcribe the nucleoprotein template. Naked pFLBH DNA is not utilized under these conditions; it also requires fraction A for specific RNA synthesis. These results confirm our belief that the NPCs are enriched in transcription factors. The specificity factors found in fraction A are present in the NPCs in a form suitable to allow promoter recognition by the RNA polymerase II and other factors in fraction C.

Although the NPCs contain RNA polymerase II, no specific synthesis is seen when ribonucleoside triphosphates are added. Specific synthesis is achieved, however, with the addition of fraction D. This suggests that fraction D contains a sigma-like protein that possibly interacts with the core enzyme thereby allowing specific initiation to occur. If this is true, it will be the first report of a sigma-like activity demonstrated in eukaryotic RNA synthesis.

Properties of Proteins Isolated from the Nuclear Matrix. The nuclear matrix (mostly insoluble protein and residual nucleic acid) is the structure that remains after treatment of the nucleus with nonionic detergent, nucleases, and high salt to remove membranes, soluble molecules and chromatin. The nuclear matrix has been implicated as being an important structural and functional component of the nucleus. Replication, transcription, RNA processing and steroid hormone binding are some of the many functions reported to be associated with the nuclear matrix. Therefore, it is reasonable that there are important DNA binding proteins that are responsible for anchoring active genes to the nuclear matrix as well as proteins that play regulatory and functional roles in transcription and replication.

The difficulty of solubilization without denaturation of proteins from the nuclear matrix has been the main obstacle to biochemical studies of these proteins. Last year we reported on the search for detergents in solubilizing the nuclear matrix proteins. We have continued these studies in greater detail and have found that two zwitterionic detergents, CHAPS and CHAPSO (cholamidopropyl derivatives of sulfobetaines), are quite effective in solubilizing nuclear matrix proteins with excellent DNA binding properties as well as protein kinase activity.

DNA binding was measured by retention of ^{3}H-labeled SV40 DNA to nitrocellulose membrane filters, electron microscopy and micrococcal nuclease digestion studies of nucleoprotein complexes. The proteins have been purified by DNA-cellulose and DEAE-cellulose chromatography. The purified proteins will be tested for their possible roles in transcription.

Two protein kinase activities, one stimulated by Mg^{2+} and the other by Ca^{2+}, have been solubilized from the nuclear matrix of both HeLa and A431 cells. Preliminary studies indicate that the phosphorylation occurs at serine and threonine, and not tyrosine. The activities are not dependent on either cyclic AMP or cyclic GMP. That the Mg^{2+}- and Ca^{2+}-stimulated activities reside on different proteins is suggested from different pH optima, salt requirements, and different thermolability. Further purification of these activities are underway.

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STRUCTURAL ASPECTS OF DNA IN ITS REPLICATION AND REPAIR

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Genotoxic chemicals that are produced during energy production and utilization react with DNA and other cellular macromolecules. The alteration in DNA as a result of such interactions leads to mutation, cancer and other toxic effects. The research objective of this laboratory is to investigate the structure and replication of DNA, and repair of DNA alterations that lead to heritable changes. Specifically, our research has two main objectives, namely, (1) investigation of the structure and replication of mammalian DNA with parvovirus DNA as a model system and (2) the role of DNA repair in mutagenesis and carcinogenesis induced by simple alkylating mutagens.

Structure of Parvoviruses. Mammalian non-defective parvoviruses, of which Kilham rat virus (KRV) is the prototype, are among the smallest viruses and contain single-stranded linear DNA genomes of about 5000 bases. The viruses, including one recently discovered for man, cause diverse diseases and birth defects in new-born rodents. The unusual resistance of these nonenveloped viruses to heat, extreme pH, detergents and organic solvents must be a reflection of a strong interaction of their capsid polypeptides. We have observed that the protein shell is impermeable to uranyl acetate used in staining for electron microscopy. We have studied the virus architecture by small angle neutron scattering at the National Center for Small Angle Scattering Research at Oak Ridge National Laboratory. Analysis of the data obtained with the empty capsids of KRV devoid of DNA, shows that the virus capsid has a radius of gyration of 105Å and a molecular weight of 4×10^6. The virus has three capsid proteins A, B, and C that have overlapping amino acid sequences. Protein A (84 Kda) has a basic pI and predicted amino acid sequence of a similar rodent parvovirus H-1. This indicates that the basic amino acids are clustered near the N-terminus. Model building and data-fitting strongly suggest that the KRV capsid contains two shells and that the inner shell is enriched for basic amino acids. Charge neutralization and condensation of nucleic acids is an important factor for the stability of viral capsids. While polyamines like spermine and spermidine, known to be present in many viruses, can be responsible for these properties, we have now shown that KRV does not have polyamines. Thus the charge neutralization, at least in part, must be carried out by the basic amino acids of the capsid proteins. The predicted basic amino acids of all three capsid proteins can account for neutralization of 30% of the total phosphate charge. We are, at present, carrying out the neutron scattering of full capsids of KRV and the results should further elucidate the structure of the DNA inside the capsid.

Identification and Characterization of Proteins Associated with KRV Replicative Form DNA. As is true for all viruses with single-stranded DNA genomes, parvovirus DNA replication involves a duplex replicative form (RF) DNA intermediate in vivo. Because of the palindromic sequences at both termini of parvoviral DNAs that can form hairpin structures, the DNA replication for viral and complementary strands is believed to be initiated at their 3' hairpin termini and thus should not involve any initiator RNA or protein. Hence the observation of Revie et al. (Proc. Nat. Acad. Sci. USA 76: 5539, 1979) that the termini of H-1 RF DNA were blocked, presumably by protein, was surprising. Since then several other workers concluded that other parvovirus RF DNA has 60 Kda proteins covalently bound to the 5' termini.

We have now observed that KRV RF DNA isolated from parasynchronous cells infected with KRV has a number of polypeptides tightly associated with it. The RF DNA does not enter agarose gels, but does enter SDS-containing gels during electrophoresis. Polypeptides of 62-66 Kda and 55 Kda are dissociated from the DNA during electrophoresis and therefore are not covalently bound to the DNA. After removal of these proteins by electrophoresis and labeling with protein-specific ^{125}I-labeled Bolton-Hunter reagent, we have shown that two polypeptides of 90 and 40 Kda are covalently associated with the RF DNA. Those proteins are released only after nuclease digestion of DNA. Immunoblotting ("Western blot") analysis of the polypeptides associated with KRV RF DNA showed that none of these cross reacts with antibody to the capsid proteins. Nondefective parvoviruses code for a noncapsid protein of 80-90 Kda that is antigenically similar in different rodent parvoviruses. The covalently attached polypeptides do not cross react with the antibody to this protein. Based on antibody cross reactivity, the 62-66 Kda polypeptides appear to be lamins of the nuclear lamina. Peptide analysis of the ^{125}I-labeled 40 and 90 Kda protein suggests that the 40 Kda protein may be a cleavage product of the 90 Kda protein.

Cell Cycle Dependence of O^6-Methylguanine-DNA Methyltransferase. O^6-methylguanine-DNA methyltransferase is a suicide repair enzyme that accepts the methyl group from the promutagenic O^6-methylguanine produced in DNA from methylation of carcinogens and mutagens. The enzyme is present in both bacteria and mammals. We have developed a sensitive and quantitative assay for the enzyme using synthetic DNA substrate containing O^6-methyl-[8-^{3}H]guanine as the only modified base. It was observed earlier that O^6-methylguanine repair in vivo is inhibited during S-phase of the cell cycle in mouse 10T1/2 cells. We have confirmed this observation using 10T1/2 cells synchronized by replating confluent cells in G_1 phase. We have monitored the distribution of cells in different stages of the cell cycle by cytofluorography. It is evident that while newly synchronized cells have a lower level of the enzyme, the reduction in enzyme level cannot account for the complete inhibition of O^6-methylguanine repair. We have concluded that the lesions in DNA become less accessible to the methyltransferase during or prior to the transition of the cells to S-phase.

Purification of O^6-Methylguanine-DNA Methyltransferase from Human Placenta. We have initiated a program of purifying the human methyltransferase to, at last, near homogeneity so that we can prepare monoclonal antibody against the enzyme. The antibody will be useful for our planned efforts to clone the cDNA and the gene of the methyltransferase. The placenta has a methyltransferase level (40-50 ng enzyme/placenta), about a tenth of that in the liver. We have partially purified the enzyme using polyethylene imine precipitation, heparin-Sepharose chromatography and chromatofocussing. Preliminary experiments suggest that ion-exchange and sizing columns in HPLC will be important additional steps for purification.

O^6-Methylguanine-DNA Methyltransferase in Somatic Cell Hybrids. Cultured mammalian cells are of two broad types, namely $Mex^+(Mer^+)$ that are proficient in O^6-methylguanine repair and $Mex^-(Mer^+)$ that do not repair the lesion. We have shown that Mex^- cells have no detectable methyltransferase (<100 molecules/cell) while Mex^+ cells contain 3×10^4 to 2×10^5 enzyme molecules/cell. We have also observed that some rodent cells, e.g., lymphocytes, are also devoid of methyltransferase. In order to map the methyltransferase gene on human chromosomes, we have, in collaboration with P. A. Lalley of the Institute of Medical Research, Bennington, Vermont, assayed methyltransferase in hybrids of Mex^+ human WI38 cells and Mex^- RAG cells of mice containing a varying number of human chromosomes. The lack of methyltransferase in all of these hybrids suggests that the Mex^- phenotype of mouse cells is trans-dominant. Further experiments are in progress with other types of Mex^+ and Mex^- cell hybrids.

O^6-Methylguanine-DNA Methyltransferase in Different Inbred Mouse Strains. We have assayed the methyltransferase activity in liver and testes of CFCW, SEC, H(101 × C3HF), C57BL 1E, and BALB/c mice provided by L. B. Russell of this Division. The enzyme level in both organs is comparable (10-11 pmol/g tissue in liver and 3 pmol/g tissue in testes) in all strains except in SEC, which has about 30% less activity in both organs. Further studies and *in vivo* toxicity and mutagenitic data are needed to establish the significance of this observation.

Synthesis of Ethyl[CH_3-^{3}H]dTTPs by Direct Alkylation. In order to study repair of alkylated thymidines in mammalian cells using synthetic DNA polymer substrates, similar to those successfully used for studying repair of O^6-methylguanine, we need alkyl dTTP with high specific radioactivity. We have now succeeded in directly alkylating [CH_3-^{3}H]dTTP (triethylammonium salt) with diazoethane in methanol and separated the alkylated dTTPs by ion-pair HPLC following removal of phosphotriesters by chromatography on DEAE-Sephadex. The advantage of this procedure is that we can obtain O^2-ethyl, O^4-ethyl and 3-ethyl dTTPs from a single reaction. The alkylated dTTPs will be incorporated into DNA substrates/templates for use in studies of miscoding and repair.

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RNA PROCESSING IN YEAST

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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 endoribonuclease of yeast has been purified about 300-fold and the type and specificity of cleavage has been studied. Cleavage of yeast mRNA and rRNA yields products that are terminated with pyrimidine 2',3'-cyclic phosphate. When the average product size is 50 nucleotides PyrA bonds constitute more than 95% of the scission sites. Double-stranded reovirus RNA, poly(U), poly(C), poly(A), and poly(A)·poly(U) are poorly cleaved, while poly(A·U) is cleaved very rapidly. Analysis of the products of poly(A·U) hydrolysis shows a very stringent cleavage of UA bonds. Gel and membrane filtration of the enzyme suggests that it is a small polypeptide (≤10,000 daltons). The enzyme can also be isolated from yeast by a peptide extraction technique using 80% ethanol. The enzyme resembles the neutral ribonucleases of mammalian tissues except that it appears to be smaller in size and has a more stringent cleavage specificity. The relationship of the enzyme to the neutral ribonucleases will require further study of its size, structure, and cellular localization.

Preliminary studies have been directed at showing the function of 5'→3' exoribonucleolytic activity. A 5'→3' exoribonuclease producing 5'-mononucleotides was first purified from yeast in this laboratory. HeLa cell fractions were analyzed and the results show that their nuclei contain the largest share of 5'→3' exoribonuclease. The unique mechanism of action of the enzyme suggests that it may be involved in removal of extra nucleotides from the 5'-termini of precursor rRNA molecules.

MODIFICATION OF STRUCTURES AND FUNCTIONS OF PROTEINS INVOLVED IN PHAGE T5 DNA REPLICATION

R. K. Fujimura B. C. Roop

Introduction. Techniques of site-directed mutagenesis have advanced to a level where it is now feasible to consider their use for the purpose of modifying proteins. Our objective is to modify structures and functions of proteins involved in DNA replication.

In vitro systems currently available for DNA replication are complex and unstable. We depend on microorganisms for production of recombinant DNA. Our objective is to establish simple, stable systems for in vitro DNA

replication that are suited for production of recombinant DNA and its mutagenesis. When established, such systems can be used in studies of effects of external agents such as by-products of energy production on the fidelity of DNA replication.

The key enzyme involved in DNA replication is DNA polymerase. Among replicative DNA polymerases, bacteriophage T5 DNA polymerase is one of the simplest yet most highly efficient enzymes. Thus we have chosen it for our studies.

Background. Phage T5 DNA is a linear duplex DNA with a molecular weight of about 7.7×10^7 and consists of more than 100 genes. Of these, only two gene products are known to be absolutely essential for DNA replication. These are the DNA polymerase coded by gene D7-D9 and the DNA binding protein coded by gene D5 (gpD5). Both of these proteins are routinely isolated to homogeneity in our laboratory. There are, in addition, several other phage gene products known to affect the onset, rate, and duration of DNA replication. Our studies suggest that some host proteins are also involved.

Our work on the characterization of the T5 DNA polymerase has shown that it is a monomeric, multifunctional enzyme (Fujimura, R. K., et al., Prog. Nucl. Acid Res. Mol. Biol. 26: 49-62, 1981). Its most preferred primer-template is single-stranded DNA with a primer. It preferentially binds to T5 DNA at a 3'-OH primer end rather than at an end of a template strand or a single-stranded region. However, it does bind to a nick of duplex DNA and utilizes a 3'-OH end of a nick as a primer for elongation and displacing the strand ahead of it. With a nicked circular duplex DNA the replication may continue until the copied segment is longer than the circumference of the initial circle. It is a highly processive enzyme; by our technique it was processive to the end of the poly(dA) used as a template (which was about 400 nucleotides long). The polymerase has a 3'→5' exonuclease associated with it which also acts processively. We have shown that a single enzyme bound at a primer end can function as an exonuclease or as a polymerase depending on nucleoside triphosphates. It can reverse the direction in the midst of hydrolysis and start polymerization. Thus, the same polymerase is capable of acting as an editing enzyme, correcting mistakes as it copies a template. By controlling this property of the enzyme one may be able to induce mutation at a specific region.

We wish to modify T5 DNA polymerase by site-directed mutagenesis. To do this we first need to isolate a segment of DNA encoding the gene. We have identified the physical locus of the DNA polymerase gene located 58.3% to 61.3% from the left end of the DNA (1). It includes conditional lethal mutants (left to right) D7, D8, am1, ts5E, ts53, am6, and D9. By use of a combination of restriction enzymes, the gene has been isolated into three DNA fragments of 1.1 kilo base pairs (kbp), 0.9 kbp, and 1.6 kbp. D7 and D8 are in the 1.1 kbp piece and D9 is in the 1.6 kbp piece.

Gene product D5 has a dual role in DNA replication and control of transcription. It is essential for the shutoff of some early transcriptions and for the expression of late genes. It is an asymmetric protein of molecular weight 28,000 and about 500,000 copies per cell, making it the most abundant DNA-binding protein synthesized in T5 phage-infected cells. It binds to both double- and single-stranded DNA, but with higher affinity and cooperativity to double-stranded DNA. Thus it differs from bacteriophage T4 gp32 and Escherichia coli single-stranded-DNA binding protein, which are known as helix-destabilizing proteins; these bind preferentially and cooperatively to single-stranded DNA.

The role of (gpD5) the product of gene D5 in DNA replication is not clear (Fujimura, R. K. and B. C. Roop, J. Virol. 46: 778-787, 1983). When nicked DNA is complexed to saturation with gpD5, both DNA polymerase and 3'→5' exonuclease activities are inhibited. E. coli exo III is also inhibited. These findings suggested that enzymes that require properly H-bonded 3' ends of DNA are inhibited by gpD5 complexed to the duplex region. Further kinetic studies of the polymerase inhibition suggested that the translocation process of DNA polymerase along a template is inhibited. The cooperativity of the binding of gpD5 may be directly correlated to inhibition of DNA synthesis. Better techniques are being developed for quantitative determination of cooperativity.

Experiments in Progress

1. Orientation of T5 DNA polymerase gene. According to genetic and physical maps we have determined, intragenic mutants are arranged in the order (left to right) D7, D8, am1, (ts5E, ts53), am6 and am9 (1). Their physical locus was determined by transforming bacteria with restriction fragments from wild type containing these loci and superinfecting with these phages that are conditioned lethal mutants. Fragments containing the appropriate locus induce highest reversion frequencies to wild type. We will have higher confidence in our data if the physical locus of the gene is determined by an additional, independent method. For this reason we have searched for shorter polypeptide of the DNA polymerase induced by various amber mutants, but have not obtained definitive results by polyacrylamide gel electrophoresis of extracted proteins. We have also assayed for polymerase activity associated with shorter polypeptides. This involves reactivation of enzymatic activities in situ in a gel. Such activities, when present, were masked by shorter polypeptides of the host DNA polymerases with polymerase activity. We are currently using immunobinding assays of the enzyme with its antibody. Anti T5 DNA polymerase was prepared with the help and advice of S. Kennel and P. Lankford (Biology Division). Results so far look encouraging and additional information on the order of transcription of the gene as well as the order of the locus of the mutants may be obtained.

2. Isolation and characterization of temperature sensitive mutants. To do site specific mutagenesis it would be helpful to identify functional domains of various DNA polymerase functions. Our strategy is to identify

such domains by means of temperature sensitive mutants. We have previously characterized one such mutant, ts53 (Fujimura, R. K. and B. C. Roop, Biochemistry 15: 4403-4409, 1976). The results suggest that the functional domains for the 3'→5' exonuclease and for the polymerase are different. The mutation at the locus for ts53 affects polymerase function but not the 3'→5' exonuclease function. Other ts mutants are being isolated by mutagenesis of DNA segments with ethyl methanesulfonate. Bacteria are transformed with such segments and superinfected with an amber mutant. We hope to get temperature sensitive mutants among the pseudo revertants. To date have obtained a few ts mutants, but only one appears to be suitable.

3. Cloning of DNA polymerase genes. Several laboratories have tried to establish a gene bank for bacteriophage T5, but without much success. Apparently it is not possible to get extensive numbers of clones by a random process. This was our reason for identifying the physical locus of the gene first. After application of several different strategies, we have finally cloned a segment which we hope has the gene. However, it does not multiply well in the current vector, M13mp18. We are currently in search of a vector that will yield copies of this. The sub-fragments of the gene are also being cloned so that they can be sequenced. Our strategy is to sequence around the locus for ts53, and then to mutagenize that site with oligonucleotide directed mutagenesis.

4. Interaction of gpD5 with DNA and its effect on DNA polymerase function. Recently, most of the efforts were spent in search for specific sites for binding of gpD5 to DNA.

To find specific sites, HaeIII digests of ϕX174 replicative form I DNA were used first. They were then fractionated according to size by RPC5 chromatography. Kinetics of DNA synthesis using HaeIII fragments as primer showed that synthesis with HaeIII-Z3 fragment as a primer was preferentially inhibited by gpD5. The HaeIII sites that determined the Z-3 fragment were also protected from HaeIII enzyme by gpD5. To date, similar studies with T5DNA have not shown any specificity of binding but more careful studies are needed.

There is a report (Ficht, T. A. and R. W. Moyes, J. Biol. Chem. 255: 7040-7048, 1980) that part of gpD5 is phosphorylated. It will be of interest to study its role in DNA replication and transcription. The 2D-gel of a gpD5 preparation showed charge heterogeneity with P_I of major fraction at 8.5 and minor fraction at 7.6. This preparation was homogenous in terms of size, and thus suggests that part of gpD5 is phosphorylated. However, we were not successful in isolation of phosphorylated gpD5.

5. DNA synthesis with short oligomers as primer. In conjunction with the protein engineering project headed by F. C. Hartman, it is of interest to determine optimal conditions for DNA synthesis with short oligomers as primers.

Published protocols using DNA polymerase preparations with unknown molecular concentrations appear empirical. We have used a T5 DNA

polymerase preparation with known molecular concentration with essentially 100% activity. This means at the optimal conditions one polymerase elongates one primer-template; the primer-templates showing this were polydA:dT_{10} and polydA:dT_{20}. The test sample of primer-template was a circular single stranded DNA with a 15-mers as a primer. The results show that the best conditions are the same as that in literature, and that the system requires an excess of DNA polymerase over primer-template. Apparently, natural single-stranded DNA tends to bind polymerase in non-functional forms.

Summary. Extensive characterizations of DNA polymerase at intramolecular levels are being carried out for T4 DNA polymerase and E. coli DNA polymerase I. We anticipate comparative studies with T5 DNA polymerase. We expect to concentrate on the structural domain essential for nucleotide condensation and to produce a polymerase more suited for in vitro DNA replication.

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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 that 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 that 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.

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 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. With the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. Using EMT, we have analyzed the 3-D *in situ* arrangement 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.

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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 three major directions: (1) X-ray crystallography of avian erythrocyte mononucleosomes; (2) smallangle neutron scattering studies of nucleosomes, HMG-nucleosome complexes, and nucleosome oligomers, and neutron crystallography of polysaccharides (H-bonding patterns); (3) molecular modeling and energy refinement of DNA-carcinogen adducts.

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 and an isomorphous heavy-atom derivative search is in progress. The second

crystal form, in space group C2, with one-half nucleosome per asymmetric unit has a similar diffraction quality.

In addition to the search for heavy-atom derivatives, other methods for phasing the $P2_1$ data have been explored. The close similarity between the two crystal forms permitted the location of the two nucleosomes in the $P2_1$ cell. A model structure placed at these coordinates was used to generate an initial set of phases. Repeated cycles of solvent flattering and Fourier inversion of the resulting map is leading to a convergent electron density map of the nucleosome core particle at 15 Å resolution. Interpretation of the 15 Å map and extension of the resolution to 6.1 Å are planned.

Crystallographic structure determination of the nucleosome will provide considerable new information about DNA packaging and regulation. It will 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 the Nucleosome and the HMG-Nucleosome Complex. Transcription of chromatin necessarily requires conformational changes at the level of the nucleosome. In a low ionic strength environment the nucleosome can be made to partially unfold and the DNA partially unwind. Small-angle neutron scattering (SANS) has been used to elucidate the conformational details of this process and the results have provided important information as to the probable in vivo mechanism of nucleosome unfolding during transcription (Uberbacher et al., Biochemistry 22: 4916-4923, 1983). Using these methods it may be possible to evaluate how chemical agents affect normal conformational changes in the nucleosome.

HMG proteins are thought to play an important role in making regions of chromatin transcriptionally active. Small-angle neutron scattering (SANS) was previously used to evaluate the conformational effects of binding HMG 14 to mononucleosomes in a stoichiometric 2:1 complex. The most recent study involved HMG 17 in a similar experiment. Analysis of the data suggests that binding HMG 17 causes the superhelical pitch of the DNA to change from 28 Å to 38 Å and the number of DNA turns around the histone core to decrease from 1.75 to 1.6. The histone core does not appear to be affected by the HMG 17 binding and the resulting changes in the DNA structure.

Materials have been produced for intended scattering studies on nucleosomal dimers and longer oligomers. These materials represent good model systems for evaluating the dynamic processes in the chromatin of higher animals.

3. Molecular Modeling and Energy Refinement of DNA-Carcinogen Adducts. The objective of this work is to determine the distortion imposed on DNA by the covalent linkage of polycyclic aromatic hydrocarbons and amines that are known environmental mutagens and carcinogens. It is likely

that the conformation of the carcinogen-DNA adducts will determine whether or not the damage is repairable, or whether a mutation that can lead to cancer will ensue on replication. This study should reveal unifying conformational features that are characteristic of mutagenic and carcinogenic adducts. Several such compounds have been studied while bound to short DNA sequences which contain high reactivity loci. These studies have demonstrated important classes of DNA distortions. This investigation will be extended to include the interactions of such compounds with nucleosomal DNA as structural details of the nucleosome become available. This will allow evaluation of the effects that histones have on induced DNA distortions and potential carcinogenesis. A new program using a Cray-1 supercomputer at Lawrence Livermore Laboratory has recently been developed to model much larger stretches of DNA.

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X-RAY DIFFRACTION

C. H. Wei

The eventual goal of X-ray diffraction studies is to elucidate and understand the structure of compounds in terms of their functions. In the past, we have carried out structural investigations of antischistosomal drugs including hycanthone and its several analogues. Our results revealed that the thioxanthene ring system is invariably roughly coplanar and that the terminal nitrogen atom of the side chain is responsible for the formation of various intermolecular hydrogen bonds, thus supporting the hypotheses made for this family of antischistosomal drugs regarding drug-DNA interaction.

Recently a new type of antischistosomal drug, oltipraz, having rather stringent structural requirements (the existence of thione sulfur and a pyrazine ring in the molecule is essential and the pyrazine and 1,2-thiol rings are to be connected directly) has been reported, and its structure has been illustrated in our laboratory (C. H. Wei, Acta Cryst. C39, 1079, 1983). This drug is unique in that it was found to exhibit a high chemotherapeutic index without the appreciable toxicity that has been observed with hycanthone. As part of our continued efforts to investigate health effects of this series of biological agents, attempts were made to grow crystals suitable for X-ray examination and to further determine the

structures of six drugs analogous to oltipraz (kindly furnished by Professor Y. Wang of the National Taiwan University, and with B. E. Hingerty of the Health and Safety Research Division.

In addition to the X-ray structural work mentioned above, efforts have also been made to isolate and purify inner histones from chicken blood that are associated with base paired DNA in nucleosome core particles. The gradient ultracentrifugation technique described by Butler et al. (Nucleic Acids Res. 6: 1509, 1977) is being used.

Furthermore, in conjunction with a pending proposal submitted last February to NIH concerning the X-ray structural investigation of an antitumor phytotoxin ricin OR (C. H. Wei, Biol. Chem. 248: 3745, 1973), in collaboration with Professor B. C. Wang of The University of Pittsburgh, the purification and crystallization of ricin OR were repeated from seeds of Ricinus communis to verify that the crystallization of the protein was reproducible.

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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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Inverted Repeats: Sites of Sequence Divergence? One major very highly repeated (VHR) DNA (~7 × 10^6 copies/genome; repeat unit = 156 bp), a family of three minor VHR DNAs (~2.8 × 10^6 copies/genome; repeat units = 71 to 74 bp), and a number of trace components account for almost 30% of the genome of a hermit crab. The repeat units of the three minor variants are defined by identical 14 bp G+C-rich inverted repeats that might form stem and loop structures. Two copies of the repeat unit (CCTA) of one of two

patent satellites of this crab occur at the center of one in seven of the G+C-rich inverted repeats; copies of the other patent satellite are found in main component DNA. To our knowledge, this is the first demonstration of small numbers of copies of a simple sequence satellite widely dispersed in a eukaryotic genome. The sequences of both the major and minor VHR DNAs are characterized by short tracts of A_n and/or T_n (n = 4-7) residues whose presence would permit the formation of perfectly matched stems separated by loops of 8 to 16 bp. The A_n and/or T_n tracts are interspersed with segments of G+C-rich DNA and are arranged differently in the major and minor VHR DNAs. Although the repeat units of the major and the three minor VHR DNAs are arranged in tandem, the composition and sequence of their bases are such that they do not form distinct bands in CsCl gradients; they are cryptic satellites.

A Complex G+C-rich Satellite: A Model System for the Definition of "Hotspots" for Mutation. The primary sequences of three cloned variants of a G+C-rich satellite DNA that accounts for 3% of the genome of a land crab have been determined. Most copies of the satellite repeat unit are defined by single EcoRI, PstI, or HindIII sites. Individual repeat units have been cloned in pBR322. The three cloned satellite variants selected for sequencing included (1) RU, (2089 bp), representative of the average size of repeat units recovered from digests of cellular satellite with any of the single site cutters; (2) TRU, (1674 bp), truncated by the presence of an EcoRI site ~300 bp from the 3' end, and (3) EXT, (2639 bp), extended by the presence of ~600 bp more DNA than the average size repeat unit. To date, it appears that every copy of the satellite may be different and that the variants are not due to cloning accidents.

Some domains of the sequences are very similar in RU, TRU, and EXT; these are composed of "garden variety" DNA. Other domains of the three variants, though clearly related to each other, are very different in specific sequences. The latter are rich in either repetitive oligonucleotides arranged in similar but not identical patterns or alternating purines and pyrimidines. Preliminary data from S1 sensitivity experiments indicate that these domains, the sites of changes in primary structure, assume unusual secondary or tertiary structures.

A Crustacean Degradative Enzyme: A Potential Indicator of Mercury Contamination? Apolysis, the separation of the epidermis from the exoskeleton in premolt crustaceans, can be induced in intermolt crabs by chilling (forced apolysis). Extracts from epidermis obtained by forced apolysis contain cathepsin D, alkaline cysteine proteinase (ACP), and chitinase. The crustacean exoskeleton is calcified and the calcium is released during breakdown of exoskeleton. ACP required activation by a reducing agent (DTT) and EDTA. The pH optimum of ACP was 8, similar to the pH of surface fluid from postmolt crabs. Antipain and leupeptin, but not Ca^{2+}, inhibited ACP activity. Chitinase was active over a broad pH range (optimum at pH 5, 60% activity at pH 7) and was inhibited by Hg^{2+} but not by Zn^{2+}, Co^{2+}, Ca^{2+}, EDTA or DTT. Many Crustacea are detritus feeders and mercury concentrated in such material may prevent normal development in

these organisms. ACP was denatured by the anionic detergent SDS but not by nonionic detergents. Cathepsin D is a lysosomal enzyme and probably has little direct involvement with the hydrolysis of extracellular proteins required for apolysis. The characteristics of the ACP and chitinase, however, correlate well with physiological conditions; both enzymes may participate in hydrolysis of protein and chitin of the membranous layer.

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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 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 caused by the purple mutant 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. The measurement of the extent of restoration was done using the Bratton-Marshall assay procedure; it was apparent that the reproducibility of this procedure was not as good as is desirable. A new assay for tryptophan dioxygenase was developed using HPLC to measure quantitatively the kynurenine or formylkynurenine that is produced. The subsequent steps leading to xanthommatin are conversion of kynurenine to 3-hydroxykynurenine to xanthommatin. The cardinal mutation also interferes with this pathway and causes the accumulation of xanthurenic acid and an unknown fluorescent metabolite. This unknown was identified as the 8-β-D-glucoside of xanthurenic acid.

In pteridine biosynthesis progress has been made as follows. The improved procedure for isolation of GTP cyclohydrolase has been improved upon yet again. The behavior of this enzyme during affinity chromatography on GTP-Sepharose is still not understood mechanistically. It appears that the enzyme can be inactivated by the column and we have devised ways to minimize this inactivation and obtain good yields of enzyme. GTP cyclohydrolase normally converts GTP to dihydroneopterin triphosphate; we demonstrated that β,γ-methylene GTP will also serve as substrate to give the corresponding analog of dihydroneopterin triphosphate. Whether this analog will serve as substrate for subsequent enzymes of pteridine biosynthesis remains to be examined.

Earlier we found the purple mutant to be due to an 80% reduction of ramiopterin synthase. In this mutant xanthurenic acid and its 8-β-D-glucoside mentioned above accumulate to higher levels than normal. When the purple and cardinal mutants are both present, the glucoside reaches even higher levels. How the purple mutation causes these changes in tryptohan metabolism and xanthommatin synthesis remains an interesting question.

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 response of Drosophila to dietary Cd^{2+} is to produce a cadmium-binding protein. The characteristics of this protein differ from the metallothionein characteristic of many organisms in that it is smaller (2000 vs 6000 = Mr). Its concentration is increased in the adult Drosophila in proportion to the amount of cadmium in the diet. Furthermore we find that strains that differ in their resistance to dietary cadmium (1 vs 3 mm = LC50) also differ in the amount of cadmium binding protein they can produce. A procedure to isolate this protein has been devised. We shall determine its amino acid composition and sequence. The information obtained so far indicates that a good correlation exists between the genetic trait for resistance and the amount of cadmium binding protein in the adult fly.

The effect of zinc ion on the structure of transfer RNA is being studied with RPC-5 chromatography. Zn^{2+}, but neither Cd^{2+} nor Mg^{2+}, causes strong retardation of tRNA on the column in proportion to the Zn^{2+} concentration. An extensive evaluation of this process is nearly completed and has included examination of pH, temperature, column packing, type of anion and cation used for elution and flow rate. The formal model that has been devised to explain the data is based on ion exchange and hydrophobic forces between the tRNA and the column matrix.

During the course of the study of tRNA on RPC-5 columns we found that the commercially unavailable Plaskon can be successfully replaced with Volfalef, a plastic bead available from France. This will be welcome news to many who use this chromatography system to study tRNA and DNA fragments.

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PHOTOCHEMISTRY OF HALOGENATED BASES

R. O. Rahn H. G. Sellin

The applications of halogen base substitution in DNA are limited only by the imagination of the investigator. Some examples include (1) virus induction, (2) chemotherapy, (3) radiation sensitization, (4) modification of recognition sites for restriction enzymes by mimicing methylation, (5) induction of Z-form DNA, (6) chromosome analysis and cytofluorimetry, (7) physical separation of DNA molecules, (8) crosslinking of protein to DNA, and (9) quantitation of DNA using radioactive iodine.

In all of these cases, the photosensitivity of the carbon-halogen bond has or could be used to advantage. A knowledge of the photophysical properties of such molecules, therefore, has relevance to these problem areas; hence the photochemistry of halogen bases in DNA continues to be a subject of continuing interest.

Our studies in the past year have been prompted by our previous attempts to identify the nature of the iodine released from IdUrd or IdCyd substituted DNA that has been irradiated at 365 nm in the presence of Hoechst 33258. Such attempts have in fact failed to show any iodine

release as measured by conventional methods. That is, when either I^- or I_3^- are analyzed for, no indication of these ions being present in solution was obtained. However, loss of iodine from the DNA does occur as indicated by the decrease in the amount of radioactive iodine 125 associated with the DNA.

To understand these results, we have begun a systematic study of the characteristics of iodine-release from a variety of halogenated bases. The results obtained over the past year indicate the following:

1. Iodine ($I\cdot$) released from monomers such as IU or IC by UV is rapidly converted into iodine ions (I^-), as measured with an ion specific probe.

2. The rate of formation of I^-, which is independent of whether oxygen or ethanol are present, can be used as a sensitive indicator of the rate of dehalogenation.

3. In the presence of I^-, iodine atoms are trapped as I_3^- which absorbs strongly at 350 nm. Stochiometric formation of I_3^- occurs in the presence but not in the absence of oxygen. Ethanol has the effect of diminishing the formation of I_3^-.

Having established a sound basis for quantitating the photochemical release of iodine from iodinated bases, we are now in a good position to apply this knowledge to the problem of understanding the photochemical behavior of these bases upon their incorporation into DNA. We are also applying this knowledge to the interpretation of the chemical changes that occur upon exposure of these same bases to ionizing radiation. Apparently, for both UV and ionizing radiation, the loss of the halogen is the initial event for sensitization due to incorporation into the DNA of both BrdUrd and IdUrd. Consequently, a common reactive species, the uracilyl radical, is responsible for the sensitized biological inactivation. Therefore, by comparing biological responses with these two forms of radiation for cells containing either Br or IdUrd in their DNA, we hope that a better understanding of molecular mechanisms in radiation biology can be achieved.

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UV-INDUCED RESPIRATION SHUTOFF STUDIES IN REPAIR- AND RECOMBINATION-DEFICIENT STRAINS OF ESCHERICHIA COLI

P. A. Swenson I. L. Norton

In the bacterium Escherichia coli there exists a network of regulatory genes concerned with induced SOS (emergency) responses brought about when the DNA is damaged by ultraviolet irradiation (UV) (254 nm) or by chemicals. All of the SOS responses, including mutagenesis and filamentation (inhibition of cell division), require the presence of the recA$^+$ and lexA$^+$ gene products. In brief, the LexA protein is the repressor of all operons involved in SOS responses. When DNA is damaged, RecA protein is synthesized, acquires proteolytic capability, and cleaves the LexA protein, thus allowing the SOS operon structural genes to be transcribed. Our area of interest is in the UV-induced recA$^+$ lexA$^+$-dependent shutoff of respiration which occurs 60 to 120 min after irradiation, depending upon the strain of bacteria used. Our objective is to understand the genetic regulation of the respiration shutoff process and so we have studied a number of bacterial strains containing mutations which affect DNA repair, genetic recombination, or both. In this work we used cells grown before and after UV at 30 or 37°C.

recA13 and lexA3 strains do not shut off their respiration after UV at either 30 or 37°C. The lexA3 mutant does not shut off its respiration after UV because the LexA3 protein is more resistant to proteolytic cleavage than the normal LexA protein. A cell with a lexA51 mutation is unable to synthesize functional repressor molecules. The gene sulA$^+$ is normally induced through derepression after UV and the gene product inhibits cell division; in an unirradiated lexA51 sulA$^+$ strain, cell division is inhibited continuously because of lack of repression of the sulA$^+$ gene and the cells die. For this reason, all work with lexA51 mutants is done with the sulA mutation present as well. sulA mutants shut off their respiration after UV at 30 but not at 37°C. Likewise, lexA51 sulA mutants also shut off at 30 but not at 37°C.

The recA$^+$ gene is normally repressed by the LexA protein but a recAo mutant synthesizes the RecA protein constitutively and, after UV, shuts off at both 30 and 37°C. When a cell is lexA3 recAo, respiration shutoff does not occur at either 30 or 37°C.

The recF$^+$ gene plays an important role in genetic recombination. The recF pathway of genetic recombination is inducible since it requires the recA$^+$ and lexA$^+$ gene products. The recF and the recF recAo mutant strains

do not shut off their respiration after UV at either 30 or 37°C. A recA-linked mutant gene srfA, suppresses repair and recombination deficiencies of recF, probably because of an altered RecA protein. UV-irradiated srfA mutants shut off their respiration at 30 but not at 37°C and the same is true for recF srfA mutants.

The recA441 gene codes for a RecA protein that has its proteolytic properties activated more easily than the normal RecA protein. At 42°C the unirradiated recA441 strain shows constitutive expression of many SOS responses. This strain shuts off its respiration after UV at both 30 and 37°C. At 42°C, neither unirradiated or irradiated cells shut off their respiration, behaving like wild type strains JM1 and AB1157.

Last year we reported that UV-induced respiration shutoff did not take place in recB or recC strains. These strains are radiation-sensitive and are deficient in genetic recombination. Each gene codes for a subunit of the RecBC nuclease, which has activities for several nucleases, as well as for helicase (DNA unwinding) activity.

A. Chadhury and G. R. Smith of the Fred Hutchinson Cancer Research Center isolated recB and recC mutant strains which are deficient in the double-strand exonuclease activity of the recBC nuclease but have normal recombination ability and normal sensitivity to UV (Proc. Natl. Acad. Sci. USA, in press). We obtained one of these unusual mutants (AC301) (recB1009) from them and found that it did not shut off its respiration after UV at 37°C. This result suggests that the double-strand exonuclease activity is necessary for UV-induced respiration shutoff but, at 30°C, shutoff did occur after UV.

It occurred to us that one of the mutations recB or recC might also be conditional in the same sense. At 37°C recB21 and recC22 strains each fail to shut off their own respiration but, at 30°C, only the recC strain fails to do so; the recB strain shows the normal UV-induced respiration shutoff response.

In summary, the following strains show UV-induced respiration shutoff at both 30 and 37°C: Wild type (JM1 and AB1157), recAo, and recA441. The following strains do not shut off at either 30 or 37°C: recA, lexA3, lexA3 recAo, recF, recF recAo, recC22, recB21 recC22. The following strains shut off at 30 but not at 37°C: sulA, lexA51 sulA, srfA, recF srfA, recB21, and recB1009 (nuclease$^-$). The group of strains that shut off their respiration after UV at 30 but not at 37°C includes strains with diverse mutations. Our present working hypothesis is that the sulA, srfA recB21 and recB1009 mutations are conditional ones, at least with respect to respiration shutoff, showing this recessive character at 37°C, but shutting off like wild type strains at 30°C. In the case of recB1009, where the double strand exonuclease is missing, and of recB21, the aberrant response at 30°C may be due to some previously unreported activity in the recBC enzyme that is conditional.

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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. Significant progress has been achieved in three areas.

1. Recycling of Cell Surface Sialoglycoproteins in HeLa Cells. Terminal sialic acid residues in living cells are externally labeled with a $NaIO_4/NaB[^3H]_4$ reaction and the cells returned to their growth medium. At subsequent times the cells are harvested and fractionated on Percoll gradients; both analytical fractionation (emphasizing recovery, to account for all of the label) and preparative fractionation (emphasizing purity at the expense of recovery) are performed, and the fractions analyzed by electrophoresis. The following pattern emerged. Surface glycoproteins are internalized with a bulk half-time of 5-8 h in steady state of growth. Once internalized they are first found in a compartment of endocytic vesicles and endosomes that is mildly acidified to pH 6. Within 15 minutes these membranes fuse with secondary lysosomes where the acidification further reduces the pH to 4.8-5.1. The specific radioactivity of the lysosomes reaches a peak in 45-90 min. If lysosomal activity is inhibited with chloroquine, the labeled glycoproteins remain in these organelles; virtually all surface proteins can be found there. In untreated cells most but not all of the glycoproteins move into the Golgi compartment in about 3 h. Significant sorting takes place at this step, some of the label being specifically lost in the lysosomes. The proteins associated with the Golgi later (5 h) fill a post-Golgi vesicular compartment. The internal compartments are twice as large as the surface from which they were labeled. Eventually, again with a half-time of 5-8 h, the surviving glycoproteins are recycled back to the cell surface. The majority, or about 70-90%, make the cycle about 20 times before they are finally degraded in the lysosomes. 5'-Nucleotidase is a surface enzyme that appears to be multiply recycled, while Na,K-ATPase appears to be degraded in its first encounter with the lysosomes. The recycling may be the mechanism by which surface glycoproteins are monitored for intactness and turned over.

2. Development of Na^+-dependent Hexose Transport in Cultured Renal Tubule Cells. In postconfluent cultures, LLC-PK_1 cells, derived from pig

kidney, differentiate the proximal tubule function of concentrating hexoses, using the energy stored in the medium/cell Na^+ electrochemical gradient. This is the mechanism by which glucose filtered with the plasma at the glomerulus is retrieved by the intact animal. The cells are capable of regulating their osmotic content so that even after concentrating hexose to high levels they undergo no volume change. If the volume regulating mechanism is blocked by suspending the cells in K^+-free Na-gluconate, and if the cells are allowed to extrude the co-transported (with hexose) Na^+ by stimulating Na,K-ATPase with the K^+-congener NH_4^+, the cells taking up hexose swell osmotically and can be separated from nontransporting cells on Percoll density gradients. Quantitative analysis of developing populations on such gradients reveals that, over a period of about three weeks, non-transporting cells are stochastically recruited into the fully differentiated subpopulation but that it may take two days for an individual cell, once initiated, to develop its full transport capacity.

3. Protein Kinase C, TPA, and Na^+-dependent Amino Acid Transport. Undifferentiated, rapidly growing LLC-PK_1 cells vigorously transport certain amino acids by their so-called "A-system." At confluence the A-system activity is stepped down to low but stable levels. Treatment of such cells with the tumor promoter TPA rapidly (10-60 min) reactivates the A-system. It is now recognized that a major, if not the only, functional receptor for TPA is the enzyme protein kinase C. This kinase is Ca^{++} and phosphatidyl serine dependent, and its Ca^{++} requirement can be drastically reduced with diacylglycerols which are normally not found in the cells. TPA substitutes for diacylglycerol and activates the enzyme at the normal low free Ca^{++} levels. We have implicated protein kinase C in regulating the A-system by observing that permeating diacylglycerols which we have shown to be effective enzyme activators will also reactivate the A-system in whole cells, a response that overlaps with and is not additive to the TPA response. In rapidly growing (and transporting) cells, protein kinase C is associated with a particulate fraction comprised of surface membranes and organelles. At confluence the enzyme is found in the cytosol. After TPA treatment of confluent cells the enzyme is lost from the cytosol and is again found associated with the particulate fraction. The redistribution of the enzyme parallels A-system activity.

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THEORETICAL AND APPLIED CRYOBIOLOGY

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

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

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 is that unfrozen fraction or 1 molal or as high as 2.8 molal.

The suspensions in our experiments had hematocrits of only 2%. However, investigators have reported that the hemolysis of red cells frozen to given subzero temperatures rises sharply when the cell hematocrit is above 40%. The water that leaves cells during slow freezing is added to the extracellular ice and at high hematocrits the volume of this effluxing cell water is sufficient to substantially alter the numerical value of the unfrozen fraction and thus possibly alter the relation between survival and unfrozen fraction. Consequently, we repeated the experiments referred to above with initial hematocrits of 0.2, 2, 8, 40, and 60% to see if the adverse effect of high hematocrits could be a result of the change in the unfrozen fraction stemming from the increased size of the extracellular ice compartment. However, this proved not to be the case. The hematocrit effect is due to something else.

During slow freezing red cells shrink osmotically and become converted into spiny-surfaced echinocytes. They also become more crowded together as water is removed from the unfrozen channels and converted to ice. The higher the initial hematocrit, the greater is the probability that the shrunken cells will be pressed against each other. During thawing they will again become dispersed. To mimic these events, we have subjected red cells to packing by high speed centrifugation (10,000 g) at -8°C in unfrozen hyperosmotic glycerol/NaCl solutions and determined their survival before and after resuspension. We found that nearly all the damage occurs during resuspension and that the more shrunken the cells are prior to the centrifugation, the greater the damage from resuspension. The simplest explanation of both this and the added damage from the freezing of high hematocrit suspension is that the surface spines of the echinocytes physically interlock during cell packing, and that damage occurs after the cells are forcibly separated during thawing or resuspension.

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 or 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 the 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.

We are now turning our attention to the rheological damage. The approach will be to subject cells in unfrozen supercooled media to deformation by rheological forces. We are beginning with the simple approach of forcing cells at high velocity through hypodermic needles at subzero temperatures. Preliminary experiments show that this produces considerable damage even though no ice is present.

Slow Freezing Injury in Mouse Embryo. An important question is the extent to which the findings on human erythrocytes are applicable to nucleated mammalian cells. As a first step in determining their generality, we have studied the survival of mouse embryos as a function of unfrozen fraction and the concentration of salt in that unfrozen fraction. Some 3300 mouse zygotes have been subjected to subzero temperatures producing a range of unfrozen fractions and a range of concentrations of NaCl in those unfrozen fractions. Also included in the study are various weight percent ratios of salt to glycerol. The concentration of cells in these experiments (i.e., the cytocrit) is so low that cell-cell contacts should be rare during the freezing. As in the case of the red cells at low hematocrit, we find that the survival of slowly frozen 8-cell embryos is not affected by the salt concentration attained and therefore not by the extent to which the cells shrink below their isotonic volume. Low values of the unfrozen fraction (U) are damaging, but part of the damage may be artifactual. Low values of U had to be achieved by placing embryos in solutions hypotonic with respect to NaCl, which caused their volume to be greater than isotonic prior to freezing. Evidence indicates that these swollen embryos may not have dehydrated sufficiently to avoid intracellular freezing.

If these findings on red cells and embryos are found to be applicable to cells in general, they will require major modifications in how cryobiologists view slow-freezing injury and its prevention. They may be especially pertinent to the freezing of whole organs, since cells in organs generally occupy 50% or more of the available space.

Banking Mutant Strains of Mice in the Form of Frozen Embryos. The Mammalian Genetics and Reproduction Section has begun to preserve some of their mutant mouse stocks in the form of frozen embryos. They consult with us on this project as the need arises.

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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 energy from biomass.

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 >100-times that for the reaction with N-2-acetyllysine with TNBS. The pH-dependencies of the inactivations by TNBS suggest that the pKa values for the two active-site lysyl residues is <8.0. With such low pKa values and high reactivities, either lysine could be the essential base that initiates catalysis by proton abstraction from C3 of ribulosebisphosphate.

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.

Several collaborations with investigators at other institutions are ongoing. These include X-ray diffraction studies of ribulosebisphosphate carboxylase from R. rubrum (David Eisenberg, UCLA), characterization of intermediates in the carboxylase reaction pathway by rapid quenching techniques (Irwin A. Rose, Institute for Cancer Research, Philadelphia),

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), and characterization of the reactions of glyoxylate and pyridoxal phosphate with ribulosebisphosphate carboxylase (N. Edward Tolbert, Michigan State University).

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Comparative Genetics Section

SECTION OVERVIEW - R. J. PRESTON

The Comparative Genetics Section maintains its overall program direction of providing information that can be directly or indirectly used to determine the genetic and carcinogenic hazards of radiation and chemical agents to man. This involves studies designed to determine the mechanism of induction of mutations (including chromosomal alterations) using a variety of approaches and assay systems, and the use of the information obtained to develop and validate new assays that will aid in the extrapolation from mutation data obtained in laboratory model systems to man.

It has become increasingly apparent that there are likely to be differences in sensitivity to the induction of mutations by radiation and chemicals in somatic or germ cells within human populations. Several programs within the Section are investigating the possible reasons for sensitivity differences and factors that can influence sensitivity.

Differences in the capacity of cells to repair DNA damage will influence the sensitivity of cells to killing and mutation induction. Studies with mammalian cells (*in vivo* and *in vitro*) and with yeast cells are directed towards understanding the mechanisms of repair of the different types of DNA lesions that can be induced by radiation and chemicals, and the cellular effects that alterations in DNA repair capacity can have. The following areas of research demonstrate the approaches being taken in the Section:

(1) A detailed analysis of the repair defective *rev3* gene mutations in yeast makes it possible to characterize the specific mutational change, and to determine the influence of the specific mutations on cellular mutagenesis.

(2) It has been demonstrated that the ability of Drosophila to metabolize dimethyl nitrosamine by the P450 system is both strain- and age-dependent. The relationship between this activity and mutagenicity is not clearly established. These types of studies are clearly important in the understanding of differential sensitivity of individuals to mutagenesis or toxicity of chemical agents requiring metabolic activation.

(3) CHO cells resistant to a low level of MNNG have been selected, and it has been shown that these lines are more resistant to the toxic effects of higher doses of MNNG than wild-type cells, but are more sensitive to MNNG-induced mutagenesis. Adaptive mutagenic responses could influence the

sensitivity of individuals to exposures to chemicals environmentally or occupationally.

(4) From studies of the toxicity of ethylene oxide in mice, a strain has been identified that is much more sensitive than others tested. The genetic basis for this increased sensitivity is being studied. Thus an animal model could help in determining whether the established differential sensitivity in humans exposed to EtO has a similar genetic basis.

(5) Studies of DNA repair mechanisms in normal human fibroblasts and cells from individuals with known DNA repair defects could lead to a better understanding of the role that minor differences in repair capacity between individuals or groups could have in differential sensitivity to mutation induction (germinal or somatic).

(6) Studies on the role of DNA repair, following radiation or chemical exposure, in the induction of chromosome aberrations could provide the basis for explaining observed differences in sensitivity to aberration induction between species and within species.

A wide range of other studies in the Section are used to provide new information on the induction of genetic effects:

(1) Anaerobic bacteria can be grown in the laboratory by utilizing oxygen-consuming membrane preparations of Escherichia coli to provide the oxygen-free atmosphere required for growth. There are clearly many applications of this simple technique including studies of oxygen toxicity and genetic studies of anaerobic organisms that are responsible for several human diseases.

(2) Molecular studies have enabled two ethylnitrosourea-induced hemoglobin mutants to be characterized, and the characterization of the expression and regulation of β-minor genes in homozygous β-thalassemic mice. Studies such as these are of great importance in understanding the mechanisms of induction of mutations and for understanding the effects of specific mutations in relation to adverse health effects.

This overview indicates some of the approaches being taken in the Comparative Genetics Section to provide background information for estimating adverse health effects in man from exposure to chemical agents and radiation. There are many advantages to comparative approaches in genetic research that cannot be met by studies on single genetic systems. By making use of the very wide range of mutants in prokaryotic and eukaryotic systems and by using in vitro systems where potential sources of variation of response can be controlled, it is possible to determine both the similarities of mutation induction and selection in very diverse organisms and to understand differences in these processes in different species or cell types. It is only from these approaches that extrapolation of the genetic effects of radiation and chemical agents from species to species and from model systems to man can be made.

COMPARATIVE MUTAGENESIS

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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 endpoints, 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)

The Comparative Mutagenesis Group has continued research with energy-related materials during this year. Emphasis has been placed on the following aspects of the health effects evaluation: (1) preliminary screening of actual products and/or effluents for mutagenesis using short-term assays; (2) validation of these results in additional batteries of tests; (3) extension of the initial results with crude materials to actual compounds through fractionation, chemical characterization and identification; and (4) specific projects involving (a) examination of the connection between mutagenesis and carcinogenesis, and (b) extrapolation of *in vitro* results to the whole animal. Funded studies (Fossil Energy) included consideration of alternate methods of treatment of fuels in order to mitigate potential health effects.

Upgrading coal-derived liquids by means other than hydrotreatment: Salmonella microbial mutagenicity (with C.-h Ho, A. R. Jones, and M. R. Guerin, Analytical Chemistry Division). 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.

This report presents the initial results of studies designed to identify and evaluate promising non-hydrotreating processes for reducing the toxicological properties of coal liquids. In these studies to date, the biological evaluation has been limited to the Ames mutagenicity assay. Other bioassays including mouse dermal tumorigenicity and aquatic toxicity are in progress.

Samples used in this study were two heavy fuel oils and one fuel oil blend obtained from the Catlettsburg, Kentucky, H-Coal Pilot Plant. From these three fuel oils, three H-coal heavy-end products (vacuum gas oil range) were generated by redistillation. The initial boiling points of the three heavy-end products were greater than 650°F.

In the exploratory study, one of the heavy fuel oil samples was subjected to nine alternative processes which were designed to reduce or eliminate the microbial mutagenicity of the oil. The processes were distillation, acid washing, deasphalting, cyclohexane/DMSO partition, charcoal adsorption, $ALCl_3$ catalytic conversion, water washing, alkaline washing, and air oxidation. Mutagenicity assays on the resulting products indicate that the first six treatments listed above show promise for removing mutagens. The fraction of the mass recovered after each of these treatments ranged from 70 to 85%.

In the evaluation study, five of the promising treatments identified in the exploratory study were applied to five H-coal liquid products. These five treatments were chosen not only because they had shown promise in the exploratory study, but also becasue they are very similar to processes generally employed in existing refineries. Each of these treatments (distillation, sulfuric acid treatment, deasphalting, pentane/furfural partition, and clay percolation) was found to significantly reduce the mutagenicity in the resulting product.

II. Chemical Mutagenesis and Metabolism in Drosophila Melanogaster (C. E. Nix and L. C. Waters)

The utility of Drosophila for evaluating the potential health effects of physical agents, ionizing irradiation, and chemical compounds, nitrogen mustards, was proven in the 1930's and 1940's by H. J. Muller and C. Auerbach, respectively. Many subsequent investigations have added to the extensive data base concerning the mutagenic effect of pure chemical compounds, complex chemical mixtures, and physical agents. Nevertheless, it has become increasingly clear that many variables influence chemically induced (also spontaneous) mutagenesis. Besides the obvious environmental variables such as nutrition, temperature and culture conditions, the genotype plays a major role. Differences in DNA repair pathways, the presence of mobile genetic elements, and metabolic activity are only a few of the possibilities of genetic control of mutagenesis. Many of these

problems can best be investigated by using Drosophila as a model system. Basic information from these experiments can serve as a model for future investigations in mammals.

Existing mutagenic assay systems in Drosophila are well suited for estimating chemically induced mutagenesis; however, refinements and improvements can be made. During the past year we have investigated various routes of mutagen exposure and developmental stage specifity. Results are incomplete but they suggest that some chemicals are more effective when fed to larvae rather than to adults.

Using stocks and techniques developed by R. F. Grell, we have initiated experiments to investigate chemically induced non-disjunction in Drosophila females. Although chromosomal non-disjunction is an important biological phenomenon, there are few in vivo assays for measuring chemical induction of abnormal chromosome behavior in females.

In order to better estimate potential health effects of xenobiotic agents we need a better understanding of how the intact organism handles such chemical (or physical) insults. We have chosen to concentrate on the description of the metabolic pathways of model chemical compounds and their genetic control. Previous reports from our laboratory have demonstrated significant strain differences in mutation frequency when adult males are treated with several simple alkylating agents. Attempts to correlate these differences with changes in mixed-function oxidase activity have met with limited success. Although we demonstrated strain differences in dimethylnitrosamine demethylase activity, there was no correlation with DMN-induced mutagenesis. In an effort to determine which metabolic system is reponsible for activating DMN to a mutagen, we have looked for prostaglandin synthase-dependent metabolism of DMN in DMN-demethylase negative strains. Although we did not demonstrate activity, we are pursuing this possibility in vivo by evaluating the effects of indomethocin, an inhibitor of prostaglandin synthase, on DMN-induced mutagenesis.

Differences in DNA repair as an explanation for strain differences in mutation frequency have been eliminated. Methylnitrosourea (MNU), a direct acting mutagen, was equally mutagenic in all wild-type strains tested. This indicated that there are no major differences in the DNA repair capacity of these strains. We have also tested mutation induction in excision repair defective (mei-9^{AT1}) males treated with MNU and DMN. The frequency of mutations (sex-linked recessive lethals) is reduced 2-3-fold in mei-9^{AT1} males. This reduction in mutation frequency is limited to the post-meiotic stages of early and late spermatids. Mature sperm and spermatogonia are equally susceptible to mutation in wild-type and mei-9^{AT1} males. It is interesting that spermatogonia are relatively refactory to mutagenesis with the frequency being 5-10% that observed in spermatids. This is not unexpected as cell selection would eliminate many lethals induced in pre-meiotic stages. The interesting observation is that differences between the two genotypes are observed in early post-meiotic stages. These data can be interpreted as evidence for the presence of an error-prone repair process in spermatids of Drosophila males. At least a

part of this function is eliminated when the mei-9^{AT1} mutation is present. Preliminary evidence suggests that other mei-9 alleles similarly reduce the mutation frequency in mid to early spermatids.

A class of membrane-bound hemoproteins, collectively called P-450, is involved in metabolism of xenobiotics. As a result of this metabolism many compounds are rendered more water soluble and usually less toxic; however, in some cases the compounds are made more toxic. Multiple forms of P-450 exist and undoubtedly account for the capacity to metabolize large numbers and diverse classes of chemicals. Whether there is an infinite number with unique specificity is currently under investigation. In any event it is clear that the biological effects of xenobiotics can be dependent on this enzyme system.

Drosophila is a unique system with which to evaluate (a) the role of P-450 in converting certain chemicals to mutagens and (b) the molecular mechanisms by which P-450 activities are qualitively and quantitatively regulated. Our recent progress can be summarized as follows:

a. The structural gene for DMN-demethylase was localized to chromosome II by the chromosomal substitution method. By mapping the gene for resistance to phenylurea and subsequent biochemical analysis of the recombinant progeny, we demonstrated that the gene(s) for phenylurea resistance and DMN-demethylase activity were identical or very closely linked. The map location was on IIR between cinnabar (cn) and curved (c) at approximately 62-64. Experiments are in progress to obtain a more precise map location. A trans-acting regulatory gene, required for maximum DMN-demethylase expression, is located on chromosome III. Using a multiply marked third chromosome we have constructed various recombinant chromosomes in order to map the regulatory factor. The effects of the structural and regulatory gene(s) are additive. We believe this is the first evidence of a regulatory gene affecting the endogenous level of a P-450 enzyme and offers a unique system for evaluating the molecular mechanisms involved.

b. Whereas mouse liver P-450 can be resolved by gel electrophesis into 7 or 8 major, distinct protein bands, only two are seen in Drosophila. One, band a, is found in all 20 or more strains examined; the other, band b, is found only in a few strains. Higher P-450 enzyme activities and phenylurea resistance are associated with the presence of band b. In fact, DMN-demethylase is found only in strains in which band b is present. The data show that DMN-demethylase is a component of band b, that the structural genes for band b are located on chromosome II close to insecticide resistant genes, and that many of the components in band b are regulated by a gene(s) on chromosome III as previously described for DMN-demethylase.

We have localized structural and regulatory genes for a subset of P-450, band b, that probably accounts for one-third of the P-450 activity of positive strains and for a specific P-450 enzyme, DMN-demethylase. Further analysis of this system should help to explain individual, strain, and species variation in the constitutive expression of P-450. In general, this project is related to all those whose goal is to evaluate, predict

and/or alter the effects of chemicals on health. During the period covered by this report we (L. C. Waters) have utilized the concepts and methodology involved in this work to initiate similar studies using mouse skin. Progress in that area is described in the report of the Toxicology Section.

III. Genetic Control of Mutagenesis and DNA Repair. (F. W. Larimer)

In the yeast Saccharomyces cerevisiae, three major epistasis groups of mutants controlling the processing of DNA damage have been recognized. One group (RAD52) is required for the repair of double-strand breaks in DNA; a second group (RAD3) directs error-free excision-repair of pyrimidine dimers and other bulky (chemical) lesions; a third group (RAD6) is required for an infidelitous form of repair necessary for nearly all induced mutagenesis.

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

Recently we isolated the REV1 gene from a cosmid library of genomic yeast DNA. Two independent isolates were obtained, one with an insert of 38 kbp, and the other with an insert of 30 kbp. Restriction mapping indicates an overlapping region of 15.5 kbp which contains the REV1 gene. Subcloning is in progress to determine the minimum fragment carrying REV1. Both single-copy cosmids confer the full Rev^+ phenotype on transformed rev1-1 strains — mutability and UV resistance are restored to the wild-type level. Curing transformed strains of the cosmids causes a reversion to the Rev^- condition. Multicopy plasmids bearing REV1 are being constructed to study dosage effects. When subcloning is complete, the transcript will be identified and we will attempt to identify the protein product in maxicells. A gene disruption/deletion will be constructed to determine if REV1 is an essential gene.

Recent results reported by Prakash for RAD6, by Freidberg for the RAD3 group, and by Shild for the RAD52 group (12th International Conference on Yeast Genetics and Molecular Biology, Edinburgh, Scotland, 1984) indicate that the molecular cloning approach is of great value to elucidate the molecular mechanisms of DNA repair and mutagenesis. In addition to the continued study of REV1, we intend to extend this approach to include REV2 as well as new Rev^- mutants that are currently being generated.

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MAMMALIAN CELL GENETIC TOXICOLOGY

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Our goal is to study mechanisms of gene mutations and to determine the mutagenic effects of environmental chemicals using mammalian cells. We anticipate that the results of our studies will eventually be used for the estimation of risk to humans exposed to chemical agents. We have accomplished the following objectives using two mutational assays developed in our laboratory. 1) The Chinese hamster ovary cells/hypoxanthine phosphoribosyl transferase (CHO/HGPRT) assay has been used to study a mutant line derived from CHO-K1-BH4 cells resistant to the cytotoxic effects of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); CHO-K1-BH4 cells were also used to study metal toxicity, the role of DNA alkylation in gene mutation, and the effect of glucuronide conjugation on the toxicity and mutagenicity of benzo(a)pyrene [B(a)P]. 2) Using the CHO/pSVgpt system, in which the CHO-AS52 cell line carries a copy of the E. coli xanthine-guanine phosphoribosyl transferase (XPRT) gene (gpt) (the mammalian equivalent of the hgprt gene) from the plasmid vector pSVgpt, we have determined the mutagen specificity of several chemical and physical mutagens at the DNA sequence level. Similar experiments have been conducted in CHO-K1-BH4 cells as a means to compare the molecular mechanisms of mutagen specificity between mutation of the mammalian hgprt gene and its bacterial equivalent, the gpt gene. The following summarizes the highlights of the projects completed.

1. Quantitative Mutagenesis and Cytotoxicity with the CHO/HGPRT Assay.

A. A nitrosoguanidine-resistant CHO mutants. In the course of studying whether pretreatment with a low dose of MNNG (0.01 μg/ml) to CHO-K1-BH4 cells would make them refractory to the cytotoxic and mutagenic effects of higher doses (0.1-1.0 μg/ml) of MNNG (adaptive mutagenesis), we isolated 8 clones of mutants resistant to the lethal effect of MNNG (1 μg/ml).

Further testing showed that 6 out of 8 mutant clones retain the phenotype of MNNG resistance after subculturing in the absence of MNNG for over 100 doublings. Two out of these 6 resistant mutants were chosen to study dose-response to 6-thioguanine resistance by MNNG in the CHO/HGPRT assay. We found that these two mutants are five times more resistant to the toxic effects of MNNG but that they are more sensitive to MNNG. Enzymic analysis showed that wild type cells, with or without MNNG treatment, exhibit no detectable O^6-methylguanine-DNA methyltransferase activity. These preliminary results suggest that either the mutants incorporate more MNNG causing more mutagenic lesions, or that the mutants are more proficient in repairing cytotoxic lesions resulting in an error-prone pathway mediated mutagenesis. Further studies including the spectrum of resistance to other mutagens and MNNG-induced DNA adducts are under way.

B. Quantitative analysis of the toxicity of sixteen metallic compounds in CHO cells. It is known that some metallic compounds are toxic to many living organisms, including man. Coal contains a variety of metallic impurities. Due to the increasing amount of coal mining and the development of coal-derived synthetic fuel systems, the potentially adverse biological effects of metallic compounds have become environmental concerns. We determined the cytotoxicity to CHO cells of 16 metal salts and correlated metal-ion softness and CHO toxicity in view of the documented correlation between the toxicities of metal ions and parameters characterizing their softness. Since CHO cells can be used in a rapid prescreening toxicity assay, we compared our results with previously obtained toxicity data for mice and Drosophila using metallic salts from the same batches.

We determined toxicity by measuring the cloning efficiency (CE) of CHO cells after exposure to the metals. CHO cells differed by a factor of 10^5 to 10^6 in their toxic response to these metal salts. Cadium(II) was the most toxic ion and Mg(II) the least toxic based on either CE50 (concentration required to reduce the CE to 50%) or D_o (concentration increment which reduced the CE by 63%). On the basis of CE50, the toxicity ranking was Ag > Tl for monovalent metals, Cd > Zn > Hg > Co > Cu > Mn > Ni > Be > Pd > Sr > Mg for divalent methods, and In > Rh > Y for trivalent metals. A similar ranking was found for D_o. For the 11 divalent metals, correlations of CE50 and D_o in the CHO cell assay and the Pearson-Mawby softness parameter for metals were reasonably strong. A good correlation exists between the results of this study on the toxic response in CHO cells and published data on toxicity in mice and Drosophila. It appears that the CHO cell cloning assay may be useful in preliminary screening of metallic compounds and as an indicator of their predicted toxicity in higher organisms.

C. An analysis of alkylating mutagenesis in CHO cells. Earlier, we used the CHO/HGPRT assay to study structure-mutagenicity relationships of 10 direct-acting alkylating chemicals. On an equimolar basis within each structurally related group (nitrosamidines, nitrosamides, alkyl alkanesulfonates and alkylsulfates), chemical reactivity with DNA (s value), mutagenicity and cytotoxicity decrease with increasing size of the alkyl

group. The methylating agents are more mutagenic and cytotoxic than the corresponding ethylating agents; however, significantly different relationships are observed if comparisons are made at equitoxic levels.

Further studies showed that both the toxicity and the production of DNA lesions by a chemical affect the determination of mutagen potency. For example, MNU is 5 times more mutagenic than ENU on an equimolar basis; however, MNU is only 1/2 as mutagenic as ENU at the equitoxic level because MNU is more toxic than ENU. DNA binding studies showed that MNU has 15 times the alkylating activity of ENU. Thus, at equal levels of total alkylation, ethylation of DNA appears to produce more mutants than the corresponding methylation. Our recent observation that a higher proportion of O^6-ethylguanine is produced after ENU treatment as compared with O^6-methylguanine formed from MNU and the longer persistence of the O^6-methylguanine adducts, relative to the N^7-alkylguanine adducts, lends support to the accumulating evidence for the major role of O^6-alkylguanine in alkylating mutagenesis.

We also studied the possible mutagenic role of alkylated dNTPs. Using a protocol for treating CHO cells with calcium phosphate, individual dNTP adducts can be introduced into CHO cells at significant levels. m^6dGTP was found to be neither cytotoxic nor mutagenic. Experiments using [8-^{3}H]-m^6dGTP indicate that this alkylated dNTP, as it occurs in the intact cell, is not a precursor for DNA synthesis. Instead, it appears that this analog can be converted to other nucleoside and nucleotide forms, one of which (O^6-methyldeoxyguanosine) can be repaired by adenoine deaminase. This modified treatment protocol seems to hold promise for the study of other dNTP adducts, as well as other highly charged, usually nonpermeable molecules.

D. Glucuronide conjugation reduces the cytotoxicity but not the mutagenicity of benzo(a)pyrene in the CHO/HGPRT assay. Benzo(a)pyrene [B(a)P] is biotransformed by the mixed-function oxidase (MFO) system to numerous metabolites some of which are cytotoxic and/or mutagenic to mammalian cells. However, conjugation of B(a)P metabolites with glucuronic acid in vivo is a major pathway of detoxication and elimination. The effects of glucuronide conjugation on B(a)P-induced cytotoxicity and mutagenicity were studied using the CHO/HGPRT assay with a rat liver homogenate preparation containing MFO system cofactors (S9-mix) and uridine diphosphate α-D-glucuronic acid (UDPGA). B(a)P metabolites proximate to the biologically active B(a)P quinones [B(a)P 6-OH] and to the B(a)P 7,8-diol-9,10 epoxide isomers [B(a)P 7,8-diol], were also assayed with S9-mix in the absence and presence of UDPGA. The addition of UDPGA to S9-mix reduced B(a)P-induced cytotoxicity but did not affect mutagenicity. B(a)P 6-OH-mediated cytotoxicity was also reduced in the presence of UDPGA. UDPGA had no effect on B(a)P 7,8-diol-induced cytotoxicity or mutagenicity. B(a)P phenols have been shown to be the preferred substrates for UDP-glucuronyltransferase enzymes. Thus, the reduction of B(a)P 6-OH-induced cytotoxicity by glucuronide conjugation may be due to the elimination of cytotoxic phenols and quinones. Since B(a)P 7,8-diol is a poor substrate for UDP-glucuronyltransferase enzymes, no effects on B(a)P-induced

mutagenicity or B(a)P 7,8-diol-induced cytotoxicity and mutagenicity were observed.

2. Molecular and Quantitative Analyses of Mutation in pSV2gpt Transformed CHO Cells (CHO/pSVgpt System).

A pSV2gpt transformed CHO cell line has been used to study mutation at the molecular level. This cell line, designated AS52, was constructed from a HGPRT deficient CHO cell line following the introduction of the plasmid vector pSVgpt. It has been previously shown that AS52 cells contain a single, functional copy of the E. coli gpt gene stably integrated into the Chinese hamster genome. Conditions for its use in the study of mammalian cell mutagenesis have been defined. The rate of spontaneous mutation at the gpt locus (2×10^{-6}/cell division), and phenotypic expression time of XPRT mutants (seven days), compare favorably with that of the hgprt locus in, and expression time of HGPRT mutants derived from, the wild type CHO-K1-BH4 cells.

AS52 and CHO-K1-BH4 cells exhibit similar cytotoxic responses to the four agents that have been examined. However, statistically significant ($p < 0.005$) differences occur in mutation induction by three mutagens. Ratios of $XPRT^-$ to $HGPRT^-$ mutants induced per unit dose of ethyl methylsulfonate (EMS), UV-light, and ICR 191 are 0.70, 1.4 and 1.6, respectively. This ratio is also considerably larger than 1.0 for X-irradiation (6.1-19, depending upon dose), but exact comparisons cannot be made due to a difference in the shapes of the dose-response curves.

A large number of HGPRT and XPRT mutant lines which arose spontaneously or following chemical treatment were analyzed by Southern blot hybridization. None of the HGPRT mutant cell lines which arose following treatment with EMS (21/21), UV-light (23/23), or ICR 191 (22/22) exhibited detectable alterations of the hgprt locus by this technique. Similar observations were made for the gpt locus in EMS- (20/22), UV-light- (21/26) and ICR 191-induced (20/24) XPRT mutant cell lines. X-ray-induced mutants, however, contained a large proportion of deletion mutations affecting both the hgprt (15/21) and gpt (26/26) loci. These results indicate that, for the most part, each agent induces similar lesions at both loci. However, a disparity was found between spontaneous HGPRT and XPRT mutants; most spontaneous hgprt mutants (18/23) exhibited no detectable alterations in DNA sequence, while most gpt mutants (14/23) contained deletions.

The technique of plasmid rescue has been used to recover pSV2gpt sequences from the AS52 cell line. Transformation of E. coli with DNA prepared from AS52 cells resulted in the recovery of ampicillin resistant (Ap^r) colonies that also exhibited the gpt^+ phenotype. In similar experiments, selection for the Ap^r marker presumably allowed recovery, in plasmid form, of adjacent mutant gpt loci from all three $XPRT^-$ derivatives of AS52 tested. While none of the plasmids recovered are useful in their present form, further refinements in cloning techniques should allow the recovery of mutant gpt sequences from the mammalian host cell line. The combined

results of this study indicate that the AS52 cell promises to be useful for future study of mutation in mammalian cells at the DNA sequence level.

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MAMMALIAN CYTOGENETICS GROUP

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The Mammalian Cytogenetics group has continued its studies designed to provide an understanding of the mechanism of induction of chromosomal alterations but with a new emphasis on how specific chromosome translocations or deletions might be induced or transmitted. This is of particular relevance since recent studies have indicated the involvement of specific translocations in the induction or development of cancers. We have also continued with an extensive study of the background frequencies of chromosome aberrations and sister chromatid exchanges (SCE) in a human population group with the intention of attempting to identify the causes of variations in frequency. The direction of the program is to provide information that can be used directly or indirectly for providing estimates of the genetic or carcinogenic hazard of radiations and chemical agents.

1. The Role of Poly(ADP-ribose) in Radiation-induced Chromosome Aberrations. In the past years it has been shown that the synthesis of poly(ADP-ribose) is involved in a variety of cellular processes: regulation of gene expression and differentiation, modulation of enzyme activities, preservation of chromatin structure, and efficiency of DNA repair. The role of poly(ADP-ribose) in DNA repair was of particular interest in our studies of the mechanism of induction of chromosome aberrations as we had shown, in a series of experiments, that chromosome aberrations could result from the interaction of DNA base damaged during their repair by an excision process. It had also been shown that DNA strand breaks were an efficient substrate for the production of poly(ADP-ribose).

3-Aminobenzamide (3-AB), an analogue of nictoinamide adenosine dinucleotide (NAD), is a potent inhibitor of poly(ADP-ribose) synthesis. Thus, our general approach was to irradiate cells, post-treat with 3-AB to inhibit poly(ADP-ribose) synthesis, and then determine if this inhibition could alter the frequencies of induced chromosome aberrations. The following series of experiments based on this general approach would allow for the determination of a role for poly(ADP-ribose) in chromosome

aberration formation and for further studies of possible differences in aberration induction by high and low LET radiation.

Human lymphocytes, prior to PHA stimulation (G_0 cells), were irradiated alone or pre-treated with 3AB (5 mM) for 30 min prior to irradiation with 200 rads of 250 kvp X rays. Post-irradiation incubation with 3AB was for 1, 2, or 6 h. Following the various treatments, lymphocytes were mitogenically stimulated with PHA, and preparations made 48 h later. There was no difference in chromosome aberration frequency for cells X-irradiated alone and those incubated with 3AB after irradiation. This is in contrast to previously reported results from another laboratory.

In contrast, if cells were stimulated with PHA for different periods of time prior to irradiation, there was a marked enhancement of the frequency of chromosome aberrations following post-irradiation incubation with 3AB. The frequency of chromosome aberrations increased with time of treatment after PHA stimulation, i.e., the further cells had progressed into G_1 at the time of irradiation, the greater the effect of 3AB treatment on aberration frequency. The enhancing effect of 3AB on X-ray-induced chromosome aberrations was lost once the cells were in the DNA synthesis or S-phase at the time of irradiation. These results show that the inhibition of poly(ADP-ribose) synthesis by 3AB can increase the frequency of X-ray-induced chromosome aberrations, provided the cells are cycling at the time of irradiation. While a definitive explanation of these results is not possible, we suggest that the inhibition of poly(ADP-ribose) synthesis leads to the inhibition of a step in DNA excision repair that results in the accumulation of gaps in the DNA. On reversing the inhibition, these gaps can interact to form chromosome aberrations in much the same way as we have shown previously using cytosine arabinoside (ara-C) or aphidocolin as inhibitors of excision repair of X-ray-induced base damage.

The reason for the confinement of the 3AB interaction with X-ray-induced damage to G_1 of the cell cycle is presently unclear. It may be, however, that the enzymes that are responsible for the step(s) of excision repair that are under poly(ADP-ribose) regulation are temporally expressed during the cell cycle. Recently, it has been shown that poly(ADP-ribose) regulates the catalytic activity of a topoisomerase purified from calf thymus. It has also been suggested that there is an involvement of topoisomerase in DNA repair. Preliminary experiments in our laboratory with novobiocin, a topoisomerase inhibitor, have demonstrated that it can abolish the 3AB enhancement of X-ray-induced aberrations. Therefore, cell cycle dependent expression of a topoisomerase that is regulated by poly(ADP-ribosyl) action may explain the interaction of 3AB with X ray damage induced in G_1. We are exploring other possibilities, but clearly there is an involvement of poly(ADP-ribose) synthesis in the repair of DNA damage that can lead to chromosome aberrations. Inhibition of this synthesis can result in increased aberration frequencies in cycling cells.

In contrast to these results with X rays, when cells were irradiated with 30 rads of fission neutrons, either in G_0 or G_1, there was no effect of post-irradiation 3AB incubation on the frequency of chromosome

aberrations. These results are very similar to those we have previously reported using ara-C as a repair inhibitor. They further enhance our hypothesis that chromosome aberrations induced by fission neutrons result from the interactions of directly-induced double strand breaks whose repair does not appear to be affected by 3AB or ara-C treatment, whereas X-ray-induced aberrations (at doses above 50 rads) result largely from the interaction of coincidentally repairing base damages. Base damage repair is by an excision process that can be affected by the inhibition of poly(ADP-ribose) synthesis. This proposed difference in mechanisms of induction of aberrations by high and low LET radiations has important consequences with regard to determination of RBE and extrapolation between species or cell types within a species.

2. Differential Sensitivity of Normal Bone Marrow and Leukemic Cells to the Induction of Radiation-induced Chromosome Aberrations. It is of importance to determine the factors that can influence the sensitivity of different cell types or cells from different species to the induction of chromosome aberrations by radiation and chemicals. That there are differences has been well documented, but the reasons for these has not been established. We have chosen to use a model system to provide pertinent information.

A cell line, M90, was established from a myelogenous leukemia of an RFM mouse. The M90 cells and in vitro normal mouse bone marrow cells were analyzed to determine if there was a differential sensitivity to X-ray-induced aberrations and/or differences in post-irradiation cell cycle progression. Cells identified as being in G_1 at the time of irradiation by their staining pattern after replication in BrdU, were analyzed for all types of chromosomal aberrations following X-ray doses of 50, 100, 150 and 200 rads. M90 cells showed a greater sensitivity to the induction of both chromosome-type aberrations (dicentrics and terminal deletions) and chromatid-type aberrations (exchanges and deletions) compared to normal mouse bone marrow cells which only had chromosome-type aberrations. The presence of chromatid-type aberrations in the M90 cells only suggested a differential progression through the cell cycle for the two cell types after irradiation. Mitotic index and flow cytoflurograph analyses were performed and showed that both cell types have a delay in progression from G_2 into mitosis but that only the normal mouse bone marrow cells have a delay in progression from G_1 into S following X-irradiation. These results indicate that the M90 leukemia cells have an increased radiosensitivity which may be due to a defect in their ability to respond to DNA damage as evidenced by their lack of a G_1 delay. This delay would allow normal cells an increased time to repair DNA damage before replication. These same characteristics have been observed in ataxia telangiectasia (AT) cells and may well represent a general feature of cells with increased radiosensitivity.

3. Neutron-induced Chromosome Aberrations in Mouse Bone Marrow Cells, and the Possible Relationship to the Induction of Leukemias. In conjunction with R. L. Ullrich of the Biology Division we are trying to establish if the induction of specific chromosome aberrations are involved in the

initiation or development of neutron-induced myelogeous leukemia and if the analysis of chromosome aberrations in bone marrow cells can be used to predict the frequency of leukemia.

In order to obtain dose response curves for asynchronous (cycling) cell populations it is essential that the position in the cell cycle at the time of exposure be determined for each cell analyzed. This enables the dose response curve to be obtained for comparable cell populations, and also for curves to be produced from cells analyzed in the different stages of the cell cycle. In order to achieve this, a tablet of BrdU is implanted into each mouse immediately after irradiation, or into unirradiated (control) animals at the same time. The particular chromosmal staining pattern observed (following a staining procedure used for chromatid differentiation in M2) enables the identification of the cell cycle stage for each analyzed cell at the time it was irradiated. Animals have been killed and bone marrow preparations made over a wide range of post-irradiation times — every 2 h from 2 to 24 h — such that the whole cell cycle is adequately sampled. At the earlier sampling times insufficient cells were available from the animals given 40 rads. Thus, the earlier sampling time data were obtained from 6 h post-irradiation animals. We also determined that we could make assessments of the appropriate sampling times for the banding studies (when no BrdU would be present) for each of the neutron doses so that equivalent cell populations would be analyzed by calculating a proliferation index. The proliferation index is a measure based on the staining patterns in BrdU-substituted chromosomes. A numerical assignment is given to each metaphase cell based upon the fraction of the cell cycle through which it had passed between irradiation and fixation. Equivalent proliferation indices for two treatments indicate that similar treatment populations are available for analysis at metaphase. It is quite clear that the sampling times required to obtain similar proliferation indices are different for different neutron doses.

All classes of chromosome- and chromatid-type aberrations are recorded, along with the stage of treatment for each cell based upon its staining pattern. The dose response curves are plotted from data that are selected on the basis of a sampling time for each dose that has given similar proliferation indices. Linear dose response curves were obtained for G_1 and S/G_2 irradiated cells.

We have also analyzed about 50 banded preparations, mostly from the 20R group. Seven deletions and five translocations have been identified, but from this small sample it is not yet possible to determine any genomic distribution.

4. The Induction of Random or Non-random Chromosome Exchanges by X Rays and Chemical Agents. We have initiated a series of studies to determine if radiation and chemical agents induce chromosome exchanges randomly or non-randomly within the genome. Since certain cancers are associated with specific chromosome alterations it is of considerable interest to determine if mechanisms exist that would provide for an increased probability of such specific alterations.

We are utilizing three Chinese hamster-mouse hybrid cell lines containing different proportions of hamster and mouse chromosomes. Hamster chromosomes contain only small amounts of repetitive DNA, whereas mouse chromosomes contain considerably higher amounts, particularly at the centromere. Chromosome exchanges have been analyzed following treatment with X rays or mitomycin C, and are recorded as involving hamster/hamster, mouse/hamster or mouse/mouse chromosomes. The frequency of chromatid deletions induced in hamster or mouse chromosomes provides a measure of the relative sensitivity of the two genomes within the hybrid cells. Following X rays the relative sensitivity of the two genomes is approximately proportional to their DNA contents and the chromatid exchanges are distributed with a similar relationship, i.e., they are randomly distributed within and between hamster and mouse genomes. Following mitomycin C treatment there is an increased sensitivity of mouse chromosomes, as measured by chromatid deletion frequency, and in the majority of cases involving the highly repetitive centromeric sequences almost all the exchanges are between mouse chromosomes. These preliminary results suggest that we have a model system for determining the mechanism of induction of specific chromosome aberrations and for the localization of such alterations.

5. Background Frequencies of Chromosome Aberrations and SCE in Human Populations. In collaboration with M. A. Bender of Brookhaven National Laboratory, we have continued our analysis of chromosome aberrations and SCE in a stratified laboratory population. In excess of 300 samples have been analyzed so far, but the data set will not be analyzed statistically until the study has been completed. We intend to look for variations in background frequency and attempt to determine possible intrinsic and extrinsic sources of variation. It is already apparent that there are some unusual observations, including single cells with several aberrations and cells containing a shattered genome. These cells might represent indicator cells of potentially increased sensitivity of an individual, but this can only be ascertained by long-term follow-up epidemilogical studies.

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DNA DAMAGE AND ITS REPAIR

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Human cells are almost constantly exposed to the environmental agents that cause alterations in their genetic material - DNA. Since damage to DNA may be harmful or lethal, it is no surprise that cells possess the ability to repair DNA. Research on mammalian systems, including human cells, has suggested that damage to DNA plays a causal role in carcinogenesis. The cellular mechanisms utilized in the repair of DNA damaged by environmental agents are responsible for maintaining the fidelity of the DNA and, therefore, are critical in the prevention of mutations and cellular transformation. We have studied these mechanisms for some time, especially 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 genetic disease xeroderma pigmentosum (XP), which are repair deficient, are used in these studies. Repair in other cell types is studied whenever such studies can contribute to the 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).

Detection and Repair of a UV-Induced Photosensitive Lesion in the DNA of Human Cells. Ultraviolet light causes a variety of alterations in the DNA of human cells. As has been noted before, the most prevalent lesion formed is the pyrimidine dimer. It's formation and biological consequences are well documented. We know that other DNA damage of unknown biological importance may occur less frequently. Recently, the formation, lethality, and mutagenic action of a minor cytosine - thymine UV-product (TC) has been studied. This TC (6-4) product is formed at one-tenth the rate of pyrimidine dimers but may be responsible for a major biological effect. More recently we have observed another alteration in the DNA of cells irradiated with far-UV light. When human tissue culture cells are given low doses of 254 nm light, essentially no measurable single-strand breaks are formed in the DNA. When these lightly irradiated cells are then exposed to near-UV light of 313 nm, breaks appear in the DNA. These breaks increase linearly with increasing doses of UV (254 nm) light up to about 50 Jm^{-2}. Pyrimidine dimers are probably not involved in the formation of the breaks since fish cells proficient in enzymatic photoreactivation of pyrimidine dimers show little reduction of 313 nm light sensitive sites following a dose of photoreactivating light (360 nm) sufficient to monomerize about 85% of the pyrimidine dimers. In all human cells tested, 20 Jm^{-2} of 254 nm light produces about 2 breaks per 10^9 dalton DNA when followed by irradiation with high doses (1.2×10^5 Jm^{-2}) of 313 nm light. In normal human fibroblasts the 313 - sensitive lesion disappears from the DNA during 5 h of incubation in growth media. As noted before, normal human cells are able to excise pyrimidine dimers from their DNA. In cells derived from patients with the DNA repair deficient genetic disease, (XP), other patterns of dealing with this product are observed. In XP cells most deficient in excision repair of pyrimidine dimers (XPA), the number of these products actually increases during a 5-h period of incubation. In cells derived from XP patients proficient in dimer excision (XP-variant), the product remains in the DNA even after 20 h of incubation. The difference seen in normal cells vs XPA in the above experiments is likely due to the ability of normal cells to perform excision repair and the inability or XPA cells to do this. However, it is well known that XP variant cells have normal excision repair. Why then do they not excise the 313 nm sensitive lesion as do the normal cells? We suggest this is because the lesion must first be modified to an intermediate that is a substrate for the excision repair system and that in XP variant cells this modification does not occur. The modified intermediate may be more sensitive to 313 nm light than the original lesion and this could explain an apparent increase with time in the XPA cells. This explanation seems to fit the observed experimental data we have at the present time.

The Use of Inhibitors in the Study of DNA Excision Repair in Human Cells. We have used agents that inhibit or interfere with DNA replication in hope of a better understanding of biochemical events involved in repair. Arabinofuranosyl cytosine is incorporated into repaired DNA and prevents strand rejoining. Aphidicolin, an inhibitor of DNA polymerase, inhibits DNA repair in confluent cells but not in rapidly cycling cells. Hydroxyurea (HU) at concentrations greater than 10 mM has a pronounced effect on the number of dimers excised over a 24-h period and this inhibitor is more

effective in quiescent cells than in log-phase cells. Novobiocin inhibits UV repair but not repair of methyl sulfonate-induced DNA lesions and is believed to inhibit a DNA gyrase activity.

Cell Growth State and Apparent DNA Excision Repair in Human Cells. Chemical and physical agents damage DNA resulting in various deleterious effects leading to cell transformation and/or death. Studies of UV light chemical to DNA has led to an understanding of the way in which damage is repaired. The steps leading to DNA repair are incision and excision of the altered base(s), DNA resynthesis, and ligation. We have used agents that inhibit DNA replication to better understand the biochemical events leading to repair. Arabinofuranosyl cytosine (ara-C) is incorporated into repaired DNA preventing strand rejoining. The number of DNA single-strand breaks (SSB) is indicative of excision repair. However, the number of breaks observed is related to the cell growth stage. Proliferating cells exhibit fewer SSB's (repaired sites) than cells that are growth-arrested due to contact inhibition. We know from other studies that the actual number of DNA sites repaired is influenced very little by the cell growth phase. The number of sites repaired, as measured by BrdU-photolysis, is not influenced by cell stage. It may be that the ratio of dCTP to ara-CTP in the nucleotide precursor pools and subsequently incorporated into the repaired site determines complete synthesis and rejoining. The efficiency of ara-C to measure DNA excision repair is somehow related to the metabolic state of the cell - probably involving DNA synthesis and the availability of DNA precursors.

The Inhibition of Ultraviolet Radiation-induced DNA Repair in Human Diploid Fibroblasts by Arabinofuranosyl Nucleosides. In collaboration with Dr. R. Snyder, Stauffer Chemical Company, Farmington, Connecticut, we have studied the antiviral compounds 9-β-D-arabinofuranosyladenine (ara-A), 9-β-D-arabinofuranosyl-2-fluoroadenine (FAA), 9-β-D-arabinofuranosyl-hypoxanthine (ara-Hx), 9-β-D-arabinofuranosylguanine (ara-G), 1-β-D-arabinofuranosylthymine (ara-T), 1-β-D-arabinofuranosyl-2-fluorocytosine (FAC), 1-β-Darabinofuranosyl-2'-fluoro-5-iodocytosine (FIAC) and 1-β-D-arabinofuranosyl-2'-fluoro-5-methyluracil (FMAU). The activities of these compounds were compared to the activity of ara-C in the inhibition of UV-induced DNA repair in log and confluent human fibroblasts. In UV-irradiated confluent human fibroblasts, ara-nucleoside treatment led to SSB's. The order of effectiveness in confluent cultures was ara-C and its derivatives >ara-A, FAA, ara-G, ara-Hx>ara-T. In rapidly cycling cells, however, sensitivity to repair inhibition was exhibited only in response to ara-C and FAC. If 2 mM HU was administered with ara-A, FAA, or FMAU, DNA strand breaks were seen. HU also increased the efficiencies of ara-C and FAC. No significant strand breaks were observed in UV-irradiated log phase cells treated with FIAC, ara-Hx, ara-G or ara-T, even in the presence of HU. This differental effect has not been previously demonstrated and must be fully understood in order to gain predictive value in therapeutic applications of the ara-nucleosides. The strong effect on non-cycling cells, generally considered to be immune to inhibitory effects of antiproliferative agents, should be taken into consideration in combination chemotherapeutic application of ara-C and DNA damaging agents.

DNA Repair Arrest by Ara-C in Log Phase and Confluent Cultures of Normal and XP-variant Human Fibroblasts. The ara-C/ara-C,HU assay was used for studies of UV-induced DNA repair in log phase and confluent cultures of normal and XP-variant human skin fibroblasts. Normal and XP-variant cells showed a wide range of sensitivity to both ara-C or ara-C/HU in log phase cultures. The maximal level of repair inhibition was observed in normal cells receiving ara-C alone after UV, but was 65% lower in XP-variant cells receiving ara-C vs ara-C/HU. Repair arrest was more rapidly reversed by increasing concentrations of exogenous deoxycytidine (dCyd) added in combination with ara-C in XP-variant compared to normal cells, especially in confluent cultures. With ara-C/HU, the level of repair inhibition was reduced further in the XP-variant than in cells exposed to ara-C alone. However, the same addition of HU had relatively little affect on dCyd competition in normal cells. Assay of dNTP pools by HPLC in these cell lines showed an elevated level of dCTP in the XP-variant lines during log phase. This is a possible explanation for lowered sensitivity to DNA repair inhibition by ara-C in these cells.

Excision Repair of Ultraviolet- and Chemically-induced Damage in the DNA of Fibroblasts Derived from Two Closely Related Species of Marine Fish. The ability of an organism to modify chemical damages is an important factor in studies of the effect of xenobiotics on aquatic systems. We have examined the capacity of fibroblasts from two closely-related marine fish, the tautog and the cunner, for excision repair of UV- and chemically-induced damage in their DNA. Two different assays, bromodeoxyuridine photolysis and the ara-C repair inhibition assay, were used to demonstrate that the levels of both ultraviolet and chemical excision repair are extremely low in these cells compared to that seen in normal human fibroblasts. In addition, while the levels of repair of N-acetoxyacetylaminofluorene (N-AAF) and ethyl methanesulfonate damage are about the same in both fish, interesting differences in the levels of repair of 4-nitroquinoline-1-oxide, methyl methanesulfonate and UV-induced lesions were seen between the two species.

Quantitation of Carcinogen-induced DNA Damage and Repair in Human Cells with the UVR ABC Endonuclease from Escherichia coli. The endonuclease complex coded for by the uvrA, uvrB, and uvrC genes of Escherichia coli (UVR ABC endonuclease) can incise DNA containing a variety of "bulky-type" lesions, such as those resulting from ultraviolet light (UV), +anti-benzo(a)pyrene diol epoxide (BPDE), and N-acetoxy-2-aminofluorene. The UVR ABC endonuclease was able to incise approximatley 85% of the BPDE-DNA adducts. The endonuclease-sensitive-sites (ESS) were determined by sedimenting the DNA in alkaline-sucrose gradients and estimating its molecular weight. Normal human fibroblasts treated with 1, 2, and 4 μM BPDE for 1 h at 37° had 5.9, 17.0, and 27.2 ESS/10^8 daltons of DNA, respectively. Xeroderma pigmentosum fibroblasts (XPA) had 20-25% more ESS at each dose. Allowing the 4 μM BPDE treated cells to perform excision repair for 12 h resulted in the removal of 60% of the initial number of endonuclease sensitive sites from normal cells and 40% from XPA cells. In cells treated with 10 J/m^2 of UV light the UVR ABC endonuclease detected 12.5 ESS/10^8 daltons of DNA. These results demonstrate the use of the UVR

ABC endonuclease in a quantitative assay for determining the number of carcinogen-induced lesions in human DNA.

Role of Calmodulin in DNA repair; Effects of Trifluoperazine on Repair of Human DNA. The interaction of calmodulin (CaM) with the repair process was studied using trifluoperazine (TFP), a potent inhibitor of CaM function. Results obtained using normal human cells in the dBrU photolysis assay indicated 2 μM TFP inhibits repair 30%. Preliminary results of experiments using ara-C indicate there are 30% more breaks in human skin fibroblasts when the cells are incubated in the presence of TFP and ara-C/HU than in those incubated with ara-C/HU only. The results suggest a possible role for calmodulin in altering the initial incision by the repair endonuclease.

Photoreactivation and Dark Repair in the Vertebrates. It has been well established by us and by other laboratories that enzymatic photoreversal of ultraviolet-induced pyrimidine dimers, i.e. photoreactivation (PR), is an inherent property of vertebrate cells including fish, amphibia, reptiles, birds and marsupials. By contrast, dark or excision repair in these species is usually greatly reduced in comparison to excision repair in human cells. Lower vertebrates generally have about 10% or less of the excision repair capacity of human cells. In recent experiments with cells from the timber rattlesnake, Cortalus horribus, we have observed an exception to this rule. In this species, excision repair capacity exceeds that of human cells. It is the first cell found that is proficient in both PR and excision repair and opens the possibility of a number of experiments involving the relevance of a variety of UV-induced DNA photoproducts to cell transformation and/or death.

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. Summaries of our progress during the past year are presented below in four interrelated areas of research.

Mechanisms of Mutations in Germinal Cells. Our prior genetic and molecular studies on mouse hemoglobins provided the basic information used to determine the structure and organization of the hemoglobin genes of mice. Such knowledge was essential in order to reveal the mechanisms of the α-globin gene deletions induced by X-radiation, the base substitutions induced by ethylnitrosourea and the spontaneous deletion of the β-major globin gene in mice. Two of these mutations have been characterized this year.

We have completed our studies on the amino acid substitution associated with an ethylnitrosourea-induced mutation at the compound β-globin (*Hbb*^{s}) locus in mice. The mutation was discovered by Dr. Susan E. Lewis, Research Triangle Institute, Research Triangle Park, NC, and sent to us for molecular studies. The mutation changed the electrophoretic mobility of one of the β-globin polypeptides encoded by one of the two adult β-globin genes of *Hbb*^{s} mice. Amino acid analysis of the variant β-globin showed that the electrophoretic mobility difference was caused by the substitution of a single amino acid (valine to glutamic acid) at residue 60. The codon for valine at position 60 is GTG; the simplest explanation for the presence of glutamic acid at residue 60 is that ethylnitrosourea induced a substitution of a single base (GTG to GAG) in the DNA. This T to A transversion is the reciprocal of one we reported last year where ethylnitrosourea was shown to have induced a CAC to CTC transversion resulting in an amino acid substitution of histidine to leucine at position 89 of an α-globin gene. Neither of these mutations has been observed previously in humans, mice, or other mammals, and their novel occurrence may be indicative of other unusual mutational events that do not ordinarily occur in the absence of specific mutagen exposure. It is noteworthy that neither of these mutations involves guanosine; ethylation

of the O^6-position of guanosine has been suggested by others as the likely cause of mutations induced by ethylnitrosourea. As additional mutations are characterized, comparisons between newly induced and naturally occurring mutations in the mouse will provide information on the occurrence of unique mutations induced by chemical mutagens.

The break points of the spontaneous deletion of the β-major globin gene of the β-thalassemic mouse have been located and fully characterized as part of our continued collaborative research with Dr. W. French Anderson, National Institutes of Health. The areas immediately flanking the deletion have been cloned and sequenced. The deletion is 3708 base pairs long, 1982 base pairs 5' and 1726 base pairs 3' to the "cap" site for the β-major globin gene. There is a novel sequence 68 base pairs long inserted at the break point. Insertion of this sequence must have been coincident with the deletion because it was not present in the homologous segment of the normal chromosome of the DBA/2 mouse in which the spontaneous deletion occurred. This novel sequence ends in a 25 base pair stretch of poly(A:T). The remainder of the inserted sequence shows marked similarity to the FB3, FB4, and copia elements of Drosophila melanogaster as well as the mouse myc oncogene. The presence of this novel sequence at the site of the spontaneous deletion suggests that the deletion and incomplete "virus" insertion were causally related events.

Regulation of Gene Expression and the Organization of Genes. Mutations at specific loci have been used to identify specific alterations in nucleotides, both within and outside the coding sequences, that affect gene expression. Most studies on gene regulation are performed in terminally differentiated cells but knowledge of these cis and trans acting control mechanisms should help us to understand better how specific genes are regulated during development and cell differentiation and to learn what might go wrong during abnormal growth and differentiation of tumor cells.

Expression of the β-minor globin gene is modulated in β-thalassemic mice. Each erythrocyte in normal adult DBA/2 mice has approximately 13.8 pg of hemoglobin. Eighty percent of the hemoglobin (11.04 pg) is the $\alpha_2\beta_2^{d\text{-major}}$ tetramer and twenty percent (2.76 pg) is the $\alpha_2\beta_2^{d\text{-minor}}$ tetramer. In homozygous β-thalassemic mice the β-major globin gene is deleted and only $\alpha_2\beta_2^{d\text{-minor}}$ hemoglobin is produced; each erythrocyte, on the average, contains 9.0 pg of hemoglobin. Thus, the two β-minor globin genes in homozygous β-thalassemic mice encode 3.26 times as much β-globin as do the same two genes in normal DBA/2 mice. Total RNA was extracted from β-thalassemic and normal DBA/2 reticulocytes, the RNA was immobilized on nitrocellulose paper and probed with a nucleotide sequence that is specific for β-minor globin RNA. The β-minor component of the RNA in the thalassemic reticulocytes was increased approximately threefold compared to the control level in DBA/2 reticulocytes. It is clear that regulation of the β-minor globin gene occurs at the level of transcription. The enhanced expression of the β-minor globin gene might be due to an alteration in DNA around the β-minor globin gene in thalassemic mice. Both normal and β-thalassemic mice have a nuclease hypersensitivity site within the second intron. Thalassemic mice possess an additional DNase I hypersensitivity

site approximately one Kb 5' to the "cap" site. Further molecular studies are required to establish whether or not these alterations in DNA are directly responsible for the enhanced transcription of the β-minor globin gene in β-thalassemic mice.

The level of expression of the homologue of the β-minor globin gene in mice of the $\underline{Hbb^s}$ haplotype can also be modulated. Mice of the $\underline{Hbb^s}$ haplotype have two β-globin genes, called β-s and β-t, but synthesize a single kind of β-globin polypeptide. It was not known whether both genes encode the same polypeptide or only one gene is expressed. The ethylnitrosourea-induced mutation at $\underline{Hbb^s}$, which caused the valine to glutamic acid substitution at residue 60 described above, occurred in the β-s globin gene. The product of the mutant gene, β-s2 globin, can be distinguished from β-s and β-t globin by electrophoresis. Moreover, hemoglobins with these β-globin chains can be completely separated from the three embryonic hemoglobins by electrophoresis. Thus, the relative levels of β-s2 and β-t globins encoded by the two β-globin genes of the mutant haplotype ($\underline{Hbb^{s2}}$) can be studied during development and under conditions of hematopoietic stress. During early fetal life, most of the globin synthesized by the two adult β-globin genes was β-t; e.g., at 11.5 days of gestation β-t comprised over 80% of the adult β-globin. The relative quantity of β-t globin decreased during development. The β-t globin represented only 34 and 27% of the adult globin in newborn and adult mice, respectively. Hematopoietic stress, induced by repeated bleeding or through anemias associated with α- and β-thalassemia, enhanced the expression of β-t from the normal 29% level to between 35 and 42%. We are in the process of testing whether these changes are associated with changes in the relative quantities of β-s2 and β-t specific RNA in reticulocytes.

We have previously reported that the congenic pair, C57BL/10 and C57BL/10.F, differ at the H-2 locus and have mean ages at death of 706 and 456 days, respectively. C57BL/10.F mice also have a reduced basal serum IgA level (63 mg/dl compared to 256 mg/dl for C57BL/10). Backcross and F_2 progenies of reciprocal F_1 hybrids were classified for their H-2 genotypes and IgA levels and their ages at the time of their natural deaths were recorded. When the F_1 maternal parent of the backcross and F_2 progeny was (C57BL/10.F × C57BL/10)F_1, rather than (C57BL/10 × C57BL/10.F)F_1 the incidence of progeny with low basal serum IgA levels was significantly higher. Thus, expression of basal serum IgA levels was controlled in part by the origin of the F_1 maternal parent. In this study mice with the lower basal serum IgA level died earlier than did mice with the higher basal level of serum IgA. In collaboration with J. A. Otten, Biology Division, we have shown that C57BL/10.F mice spontaneously shed an endogenous ecotropic retrovirus. This virus might be responsible for both the suppression of the basal serum IgA level and the shortened life span of the F_2 and backcross progeny of (C57BL/10.F × C57BL/10)F_1 mothers. We have shown that endogenous ecotropic retrovirus sequences are reintegrated in DNA of somatic cells isolated from the lymph node and spleen of C57BL/10.F mice. We are presently investigating whether these retroviral sequences are reintegrated into specific lymphoid populations of the differentiating

immune system. If so, the ecotropic virus might suppress the basal serum level of IgA, which results in life shortening.

Physiological and Pathological Effects of Specific Mutations. Hematological studies in mice of the $\underline{Hbb}^{s2}$ haplotype did not detect any overt abnormalities associated the valine to glutamic acid substitution at residue 60 of the β-globin. However, the ratio of α/β globin synthesis was found to be 1 in $\underline{Hbb}^{s2}$ mice that were also heterozygous for α-thalassemia (we expected this ratio to be about 0.75). This result suggested either that β-globin synthesis is deficient in these mice or that the β-globin being synthesized is unstable and rapidly degraded. The globin monomers are known to be less stable than α/β dimers or $\alpha_2\beta_2$ tetramers; therefore, the excessed β-globins in heterozygous α-thalassemic, homozygous $\underline{Hbb}^{s2}$ mice might be degraded by proteolysis if the valine to glutamic acid substitution makes the β-s2 globin unstable. Direct tests were done to compare the stability of $\alpha_2\beta_2{}^{s2}$ and $\alpha_2\beta_2{}^{s}$ hemoglobins of mice of the $\underline{Hbb}^{s2}$ and $\underline{Hbb}^{s}$ haplotypes. The analyses showed that the former hemoglobin was less stable. In collaboration with Dr. Joseph Fuhr, University of Tennessee Memorial Research Center, we found that the $\alpha_2\beta_2{}^{s2}$ hemoglobin had a higher affinity for oxygen (P_{50} at 35 mm of Hg) than did $\alpha_2\beta_2{}^{s}$ hemoglobin (P_{50} at 44 mm of Hg). The position of the substitution of valine to glutamic acid is three amino acids away from the heme binding histidine at residue 63; it lies within an amino acid sequence in which five of eight amino acids are positively charged. The presence of the negatively charged glutamic acid residue within this sequence of positively charged amino acids apparently weakens the non-covalent bond between heme and β-s2 globin and the nascent chain is more susceptible to proteolysis.

The above observation prompted us to examine the oxygen binding affinity of hemoglobin that contains the histidine to leucine substitution at residue 89 in the α-chain. This substitution is located two amino acids away from the heme binding histidine at residue 87; it lies within an amino acid sequence in which four of six amino acids are positively charged. This hemoglobin also has a higher affinity for oxygen. Studies will be done to determine whether hemoglobin with the mutant form of α-globin is less stable than normal hemoglobin and matings are being made to determine the combined effects of these mutations in mice.

A longitudinal study on the hematology of a group of β-thalassemic mice and their heterozygous and normal littermates has shown that the anemia became more severe in older thalassemic mice. Blood hemoglobin in homozygous β-thalassemic mice fell from 10 g/dl to less than 8 g/dl between 4 and 15 months of age. The technique of ^{51}Cr labeling of erythrocytes was used to show that the mean life span of erythrocytes from homozygous β-thalassemic mice was only 16 days when transfused into normal isogenic mice and only 20 days when transfused into isogenic mice that had been splenectomized. In comparison, erythrocytes from heterozygous β-thalassemic mice and normal littermates had mean life spans of 39 and 40 days, respectively. The spleen of homozygous β-thalassemic mice weighs six times as much and contain four times as many hematopoietic stem cells as does the spleen of littermate heterozygotes and control mice. Splenic

hematopoiesis probably contributes to the production of the extra erythrocytes required to sustain erythrocyte homeostasis in β-thalassemic homozygotes in which the mean erythrocyte life span is reduced to less than 50% of that in normal mice.

Survival data on a small population indicate that the mean age at death will be less than 20 months for homozygous β-thalassemic mice and about 24 months for heterozygotes and controls. The fact that the homozygotes survive into adulthood and remain moderately healthy makes them a very suitable animal model in which to develop and test alternative techniques of gene therapy that everyone hopes can be successfully applied to the treatment of human thalassemia. Histological sections show that several tissues in homozygous β-thalassemic mice have large quantities of stainable iron, which might also make these mice useful in in vivo tests of the effectiveness and possible long-term side affects of iron chelators being used to treat human patients.

Drs. Janan T. Eppig and Jane E. Barker, The Jackson Laboratory, have shown that 5 to 15% of the cells in mice with the inherited Hertwig's anemia exhibit abnormal mitosis. These cells contain more than the diploid but less than the tetraploid number of chromosomes. Cells with less than 40 chromosomes were not scored because one cannot be sure whether or not some chromosomes are lost during the preparation of chromosome spreads. In collaboration with R. Mann and R. E. Hand, Jr., Biology Division, the quantity of DNA in propidium iodide stained hematopoietic cells of these mice has been examined by flow cytometry. The results showed that the distribution of hyperdiploid cells is definitely not random and that there are very few hypodiploid cells in the hematopoietic tissues of mice with Hertwig's anemia. These results indicate that many hyperdiploid cells survive but the hypodiploid cells do not. Cytological analyses are being done to determine whether specific chromosomes are represented more frequently among the hyperdiploid cells.

Studies on the Toxicity and Mutagenicity of Ethylene Oxide in Mice. These studies are being done in collaboration with S. Lock, Biology Division. Additional data have been obtained on the frequency of 6-thioguanine resistant (putative mutant) pluripotent stem cells in the bone marrow of mice exposed to ethylene oxide and C57BL/6 controls. Five mutant colonies have been identified among the 562,100 pluripotent hematopoietic stem cells analyzed from mice exposed either for 1, 2, 4, 8 and 14 days or for 4, 6, 8 and 10 weeks (mutation frequency of $0.89 \pm 0.38 \times 10^{-5}$) compared to three mutant colonies among the 483,000 stem cells analyzed from controls (mutation frequency of $0.62 \pm 0.32 \times 10^{-5}$). Four of the mutant colonies came from mice exposed to ethylene oxide for 4 and 6 weeks. R. Mann, Biology Division, has used data from this experiment to calculate the critical sample sizes for determining the statistical significance of mutation frequencies where the true mutation frequencies are not known. The use of larger control sample sizes reduces the size of the treated sample necessary to rule out four-, three-, or twofold increases of the mutation frequency in the treated group. Using a 1,000,000 control group, a significance decision on a two- to fourfold

increase in the spontaneous mutation frequency can be obtained using treated sample sizes of 124,313 to 356,400 stem cells. With modest effort and cost such sample sizes can be obtained since the average number of pluripotent hematopoietic stem cells in the bone marrow of each C57BL/6 mouse is 13,635.

Inhalation of ethylene oxide affects the hemopoietic system of mice. Hematocrit, red cell number, bone marrow cellularity and the number of pluripotent hemopoietic stem cells are about 90% of values for controls. Fetal tissues are often more sensitive to chemicals than are the same tissues in adult animals so we have begun to explore the effects of ethylene oxide on the developing hemopoietic system in the fetal liver of mice. Pregnant females (13.5 and 14.5 days post copulation) were exposed to 255 ppm of ethylene oxide, 6 h a day for 4 days. This exposure caused a 40% reduction in fetal weight, a 35% reduction in fetal liver cellularity, a 32% reduction in the incidence of pluripotent hemopoietic stem cells in the liver and a 57% reduction in the number of stem cells in fetal liver. The data show that the developing hemopoietic tissue of the fetus is indeed more sensitive to the cytostatic effects of inhaled ethylene oxide. Differential sensitivity to mutagens and toxic substances of fetal and adult tissues should always be considered in making estimates of the risk to women of exposure to volatile chemicals.

A small percentage of humans are highly sensitive to ethylene oxide. The basis for this sensitivity is presently unknown and an experimental animal model to test for extreme sensitivity to ethylene oxide has not been identified. During studies to test for the effects of ethylene oxide in mice with a variety of genetic deficiencies, we found a strain that was much more sensitive than others tested. F_1 hybrids between the sensitive and a resistant strain are resistant to ethylene oxide, thus sensitivity is a recessive trait. Classical genetic tests are being used to map and identify the genetic factor(s) associated with sensitivity to ethylene oxide in mice. It should then be possible to determine whether humans who have different sensitivities to ethylene oxide show similar genetic differences.

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ANAEROBIC MICROBIOLOGY

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During this reporting period we have continued to use our novel methods for producing anaerobic conditions in studies of oxygen sensitive bacteria. These bacteria are of interest because they can be used to produce fuels and solvents, are responsible for several important diseases and can be informative in studies of the mechanisms of oxygen toxicity and radiation inactivation.

We have demonstrated that techniques based on the oxygen-reducing properties of Escherichia coli membrane preparations are very efficient for isolating new strains of anaerobes from soil samples. Acetone and butanol producing organisms, most probably examples of Clostridium acetobutylicum, were found in 54 out of 64 soil samples examined. This frequency is significantly higher than found by conventional techniques. The concentrations of acetone and butanol produced by these isolates were comparable to those observed for our best laboratory reference strain. The details of this work are contained in a manuscript submitted for publication in Applied and Environmental Microbiology.

We have also used the oxygen-consuming membranes in collaborative experiments with members of the Chemical Technology Division. Preliminary results indicate that removal of oxygen by the membrane fraction increases the rate at which the mixed populations of microorganisms found in sewage sludge or experimental digestors can degrade radioactive cellulosic wastes. This work is continuing in order to optimize the process and determine which organisms are most affected by the presence of the membrane fraction. We have completed experiments in collaboration with members of the University of Tennessee Veterinary Hospital. These experiments establish that media made anaerobic by use of the Escherichia coli membrane fraction are useful for detecting anaerobes in clinical samples. We have initiated experiments in collaboration with investigators at Cornell University to explore further the ability of the membrane fraction to stimulate the growth of methanogenic bacteria.

In collaboration with M. E. Bolling and W. E. Masker (Biology Division, ORNL) we have established that an Escherichia coli polA(Ex) mutant which is normally inviable at 43°C can be induced to grow and divide

temperature. This activity of the membrane fraction depends not only on anaerobiosis but may also require some material, as yet unidentified, that is a normal component of the fraction. These observations are similar to ones made earlier by our group when we established that the recovery of irradiated "lon" mutants of Escherichia coli, in the presence of membrane fraction, depended both on anaerobic conditions and on another alteration of the environment brought about by some component of the fraction. The details of this work are contained in a manuscript accepted for publication in the Journal of Bacteriology.

Our efforts to understand the mechanisms of oxygen and radiation damage in microorganisms are pursued by two kinds of experiments. In one of these, oxygen sensitive mutants of the normally facultative bacterium Escherichia coli, are isolated after treatment with a mutagen. The mutants obtained are then characterized genetically and biochemically. During this reporting period we have developed the first techniques for isolating such mutants and have screened several thousand clones. Five suspect oxygen-sensitive mutant clones have been obtained after ultraviolet light treatment of the parental culture, AB1157. These "mutant" clones grow slowly or not at all in the presence of air. As far as they have been tested, they retain the amino acid requirements and streptomycin resistance of the parental strain. Further characterization of them should help determine the enzymatic functions necessary for an organism to prosper in an oxygen-containing atmosphere.

A second approach to the study of oxygen and radiation sensitivity mechanisms involves the use of strains of the anaerobic bacterium Clostridium acetobutylicum. We have reported that the strains of this organism that are most sensitive to oxygen are also most sensitive to ionizing radiation. This suggested that there might be a common mechanism by which these two agents inactivate or that the same repair mechanisms might be effective. We have therefore performed experiments to explore the molecular lesions produced in the DNA of Clostridium acetobutylicum by oxygen and ionizing radiation and have observed that the apparent average molecular weight of DNA increases with increasing exposure times of the whole cells to air. The data were obtained by lysing cells, after exposure to air, on alkaline sucrose gradients and performing the appropriate centrifugation. The apparent average molecular weight in unexposed cells is 80×10^6 and reaches a value of 300×10^6 after air has been passed through the culture of cells for 60 seconds. We have also observed that, if the cells are returned to an oxygen free environment, this unusual "damage" is repaired and the average molecular weight is reduced. Before performing these experiments, it had been anticipated that if air exposure has any effect on the DNA of these anaerobes it would result in a decrease in average molecular weight. The unusual observation of an increase in average molecular weight has been observed in many experiments and the phenomenon is not yet understood.

As indicated in earlier progress reports, we are attempting to develop systems that will facilitate the genetic engineering of anaerobic micro-organisms. To do this we must develop a collection of stable mutants. Our

experience, and that of others, has been that vegetative cells of Clostridium are unusually refractile to mutagenisis by the commonly used physical and chemical mutagens. We have now developed a system in Clostridium butyricum that yields mutants in reasonable numbers. Spore suspensions of this organism have been exposed to large doses of ionizing radiation and antibiotic resistant mutants have been obtained. The frequency of mutants approximates a linear function of dose. It remains to be seen if this technique will work for spores of Clostridium acetobutylicum.

During this reporting period we have also explored some additional applications of the oxygen reducing properties of the E. coli membrane fraction. The membrane fraction has been bound to various supporting materials (sepharose acrylamide, etc.) and suspended in columns of different configurations. Oxygenated 10 mM Tris buffer containing a small amount of sodium lactate has been pumped into these columns and the oxygen content of the effluent measured. The most effective column so far devised is an 18 cm column capable of delivering deoxygenated buffer at a rate of 3 ml/minute for at least 3 weeks.

In addition to the research accomplishments discussed above, our group has continued to operate the Biology Division Fermentation Facility. We have grown large quantities of various microorganisms for various division members and have also grown cells for investigators at the University of California, San Diego; Wesleyan University; the University of Seoul; the University of South Carolina; and a small company (Energenetics) attempting to produce fuels by the anaerobic fermentation of cheese whey.

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VALIDATION OF A SHORT TERM ASSAY FOR TERATOGENS: FETAX (Frog Embryo Teratogenesis Assay: Xenopus)

J. N. Dumont

The FETAX (Frog Embryo Teratogenesis Assay: Xenopus) system is a rapid, short term, inexpensive assay that can detect with reasonable accuracy compounds that are potential teratogens. In addition to providing guidance prior to more costly testing, the FETAX system is a useful tool for detailed studies on the underlying effects of teratogens on developing tissue and organ systems. This is especially useful when one realizes that not all teratogens have the same mode(s) of action: some produce adverse

developmental effects by provoking the genetic makeup of cells, others interfere with normal cell division, still others produce toxic effects that kill cells or cell populations.

In recent years there has been intense interest in the scientific community regarding the development of short term, inexpensive in vitro assays for the detection of teratogens. Some have been developed but, aside from the more costly models that use mammalian organ or embryo culture, only FETAX uses whole, viable, vertebrate embryos. The FETAX system is simple to use, has easily detected end-points, gives quantitative dose-responses, and provides a measure of both embryo toxicity and teratogenicity. Thus the assay is useful for assessing potential toxicity of compounds or complex mixtures likely to be found in the environment but, in addition, provides data that can be used to rank compounds on the basis of their potential teratogenicity.

Rankings of potential teratogenicity of materials are based on the TERATOGENIC INDEX (TI) - a number generated from the ratio of the LC_{50} and the EC_{50} (EC_{50} - concentration that produces 50% abnormal embryos among those that survive the 96 hour test period). TIs of 1.0 or less indicate that the compound (or other test material(s) which may be complex mixtures such as might be found in environmental spills) is embryotoxic. TIs greater than 1.0 indicate increasing teratogenicity. The larger the value of the TI, the higher the potential for teratogenic activity. Some compounds have very high TI values, e.g., 5-fluorouracil, 33; aminonicatinamide, 20; thiosemicarbazide, 28; nickel chloride, 36; others give more moderate values, e.g., retinoic acid 6.6; trypan blue, 4.5; ethylene oxide, 7.6. These compounds are considered highly teratogenic. Compounds that are considered embryotoxic (and non-teratogenic) include disulfram (TI = 0.33), amaranth (TI = 0.52), and Lindane (TI = 1.0). Thus, on the basis of the TI values, compounds can be ranked according to their potential embryotoxicity/teratogenicity and the ranking used to make priority listings for further testing in more expensive and long term systems.

An important aspect of the use of any short term system for drawing implications about teratogenicity (or other expressions of toxicity) is the validity or reliability of the system. The FETAX system has been tested with 41 known mammalian teratogens. It was able to predict with 85% accuracy (false negative rate = 15%) the teratogenicity of these compounds. Those it failed to identify as teratogens include caffiene, captan, phenylalanine, and cytochalasin. In some of these cases there is still question about the correct classification (teratogen vs non-teratogen) of these compounds. Others, like cytochalasin, are known terataogens but are so toxic to early developing embryos that cell division is prevented. Nine non-teratogens have also been examined. Some of these compounds (e.g., propylene glycol, saccharin, sodium cyclamate) while not very toxic, require very high concentrations to produce teratogenic effects (false positives). Nevertheless, the accuracy (true positive) rate of 85% on the compounds tested indicates that the FETAX system is a useful predictive tool for the identification of potential teratogens. The data also suggest

that the assay system is capable of identifying those materials that may be truely toxic as opposed to teratogenic.

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Mammalian Genetics and Reproduction Section

SECTION OVERVIEW - LIANE B. RUSSELL

The work of the Mammalian Genetics and Reproduction Section concerns itself almost exclusively with the germ line. Effects of chemical and physical agents are studied in the germ cells of treated mice or in offspring derived from such cells. Methods for the measurement and evaluation of such effects are developed and validated. Basic changes in the genetic material are analyzed, and sometimes exploited as tools in the study of gene structure and expression.

While many new studies have been initiated since last year, and ongoing studies have progressed satisfactorily, the major themes of the Section's research remain the same: (1) The frequency of induced heritable damage. This includes the detection of inherited lesions representative of various endpoints, and the study of how their relative frequencies depend on biological and physical variables. (2) Basic genetics and cytogenetics, including the genetic and fine-structure analysis of induced and spontaneous mutational changes, and studies of chromosome behavior in the germline. (3) Developmental genetics and the study of phenotypes. We are concerned not only with the frequency of mutations but also with their effects, particularly such dominant effects as reduced viability, morphological anomalies, and inherited sterility. (4) The interaction of chemical agents with germ-cell DNA. This includes detection of primary DNA damage in gonads by means of both unscheduled DNA synthesis (UDS) and indices of fragmentation, and the measurement of molecular doses in germ cells. (5) Reproductive biology. The study of normal and abnormal gametogenesis, germ-cell transport, fertilization, early embryonic development, and the effects of various agents on these processes is inextricably tied to mammalian genetics.

There is no sharp division between these themes; on the contrary, there are many interfaces and some reciprocal relationships between basic and more applied aspects of the work. For example, the altered genes and chromosomes scored in mutagenesis experiments can provide excellent tools for studies on gene structure and expression; in turn, a more basic understanding of the nature of the mutational lesions induced can help elucidate the manner of action of certain chemical or physical agents. Similarly, the testing of specific chemicals can be carried out in such a manner as to contribute to method development or to our understanding of the detailed properties of the biological material.

Method development and validation have proceeded in a number of diverse areas, notably the following:

- Handling of "cluster" mutants, specifically, development of a method for estimating the number of independent events when mutation rate and progeny per male are both high; and the testing of cluster-handling methods by computer simulation.

- Extension of tests of alkaline-elution procedures for sperm DNA to include chemicals of the S_N-1 as well as S_N-2 type of reaction mechanisms, and to sample various germ-cell stages. Parallelisms demonstrated between the patterns of DNA elution and transmitted clastogenic damage are hopeful signs that the method might become applicable to human monitoring.

- Broadening of inhalation studies for examining genetic risk.

- The measurement of late intrauterine death in the detection of presumed nondisjunction events.

- Use of an interspecies cross to demonstrate that a lethal mutation which, in the heterozygote, is deficient for LDH-A activity in fact has a deletion of genetic material (i.e., of a restriction fragment shown to contain the Ldh-1 gene).

- Utilization of a set of overlapping deletions previously analyzed by complementation mapping to exploit analysis of a genomic region to which entry has been gained via a stably integrated murine leukemia virus.

- Demonstration that the relation between frequency of translocations scored cytologically in meiocytes of exposed males and frequency of transmitted translocations follows no clear pattern.

- Review of 14 mammalian germ-line tests and 7 tests for gonadal exposure; examination of selection of tests.

Although the testing of chemicals is not a major objective of the Mammalian Genetics and Reproduction Section, a number of substances were again investigated this year. In addition to some of the standard alkylating agents, which, along with radiation, were used as tools in the investigation of biological processes or for method development, the agents studied for various genetic effects were ethylene oxide, dibromochloropropane (DBCP), hexamethylphosphoramide (HMPA), ethanol, urethane, 6-mercaptopurine, pyrene, benzo[a]pyrene, 2-acetylaminofluorene, 4-acetylaminofluorene, and ^{239}plutonium. The related compounds, ethylnitrosourea (ENU) and methylnitrosourea (MNU), continued to be used for in-depth studies to explore the biological variables that affect mutagenicity in mammalian germ cells.

Since mammalian germ-line tests, among all mutagenicity assays, are uniquely qualified to provide information pertinent to genetic risk

assessment, we have always considered the latter to be among the major missions of the Section. Several of the studies already alluded to have some bearing on genetic risk assessment, but the following are particularly germane to the subject.

- The effects of ethylene oxide are being studied over a range of concentrations and exposure times for a variety of genetic endpoints.

- The heritable translocation frequency per R of X rays has for the first time been reliably determined for the cell stage of greatest pertinence to risk in men, the spermatogonial stem cell.

- The amount of radiation-induced dominant damage in spermatogonia that results in death between conception and weaning age has been shown to be 19 per million liveborns per R of low-LET irradiation. Most of this death occurs during the early intra-uterine period.

- Additional evidence has been presented that ethyl alcohol consumed shortly after copulation may induce nondisjunction.

- Synergistic interaction of unlinked skeletal mutations has been demonstrated. Such interactions of dominants may be the cause of some human genetic disorders that are presently thought to have autosomal recessive inheritance.

Findings in several of the experiments provided information about the properties of the biological system and about the nature of its interaction with environmental agents. Some examples follow.

- Strain differences have been demonstrated for X-ray induction of "dominant lethals" (segregation products of induced translocations) in spermatogonial stem cells.

- The effect of strain on dominant-lethal frequency of the untreated females mated to exposed males, has been shown to exist for yet another chemical, MMS.

- A major strain difference has been found in the rate at which N7-ethyl guanine is removed from testis following ENU treatment.

- Results of experiments in which exposures to X rays were followed, at various intervals, by ENU treatment provide tentative evidence that ENU exerts much of its mutagenic effect in S-phase spermatogonia.

- The critical period for ethanol-induced nondisjunction is probably the time of completion of the second meiotic division of the oocyte.

- Mitotic activity in spermatogonia surviving an acute X-ray exposure shows evidence of cell synchronization, probably resulting from the killing of sensitive stages in the cell cycle.

- Additional data confirm the absence of circadian rhythms in spermatogonial stem cells. There is no effect of time of day on mitotic index, radiation sensitivity, or DNA synthetic activity.

- A study of seminiferous tubule whole mounts has confirmed the slight decline with age in spermatogonial stem-cell numbers reported last year on the basis of sectioned material.

- The concentration of inhaled ethylene oxide may determine the shape of the dose curves for induced chromosome aberrations.

Mutations and chromosome aberrations generated in mutagenesis experiments continue to provide valuable material for basic studies in genetics and cytogenetics.

- Among the mutations involving one of the specific loci (p), we identified a deletion that also involves the nearby structural gene for lactate dehydrogenase A. Molecular studies have shown that tissue-specific levels of this isozyme are controlled pretranslationally.

- Overlapping deletions involving the specific-locus marker (d) are proving valuable in illuminating the organization of a locus associated with a stably integrated murine leukemia virus.

- X-autosome translocations have been rare but highly valuable rearrangements because autosomal genes become subject to X inactivation. The majority have been studied at our laboratory, and we have now reviewed their various characteristics. A newly arisen X-autosome translocation will be particularly valuable in shedding light on inactivation mechanisms.

- Electron-microscope studies of pachytene spreads suggest that spontaneous sex-vesicle loss may be a significant cause of missing paternal sex chromosomes in the conceptus, and may at least partially account for the predominance of $X^{M}0$ over $X^{M}X^{P}Y$, and of $X^{M}0$ over $0X^{P}$.

- Analyses of synaptonemal complexes in male X-autosome translocation carriers, as well as other evidence, lead to the hypothesis that X-Y pairing is nonhomologous, with crossing-over prevented by "precocious" desynapsis.

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 made it feasible to study the biological and treatment factors that can affect the rate of chemically induced mutations in mammalian germ cells. Last year we reported on an extensive dose-response curve, which falls below linearity in the low-dose range, on the major effects of sex and germ-cell stage, on the mutational spectrum (distribution among the 7 loci), and on the use of large-dose fractionation to augment mutation rate in spermatogonia. Some 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 an experiment in which 20 fractions of 5 mg/kg of ENU (total dose 100 mg/kg) were injected at 3- or 4-day intervals over a period of 10 weeks. The totals obtained to date are 10 mutations in 22,337 offspring. The induced mutation rate (experimental minus control) is 74% of the induced rate obtained earlier in a 10 × 10 mg/kg fractionation experiment. The reduction is not statistically significant, but suggests that repair may be greater with the smaller dose fractions.

ENU exposure of pre-irradiated males. Two separate sets of experiments were conducted. The first was done to test whether a dose of X rays (100, 300 or 500R) would augment the mutational effect of a dose of 100 mg/kg of ENU injected 24 h later. (Splitting a dose of X rays in this fashion greatly increases the mutation rate in spermatogonia.) The total results now obtained from the three experiments with the different X-ray doses, followed by the ENU dose, are 23 mutations in 9,533 offspring. This can be compared with 35 mutations expected on the basis of additivity, i.e. the sum of radiation-induced, ENU-induced, and spontaneous. The observed number of mutations falls below expectation in all three groups. Thus there is no evidence of an augmenting effect. In fact, the total mutation frequency is on the borderline of being significantly below the frequency expected on the basis of additivity ($P = 0.07$).

It is clear from the above experiment that the post-irradiation interval that sensitizes spermatogonia to a second radiation exposure does not sensitize them to ENU. A possible explanation lies in the fact that the proportion of cells in S phase 24 h after X-ray exposure is lower than normal. If, as seems plausible, this is the phase most sensitive to ENU-induced mutation, then one might expect a result less than additive with the regimen used in the above set of experiments.

A second set of experiments was started to test the hypothesis that ENU is most effective in S phase. 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 given a dose of 100 mg/kg of ENU at these intervals after these doses of X-rays. The data are not yet extensive enough for a firm conclusion. With the 150 R X-ray exposure 11 mutations have been obtained in 3,453 offspring, and 11.5 mutations would have been expected on the basis of additivity. In the 300 R X-ray experiment, with the 5-day interval before ENU treatment, 16 mutations have been observed to date in 3,017 offspring, whereas only 11 would have been expected on the basis of additivity. Thus these latter results are at least in the direction expected on the above hypothesis.

Results with females exposed to ENU. In a continuation of the experiment in which females were injected with 100 mg/kg of ENU, we have obtained 1230 offspring from matings made in the third to sixth week after injection. No mutations were observed. We also obtained an additional 6,978 offspring from matings made after the sixth week postinjection, and one mutation was observed. Combining these last data with all earlier data from arrested oocytes exposed to ENU still fails to give a mutation rate significantly higher than the control.

In order to test the possibility that the failure to induce mutations in females exposed to 100 mg/kg of ENU might be due to oocyte selection (i.e., that the only oocytes that survive might be those in which the nucleus received a lower than average ENU concentration), we have started an experiment with a dose of 50 mg/kg. To date this has yielded 2,123 offspring from matings made in the first 6 weeks after injection and 2,110 offspring from later matings. No mutations were observed in either group.

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DEVELOPMENT OF A NEW METHOD FOR ESTIMATING THE NUMBER OF INDEPENDENT EVENTS SAMPLED IN SPECIFIC-LOCUS EXPERIMENTS CONTAINING CLUSTERS

P. B. Selby

A group of mutations of a given type, found among the offspring of a single mouse in a specific-locus experiment, is referred to as a cluster. Clusters have been found in many specific-locus experiments, and W. L. Russell (In: Peaceful Uses of Atomic Energy, Intern. Atomic Energy Agency,

Vienna, pp. 487-500, 1972) proposed a method for analyzing data statistically when clusters occur. His method works well if the frequency of induced mutations is low enough to make it highly improbable for two independent mutations to occur among the offspring of any one parent. Recently, however, some chemical mutagens have been found to yield much higher mutation frequencies than those induced by radiation. In addition, the average number of offspring per treated male is often much larger in mutagenesis experiments with chemicals than with radiations. W. L. Russell (Proc. Natl. Acad. Sci. USA 79: 3589-3591, 1982) therefore suggested that some apparent clusters might actually be groups of independent mutants. If such "false" clustering accounted for an important fraction of the clusters seen in an experiment, application of Russell's earlier method of correcting for clusters in statistical analyses would decrease the chances of rejecting the null hypothesis in a mutagenesis study.

In order to overcome this problem, we have devised a method for calculating the expected numbers of true clusters and false clusters for a given experiment. Our method uses the probability function of the binomial distribution, and it requires information from the experiment as to the mutation frequency per locus and the mean number of offspring produced per experimental animal. The calculation also yields best estimates of the numbers of independent mutations and independent offspring to be used in statistical tests. We have written a program in APPLESOFT II BASIC that carries out the calculations (which are extremely tedious when made manually) and have used it to analyze results from some of the specific-locus studies carried out in our Section. In some experiments, many of the clusters were found to be false clusters.

APPLICATION OF COMPUTER SIMULATIONS TO IMPROVE UNDERSTANDING OF HOW TO ANALYZE DATA WHEN CLUSTERS OF MUTATIONS ARE PRESENT

P. B. Selby

In order to improve our understanding of how best to analyze data when clusters of mutations are present (see preceding report), we have written a computer program in APPLESOFT II BASIC that permits detailed simulations of specific-locus experiments. Variables that can be set in the program are the mutation frequency, the spectrum of mutations, the number of stem cells surviving treatment, and the number of offspring sampled per male. Pseudo-random numbers (of a type well-suited for making probability decisions) are generated to determine whether mutations occur and how frequently each independent mutation is sampled. The simulations make it possible to test the validity or accuracy of various methods of analysis that we or others have used. For example, simulations demonstrate that the new method for determining how many independent events have been sampled (see preceding report) is superior to the method that we have been using when correcting for clustering in statistical analyses. The simulation program will have various other applications.

SPECIFIC LOCUS STUDIES WITH FOUR CHEMICALS

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The data base for chemicals investigated in the mouse specific-locus test is being steadily enlarged. This year, we completed experiments on two chemicals (DBCP and HMPA) and got well underway with studies on two additional ones (urethane and 6-mercaptopurine). The work is being carried out under contract with the National Toxicology Program.

1. 1,2-dibromo-3-chloropropane (DBCP). The nematocide DBCP produced negative results in a specific-locus test for gene-mutation induction in the germ line of male (101 × C3H)F_1 mice. Most of the males were treated with 5 daily intraperitoneal injections of 80 mg/kg DBCP in corn oil (total exposure, 400 mg/kg); a few received lower exposures. Among 39,519 offspring derived from treated spermatogonial stem cells, there were 2 mutants, an incidence almost identical to the spontaneous one. This result rules out (at the 5% significance level) an induced mutation frequency greater than 2.0 times the historical control rate. Among 6,240 offspring derived from treated post stem-cell stages, there were no mutants, ruling out a multiple of 8.0 times the control (an inconclusive result). While clear fertility effects of DBCP have been found in other mammalian species, the fertility of the treated (101 × C3H)F_1 males was not affected. It is therefore possible that the negative genetic results may be due to superior repair capacity of spermatogonial stem cells or to a failure of (101 × C3H)F_1 mice to metabolize DBCP to active intermediates. Experiments are in progress to obtain evidence on the latter of these possibilities.

2. Hexamethylphosphoramide (HMPA) is a Drosophila mutagen which has yielded mixed results in mammalian in vitro tests. A single i.p. injection of 2000 mg/kg HMPA in HBSS [found by us to be the MTD for (101 × C3H)F_1 males] produced no fertility disturbances at any post-exposure interval. The spermatogonial stem-cell results (no mutants in 22,379 offspring) are clearly negative; the minimum multiple of the historical control rate that is ruled out by the 2000 mg/kg results is 1.5 (at the 5% significance level). For post stem-cell stages, the results for the small sample are inconclusive (0 mutants in 2901 offspring) and rule out a multiple of 18.2× control rate.

3. Urethane. Urethane is a rodent carcinogen that has given widespread, though erratic, evidence of mutagenicity and carcinogenicity. Of greatest interest is the recent 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 carried out at the MTD of 1750 mg/kg, urethane being dissolved in HBSS and administered by single i.p. injection.

A mild depression in average number (but not size) of litters derived from matings made during the first postinjection week probably results from slight morbidity of the males following urethane treatment. Productivity data give no evidence for major spermatogonial killing.

The mutation-rate result for either spermatogonial stem cells (no mutations in >13,000 offspring) or post stem-cell stages is clearly negative by Gene-Tox criteria (i.e., not significantly different from the historical control rate and less than 4 times this rate). Although over 16,000 offspring were scored for the sum of all post stem-cell stages, the sample for any individual week was no larger than 2600. Each "zero" result by itself was thus "inconclusive" by the Gene-Tox criteria. The post stem-cell data are, however, already sufficient to indicate that, in all germ-cell stages, the effectiveness of 1750 mg/kg urethane is at least one order of magnitude below that of the mutagenically optimum exposure to ENU.

4. Mercaptopurine (6MP). Earlier studies with this base analog have indicated that part of the fifth and sixth weeks following exposure of males may be critical with respect to induction of dominant lethals, sex-chromosome trisomics, and cellular damage detected in the testis. It was of interest to determine whether a similar sensitivity pattern might exist for gene mutations.

6MP was dissolved in 0.03 M NaOH and administered by single i.p. injection at 150 mg/kg. This exposure results in ca. 30% death, most of which occurs within 24 h. Each treated male was given a fresh set of two T-stock females each week until the end of the 7th week postinjection, except that during weeks 5 and 6 females were replaced in the middle of the week as well, providing additional opportunities for sampling the putatively sensitive germ-cell stages. In the first of several replicate experiments, males were allowed to continue in the rotation/mating scheme from the eighth week onward to produce offspring derived from treated spermatogonial stem cells.

Productivity data indicate a slight drop in litter size and number of litters for matings made 29-38 days postinjection, generally confirming the results of earlier dominant-lethal studies. Data for the weeks following the seventh give no evidence for a sterile period and thus argue for the absence of major stem-cell killing.

No mutants were found among 13,937 offspring derived from treatment during post stem-cell stages. For the sum of all such stages, the result is negative by Gene-Tox criteria, i.e., the induced rate is <4× historical control. For any one post stem-cell stage by itself, the multiple ruled out is, of course, much larger. The mutation frequency for spermatogonial stem cells does not differ significantly from historical control frequency, but the multiple ruled out is slightly larger than 4. Additional data now being collected may shrink this multiple.

MEASUREMENT OF GERM-LINE MUTATION FREQUENCIES FOLLOWING PROLONGED ETHYLENE OXIDE INHALATION AND APPLICATION OF THE RESULTS TO ESTIMATION OF HUMAN GENETIC RISK

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Because of widespread human exposure, the industrially important chemical ethylene oxide, EtO, poses a potential risk to future generations. EtO has been shown to produce various types of genetic lesions in a number of test systems, but none of the earlier studies has addressed the possible induction of gene mutations in the mammalian germ line; nor has it involved spermatogonial stem cells, the cells of greatest importance in assessing genetic risk to men, since they can accumulate damage for the whole reproductive lifespan of an individual without being eliminated as are exposed post stem-cell stages. Results of a specific-locus experiment with inhaled EtO were used to calculate risk from transmitted gene mutations.

Male mice exposed in an inhalation chamber accumulated total EtO exposures of ~101,000 or ~150,000 ppm.h in 16-23 weeks. The spermatogonial stem-cell mutation rate at each exposure level, as well as the combined result, did not differ significantly from the historical control frequency. At the lower and higher exposure levels, the results ruled out (at the 5% significance level) an induced frequency that is, respectively, 0.97 and 6.33 times the spontaneous rate; the combined results ruled out a multiple of 1.64.

The relationship between mouse spermatogonial stem-cell mutation rates and EtO-induced testis ethylations was compared with the relationship between Drosophila poststem-cell mutation rates and sperm ethylations (Lee, W. R., Report for EPA Cooperative Agreement CR-806876, 1980). The comparison does not rule out equal mutability per ethylation; but it cannot prove parallelism. An evaluation of the so-called "parallelogram" approach that proposes to extrapolate non-mammalian mutation data to man on the basis of molecular dosimetry will require a more efficient alkylator than EtO and the use of comparable germ-cell stages.

More meaningful conclusions may be drawn by utilizing the data for direct estimation of human risk by expressing the induced mutation frequency that is ruled out (at the 5% significance level) as a multiple of control rate, and extrapolating to human exposure levels. The probable absence of major stem-cell killing (and thus, possible cell selection) by EtO indicates that such extrapolation probably does not produce an underestimate. For a human exposure concentration of 0.1 ppm on working days during the reproductive lifespan, the mouse experimental results rule out (at the 5% significance level) an induced spermatogonial stem-cell gene mutation rate greater than 8% of the spontaneous rate; for 1.0 ppm, they rule out an induced rate roughly equal to the spontaneous rate. The induced rate for any one post stem-cell stage would have to be about three

orders of magnitude higher than that for stem cells to constitute an equivalent risk.

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HERITABLE TRANSLOCATION AND DOMINANT-LETHAL MUTATION STUDIES IN MICE EXPOSED TO INHALED ETHYLENE OXIDE

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L. A. Hughes[2]
J. R. Inman

Currently, there is considerable interest in the determination of the magnitude of the health hazard posed by inhaled ethylene oxide (EtO). EtO is mutagenic in several genetic test systems. Experiments with mice (independently by W. M. Generoso and R. B. Cumming) and rats (Embree, J., et al., Toxicol. Appl. Pharmacol. 40: 261-267, 1977) showed that short-term inhalation exposure to high concentrations of EtO, or i.p. injections of EtO, induced high levels of dominant lethals in postmeiotic male germ cells of both species. More important, we found that i.p.-injected EtO induced heritable reciprocal translocations in mice (Generoso, W. M., et al., Mutat. Res. 73: 133-142, 1980). In the assessment of genetic risk to human beings, data on heritable genetic changes induced in mammalian germ cells provide the most definitive mutagenicity information. Accordingly, we have in progress a study to determine the shape of the dose-effect curves for heritable translocations and dominant-lethal mutations induced in male mice by inhaled EtO. In addition, we are conducting a study to determine the sensitivity of female germ cells to the induction of dominant lethals by inhaled EtO.

In one of our studies, (101 × C3H)F_1 male mice, 10-12 weeks old, were exposed 6 h per day to EtO in air at concentrations of 165, 204, 250, or 300 ppm for a total of 11 weeks. For the first eight weeks, the exposures were given on the 5 working days and then daily for the last three weeks. During the 11th week of exposure, males were caged with females overnight and exposed to EtO during daytime. Matings were continued for one to two weeks after the end of the exposure. The majority of the pregnancies were allowed to go to term in order to produce first-generation progeny to be tested to determine the induced rate of heritable translocations. The remainder of the pregnancies were terminated 12-15 days after mating for analysis of induced dominant-lethal effects.

In another experiment, males were exposed 6 h per day to EtO in air at concentrations of 500 or 400 ppm for four consecutive days. After the last

exposure, the males were mated to unexposed females serially up to 12 days. Mated females were killed for uterine analysis at 12 to 15 days in gestation.

Both the long-term and short-term studies are still in progress, but results to date indicate that the two modes of exposure yield differently shaped dose-effect curves. With the short-term exposure, the current dominant-lethal data indicate a non-linear response. The responses for the 400 ppm concentration were consistently only about half of those for 500 ppm. With the long-term exposure, on the other hand, there appears to be a linear dominant-lethal dose-response. Frequencies of dominant lethals were 18%, 22%, 31%, and 37% for concentrations of 165, 204, 250, and 300 ppm, respectively. Similarly, the frequencies of sterile and partially sterile male progeny (so far, available only for two of the four concentrations) indicate a dose-response curve that approximates linearity. Heritable translocation frequencies were 3.32% (38/1143) and 5.58% (57/1021) for 165 and 204 ppm concentrations, respectively. This finding suggests, either that the long-term exposure affected the ability of mice to detoxify EtO, or that continuous exposure of germ cells at the stem-cell stage and throughout the entire spermatogenic cycle could have altered these cells in ways that increased their sensitivity.

Preliminary data indicate that EtO may also have dominant-lethal effects in maturing oocytes. (C3H × C57BL)F_1 females were exposed daily (6 h per day) on weekdays for two weeks to 300 ppm EtO, and then mated for the first four days following treatment to unexposed males. Uterine analysis of pregnant females 17 days after mating revealed a marked increase in the incidence of resorption bodies (dead implants) among the EtO-exposed females (44%, vs 7% in the control group). This study is continuing.

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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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Recently, we completed two heritable translocation studies with X-rays on spermatogonial stem cells, the most important cell stage in males for risk considerations. One study determined the shape of the dose-response curve and the other the effects of dose-fractionation. Data obtained from

these studies, (which are by far the most extensive, to date, for heritable translocation induction in this germ-cell stage) for the first time allows the estimation of the magnitude of risk per unit exposure to acute X irradiation. The expected increase in heritable translocations per rad of acute X rays is 3.89×10^{-5} per gamete.

This year, we started 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 at 30 R/min, the other to either 150, 300, 600, or 1200 at 0.7 R/min. Each group consists 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. Offspring of matings made 60 days or longer postirradiation are being collected and tested for the presence of translocations. So far more than 6000 offspring have been produced.

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ANALYSIS OF SEGREGATION PRODUCTS FROM RADIATION-INDUCED RECIPROCAL TRANSLOCATIONS IN SPERMATOGONIAL STEM CELLS

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The frequency of reciprocal translocations scored in meiocytes descended from irradiated spermatogonial stem cells has been reported to be twice as high as the frequency of translocations transmitted to first-generation progeny (Ford, C. E., et al., Cytogenetics 8: 447-470, 1969). The authors interpreted this result in terms of a selective process that is operating on diploid genomes, but perhaps taking effect in the haploid spermatids or spermatozoa. M. Crocker (Mutat. Res. 103: 339-343, 1982), on the other hand, has suggested that the frequency of translocations scored cytologically may be spuriously elevated as a result of metaphase-I delay (i.e., that spermatocytes carrying translocations take longer to pass through metaphase I). Neither of the suggestions was based on direct evidence.

In collaboration with Drs. J. G. Brewen and R. J. Preston, we have explored the relation between the frequency of transmitted translocations and of translocations scored cytologically in exposed males. If the interpretation of Ford et al. is correct, the ratio should be 1/8 (i.e., 1/2 of the roughly 1/4 expected on the basis of segregation). In effect, we found the ratio to range from 0.095 to 0.248 for various single and fractionated doses (Generoso, W. M., et al., Mutat. Res. 126: 177-187, 1984). Tests of the hypotheses that (a) the observed frequencies are the same as those expected on the basis of no selection (i.e., transmitted = 0.25 of cytologically scored), and (b) that the observed frequencies are only one-half of those expected (i.e., as reported by Ford et al.) showed that there was no specific exposure-related pattern that would suggest a close association between cytologically scored and transmitted frequencies. Thus, no reliable extrapolation can be made from cytological findings in exposed males to what might be expected among progeny.

Considering the fact that the cytological scoring must have missed a number of cells that had translocations, it does appear that, in general, the observed frequencies of transmitted translocations are lower than the frequencies expected on the basis of segregation alone. This discrepancy may mean that either certain of the assumptions are not operational or that forces not related to segregation enter the picture.

We are currently studying this problem using two approaches. In the first one, we are combining a sizeable dominant-lethal experiment with a cytological study of the testes of the exposed males. Since spermatogonia are irradiated, the dominant-lethal effect is presumably the result of meiotic segregation (of unbalanced gametes) from translocations induced in the stem cells. Our choice of the optimum exposure conditions and size of experimental groups was greatly aided by our earlier study on dose-fractionation effects (Generoso, W. M., et al., Mutat. Res. 126: 177-187, 1984). Two types of ~12-week old male mice, $(C3H \times 101)F_1$ and $(SEC \times C57BL)F_1$, were simultaneously exposed to four 500 R doses of acute X rays separated by four weeks. Control males were not sham-exposed but were randomly separated from experimental mice prior to start of exposure. Males were mated to highly fertile $(C3H \times C57BL)F_1$ females during the period 153-159 days after the last-X ray exposure. Thus, there were four groups (experimental and control for each of the two stocks), each with 81-91 pregnant females analyzed for fetal lethality. As we had expected for the $(C3H \times 101)F_1$ males on the basis of earlier heritable-translocation and cytological data, the incidence of dominant-lethality was observed to be 25%. Unexpectedly, however, the frequency observed for the $(SEC \times C57BL)F_1$ stock was only 7%. This difference is highly significant. Since the two types of males were irradiated together, the difference in dominant-lethal response cannot be attributed to difference in dose received. Random samples of exposed and control mice from each of the two stocks were killed for cytological study of meiocytes shortly after they were mated for the dominant-lethal study. Cytological scoring of coded slides is currently underway.

The second approach takes advantage of the ongoing γ-ray dose-rate study of heritable translocations (see preceding report). This project provides an excellent opportunity for studying the relationship between translocations scored cytologically in irradiated males, transmitted balanced exchanges (heritable translocations), and unbalanced segregants (lethals). Offspring to be tested for heritable translocations are being collected over a period of time following spermatogonial stem-cell repopulation, while the cytological and dominant-lethal studies are underway with the same parental males at various intervals during the period in which progeny are produced.

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MECHANISMS FOR INDUCTION OF CHROMOSOME ABERRATIONS IN MALE GERM CELLS

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P. W. Braden

A long-term project has been underway 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 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 ethylene oxide 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, primarily alkylate 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. Recently, it was shown by A. D. Tates and co-workers (Mutat. Res. 107: 131-151, 1983) that phosphotriesters in rat liver cells are very stable also, and that the persistence

of these adducts paralleled the formation of micronuclei (generally believed to be indicative of chromosome-breakage events). 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 cystosine.

In an attempt to provide additional evidence bearing on this hypothesis, we are currently comparing 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. Since G. A. Sega's estimate of the half-life for spontaneous hydrolysis of N-7 alkylguanine is about 7 days, 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.

We have completed a preliminary study to determine the MMS doses that will induce about 50% dominant-lethal mutations in the two mating intervals. In the course of this study, it was observed that the dominant-lethal frequency after MMS treatment of mature sperm from (SEC × C57BL)F_1 males was affected by the strain of the untreated females: the yield was clearly higher with T-stock than with (101 × C3H)F_1 females. We, therefore, decided to use T-stock females in the comparative study. With such females, the doses that produced about 50% dominant-lethal mutations are 40 mg MMS/kg for late spermatid-early spermatozoa stages and 120 mg/kg for mature sperm. The comparative heritable translocation study is in progress.

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ETHANOL-INDUCED LATE FETAL DEATH IN MICE EXPOSED AROUND THE TIME OF FERTILIZATION

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K. T. Cain W. M. Generoso

A recent report by M. H. Kaufman (Nature 302: 258-260, 1983) that ethanol induces chromosomal nondisjunction in mice attracted considerable attention because of its implication that ethanol consumed around the time of sperm entry into the egg might be the cause of certain types of chromosomal defects that are commonly observed in human spontaneous abortions or neonatally. Kaufman found up to 20% of aneuploidy (monosomy + trisomy) in embryos of females that had been exposed to ethanol after copulation. Since, in the mouse, all monosomic and trisomic conditions of autosomes are lethal by the time of birth, we anticipated that the treatments used in Kaufman's experiments would result in a detectable increase in intrauterine death. Accordingly, we investigated the dominant-lethal response of female mice given 1 ml of 12.5% ethyl alcohol orally shortly after mating. In addition, we conducted a corresponding cytological analysis of the first-cleavage metaphase.

For the dominant-lethal study, the mating procedure was designed so that the time of fertilization in relation to the time of treatment was known. Males and females were caged together for 30 minutes each morning, beginning shortly after the start of the light period. As determined in previous studies, the ovulated eggs are already in the ampullae at this time. Females were checked for the presence of vaginal plugs at the end of the mating period, and were given, via oral intubation, 1 ml of 12.5% ethanol or distilled water, either 1.0 or 1.5 h after the end of the mating period. (We had found in an earlier study that sperm entry into the egg starts about 1.75 h after the end of such a mating period.) Females were killed for uterine analysis 12 days after mating.

In the cytological study, the females were superovulated by injection with 5 I.U. of pregnant mare serum (PMS), followed 48 h later by 5 I.U. of human chorionic gonadotropin (HCG). Immediately after injection of HCG, females were caged with males overnight. Those which had vaginal plugs the next morning were given either 1 ml of 12.5% ethanol or 1 ml of distilled water orally at 13.5 to 14.5 h after HCG (i.e., about 1.5 - 2.5 h after the expected time of ovulation). Since sperm entry starts about 1.75 h after mating, the oocytes were in the process of completing the second meiotic division at the time of treatment. (Kaufman administered ethyl alcohol 13.5 h after HCG.) Mated females were injected intraperitoneally with 2.5 mg/kg of colchicine 9-10 h after ethanol administration and were killed the following morning (about 15 h after colchicine treatment). Eggs were flushed from the oviduct and prepared for cytogenetic analysis. In all cases, only one egg was fixed per slide. The slides were coded and scored without knowledge of their derivation.

The incidence of late death (day 11 postconception or later) was found to be significantly increased when ethanol was administered 2 h following

the end of the mating period (21/1106 vs 5/1008, p = 0.002), but not when the interval was shorter. Measurements of early death were not sensitive enough to show an effect of ethanol treatment because of the high control frequency. Limited cytological data showed an incidence of induced trisomy in line with the excess frequency of late death, but the trisomy incidence by itself was not significantly different from that in control. It appears probable that these ethanol-induced late deaths are primarily the result of nondisjunctional events occurring during the time when the highest levels of ethanol in the oviduct are expected, a time when the majority of the oocytes were completing the second meiotic division. In support of this are the findings of several investigators that indicate that most autosomal trisomies in the mouse survive into the second half of the gestation period.

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

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

Last year we reported on a computer analysis of the litter-size-reduction (LSR) results from 14 earlier specific-locus experiments involving 158,490 litters (approximately 900,000 offspring). 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. Following a more detailed statistical analysis, we have now reached the following conclusions.

There is a humped dose-response curve at the dose-rate of 90 R/min, 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. The analysis also demonstrates 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 0.00294% ± 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 humans suggests that if men were exposed to 1 R of low-LET low-dose-rate radiation, the number of deaths before late childhood caused by induced dominant mutations among their children would be about 19 per million liveborn. This number can be added to the earlier estimate of an approximately equal number of viable disorders in all body systems as based on dominant skeletal mutations. This yields a total estimate of induced dominant damage, but much of this addition can be shown to represent death in very early embryonic life that would not be recognized in humans.

Other analyses of earlier specific-locus experiments are also in progress now that this mass of data has been loaded into ORNL's PDP-10 computer. One of these, a study of the amount of induced death occurring between birth and weaning, is nearing completion.

SYNERGISTIC INTERACTIONS OF TWO RADIATION-INDUCED DOMINANT SKELETAL MUTATIONS

P. B. Selby
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G. D. Raymer

Double heterozygotes were constructed for the mutations provisionally identified as Ccd (Cleidocranial dysplasia) and Dsh (Short digits) to determine whether these mutations might together cause malformations that neither one can cause alone. We have earlier shown that these 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 several abnormalities that were far more extensive than expected on the hypothesis of additivity of effects. For example, the area of the hole in the skull was more than twice as large as the sum of the areas found in the single heterozygotes. The number of bones in the interparietal region was 1.0 ± 0 [SE] for non-mutant segregants, 1.0 ± 0 for Dsh/+, 2.6 ± 0.4 for Ccd/+, and 8.0 ± 0.7 for double heterozygotes. Interactions were not always in the direction of enhancement; thus, Ccd often entirely suppresses the ability of Dsh to cause large boney plates on many of the ventral ribs.

The offspring examined to date had parents that were not inbred. Interactions between Ccd and Dsh will also be looked for in crosses of mice congenic on the C57BL/10 background.

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.

TESTS FOR HERITABLE GENETIC DAMAGE AND FOR EVIDENCE OF GONADAL EXPOSURE IN MAMMALS

Liane B. Russell

The completion of the Gene-Tox data base has provided tools for determining the reliability with which various short-term mutagenicity tests predict the ability of an agent to induce heritable genetic damage. Mammalian germ-line (MG) tests are thus increasingly being used to provide standards for judging the predictive capabilities of other tests.

Another major function of MG tests is genetic risk assessment. It is generally recognized that even if one or more non-MG short-term tests should prove to be predictive of heritable genetic damages in mammals, the task accomplished would be limited to hazard identification. There are several reasons for this. The mammalian germ line contains a complex assortment of cell types whose responses to a given mutagen can vary greatly for any given genetic endpoint, and not necessarily in a correlated fashion for different endpoints. Spermatogonial stem cells, the stage of greatest importance for genetic risk assessment in males, appear to possess considerable capacity for genetic repair, which may manifest itself in nonlinear dose-response curves. Finally, mutation rates for various types of genetic lesions must be translatable into types and quantities of phenotypic damages expected, and this type of translation probably differs according to the nature of the genetic lesion.

Because of the increased emphasis on enlarging the body of information that can be provided by studying genetic damage induced in the mammalian germ line, we have reviewed and summarized 14 MG tests and their variants. These tests are of two general types: (a) those designed to detect certain classes of genetic damage (gene mutations, chromosome breakage and/or rearrangement, and chromosome mis-segregation), regardless of whether or not the endpoint scored has any significance to human health, and (b) those designed to detect phenotypes that have human health implications, while the nature of the genetic damage is not usually known. The selection of tests to meet various objectives was examined.

Exposure to an environmental agent that does not result in the presence of this agent (or its derivatives) within reproductive cells does not constitute a genetic hazard, regardless of how mutagenic the chemical may be in somatic cells. Tests for gonadal exposure are, therefore, important as preliminaries or components of studies on germ-cell mutagenicity. We have reviewed and discussed seven such tests and their variants.

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2. Russell, L. B., C. S. Aaron, F. de Serres, W. M. Generoso, K. L. Kannan, M. Shelby, J. Springer, and P. Voytek. Evaluation of mutagenicity assays for purposes of genetic risk assessment. Mutat. Res., in press.
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ANALYSES OF SPECIFIC REGIONS OF THE MOUSE GENOME

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Specific-locus mutation-rate studies generate mutations, many of which have been maintained as stocks and analyzed genetically. Some are now providing valuable tools for basic studies, and others undoubtedly will in the future. The stock-maintenance program (including embryo freezing) is thus an important component of the Section's effort.

1. Mutations Involving p: Studies of Lactate Dehydrogenase Regulation

Mutations involving the p locus (one of seven markers in specific-locus tests) were used as tools in experiments whose ultimate objective is the study of tissue-specific regulation of the enzyme lactate dehydrogenase. Because of the known relatively close linkage of p (pink-eye dilution) and Ldh-1 (lactate dehydrogenase A) on Chromosome 7, about 40 p-lethal and p-sublethal stocks were investigated for LDH-A deficiency. One of the p-lethal strains was found to have reduced LDH activity in various tissues, as well as altered isozyme patterns in kidney.

Attempts to identify a restriction fragment that contains the structural gene for LDH-A at first encountered some complexities. When mouse genomic DNA was digested with any one of various restriction enzymes, a rat cDNA probe was found to hybridize to multiple fragments, which (on the basis of subsequent findings) were concluded to be pseudogenes or other homologous sequences located on various mouse chromosomes. To overcome this complication, we utilized a mapping panel of mouse-hamster somatic cell hybrid lines some of which were shown to contain mouse Chromosome 7 (or the appropriate portion of Chromosome 7 from an X-7 translocation generated at our laboratory), and some of which lacked this chromosome. By probing EcoRl-digested DNA from this panel with the rat LDH-A cDNA, we

found the presence of a 13.5 Kb band to be completely correlated with the presence of the appropriate portion of Chromosome 7.

Having identified the restriction fragment containing the Ldh-1 gene, we were able to investigate whether the p-lethal mutant that is deficient in LDH-A activity in fact has a deletion of genetic material. The p-locus mutant (Mus musculus) was crossed to mice of the feral species, Mus spretus. EcoR1-digested DNA from offspring as well as from parents was probed with a fragment of a mouse LDH-A cDNA containing a portion of the coding region of Ldh-1. Those offspring that showed reduced LDH-A activity in various tissues were found to lack the 13.5 Kb band characteristic of the mutant (in Mus musculus), thus confirming that the mutation is a deletion.

In adult mice, the A vs B subunit composition of the tetrameric LDH varies markedly with the tissue, as does the amount of LDH relative to total protein. Having found an Ldh-1 deletion (Ldh-1 codes for subunit A), we were able to show that, in each tissue, LDH activity is reduced to a level consistent with inactivation of one of the two genes encoding LDH-A. By using LDH-A cDNA clones in Northern analysis to estimate levels of LDH-A mRNA in various tissues, it was possible to conclude that the enzyme levels are controlled pre-translationally.

2. Mutations Involving d: Using a Provirus as a Marker to Gain Molecular Access to the Region. A large number of radiation-induced mutations involving d and/or the closely-linked se loci had been genetically analyzed by us several years ago (Russell, L. B., Mutat. Res. 11: 107-123, 1971) and a complementation map constructed. DNA from some of these mutants has now been analyzed in a collaborative study with Nancy Jenkins, Neal Copeland, and E. M. Rinchik (Univ. Cincinnati) which makes use of the finding that the original spontaneous d mutation resulted from the stable integration of an ecotropic murine leukemia virus into Chromosome 9.

The entire provirus associated with the d allele is contained in an 18-Kb EcoR1 fragment which has been cloned. A Pst I subclone (for a region in genomic DNA outside the virus) has been used as a probe for EcoR1-digested DNA from various mutants. Such a probe hybridizes to 18.0 Kb fragments in d/d animals and to 9.0 Kb fragments in the wild type (no virus; flanking DNA only). Several independent mutant stocks carrying d se deletions opposite d [i.e., Df(d se)/d] were found to have an 18.0 Kb band only, which is derived from the d chromosome, i.e., the sequence homologous to the probe (or the major part of it) was missing from the deficient chromosome. The same was true for several d^{pl} (prenatally-lethal dilute) mutants made heterozygous with d (i.e., d^{pl}/d). Other d^{pl} mutants had an 18.0 as well as a 9.0 Kb band, i.e., the sequence homologous to the probe was not deleted and, since the mutation had originally been induced in a wild-type chromosome, the virus was not present. One d^{pl} mutant is of particular interest, since d^{pl}/d animals have, in addition to the 18.0 Kb (d) band, a 4 Kb band. The sequence homologous to the probe is still present, but another portion of the EcoR1 fragment has been deleted. This

mutant is therefore serving to localize one end of the deletion within the characterized DNA. Offspring from crosses of this mutant to other d se-region mutations will be informative in view of the previously constructed complementation map.

A MUTATION OF THE STRUCTURAL LOCUS OF TYROSINASE IN DROSOPHILA

E. H. Grell

Because it is involved in melanin synthesis, tyrosinase has been the subject of study in several organisms. In mammals, the c locus specifies the structure of tyrosinase. In Drosophila, the genetics of tyrosinase has not been clarified. Several mutations that affect melanization do not affect tyrosinase, and several mutations that affect tyrosinase activity do not affect melanization. The tyrosinase system has been shown to be complicated. It is usually present as an inactive proenzyme, and at least six proteins are involved in activation.

Drosophila melanogaster and D. simulans contain tyrosinases with different electrophoretic mobilities. We have not detected allelic differences of electrophoretic mobility within a species. Because hybrids between the two species are sterile, a genetic analysis cannot be carried forward in the conventional way. To circumvent hybrid sterility, a series of "partial hybrids" were constructed. These flies are heterozygous for all of the chromosomes of the two species, except for one arm which is homozygous for one of the species. The "partial hybrids" are derived from the fusion of complementary egg and sperm genotypes which are disomic and nullisomic for a particular chromosome arm. This analysis showed that the gene determining tyrosinase mobility is located on the right arm of the second chromosome.

In D. melanogaster, a mutant was discovered which causes the tyrosinase-containing cells of larval hemolymph to be shrunken and dark. The mutant was named Black cells (Bc). Bc homozygotes have almost no tyrosinase and greatly reduced amounts of DOPA oxidase activity. Bc/+ animals have about one-half of the tyrosinase activity of +/+. In heterozygotes of Bc and D. simulans, only the simulans form of the enzyme is detected by electrophoresis. This is a good indication that Bc does not have a functional tyrosinase structural locus. Bc is located on 2R.

At this point it is possible to collect mutations and deletions of the gene for tyrosinase. A rapid test for tyrosinase activity has been developed and appropriate stocks constructed. Since the enzyme appears to be complicated regarding biochemical information, the genetics is likely to be complex. It is also feasible to tag the gene with P elements for the purpose of cloning.

X-AUTOSOME TRANSLOCATIONS IN THE MOUSE: THEIR CHARACTERIZATION AND USE AS TOOLS TO INVESTIGATE GENE INACTIVATION AND GENE ACTION

Liane B. Russell
N. L. A. Cacheiro[2]
T. Ashley[2]
C. S. Montgomery
J. W. Bangham

Mouse X-autosome translocations [T(X;A)'s], which lead to mosaicism for autosomal gene inactivation, played a role in developing the single-active-X hypothesis. Of 17 translocations observed, 16 have now been at least partially studied genetically and/or cytologically, 12 at this laboratory. All but one were induced by mutagens in postmeiotic germ-cell stages. Eight have involved chromosome (Ch) 7, four Ch 4, and one each Ch's 2, 11, 12, 16, and 17. While part of the nonrandomness of distribution results from bias of detection, some may be due to selection against T(X;A)'s that involve certain autosomal regions.

One of the rearrangements is an insertion of an inverted Ch 7 segment into the X; the others are reciprocal translocations. Genetic mapping indicates crossover suppression near breakpoints of some of the T(X;A)'s, affecting primarily the short arms. Breakpoint order is the same in the genetic, mitotic, and meiotic maps, but relative distances indicate more recombination in distal than central portions of the chromosomes. Eleven of the T(X;A)'s provide long marker chromosomes (>Ch 1), and at least 5 a short marker. Unbalanced types (duplications) survive postnatally in only two of the rearrangements. Several of the T(X;A)'s fairly frequently produce XO;2A daughters.

In one T(X;A), X^T is virtually always active. For all others, all informative genes that have been investigated in the A and/or X portion of the long chromosome are subject to inactivation. In the reciprocal chromosome, on the other hand, no inactivation was found in one of the translocations in which several informative genes were studied. The latter result, and most cytological findings, make it likely that there is a single site (or contiguous region) in the central part of the X that is responsible for primary differentiation of the X's. The site can be represented by three alternative stages.

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

The mosaic composition of a T(X;A) female with respect to a given autosomal gene is determined by (1) whether primary differentiation is random, (2) how the spreading effect is accomplished, and (3) whether selection against functionally unbalanced cells occurs. Depending on the autosome involved, selection is different in different tissues, probably

because certain genes critical to the development of certain cells are functionally hemizygous in some rearrangements but not others.

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

All T(X;A)'s produce sterility in balanced heterozygous males, with spermatogenesis not proceeding past meiotic metaphase. Several suggested explanations have been eliminated through experimental approaches.

One of the X-7 translocations, T(X;7)16Rℓ, which was recently discovered in the progeny of an ENU-treated male, has been partially studied since the publication of the paper cited below. Preliminary analyses of QM-banded mitotic chromosomes (N. L. A. Cacheiro) and synaptonemal complexes, i.e., pachytene chromosomes (T. Ashley) indicate that the Chromosome-7 breakpoint may be located between the R2 and R3 breakpoints, but closer to the latter. This makes the translocation of particular interest, since it should place the c locus and other useful markers onto the short translocation product and thus either corroborate or disagree with the findings from R2, which had provided evidence in favor of a single inactivation site (or region) in the X. Preliminary data indicate that the c-locus is, in fact, not inactivated in R16, despite the closeness of the breakpoint to c.

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X-Y CHROMOSOME ASSOCIATION: EVIDENCE FOR NON-HOMOLOGOUS PAIRING

T. Ashley[2]

According to the classical view, there is a homologous pairing segment of varied length in the X and Y chromosomes of all mammals, with one or more obligatory chiasmata in that segment insuring proper disjunction. Evidence is presented for an alternative hypothesis, according to which X-Y pairing is nonhomologous, with crossing-over prevented by "precocious" desynapsis, and normal disjunction assured by achiasmatic end association.

Several genes on the mouse X chromosome are, (on the basis of crossover distance with common markers) located distal to the R5 breakpoint of our T(X;7)5Rℓ. Another X-linked gene maps distal to the distal In(X)1H

breakpoint. All four genes are thus located in a portion of the X that regularly synapses with the Y; yet no corresponding loci are known on the Y. In man, 14 DNA fragments map to a portion of the X that pairs with the Y, but no corresponding sequences have been found in the Y. Conversely, sequences present in both X and Y are located in physically non-corresponding regions of the two chromosomes.

Our electron-microscope studies of synaptonemal-complex behavior in two X-autosome translocations [T(X;7)5Rℓ and T(X;7)6Rℓ] have demonstrated that the X and Y can synapse with Chromosome 7, i.e., non-homologously. Regions of normal X-Y pairing as well as regions in which X-Y pairing never occurs are involved in this nonhomologous synapsis with an autosome.

Maximum synapsis of the sex chromosomes in mouse and man appears to occur during only the earliest substage of the synaptonemal-complex period, and is soon followed by desynapsis, which leaves a mere terminal association of the two axes. It is suggested that the X and Y desynapse prior to the time at which crossing-over occurs in the autosomes, and that it is this early desynapsis which prevents nonhomologous "crossing over" in the sex chromosomes.

X-Y CHROMOSOME ASSOCIATION: SEX-VESICLE LOSS AS A POSSIBLE CAUSE OF XO CONCEPTUSES

T. Ashley[2] Liane B. Russell

We have earlier shown (Russell, L. B. In: Chemical Mutagens - Principles and Methods for Their Detection, Vol. 4, ed. by A. Hollaender. Plenum Press, New York, 1976, pp. 55-91) that the frequency of spontaneously occurring X^M mice, instead of equalling that of X^MX^PY (as would be expected if the origin were meiosis-I nondisjunction), exceeds it by a factor of about 30. (X^M and X^P designate maternally- and paternally-derived X chromosomes.) Furthermore, X^MO is about 5-10 times as common as OX^P. Similar unbalances occur in man. We suggested that the excess of XO over XXY may be due to losses occurring during or shortly after fertilization, and that the paternal contributions to the zygote are more vulnerable than the maternal.

An alternative hypothesis is now advanced on the basis of EM studies of synaptonemal complexes (SCs) in pachytene spermatocytes, prepared by spreading techniques that allow observation of whole nuclei. Analysis of over 500 spermatocytes revealed at least 8 cells with free sex vesicles, i.e., X and Y axes (and associated chromatin), separated by a distance in excess of 2 nuclear diameters from the nearest nucleus. Individual autosomal SCs have not been found in similar isolation. There were also several nuclei lacking sex vesicles but complete with regard to the autosomal SC complement. Assuming the maximum coincidence between these two reciprocal states, the overall frequency was about 1.8%, but in one

animal it was as high as 6.7%. Some years ago similar conditions had been observed (without being tallied) in a population of mice that sired a relatively high frequency of XO daughters.

Several authors have reported that the sex vesicle is found adjacent to the nuclear membrane and occasionally "bulges" toward the cytoplasm in sectioned material. There is no RNA synthesis in the sex vesicle of normal males, and (in contrast to the condition in autosomes) little if any incorporation of tritiated uridine, indicating that the vesicle is not active in the progress of meiosis.

These various observations suggest that the sex vesicle may be lost during late pachytene, and that the resulting cell will proceed through meiosis and spermiogenesis, giving rise to sperm lacking a sex chromosome and producing X^MO conceptuses. The proposed mechanism is presumably not the only source of X^MOs, but may make an important contribution to their overall frequency.

DNA REPAIR STUDIES IN MAMMALIAN GERM CELLS

G. A. Sega J. G. Owens

As part of an International Program for the Evaluation of Short-Term Tests for Carcinogenicity we have been evaluating the ability of four test chemicals to induce unscheduled DNA synthesis (UDS) in the germ cells of male mice. The chemicals being used are pyrene (P), benzo[a]pyrene (BaP), 2-acetylaminofluorene (2 AAF) and 4-acetylaminofluorene (4AAF). Methyl methanesulfonate (MMS) was used as a positive control.

A series of dosages were given with each chemical, up to the highest level the mice could tolerate. Usually, the chemicals were dissolved in dimethyl sulfoxide (DMSO), and given to the animals by gavage. Testicular injections of [^{3}H]dThd (18 μCi/testis) were given along with the chemicals. Sixteen days after treatment, sperm were recovered from the caudal epididymides and assayed for a UDS response using liquid scintillation counting.

The results clearly showed a good dose-response curve with MMS, the positive control. None of the four test chemicals gave a positive UDS response at any dose used when DMSO was the carrier. (A positive response would have to be at least 2-3 times the control values.)

However, at the highest doses of BaP used, we had to use corn oil as a carrier because of solubility problems. Doses of either 300 and 400 mg/kg produced a strong UDS response in the germ cells. Although there was quite a bit of variability between animals (5 for each dose), the UDS response of the germ cells with 400 mg BaP/kg was significantly higher than with 300 mg BaP/kg. We are now in the process of repeating all of the BaP exposures,

using corn oil as the carrier. Exposures will also be by i.p. injection as well as gavage. This work is important since it will give us additional information on how the method of chemical exposure of test animals may affect the amount of biological damage that is produced.

DNA DAMAGE IN MAMMALIAN SPERM ASSAYED BY ALKALINE ELUTION

G. A. Sega E. E. Generoso

As a further means of assessing the damage produced in mammalian germ cells by model mutagens, we are using an alkaline-elution procedure to measure DNA strand breaks in developing sperm stages of the mouse. For each experiment, one group of hybrid males have their germ-cell DNA pre-labeled with ^{3}H-thymidine (^{3}HdT) and are subsequently exposed to the test mutagen. A second group of hybrid males have their germ-cell DNA pre-labeled with ^{14}CdT 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 pass rapidly through the filter, while normal-sized DNA takes much longer to pass through the filter. The amounts of treated and control DNA eluted are then determined using liquid scintillation counting techniques.

When mice were exposed to ethylene oxide (EtO), the amount of sperm DNA eluted was highest during the second week posttreatment. Our chemical dosimetry experiments with EtO have shown that spermatozoa sampled in the second week posttreatment (mid- to late-spermatid stages at the time of treatment) show a peak level of EtO alkylation. This increased level of alkylation was shown to be the result of increased protamine alkylation but not of increased DNA alkylation. The genetic pattern of induced dominant lethality produced by EtO also shows a peak effect in sperm sampled in the second week posttreatment. Thus, following EtO exposure, there is overall good agreement between the pattern of DNA elution, the pattern of genetic damage, and the pattern of protamine alkylation.

The eluted amount of EtO-treated sperm DNA drops off in the third week after exposure, and by 23 days posttreatment there is no measurable increase in elution of the treated DNA above the level of the controls. The drop-off in the third week after exposure is attributed to active DNA repair processes that we have demonstrated to be occurring in these stages. (We have never observed the occurrence of any DNA repair in sperm stages sampled during the first two weeks following mutagen exposure.)

Ethyl nitrosourea (ENU) has also been studied in our alkaline elution experiments. Unlike EtO, ENU reacts by an S_N-1 type mechanism and produces

relatively more alkylation at less nucleophilic sites such as the O^6-position of guanine and the phosphate groups in the DNA backbone. Also, the interaction of ENU with protein sulfhydryl groups is considerably less than that of EtO. For the alkaline elution experiment with ENU, the exposure was a 100 mg/kg i.p. injection. The amount of sperm DNA eluted was considerably less after ENU than after EtO treatment, and ENU, unlike EtO, produced no increase in DNA elution during the second week post-treatment.

It is of interest to note that ENU does induce dominant-lethal mutations in the germ-cell stages we are sampling, although at relatively low levels. There is, however, no increase in the dominant lethals induced by ENU in the second week posttreatment such as that observed with EtO. The alkaline elution pattern we have measured in the developing mouse sperm with ENU is thus consistent with the pattern of dominant lethals that ENU produces in meiotic and postmeiotic stages in the mouse.

The good correlation found for both EtO and ENU between the pattern of DNA elution in developing sperm of treated mice and the pattern of induced genetic damage. This adds weight to the possibility of using alkaline elution to monitor human sperm for DNA breakage which can be correlated with genetic damage.

CHEMICAL DOSIMETRY STUDIES IN MAMMALIAN GERM CELLS

G. A. Sega
P. A. Brimer
L. G. Cox[7]

Chemical dosimetry using radioactively labeled chemical mutagens continues to provide important information about the molecular events going on within germ cells after mutagen treatment. It is an extremely sensitive procedure and can be used to measure binding of chemical agents to germ cells and to germ-cell DNA and proteins at exposure levels that are orders of magnitude lower than those needed to produce a statistically significant genetic effect. As dosimetry data are combined with other genetic and cytogenetic data using the same chemicals, we are learning more about the relationship between the extent of chemical damage in the germ cells and the amount of genetic damage expected at realistic exposure levels for humans.

Work has continued with ethyl nitrosourea (ENU). Both (101 × C3H) and BALB/c males were given i.p. injections of 0.8 mg [^{3}H] ENU/kg in order to extend the dosage range of ENU down to lower levels. BALB/c animals were used since some of our earlier work had shown that unscheduled DNA synthesis (UDS) induced in the BALB/c germ cells by alkylating agents such as methyl methanesulfonate (MMS) was only about 1/4 of that induced in the (101 × C3H) strain. As this could be a reflection of differences in DNA repair capability in the two strains of mice, we wanted to study the rates

of removal of lesions from the testis DNA of the (101 × C3H) and BALB/c mice after exposure to [^{3}H] ENU.

The total initial alkylation level of DNA after exposure to 0.8 mg ENU/kg was about the same in the two strains of mice. At 1 h post-injection, the ethylations per 10^6 deoxynucleotides (Et/dN) were 0.104 in the (C3H × 101) strain and 0.091 in the BALB/c mice. At this exposure level, there was no significant difference between the strains of mice in the rate of removal of lesions. In addition, it was found that, within experimental uncertainties, there was a linear relationship between ENU exposure and total alkylation of testis DNA over an exposure range of 0.8 to 100 mg/kg.

Since there was no strain difference after an ENU exposure of 0.8 mg/kg, we injected additional males of the two strains with 100 mg [^{3}H] ENU/kg and looked specifically at the rates of removal of N7-ethylguanine (N7-EtG) from the testis DNA. By 2 and 4 days posttreatment, a 43 and 75% drop, respectively, in the level of N7-EtG was noted in the testis DNA of the (101 × C3H) mice (as compared to day-1 post-treatment). By contrast, the amount of N7-EtG in the testis DNA of the BALB/c mice remained at roughly the same level 2 days posttreatment as it had been at 1 h. Since N7-EtG is the major DNA base lesion produced by ENU, and since it is a lesion that can elicit a UDS response because of excision repair (unlike O^6-EtG which is repaired without new DNA synthesis), these results are consistent with our observation that the germ-cell UDS response following exposure to a monofunctional alkylating agent is greater for (101 × C3H) than for BALB/c males.

Results of experiments such as those reported here have important implications for mammalian mutagenicity studies in general. If some strains of mice are better than others in repairing DNA damage within the germ cells, then testing of potential mutagenic agents in these different strains may produce significantly different levels of genetic effects.

POSTIRRADIATION DIVISION OF SURVIVING SPERMATOGONIAL STEM CELLS

E. F. Oakberg C. C. Cummings

There has been a long-standing debate over the mitotic activity of the spermatogonia surviving radiation exposures, but no systematic investigation on this subject has been carried out; observations have been incidental to other objectives such as the study of cell survival. Conclusions ranged from prolonged mitotic inhibition (Erickson, B. H., Radiat. Res. 86: 34-51, 1981) to accelerated division of the surviving spermatogonial stem cells (Huckins, C. and Oakberg, E. F., Anat. Rec. 192: 529-542, 1978). We thought it probable that mitotic activity might oscillate, and that time of sampling might have significant effects on the mitotic index observed.

Hybrid male (101 × C3Hf)F_1 mice, 12 weeks old, were given an exposure of 300R X-rays and killed at 3 h intervals from 3 to 24 h, at 6 h intervals from 30 to 48 h, at 12 h intervals from 48 to 72 h, and at daily intervals from 96 to 192 h. A peak of mitosis was observed at 12 to 18 h post-irradiation, followed by almost no mitotic activity at 21 to 42 h, then an abrupt increase to a plateau of about 1% at 48 to 72 h, a further increase to 2.1% at 96 h, lower values at 120 and 144 h, and a third peak (the highest value, 2.6%) at 168 h. Thus, there appear to be peaks and valleys in the mitotic index curve, most likely as a result of cell synchronization resulting from killing of sensitive stages of the cell cycle and, possibly, retardation of other stages. It is clear that time of sampling will strongly influence conclusions concerning mitotic activity after irradiation. The whole-mount technique will now be used to identify the cells that divide at the respective peaks.

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LACK OF CIRCADIAN RHYTHMS IN SPERMATOGONIAL STEM CELLS

E. F. Oakberg C. C. Cummings

In the previous report (ORNL-6021, pp. 125-126, 1983) we presented the conclusion that spermatogonial stem cells showed no circadian rhythms in mitotic index or in sensitivity to radiation. A significant effect of time of day on DNA synthetic activity, however, was observed, but the pattern appeared to be random rather than typical of a circadian rhythm. This experiment has now been repeated, and again there was a significant effect of time of day, but the results were not in agreement with those of the initial experiment (correlation coefficient of -0.12). Data from the two experiments were pooled for statistical analysis, and a significant difference between times sampled (x^2 28.6, 7 df, $P < 0.001$) was observed; but the pattern appeared to be random, and to result from extra-binomial variability. Therefore, we have concluded that there also is no effect of time of day on the number of spermatogonial stem cells in DNA synthesis.

Stage of the cycle of the seminiferous epithelium, unlike time of day, did show an effect on mitotic index. The frequency of 2.7% observed at stage 5 was significantly higher than the frequency at any other stage. DNA synthetic activity also showed a repeatable pattern, with the lowest frequencies of labeling found at stage 1, and the highest values at stages

3 and 4. It had been observed earlier that the long-cycling compartment of the A_s spermatogonia preferentially incorporate label at this time.

Stem cells in different organisms and tissues possess certain characteristics, such as a low mitotic rate and resistance to noxious agents, wherever they occur. Other stem-cell properties, however, appear to be tissue specific; among these are times of maximum and minimum division rates and amplitude of the effect. Our present data suggest no circadian rhythm for spermatogonial stem cells. However, the A_s (Asingle, Astem) spermatogonial population contains both fast- and slow-cycling cells, and though the slow-cycling spermatogonia appear to be the predefinitive stem cells, and the more numerous fast-cycling cells already differentiated, proof of this has been elusive. It is possible that a circadian rhythm in the true stem cells could have been masked by mitotic activity of the more numerous fast-cycling A_s spermatogonia.

CHANGES IN THE SPERMATOGONIAL STEM CELL POPULATION WITH AGE

E. F. Oakberg C. C. Cummings

Contrary to common belief, gonocytes in the male mouse initiate division by beginning DNA synthesis on the day of birth. Cells with morphology characteristic of the A_s (stem) spermatogonia appear by 4 days of age. Mitotic activity of the stem cells is higher in juvenile animals than in adults; cell cycle properties characteristic of the adult are established by 21 days and maintained until 23 months of age (Oakberg, E. F., Proc. Vth Workshop on Development and Function of the Reproductive Organs, July 6-9, 1981, Excerpta Medica, pp. 149-152).

On the basis of cell counts in sections, we had observed a decline in stem-cell numbers between 12 weeks and 10 months of age, with no further change through 23 months. This result now has been confirmed by a study of tubule whole mounts, where stem-cell numbers at 11 and 23 months were 85 and 88%, respectively, of the 12 week value. This agrees well with our estimates of 85 and 79% obtained from analysis of sections, and demonstrates that, although some A_{pr} and A_{al} spermatogonia may be misclassified as A_s spermatogonia in sections, internal comparisons based on sectioned material are valid.

Toxicology Section

SECTION OVERVIEW - H. R. WITSCHI

The past year was a quiet one. It also was productive. At present there are 12 doctoral staff and three students in the Toxicology Section. During the last year, members of the Section authored 27 papers in peer-reviewed journals, wrote 9 review articles or book chapters, and issued 3 ORNL Technical Reports. Several papers report on work done in collaboration with members from other sections in the Biology Divison or with scientists from other ORNL Divisions (Health and Safety Research Division and the Analytical Chemistry Division). As was pointed out in previous annual reports, the Toxicology Section is not an isolated entity within the Biology Division or within ORNL. Staff in the Section always have and will continue to collaborate.

Collaboration is not limited to ORNL. There are strong ties with The University of Tennessee, not only through the U.T.-Oak Ridge Graduate School of Biomedical Sciences, which continues to provide education for some students within the laboratories of the Toxicology Section, but also through other University Departments. From the Department of Pathobiology of the Veterinary School, we have one student and a faculty member who do most of their experimental work at the Biology Division. In exchange, we take advantage of their expertise in animal pathology, an indispensable asset to a program in experimental toxicology.

Nevertheless, the toxicology program remains comparatively small. In studies on how chemicals may affect human health, the Section's efforts are largely limited to three fields: (1) studies on the pathogenesis of acute and chronic lung damage, (2) acute and chronic skin disease, and (3) proper functioning of the immune system.

At present, one of the most complete and most advanced programs involves the work which characterizes and explores induction and progression of the neoplastic process in the tracheal epithelium. It has now become technically feasible to expose grafted tracheas continuously or intermittently to controlled amounts of potential carcinogens. It has also become possible to continuously harvest cells from the exposed tissue. This allows one to follow, step by step, how normal cells are transformed into atypical cells and, eventually, into cancer cells. Biochemistry studies have kept pace with the model and important findings have been made that will eventually relate disturbed biochemial function to abnormal cell behavior.

Another exciting development is that it has become possible to identify, with chemical techniques, macromolecules that have been exposed to and modified by benzo(a)pyrene. Benzo(a)pyrene is ubiquitous and is thought to be a human carcinogen. We may eventually be capable of assessing actual human exposure to an environmental carcinogen by the simple expedient of measuring benzo(a)pyrene adduct formation in hemoglobin. Much work needs to be done to validate both the conceptual approach and the technical procedures involved. Nevertheless, the prospect of having a quantitative method that allows us to estimate exposure and possibly risk remains exciting.

While we have animal models for many human cancers, really good models for the leukemias, particularly the chronic myelocytic form, are still missing. Ongoing work on RFM mice promises to eventually provide us with a well-characterized animal model, an importance advance for those of us who study the pathogenesis of acute and chronic lung injury. It is commonly assumed that toxic lung damage suffered by leukemia patients treated with chemotherapeutic agents is caused by direct toxic action of the drug. However, the possibility exists that dying leukemic cells, killed by cytostatic drugs, release factors that are ultimately responsible for lung damage. This important problem can be studied when a reliable animal model of chronic myelocytic leukemia is developed. At the same time, we are learning more and more about how toxic inhalants, particularly ethylene oxide, affect the bone marrow and thus interfere with the production of both white and red blood cells.

Our knowledge on how chemicals penetrate and are metabolized by the skin continues to increase and advance at a rapid pace. Much of the progress is based on technical improvements in the methodologies. Soon it should be possible to make direct comparisons on data obtained by studying animal skin with data applicable to human skin. This will allow us to come closer to one of the premier goals of toxicology: to have a sound basis on which to extrapolate toxic effects from mouse to man.

A few years ago it was thought that the development of fibrotic lung disease could be explained by a deeper understanding of the early behavior of cell populations in the lung. The model at that time was based on the amplification of butylated hydroxytoluene (BHT)-induced lung damage by oxygen. We are no longer certain that this mechanism is of such critical importance. A reevaluation of the early events associated with toxic lung injury failed to confirm that all forms of damage proceed in a similar fashion, regardless of the causative agent. Rather, it seems that the pattern of cell proliferation after acute lung injury depends upon the specific toxic agent involved. This observation has curtailed previous speculation and we will continue to gather additional data until we have enough information to formulate a new hypothesis.

Many other observations have been made in the field of experimental pulmonary toxicology. For example, it is conceivable that oxygen might be the only agent that can be delivered in equivalent doses to animals of different size and weight (one cannot do more than breathe 100% O_2 for

several days). It appears to be metabolized into the same few molecular species in all animal species tested. Exposure to 100% O_2 allows us to study how the lungs of different species react to the same toxic agent. We found that mouse lungs respond to hyperoxia primarily with epithelial cell hyperplasia, whereas in rat lungs mostly endothelial cells proliferate. Interestingly, oxygen toxicity in man is characterized by epithelial cell proliferation. It may be tentatively concluded that mouse lung in its response to oxygen resembles that of man more than that of the rat.

Finally, we still do not know whether or not the enhancement of tumor development in mouse lung is dependent on cell hyperplasia. Using one carcinogen, urethan, it was possible to show that indeed tumors can be promoted by BHT even if over-all alveolar cell proliferation is abolished. However, with another carcinogen, 3-methylcholanthrene, the effect of the metabolic inhibitor used to prevent diffuse alveolar cell proliferation also diminished the number of tumors formed. It remains uncertain whether lung tumors can be promoted in the absence of general cell hyperplasia. On the other hand, we are accumulating evidence that BHT may enhance tumor development in the gastrointestinal tract — an unexpected finding.

Such is the progress made in experimental toxicology — slow, painstaking and quite often going in a wrong direction, but then, at other times, fast, exciting, and unpredictable. As usual, the past year has brought us a mixture of both.

SYSTEMIC TOXICOLOGY

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The Systemic Toxicology Group continues to study mechanisms of acute and chronic lung injury, especially the enhancement of tumor development by phenolic antioxidants and the inhalation toxicology of beryllium. In parallel to these studies, we maintain a toxicity testing program which, for the last year, has mostly focused on studies on the chronic dermal toxicity of coal conversion products.

(a) Mechanisms of Acute and Chronic Lung Injury. Injection of the phenolic antioxidant butylated hydroxytoluene (BHT) into mice produces acute diffuse lung injury. During the last few years we have conducted a large number of studies in which we documented the potentiation of BHT-induced lung damage by oxygen, the persistence of lesions for up to one year, and the contribution of shifts in cell populations to the development of diffuse interstitial pulmonary fibrosis. We also began to examine some of the molecular mechanisms which might underlie or accompany the production of excess collagen in a fibrotic lung. Current concepts of collagen

metabolism suggest that fibroblasts tightly control collagen production. One of the possible mechanisms of control is via the cyclic nucleotides, cAMP and cGMP. Beta-adrenergic agonists, by elevating intracellular cAMP levels, have been shown in vitro to suppress fibroblast collagen production, while beta-adrenergic antogonists were effective in removing this suppression by blocking the rise in cAMP. In a study with mice, we found that administration of the beta-adrenergic antogonist, propranolol, at a dose demonstrated to decrease the ratio of cAMP to cGMP, resulted in an elevation in total lung collagen in vivo. The increase in collagen was evident only when propranolol was administered prior to and during acute lung damage induced by either butylated hydroxytoluene, bleomycin, or high concentrations of oxygen. There was no increase in lung collagen when propranolol administration was delayed until after injury or when given to an undamaged lung. The data suggest that beta adrenergic blockade by propranolol may cause the fibroblasts, which are normally involved in the reparative process, to lose a mechanism for regulatory control that results in excessive deposition of collagen. Although this is an attempt to study in vivo mechanisms of lung injury at a molecular level we have also begun, at the same time, to develop a system in which mechanisms of injury can be studied with isolated cell systems maintained in culture (in vitro). To do this, we use a model of interstitial lung fibrosis produced by the anticancer drug bleomycin. Usually interstitial pneumonitis is a disease characterized by an interstitial and alveolar infiltration of macrophages, neutrophils and lymphocytes. As the disease progresses there is a derangement of alveolar structures as a result of alterations in quality, form, and location of collagen. This eventually results in an endstage fibrotic lung. The inflammatory component is the likely controlling factor in the pathogenesis of usual interstitial pneumonitis. Other investigators have shown that macrophages obtained from patients with this disease spontaneously secrete a macrophage-derived growth factor which stimulates fibroblasts in culture to proliferate.

We have found that the combination of single low dose of intratracheally instilled bleomycin followed by exposure to hyperoxia provides an excellent model of this disease in hamsters. Using this model, it has been observed that the macrophages increase during fibrogenesis and that this increase is a result of a continued influx of new macrophages. In addition, the phagocytic function of these macrophages is less efficient. We plan to examine macrophages from these animals to see if they secret a growth factor similar to that described in man.

We have attempted to establish cultures of lung fibroblasts from adult hamsters, adult rats, and fetal (about 17 days) rats from minced trypsinized tissues. Cultures from adult hamsters were established (up to 12 passages) but consisted of a mixture of epithelial cells and fibroblasts with a doubling time of about one week. Both of these characteristics were unacceptable for our purpose. Two attempts to establish usable cultures from rat fetal lungs failed for unknown reasons. However, primary cultures of fibroblasts were prepared from adult rat lungs. Presently, the culture is in its 8th passage and appears to consist mainly of fibroblasts. We have, however, found that the combination of low dose bleomycin followed by

hyperoxia exposure results in a progressive fibrosis in rats similar to that observed in hamsters. Since we have been more successful in obtaining a rat fibroblast line, we will proceed with using the rat macrophages to determine the presence of macrophage-derived growth factors on this primary fibroblast line.

While studies such as those described above will provide an insight into molecular mechanisms of lung disease, another series of experiments explores, at the cellular level, the development of toxic lesions in the lung produced by anticancer drugs such as bleomycin, cyclophosphamide, busulfan or carmustine. The drug on which we have the most complete data base is cyclophosphamide. We have completed a chronic study with this agent. Male BALB/c mice received a single intraperitoneal injection of 100 mg/kg of cyclophosphamide. Within 3 weeks there were scattered foci of intraalveolar foamy macrophages. With time, these foci increased in size and, one year later, occupied large areas in all lung lobes. Diffuse interstitial fibrosis was also observed. Chemical determinations showed that, in the lungs of animals treated with cyclophosphamide, collagen accumulated steadily over the entire one year period. One year after cyclophosphamide pressure-volume curves measured on excised lungs were abnormal, indicating that lung compliance was altered and total lung volumes were decreased. Thus, a single injection of cyclophosphamide produced an irreversible and progressive pulmonary lesion.

These observations prompted us to study in more detail the early lesions produced in mouse lung by a single dose of cyclophosphamide. Mice were injected with ^{3}H-labeled thymidine at different days after a single dose of cyclophosphamide and killed one hour later. Lung tissue was prepared for autoradiography and the number and types of labeled cells were counted. Usually, acute lung damage is followed immediately by a burst of DNA synthesis in the epithelial cell lining the alveoli. This burst of synthesis is followed later by proliferation of capillary endothelial cells and interstitial cells. This pattern was not seen after cyclophosphamide. Cell proliferation was delayed until 10-14 days after administration of the drug. Perhaps even more importantly, cyclophosphamide produced an almost complete block of DNA synthesis in the type II alveolar epithelial cell population. This is a new and unusual observation. Whether inhibition of epithelial cell proliferation by cyclophosphamide is causally related to the development of the progressive lung lesion seen after injection of one single dose of the drug remains to be established.

Two other observations were made in animals treated with cyclophosphamide: the activity of enzymes such as lactic dehydrogenase, alkaline or acid phosphatase, and angiotensin converting enzymes can be measured in the bronchoalveolar lavage fluid. It is usually assumed that increased activity of these enzymes accompanies toxic lung injury. In our experiment, however, we did not find a substantial increase in enzyme activity in bronchoalveolar lavage fluid. Autoradiography and assessment of DNA synthesis in lung was thus a more reliable indicator of lung damage than was a biochemical measurement. The second observation was that radiolabeled cyclophosphamide becomes, within 2 h after injection, covalently

bound in lung tissue. This observation will allow us to visualize the covalently bound drug with autoradiography and to obtain some information on the exact site at which cyclophosphamide accumulates in the lung.

Cell kinetic studies currently in progress may help us answer a crucial question: Which animal model of lung disease most resembles the human condition? Rodents are most frequently used to study acute interstitial lung injury, but numerous observations made in rats appear unique to that species. Do rats, mice and hamsters respond differently to acute lung injury? To answer this question we exposed 5 animals of each species to 100% oxygen for 48 h. After exposure, osmotic pumps, which release ^{3}H thymidine over one week, were implanted sc. After one week we determined which cell populations had incorporated labeled thymidine into DNA during the recovery period. We found that repair in rats is manifested by a high LI (percentage of labeled pulmonary cells) indicative of endothelial cell proliferation. Mice and hamsters have a lower LI, indicative of type II pneumocyte proliferation in mice, while in hamsters, macrophages and pneumocytes proliferate. Morphometric study in man suggest that type II pneumocytes increase after lung injury. From this experiment we conclude that the response to oxygen injury in mice most resembles that seen in man.

In a second series of experiments we analyzed in detail a recently developed model of human adult respiratory distress syndrome. Hyperoxia exposure poentiates lung injury seen in bleomycin-induced interstitial disease. Hamsters were treated with 5U/kg bleomycin (bleo), instilled intratrachealy, immediately followed by exposure to 80% O_2 for up to 96 h. Regardless of duration of O_2 exposure, 73% of the animals died between 72 and 96 h after instillation. All manifested gross pulmonary edema, histologic interstitial fibrosis, alveolar edema, and hyaline membranes. We conclude that as little as 12 h of exposure to 80% O_2 potentiates bleomycin-induced pulmonary toxicity, and that longer hyperoxia exposures will not further enhance this toxicity. With bleo and 24 h of hyperoxia, the onset of pulmonary edema is delayed until 72 h after instillation, coincident with mortality.

In conclusion, our studies have examined in greater depth possible mechanisms of the pathogenesis of acute and chronic toxic lung injury both at the biochemical and at the cellular level.

(b) Enhancement of Tumor Formation by Hindered Phenolic Antioxidants. Almost 10 years ago it was shown that BHT enhanced the development of lung tumors in mice. It has become obvious that the cumulative, biologically active, dose of BHT in the mouse lung tumor system is quite low. Earlier methods revealed that a total of 6 ip injections of 50 mg/kg BHT or feeding 7,500 ppm of BHT in the diet for two weeks enhanced the development of lung tumors. During the past year, we established that in strain A mice treated with a single dose of 3-methylcholanthrene and exposed for 2-4 weeks to a concentration of 500 or 1000 ppm BHT in the diet, the number of lung tumors was increased 4 months later. The total dose of BHT ingested during the shortest (3 week) exposure to 500 ppm averaged 470 mg/kg, corresponding to

an intake of 35 mg/kg/day. This is about 70 times higher than the unconditional acceptable daily intake of BHT for man, which presently is set at 0.5 mg/kg body weight — a daily intake at an approximate concentration of 30 ppm BHT in the total diet. While the concentration of dietary BHT in our experiment (500 ppm) exceeded the BHT concentration in the human diet by a factor of almost 20, and the cumulative daily intake of 35 mg/kg/day was about 75-fold higher than the acceptable daily intake, it must be remembered that the enhancing effect on tumor occurrence was obtained after only a 2 week exposure. This corresponds to about 1/75th of the normal life span of a mouse. Thus, we recommend that the minimal exposure conditions found to enhance tumor formation be compared to the proposed levels of BHT required and/or claimed to have some protective action against certain chemical carcinogens. Such a comparison makes it obvious that, in animals pre-exposed to a carcinogen, the amounts of BHT required to enhance tumor formation are lower by several orders of magnitude than are the amounts of BHT needed in the diet to provide a marginal beneficial effect.

While the precise mechanism of action of BHT remains unknown, some progress was made toward understanding important cellular events associated with enhanced mouse lung tumor development. Mice were injected with urethan (1000 mg/kg ip) and then given 4 weekly ip injections of BHT (300 mg/kg), a treatment known to enhance development of lung tumors. The cumulative labeling index of alveolar wall cells was determined during the first 6 weeks of the experiment. There is a significantly increased proliferation of cells in the alveolar zone that occurs after only the first 2 injections of BHT. In a second experiment, mice were treated with urethan and then with an inhibitor of mixed function oxidases, piperonyl butoxide (800 mg/kg), followed one hour later by BHT. Administration of piperonyl butoxide completely prevented the overall proliferation of cells in the alveolar zone that is normally seen with BHT alone. Analysis of the tumor data indicates that (a) BHT increases the number of tumors found the week after urethan regardless of whether the animals had been treated with piperonyl butoxide or not and (b) that piperonyl butoxide alone has an effect on tumor development. Thus, we could not conclude with absolute certainty that the enhancing effect of BHT on lung tumor development in mice was independent from diffuse cell hyperplasia in the lung. The ambiguity in the data required a different study. In another experiment we used 3-methylcholanthrene as the initiating agent and found that piperonyl butoxide again abolished cell proliferation and that BHT had a small, but significant, enhancing effect on tumor development. However, this observation was overshadowed by the unexpected observation that piperonyl butoxide alone greatly inhibited tumor development. For the time being, our data do not allow exclusion of alveolar cell hyperplasia as a mechanism in BHT-mediated enhancement of mwuse lung tumor development.

In studies using butylated hydroxyanisole (BHA) we have not found a modification of lung tumor formation in mice. A diet containing 0.75% BHA failed to enhance the development of lung tumors in A/J mice treated with a single dose of urethan, benzo(a)pyrene [B(a)P] or dimethylnitrosamine. Prefeeding animals with BHA partially protected animals against the

tumorigenic effect of urethan and B(a)P. Partial protection was also seen in animals given B(a)P and then fed BHA in the diet. Two isomers of BHA (3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole) were synthesized and injected ip. They failed to enhance lung tumor development. It is concluded that BHA is not a promoting agent as is BHT for lung tumors in mice.

Not all cancer researchers are ready to accept findings made with lung tumors in mice as relevant for human carcinogenesis. This poses some conceptual and practical difficulties if one attempts to judge the value of our lung tumor enhancement studies for human risk. This consideration has prompted us to initiate a series of experiments designed to determine whether exposure to BHT in the diet influences the development of colon tumors. Colon tumors have been induced in mice and rats with two different carcinogens, dimethylhydrazine and methylnitrosourea. Since colon tumors are the second most frequent human cancer and since BHT is a common food additive, we look forward to the results of these studies with considerable interest.

Although we still do not know the mechanism(s) by which BHT enhances lung tumor development in mice, in skin it actually inhibits tumor promotion. It is thought that BHT, an antioxidant, interferes with generation of oxygen free radicals, an event likely to be of critical importance in skin tumor promotion. Thus, we have decided to study the role of oxygen free radicals in lung tumor development. For this study, male A/J mice were injected with urethan (1000 mg/kg) or saline and one week later were placed into either a 70% O_2 atmosphere for up to 16 weeks or kept in room air. All animals were killed 17 weeks after urethan injection and the number of visible tumors counted. To our surprise, we found that tumor growth had been inhibited by 70% O_2. One explanation is that high O_2 inhibits pulmonary type II epithelial cell proliferation; the type II cell is the progenitor of most adenomas following urethan injection. Another possibility is that high O_2 produced elevated superoxide dismutase levels which would decrease the likelihood of free radical formation. Thus, our studies of free radicals, one of the proposed mechanisms of tumor promotion, continue.

(c) The Toxicology of Inhaled Beryllium. The acute response of mice and rats exposed to a aerosol of $BeSO_4$ is characterized by studying the DNA synthesis in alveolar type II epithelial, interstitial or capillary endothelial cells. Male Fischer 344 rats and male BALB/c mice were exposed in a nose-only inhalation chamber for 1 h to a concentration of 13 μg Be/L. Animals were killed at intervals over 21 days. Labeling index (alveolar parenhcymal cells labeled with ^3H thymidine/total cells counted) peaked at day 5 for mice and day 8 for rats. Morphologically, the greatest tissue reaction was observed in rats at day 10-14. The reaction involves an increased interstitial macrophage population, prominent Type II pneumocytes, alveolar fibrin, hemorrhage, and alveolar macrophages with ragged cell membranes and microvesicular cytoplasm. In a separate study, bronchoalveolar lavage analysis showed maximum levels of lactate dehydrogenase on day 8 in rats and day 5 in mice. Acid phosphatase peaked at day

3 in rats while alkaline phosphatase was highest on day 3 for both rats and mice.

We have recently made an observation of potentially far reaching consequences. In several elegant studies, Dr. Jay Joshi, Department of Biochemistry, University of Tennessee, has shown that ferritin has an extremely high affinity for Be. If rats are treated for 3 days with ferric ions, ferritin synthesis in the liver is induced. When we injected ferritin-treated rats with lethal doses of $BeSO_4$ iv, we found that it took twice as much Be to kill the animals than it did to kill untreated animals. The role of ferritin as a Be-complexing agent and thus a potential endogenous protective agent in acute Be toxicity will be studied further.

Toxicity Testing Program. Energy technologies associated with coal conversion processes produce substances in the general environment and the workplace that are potential health hazards. Evaluating the potential of these hazards requires establishing and updating a data base using both acute and chronic tests involving experimental animals. Hence we have developed facilities for testing substances for their acute and chronic toxicity. The acute tests include oral and intraperitoneal LD_{50}'s in mice (using relatively few animals), acute dermal toxicity in rats, skin and eye irritation in rabbits, and delayed-type hypersensitivity in guinea pigs. The chronic test is conducted with mice and consists of multiple dermal applications (2 or 3 times a week) of a substance for a period approaching the mean life span. Although the principle end points of the chronic test are tumor incidence and mean time-to-tumor, the protocol allows for determination of other dermatotoxic effects as well as a broad histopathologic assessment of effects on each animal. The procedures used for acute and chronic tests are basically those employed in other toxicologic laboratories and by and large conform to procedures set forth in the OECD Guidelines for Testing Chemicals (Paris, 1981).

During the current reporting period, our efforts have centered on the dermatotoxic effects of chronically applied coal conversion products using two protocols. The 'standard' protocol A involves painting the skin of mice 3 times a week for 18 months with 50 microliters of 25, 50, or 100% preparations of each substance using 50 mice per dose level. Protocol B consists of applying 50 microliters of a high dose (usually 100%) of a substance to the skin of mice 2 times a week for 12 months using only 10 animals per substance. The latter procedure was intended to be a relatively quick and inexpensive screening test to provide an initial estimate of tumorigenic potential of a substance.

Using the standard protocol A, we are currently testing the dermatotoxicity of the following derivatives of Illinois No. 6 coal: a blend of light and heavy fuel oils, high and low level hydrotreated preparations of that blend, and reformed naphtha (gasoline) from the blend. After 11 months of dermal application, the data show that high or low hydrotreatment of the blend substantially reduced its strong tumorigenic potential. Likewise the naphtha derivative of the blend was essentially nontumorigenic for mouse skin. Concomitant dermal tests of petroleum

derived gasoline and No. 2 home heating oil indicate that even at the 100% levels, skin tumors were not induced, although the latter is proving to be quite injurious to skin at the higher dose levels.

We are presently using the screen test (protocol B) to assess the tumorigenicity of coal oil derivatives resulting from various treatments such as distillation, hydrogenation, solvent extraction, and acid-base washes. These derivatives (prepared by the Analytical Chemistry Division of ORNL) are being tested to determine whether any of the treatments alter the tumorigenicity of the coal oils.

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INDUCTION AND PROGRESSION OF NEOPLASIA IN TRACHEAL EPITHELIUM

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The respiratory tract of man is continually accosted by hazardous agents in the environment that are potentially carcinogenic, cocarcinogenic, or which may promote the development of lung cancer. Many of these hazardous agents are introduced into the environment as particulates, gases, aerosols, and in complex mixtures during the refining and utilization of most energy sources. There is a great need to develop mammalian model systems that will test for these potentially hazardous agents 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. The tracheas of rats are used in these studies because of the structural similarity of the epithelium to the human bronchus.

In Vivo Exposure to the Test Agents. We recently developed an open-ended, flow-through tracheal implant (FTTI) model to study the induction and pathogenesis of lung cancer. In this model a 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. A major project was started during the past year to study in the FTTI model the carcinogenicity, co-carcinogenicity and promotability of benzo(a)pyrene [B(a)P] (a known carcinogen in the environment) and formaldehyde (HCHO) (an ubiquitous environmental pollutant), when these agents are given alone or in various combinations. To begin this study, the acute morphological responses of the FTTI to twice weekly exposures of different amounts of B(a)P were examined. Doses of 20, 40, 80, 160 and 320 μ g B(a)P were given 2×/wk for 2, 4 and 8 weeks and the same total doses given over different lengths of time compared. At each time point 4 FTTI from each exposure group were taken for histopathologic assessment. At the lowest amount of B(a)P [(20 μg) given 2×/wk for 2 wks (total dose 80 μg)], 40% of the epithelium remained normal mucociliary. After 8 wks of twice weekly 20 μg B(a)P (total dose = 320 μg), 25% was still normal, while the rest of the epithelium had only a mild basal cell hyperplasia with minimal amounts of transitional or squamous metaplasia. If, however, the total dose of 320 μg was given within 2 wks (80 μg, 2×/wk), less than 10% of the epithelium remained normal mucociliary while more than 60% had mild basal cell hyperplasia and 10% transitional and squamous metaplasia. After 8 wks exposure to 80 μg, 2×/wk [total dose = 1280 μg B(a)P], 30% of the epithelium was stratified hyperplasia and 20% transitional + squamous metaplasia. When 1280 μg B(a)P is given within 2 wks (320 μg, 2×/wk), more than 50% of the epithelium becomes a transitional + squamous metaplasia within this shorter time. These are examples of the increased acute toxicity of carcinogen doses given over shorter periods of time, and they serve to demonstrate the value of this model for studying the effects of carefully controlled exposure regimens on the induction and progression of neoplasia in airway epithelium.

A few years ago, we devised an apparatus for exposing the FTTI to humidified HCHO gas generated from solid HCHO, and began to develop exposure regimens. During the past year we improved on the design of this apparatus and carried out additional HCHO exposures to verify our earlier results. FTTI were exposed to 1.13, 1.4 and 3 ppm for 1 h, 2×/wk for 2 wks. Increasing doses of HCHO induced increasing areas of hyperplasia, transitional metaplasia and a flattened, regenerating epithelium with a very high mitotic index. The latter is intrepreted as repair of eroded epithelium at too high an exposure. Therefore, the exposure was reduced to 0.75 ppm/trachea and tested in combination with B(a)P to establish exposure conditions for co-carcinogenesis studies. FTTI were exposed once a week to 80 μg B(a)P followed 3 days later by 0.75 ppm HCHO/trachea for 4 wks [total B(a)P - 320 μg] and 8 wks [total B(a)P = 640 μg]. The 4 wk-exposed FTTI have been assessed. The histological types in the epithelium were: 36% basal hyperplasia, 27% stratified hyperplasia + transitional metaplasia, 10% squamous metaplasia, 19% flattened regenerating, 6% eroded. Because of the small amount of eroded epithelium present, we have reduced the HCHO further, and are now testing 2 ppm HCHO (0.25 ppm/trachea) in combination with 80 μg B(a)P weekly.

In Vivo-In Vitro Model. In vivo models are essentially limited to morphological endpoints. To overcome this limitation a combined in vivo-

in vitro model of carcinogenesis was developed several years ago, which makes it possible to study a gamut of cellular and biochemical changes in cell populations derived from the tracheal tissue at different times after carcinogen exposure *in vivo*. The approach is to expose tracheal implants to known doses of carcinogen for 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 anchorge-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, dimethylbenz(a)anthracene (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, 1.5, 5.0 and 7.2 SPC were induced in the tracheas. Other dose-dependent effects 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.

The DMBA exposure studies serve as a prototype for examining the induction of altered cell populations (SPC) by B(*a*)P and HCHO using exposure protocols described in the *in vivo* exposure section above. To this end, FTTI were exposed to 160 μg B(*a*)P 2×/wk for 4 wks (total dose = 1440 μg) and the tracheas sacrificed at 8 wks for analysis of the induction of altered cell populations. This exposure induced 1 SPC/trachea. With this data as reference, three new experiments have been initiated: 1) To determine whether the same total dose of B(*a*)P given over a longer period is more carcinogenic, FTTI are being exposed 2×/wk to 40 μg B(*a*)P for 18 wks (total dose = 1440 μg). 2) To establish a dose effect and also a B(*a*)P mount that is highly carcinogenic when given over a 4 wk period, FTTI are being exposed to 320 μg 2×/wk [total dose = 2880 μg B(*a*)P]. 3) To begin to examine possible cocarcinogenic interactions between B(*a*)P and

HCHO, FTTI are being exposed weekly to 80 μg B(a)P and 0.25 ppm HCHO/ trachea for 8 wks. In each of these experiments, the tracheas will be analyzed for the number of SPC induced/trachea and markers for the progression of neoplasia will be determined to compare the carcinogenic potency of the different exposure regimens.

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 determining the metabolic differences between the pyruvate-requiring normal tracheal epithelial cells and the carcinogen-altered cells. A few of our key findings are the following: 1) We found earlier that an increase in uptake of [^{14}C]-2-deoxyglucose was not an early marker of neoplasia nor was it a definitive marker of tumorigenicity in tracheal epithelial cells as claimed for some cell types. [^{14}C]-2-deoxyglucose uptake was actually lower in preneoplastic cells (SPC) as compared to normal cells. This uptake remained low in some tumor cells, but markedly increased in others. The latter were from rapidly growing, undifferentiated carcinomas. 2) We have recently found that glucose is oxidized at a higher rate in normal primary cultures (NPC) than SPC, and that there is greater incorporation of glucose in some cell fractions of the NPC. 3) The most striking finding was that NPC take up 5 times more [^{14}]-pyruvate than SPC in some cell fractions. To determine if changes in some key enzyme activities reflect these metabolic changes, several enzymes were recently studied. Lactic dehydrogenase activity was higher in NPC than SPC, thus paralleling the higher glucose utilization. Phosphofructokinase was not significantly different. The most important finding was the low level of NADP-linked malic enzyme activity, which catalizes the formation of pyruvate from malate, found in the mitochondrial fraction of NPC compared to SPC. This observation again emphasizes the differences in pyruvate metabolism between normal and carcinogen-altered tracheal epithelial cells.

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INHALATION TOXICOLOGY

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Exposure to ethylene oxide is considered to be a significant health hazard. Over the past year we have continued to examine its potential hazard from two different angles: (a) In collaboration with R. A. and D. M. Popp we have begun to investigate the comparative sensitivities of mice that are homozygous or heterzygous for a number of different genetic disorders and (b) we are studying the effects of pulmonary fibrosis on the toxicity of ethylene oxide in rats and mice.

In the first study survival rates of animals with different genetic traits are being compared. Groups used for this study included α-thalassemic, heterozygous and homozygous β-thalassemic, α-1-antitrypsin deficient, beige and control C57BL/6J mice. It was found that one strain of mice was more susceptible to the toxic effects of ethylene oxide; over half the animals died after 10 or less exposures and none survived beyond the nineteenth exposure. On histopathological examination of tissues from the sensitive animals that were necropsied soon after death or that were specifically sacrificed after 1 to 5 exposures, it was observed that there was a diffuse alveolar hemorrhage with perivascular chronic inflammatory cells and some evidence of pulmonary edema. There was also evidence of microscopic changes in the liver including focal hepatocellular degeneration, pyknosis and microvacuolation of hepatic cells. One animal also had a cystic kidney but it is not certain that this was treatment related. Initial conclusions from this study are that certain genetic traits render mice more susceptible to toxicity of an inhaled environmental contaminant while other genetic traits do not alter susceptibility. Further studies using offspring derived from mating the sensitive animals with normal C57BL/6J mice is currently underway to try and determine whether enhanced susceptibility is a recessive or dominant trait. The implication of this work for risk estimation in humans is potentially of great significance.

Most inhalation studies are carried out using animals in prime health. In the human population, however, many workers have underlying health problems that do not prevent them from working but may make them more susceptible to chemicals to which they are exposed during their normal working day. We have induced pulmonary fibrosis in both rats and mice in order to study whether pre-existing lung damage can alter the changes in the hematopoietic system that are caused by ethylene oxide. Fibrosis was induced in rats by a single intratracheal injection of bleomycin and a single intraperitoneal injection of butylated hydroxytoluene was used to produce the fibrosis in mice. In both species exposure to ethylene oxide was commenced approximately 4 weeks after the pretreatment and continued for 5 days per week (6 hours per day) over two weeks. At the end of exposure animals were sacrificed and bone marrow cellularity was determined. In collaboration with R. E. Hand, flow cytometric techniques were used to define the relative size of the subpopulations of marrow granulocytes and lymphocytes and also to carry out cell cycle analysis. Preliminary results indicate that the presence of fibrosis did not, per se, alter the hemotoxicity of ethylene oxide; however, there does appear to be some species difference; mice appear to be the more sensitive.

To further examine lung damage and to what extent pulmonary function tests define the degree of that damage, a collaborative study with A. F. Tryka is underway to determine whether there is any correlation between the diffusing capacity as defined by morphometric analysis with the in vivo measurement of diffusing capacity using carbon monoxide. A chemically induced progressive lesion in rats is being used and animals are being tested at 30, 60 and 90 days for carbon monoxide diffusing capacity. A battery of other pulmonary function tests including various lung volumes, pulmonary resistance and multi-breath nitrogen washout are also being employed. Immediately after being tested the animals are sacrificed and the lungs removed for use in the morphometric analysis.

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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, and (2) dosimetry of polycyclic hydrocarbons (PAH) = 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. Previously we have reported on the development and application of *in vitro* systems for maintaining intact full-thickness mouse skin in organ culture for use in dermatotoxicology studies. Under the culture conditions, metabolic viability and structural integrity of the tissue can be maintained for periods of up to 48 h. Also an important property of our culture systems is that chemicals of interest can be applied to the skin surface in a manner similar to *in vivo* exposure and the material of interest reaches the epidermal cells by diffusion through the various strata of the skin before partitioning into the culture medium.

Using this culture system, studies have shown that the translocation of benzo(*a*)pyrene [B(*a*)P], a model PAH, across full thickness mouse skin into the culture medium was dependent upon the viability and metabolic status of the skin. Detailed examination of the culture medium 24 h following topical application of B(*a*)P to mouse skin in organ culture showed that essentially only metabolites of B(*a*)P were present. The results indicated that topically applied B(*a*)P was metabolized by the skin during its passage through the skin. Thus, percutaneous absorption of

B(a)P in mouse skin was coupled to its cutaneous metabolism. Furthermore, other studies also show that absorption is modulated by factors which influence the activities of the drug metabolizing enzymes. Induction of drug metabolizing enzymes by systemic pretreatment with such inducers of mixed function oxidases as TCDD and 3-methylcholanthrene (3-MC) resulted in a several-fold increase in the rate and overall permeation of B(a)P in mouse skin in organ culture. Conversely, in experiments where respiration in the cultured tissue was prevented by the introduction into the culture medium of metabolic inhibitors such as KCN, permeation of B(a)P was significantly reduced.

Strain differences were observed in the in vitro skin permeation of B(a)P. Following in vivo pretreatment with 3-MC, increased permeation was apparent in the skin from the "inducible" C57BL/6 mice, while no changes were observed in the skin from the "noninducible" DBA/2 mice. Also, the permeation in hairless strains of mice appears to be significantly lower than in normal haired mice. This suggests the possible involvement of the philosebaceous apparatus in the penetration of foreign agents across the skin. Preliminary examination of frozen sections of mouse skin by fluorescence miroscopy 2 h following in vivo topical application of B(a)P showed that in the normal (C3H) mouse fluorescence due to B(a)P was equally intense along the epidermis and within the hair follicles and sebaceous glands. On the other hand, in the skin from the nude mouse, with its "defective" philosebaceous apparatus, fluorescence from B(a)P was localized primarily along the epidermis. Little or no fluorescence was detected in the skin appendages, although weak fluorescence was observed in some superficial sebaceous glands and hair follicles. These observations indicate that the philosebaceous apparatus of the skin may play an important role in the percutaneous absorption of B(a)P.

In contrast to B(a)P, studies with testosterone and benzene showed that the rates and overall in vitro permeation of these compounds in mouse skin were significantly greater than for B(a)P and they were not affected by the viability and metabolic status of the tissue. Similar permeation results were observed in viable and nonviable tissues; and no increase in permeation was apparent following enzyme induction. Also strain differences were not observed. This suggests that for these compounds, transepidermal diffusion was the main determinant in their percutaneous absorption. However, a closer examination of the culture medium from viable mouse skin, 24 h after topical application of testosterone, showed that both metabolites of testosterone as well as the parent compound were present in the culture medium. Examination of the corresponding medium from nonviable tissue, on the other hand, detected essentially only testosterone in the culture medium. These results indicate that in the skin there is "first pass" metabolism of some topically applied chemicals. This may have important consequences on the subsequent fate and physiological disposition of topically applied xenobiotics.

An important component in the metabolism of xenobiotics are the activities of the microsomal P450 mix-function monooxygenase enzyme system (MFO). Therefore an understanding of the properties of this enzyme system,

and the mechanisms by which the enzyme activities are regulated in skin, is of fundamental importance in evaluating the metabolic and functional capability of the skin towards topically applied xenobiotics. In this past year we have initiated studies of the MFO enzyme system of mouse skin. MFO activities, as measured by benzo(a)pyrene hydroxylase (BPH) and ethoxycoumarin deethylase (ECD), in mouse skin are very low but these enzyme activities were inducible by inducers such as TCDD. Depending on the strain of mouse, BPH and ECD activities per mg of microsomal proteins in skin can be, respectively, 0.5-15% and 0.6-2% that of the liver. The relative levels of these two activities in the skin can be different from their relative levels in the liver from the same animal; this suggests possible differences in the regulation of the expression of these activities in the two organs. Attempts to measure other MFO activities in the skin have not been successful. This is partly due to the insensitivities of the assays, although it is possible that activities such as p-nitroanisole demethylase and benzphetamine demethylase may not be expressed in the skin. Observed differences in the electrophoretic patterns in the P450 region of microsomal proteins from skin and liver are suggestive of this possibility. Preliminary results also suggest that in some strains of mice measurable enzyme activities in microsomes from dorsal skin can be fivefold higher than in microsomes from ventral skin, while in other strains no differences are apparent. Strain differences in the enzyme activities of the skin of threefold to tenfold were also observed, but such strain differences were not apparent in the liver. These initial studies on the MFO enzyme system of mouse skin support our contention that cutaneous metabolism may have significant influence on the toxicokinetics of some topically applied xenobiotics. However, to what extent cutaneous metabolism may play a role in the percutaneous absorption of xenobiotics remains to be investigated further.

Local toxicity can have a profound effect on the functional capabilities of the skin. Therefore an assessment of the cutaneous toxicity of topically applied chemicals should be an important aspect in skin permeability studies. Previously we have demonstrated that mouse skin maintained in our culture system responds to toxic insult with morphological and biochemical changes. The results indicated that biochemical changes such as release of intracellular enzymes from damaged cells into the culture medium may be useful indicators for assessing toxicity in skin in vitro. Recent studies in our laboratory using a series of chemicals with different degrees of irritant potential have shown that, following topical application, the epidermal lesions observed in the skin maintained in vitro were comparable to those seen in vivo. Moreover, there appears to be a good correlation between the magnitude of the histological lesions and the level of intracellular enzyme activities in the culture medium. These results support our initial observation that enzyme leakage could be a useful tool in assessing cutaneous toxicity in vitro. Other techniques are also being investigated. For example, stereologic analysis of skin components is currently being evaluated. We hope that eventually morphometric techniques may be employed as a tool in quantitative dermatotoxicological investigations.

The culture systems developed for mouse skin can be used for skin samples from other mammalian species. Based on histological examination and the ability of the cultured tissue to metabolize glucose, our studies have demonstrated that skin preparations from rat, rabbit, guinea pig and man can be maintained metabolically viable and structurally intact under our culture conditions. Moreover, the results from comparative in vitro permeation and cutaneous metabolism studies with skin samples from these species were qualitatively similar to the results from studies with mouse skin, that is, B(a)P and testosterone were metabolized by the skin during their permeation through the skin following topical application. However, quantitatively, permeation in the skin from these species was significantly lower than in mouse skin. These initial studies illustrate one of the major advantages of our in vitro approach; that is, comparison of the functional capabilities of human and animal skin can be made under identical and defined conditions. This, therefore, provides an opportunity for developing a rational basis for extrapolation from animals to man.

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 discovery 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, benzo(a)pyrene, 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 compound with DNA

can be used as a measure of the "biologically-effective" dose for B(a)P carcinogencity.

We are currently also investigating the suitability of hemoglobin as a cellular molecule for estimating carcinogenic risk of B(a)P (and possibly PAHs). Our preliminary data with mice exposed topically to B(a)P show: (1) The interaction of B(a)P with hemoglobin is highly specific, and occurs, presumably, via the anti diol epoxide; the same ultimate carcinogenic form of B(a)P interacts with DNA in the target tissue at the site of application. (2) The dose response of binding to hemoglobin appears to be similar to that of DNA, thus reflecting the "in vivo" carcinogenic dose of B(a)P. (3) The adduct, once formed, is stable and easily measured. (4) Hemoglobin is readily available. Future experimentation will focus on the feasibility of using human hemoglobin as a cellular molecule for estimating benzo(a)pyrene exposure.

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RADIATION IMMUNOLOGY

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Cancer remains a major health problem. It is also the primary late effect of ionizing radiation in man. Our objective is to gain insight into tumor-host relationships and to establish the role of the immune system and immunologic dysfunction in the induction and control of radiogenic malignancies. Currently we are evaluating sublethally irradiated (300R) RFM mice as a needed animal model for chronic myelogenous leukemia in man. Two primary areas of investigation are the modification of myeloid leukemia incidence in RFM mice and the modulation of immunogenicity of myeloid leukemia cells.

Modifying Leukemia Incidence in RFM Mice. While it is recognized that the incidence of any type of leukemia is genetically determined and

primarily monoclonal in origin, host factors are readily demonstrated. Significant differences occur in male and female animals and the incidence and induction period is dramatically altered by sublethal irradiation in the susceptible RFM strain. Our gross pathology findings demonstrate a 50% incidence of thymic lymphoma in 300R irradiated female RFM mice and a surprisingly low 5% incidence of myeloid leukemia. In male mice the numbers are 27% and 10%, respectively. In any effort to reduce early-occurring thymic lymphoma and have more animals at risk for later-occurring myeloid leukemia, mice have been thymectomized at 4 weeks of age and exposed to irradiation 3 weeks later. The incidence of thymic lymphoma has been completely eliminated, non-thymic lymphoma is rare, but the incidence of myeloid leukemia has increased to only 10% and 25% in female and male mice, respectively. We had previously reported that injection of bone marrow significantly delayed the onset of thymic lymphoma. To determine if this extended induction period was sufficient to allow more animals to develop myeloid leukemia, mice received bone marrow cells immediately following radiation exposure. At 500 days of age approximately 60% of the mice were dead. Moreover, at this time we are not observing an increased incidence of myeloid leukemia. These efforts provide insight into inductive mechanism(s) common to both thymic lymphoma and myeloid leukemia where different target cells are implicated.

A progressive decrease in the incidence of thymic lymphoma is seen when animals are exposed to ionizing radiation at older ages. It is generally accepted that myeloid leukemia does not express a similar age-related decrease in susceptibility. Accordingly, irradiation of mice has been delayed and animals were not exposed until six months of age in an effort to increase the incidence of myeloid leukemia. Two additional approaches to selectively modify leukemia incidence are being pursued. In the first, following irradiation, animals are treated with phorbolesters which are tumor promoters and have been reported to be potent inducers of differentiation in a promyelocytic cell line. Furthermore, we have noted that animals under extended treatment with TPA often show extensive myeloid hyperplasia. In the second approach, mice are being treated with cyclosporin, a powerful immunosuppressant that acts on T cells but has remarkedly low myelotoxicity, following irradiation. While one of the objectives of these experiments is to increase the incidence of myeloid leukemia thereby providing a better animal model for additional experimental studies, needed insights into the interrelationships among leukemias of different cellular origin during radiation leukemogenesis and the modulating role of both immunologic and nonimmunologic mechanisms will also be obtained.

Modulation of Immunogenicity of Myeloid Leukemia Cells. Effective immunization procedures do not exist for myeloid leukemia in man. Implicit in the role played by immunologic mechanisms in radiation leukemogenesis is that initiated cells have or acquire sufficient immunogenicity so that the host can recognize them as foreign. Although most leukemias are generally accepted as monoclonal at their inception, variants are generated as a result of the genetic instability of neoplastic cells; e.g., karyotypic heterogeneity and diversity within tumors with respect to cellular

morphology, growth rates, cell surface markers, drug sensitivities, etc. have been reported. Some of these variants (clones) apparently are eliminated by extra- and intra-tumor selection pressures and the more favored variant survives and proliferates. The ability to isolate, select, and grow subpopulations of cells with different biological characteristics from what we now know to be heterogeneous tumor cell isolates or lines is of great importance in our efforts to potentiate the immunogenicity of leukemia. Current efforts in this area focus on potentiating tumor immunogenicity of different myeloid isolates by (1) examining quantitative and qualitative immunogenic differences between the primary isolates and in vivo and in vitro propagated tumor cell lines, (2) cloning to determine the degree of immuno-heterogeneity within a given isolate and to clone and test for immunogenic clones, (3) immunization with selected immunogenic clones in challenge-protection studies against the non-immunogenic or weakly immunogenic primary myeloid isolate, and (4) determining antigenic cross reactivity among different clones isolated from the primary isolate as well as from different cell lines established from the same or different isolates.

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Cancer Section

SECTION OVERVIEW - R. J. M. FRY

The Cancer Section is now small, but it gains strength and breadth from interactions and collaboration with members of other sections in the Division and with colleagues in other institutions.

Cancer involves change(s) at the genetic level that is currently characterized as an activation of oncogenes. But such change(s), that holds the attention of many scientists, is either ignored by the cell or the activity is suppressed in many tissues for many years. Presumably cancer cannot be induced without early changes at the gene level, but cellular, tissue, and systemic factors clearly play a role (and sometimes a predominant role) in whether or not the cancer becomes overt and important clinically. Thus, our studies range from those at the molecular level and the control of gene expression to those concerning cell interactions and the role of immune responses. Since the agents capable of inducing cancer are multiple, the approaches must encompass the specific characteristics of chemical carcinogens, ionizing radiation, ultraviolet radiation (UVR) and viruses.

The approach of our molecular biological studies is centered on the role of activation of transposable gene elements. Since transposition may play such an important part in the expression of these gene elements, it is clear that the mechanisms controlling the movement and, in particular, the insertion of such gene elements is a central question. Radiation is a very useful tool for investigating how known induced pertubations influence the movement and expression of these elements.

One set of our studies has resulted in the identification of a so-called retroposon that has been named OBY, for the investigators in the Division: C. Y. Ou, L. R. Boone and W. K. Yang. Other studies using restriction enzyme techniques have identified what appear to be retroviral genes that may correspond to prokaryotic transposons.

It is often stated that cancer has exerted little or no evolutionary pressure, in part, because cancer is, largely, a late life disease that occurs after the reproductive years. However, conservation in the genus Mus, of the loci involved in restriction of murine leukemia viruses, especially Fv-1 locus on chromosome 4, is perhaps an example of how viruses associated with cancers have influenced the evolution of the genome. The manner in which the gene products of the Fv-1 locus restrict integration of the retroviral genome into the cellular genome is an area of continuing research.

We are carrying out a concerted study of radiation-induced myelogenous leukemia. This type of leukemia has been a cornerstone of studies of radiation-induced cancer in man. Our studies of this leukemia at the gene level involve molecular cloning in order to isolate a germ-line associated murine leukemic virus copy. The relevant locus has been mapped and found to be on chromosome 5. The question is whether the additional copies, that appear to be associated with and specific for certain types of reticular tissue tumors but not with lymphomas, are causally related to the induction of the leukemias or are an incidental event. The fact that a specific translocation may be a factor of these leukemias raises the question of whether or not radiation induces leukemia by inducing the relevant and specific translocation. The task of answering this question is underway. There is a sex-dependent susceptibility for the induction of myeloid leukemia. The absence of a corresponding sex-dependent difference in the leukemia-associated chromosome aberrations will point to the importance of other host factors. Lastly, the induction by neutron irradiation is being studied at the whole animal level and these studies will provide dose-response relationships as well as data required for modeling neutron radiation responses.

The other radiation carcinogenesis studies fall into two major groups. First, there are investigations of various facets of the mechanisms of cancer induction. Much of the information that is derived is applicable to carcinogenesis in general. The molecular and chromosomal studies discussed above fall into this category. The second group of studies includes those that are concerned with risk estimates, either by providing data required for determining quantitative responses or testing models. For example, we want to determine if a relative risk model is more appropriate than the alternative absolute model and if induction of a cancer is an independent event.

One of the dogmas of carcinogenesis is that cancer induction is an all-or-none change and yet the degree of malignancy of some induced cancers appears to be dose dependent. Unless the lesions induced by neutrons are qualitatively different, the greater malignancy of the tumors induced by neutron radiation, compared to gamma radiation, must reflect a dose dependency or relative biological effectiveness. We are testing the validity of the presumption that induction is an all-or-none-event.

In order to dissect the many factors of carcinogenesis, two epithelial *in vivo*/*in vitro* model systems have been developed. These are the mammary and tracheal systems which make it possible to investigate cell-cell interactions, progressions and regressions.

The studies of the induction of cancer by UVR are concerned with mechanisms, especially the immune and hormonal responses factors that determine the expression or suppression of initiated and potential cancer cells. There is wide acceptance that the immune system plays an important role in skin cancer. However, most of the experimental evidence in support of this idea has been obtained from studies of sarcomas and not carcinomas.

Another group of studies is designed to determine interactions between exposure to ionizing radiation and UVR, and UVR and chemical agents.

Normal growth ends in differentiation, a process involving precise control of gene expression, whereas altered gene expression is the cardinal change in cancer. The studies in this field have concentrated on the enzyme tyrosine aminotransferase (TAT). A number of cloned cDNAs have been identified in the search for the TAT gene. One of particular interest, denoted gene 33, while clearly not the TAT gene has some common features in relation to the control of the associated mRNA, whereas the products of gene 33 are not. The changes with development and some of the responses to hormonal regulation are similar but do show an interesting difference in the response to glucocorticoids. The differences in hormonal regulation of the closely related "genes" provides a useful tool in probing the mechanisms as well as the underlying gene structure that determine hormonal regulation. The collaborative search for the chromosomal location of TAT gene and gene 33 not only illustrates the value of the interaction between research groups but should both answer and raise some questions about the relative location of genes with some common hormone regulation.

The development of monoclonal antibody techniques has provided a powerful method to those interested in detecting, following, and elucidating the changes in cell surface proteins that are characteristic of malignant or toxic changes in cells. The initial change in surface antigens in malignantly transformed cells allows not only their identification but also a surveillance of the changes that accompany tumor progression. The Molecular Immunology Group has used these techniques to study the differences between benign and malignant lung tumor cells of mice. For practical reasons, in vitro cell systems are more suitable for some of these studies while the rat trachea epithelial system is being used to detect initial, sequential, changes after exposure to chemical carcinogens and spontaneous and changes later in chemical carcinogen-induced tumor cells.

Theoretically, monoclonal antibodies provide an addition to the therapy armamentarium but as is indicated by the Molecular Immunology Group there are considerable problems in obtaining effective therapy.

A new activity of the Molecular Immunology Group has been the development of monoclonal antibodies to dioxins that could be used to assay the content and clearance of dioxins in humans.

The processing of chemical carcinogens shows some important tissue- and species-dependent differences. An understanding of mechanisms and the support of risk estimates requires a detailed knowledge of the metabolic pathways that carcinogens follow. Interferences with these pathways, and therefore prevention of the unwanted sequelae, can only be attacked logically if the mechanisms of metabolism are understood. Thus, studies of the metabolism of chemical carcinogens are required to elucidate mechanisms of chemical carcinogenesis, to establish risk estimates, and to develop methods of chemo-prevention.

Despite similarities in activation of electrophilic species, and the nature of the binding of the ultimate carcinogens, there are marked differences in the susceptibility to various chemical carcinogens at the cell, tissue, and species level. This is a time when considerable trust is placed in rapid tests, the results of which are equated with a presumption of carcinogenicity. The sense of such an approach must be substantiated, and if it is not, the approach should be changed. A good example of the potential pitfalls in the use of microbial mutations has been demonstrated by the results with 6-nitrobenzo(a)pyrene. This compound is a mutagen in the Ames test but is a very weak carcinogen. Three studies on the metabolic pathway of this compound noted in the following reports help to explain the difference and open up questions of the role of the dihydrodiols in mutagenesis and carcinogenesis. Another aspect of the comparative susceptibility or resistance to cancer induction relates to the question of whether all cells are at risk or are there subpopulations of cells that have different metabolic capabilities? Initial results with variants of a tumor cell line show that there are differences in the metabolism of benzo(a)pyrene between the variants. The heterogenicity of the cell population has been determined in a collaborative study with the Cytometric Group using B(a)P fluorescence as the determinant for cell sorting. If such differences held in normal cell populations it would indicate that subpopulations with certain characteristics in their metabolic activity would be at greater risk to initiation by certain chemical carcinogens. The answer to that question is currently being sought.

The Cytometric Group has continued to develop and become an essential part of a number of projects. One of these, the collaborative study with the Chemical Carcinogenesis Group on the sorting of cells based on their handling of B(a)P, has been discussed above. In another collaborative study with R. L. Tyndall (Environmental Sciences Division), a technique has been developed for detecting Legionella bacteria in water samples using flow cytometry. This development promises to provide a rapid method of monitoring the many potential resevoirs of the organism.

MOLECULAR GENETICS OF CARCINOGENESIS

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The overall objective of this research is to understand the genetic mechanisms of carcinogenesis at the molecular level. With a working hypothesis that gene rearrangement and altered phenotypic expression of neoplastic cells are associated with, if not initiated by, the activation

of specific transposable gene elements in cellular genome, we have focused our major research efforts on three specific projects: (1) isolation and characterization of potentially transposable gene elements from the mouse genome; (2) elucidation of some host genetic mechanisms for controlling the insertion events of transposal genes; (3) and determination of the effects of environmental carcinogenic insults on these specific gene elements.

Novel Features of Endogenous Murine Leukemia Virus-related Retroviral Genes. In the chromosomal DNA of the mouse there are none to three copies of genes responsible for the production of ecotropic murine leukemia virus, a few copies of genes responsible for the production of xenotropic murine leukemia viruses, and 30-50 copies of disperse genes which show a high degree of sequence homology with the murine leukemia virus genes. The later have not been known to express by themselves as a retrovirus. By employing the recombinant DNA approach, we have isolated various molecular clones containing the murine leukemia virus-related sequences from the mouse cell genomic DNA and disclosed a few characteristic features about these endogenous retroviral genes. First, the 30-50 individual copies of disperse genomic retroviral genes are distinct from ecotropic and xenotropic murine leukemia viruses in that most, if not all, contain a unique sequence of 170-200 base-pairs in the U3 region of their LTR's (2). Second, by using flanking cellular unique sequences as molecular probes for DNA gel blot analysis it was found that these MuLV-related genes are commonly present in some inbred mouse strains at the same genomic sites but absent in others, suggesting that the incorporation of these retroviral genes into the germ line of the mouse is a relatively recent evolutionary event (3). Third, nucleotide sequence analysis of the primer-binding site in 5 individual molecular clones revealed sequences complementary to the 3' end 18 nucleotides of glutamine tRNA (Nikbakht et al., manuscript in preparation). Thus, the endogenous proviruses, which apparently use glutamine tRNA as a primer, are different from murine ecotropic and xenotropic murine leukemia viruses which utilize proline tRNA as the primer for reverse transcription. Fourth, nucleotide sequence analysis of the complete gag gene region was performed with a particular clone, AL-10, derived from cellular DNA of the BALB/c strain mouse. The nucleotide sequences obtained indicate that it contains a functional open-reading frame, similar to those of ecotropic Moloney or AKR murine leukemia viruses, but with sequence variation being most marked in the p12 protein, next in p15 protein and p10 protein, and least marked in p30 protein. It was also found that the AL-10 provirus contains the distinct B-tropism determinant at amino acid residues 109 and 110 of gag p30 (3). Fifth, in nucleotide sequence analysis of cellular flanking sequences immediately upstream of AL-10 retroviral LTR, we noted the presence of a Alu-like B-2 repetitive sequence linking to a unique 63 base-pair segment which contains RNA polymerase III recognition signal (2). The 63 base-pair segment was recognized to represent a new rodent "retroposon" class and was named "OBY" (Roger, J. H., Internat. Rev. Cytol., Suppl. 17, 1984).

Studies on the MboI/LTR Sequences: Discovery of an Apparently Novel Class of Retroviral Gene Elements (Ou et al., two manuscripts in preparation). In the beginning of this research project, we already had experimental evidence indicating that an unique ~200 base-pair segment we

inserted in the LTR U3 region of endogenous MuLV-related proviruses of the mouse represents a class of interdispersed middle repetitive sequences. These sequences are called MboI/LTR repeats, on the basis that MboI restriction enzyme digestion generates discrete sizes of specific DNA fragments containing these repetitive sequences and that at least some of the repetitive sequences are also present in solitary LTR forms, similar to those described by Wirth et al. (Proc. Natl. Acad. Sci. USA 80: 3327-3330, 1983). There are 2000 to 5000 copies of these repetitive sequences per haploid genome of the mouse; whether or not all of these sequences are present in the LTR forms remains to be determined. Based on this consideration we screened an EcoRI restricted gene library of BALB/c strain mice and isolated 96 individual lambda phage recombinant clones which showed hybridization with the unique 170 base-pair segment of the LTR of pA1-10 MuLV-related DNA clone (pAL10-170). These individual recombinant DNA clones were analyzed by the dot-blot hybridization assay to search for the content of common sequences and the pAL10-170 homologous sequences. The idea is that the search for additional common sequences may lead to identification of clones that also contain retroviral structural genes. Positive results were obtained with this experimental approach, namely, we have found structural genes associated with the MboI-LTRs. A particular clone, IS23, was studied in detail by restriction enzyme mapping. It showed a 5.5 Kbp retroviral structure with two MboI-type LTRs on both ends and was flanked by 4 base-pair direct repeats. The internal putative structural sequences of IS23 showed very little cross-hybridization with MuLV structural gene sequences (gag, pol, and env) in gel blot analysis - like the dissimilarity between the MboI type LTR and the ecotropic MuLV LTR. Nucleotide sequencing analyses of the IS23 internal fragments are in progress, although the data obtained at this writing are limited to 2 stretches of 200-300 base-pairs. A computer homology analysis using known sequences of Moloney MuLV for comparison has revealed that one stretch is equivalent of a portion of p10 gag and the other to the 5' pol region. These results are significant in that, if solitary MboI type LTRs would be equivalent to prokaryotic "insertion sequence" (IS) elements, the apparently novel retroviral genes represented by IS23 clone might correspond to prokaryotic transposons.

Mechanism of Fv-1 Restriction and Other Host Gene Control of Endogenous Murine Leukemia Viruses. For the past decade, we have studied various aspects related to the restriction of murine leukemia virus by the Fv-1 locus that is present on the chromosome 4 of all laboratory strains and wild populations of the mouse. From our experimental results of DNA transfection experiments and retroviral DNA intermediate analyses (Yang et al., Prog. Nucleic Acid Res. Mol. Biol. 29: 1-4, 1983), it is clear that the Fv-1 gene products exert their effect on the reverse transcription process of retroviral genome, and thus incapacitate the ability of retroviral DNA intermediates to integrate into the cellular genome. This indicates that the retroviral gene integration process is not only essential for the replication of these viruses but also that it is important to the leukemogenesis process induced by these viruses. The target of Fv-1 restriction is apparently a virion component that plays

important roles in reverse transcription and gene integration, and that can interact with specific Fv-1 gene products and thereby determine the N- and B-host-range tropism of the virus. We have molecularly cloned the genome of N- and B-tropic virus into DNA clones (Boone et al., J. Virol. 45: 484-488, 1983; Boone et al., Prog. Nucleic Acid Res. Mol. Biol. 29: 205-214, 1983; Liou et al., J. Virol. 46: 288-292, 1983). Isogenic recombinants of N- and B-tropic viral genomes were constructed in vitro; those recombinant viruses were isolated by DNA transfection in mouse cells and examined for host-range properties. These studies demonstrated that genetic determinants of N- and B-tropism are allelically located within a DNA segment encompassing the coding regions of gag p30, gag p10, and about 100 base-pairs of the 5' end of pol gene (Boone et al., J. Virol. 48: 110-119, 1983). Nucleotide sequences of this DNA segment as well as the derived amino acid sequences were obtained for comparison between N- and B-tropic viral DNA clones. It was found that 109th and 110th amino acid residues of p30 are glutamine arginine in N-tropic viruses and threonine-glutamic acid in B-tropic viruses and that the 159th amino acid residue of p30 was glutamic acid in N-tropic viruses and glycine in B viruses (1). These results provide conclusive evidence supporting previous studies by others that virion core p30 protein is associated with N- and B-tropism of murine leukemia viruses.

The p30 polypeptides of N- and B-tropic viruses that were predicted from the nucleotide sequence reading frames were analyzed for the presence of possible secondary and tertiary structures. It appears that the p30 protein contains a hydrophilic histone-like C-terminus, a relatively hydrophobic N-terminus, and a mixed middle portion. Thus, it is possible to speculate that the C-terminal portion of p30 binds to a nucleic acid structure that is important for viral reverse transcription and integration, while N-terminal portion of p30 may be involved in protein-protein interaction. Perturbation of the middle portion of p30 by interaction with Fv-1 gene products might lead to disruption of nucleic acid binding activity of the p30 C-terminal peptide structure or the interference of protein interacting activity of the p30 N terminus.

Radiation-induced Leukemias of RFM/Un Mice. RFM/Un mice are used as an experimental model because radiation induces an increased incidence of myeloid leukemias in these mice similar to man (Tennant et al., Prog. Nucleic Acid Res. Mol. Biol. 29: 75-86, 1983). In a previous study, we found that a transplantable myeloid leukemia line, established by Upton and coworkers from irradiated RFM/Un mice, contained a few copies of ecotropic murine leukemia virus sequences in addition to the one associated with the germ line of this mouse strain. This suggested that the germ-line copy of ecotropic murine leukemia virus gene might have been activated and propagated either by insertion into additional DNA sites during the process of radiation-induced myeloid leukemogenesis or in the transplantation process. To investigate this phenomenon, we first isolated the germ-line associated murine leukemia virus copy by molecular cloning (Liou et al., J. Virol. 46: 288-292, 1983) and located it to the chromosome 5 of the mouse. A series of primary tumor tissues as well as normal tissues have been collected from irradiated RFM/Un mice. Many reticular cell sarcomas,

some myeloid leukemias, and some thymic lymphomas were collected and verified histologically. DNA gel blot analyses revealed that both the primary tumor tissues of myeloid leukemias and most of the reticulum cell sarcomas contain a single or a few additional copies of ecotropic murine leukemia virus gene, whereas no additional copy was found in any of the thymic or other lymphomas. A recombinant phage gene library was prepared from Hind III DNA fragments of a radiation-induced myeloid leukemic spleen. Two molecular clones containing somatically acquired ecotropic viral sequences were isolated for further characterization of the integration site in the leukemic cells.

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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, how they are regulated by hormones and other specific effectors, and how they are altered in the dysfunction of gene expression in cells transformed to malignancy. 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.

Preparation of Reagents: Molecular Cloning. We have now isolated and characterized six discrete cloned cDNAs cognate to mRNAs whose expression is sensitive to glucocorticoids in rat liver. The mRNAs range in size from 4.6 to 2.0 kb. One is diminished in amount after steroid treatment while the others exhibit the more common induction response. In earlier progress reports we provisionally identified one of the cloned cDNAs (in plasmid No. 33) as cognate to tyrosine aminotransferase (TAT), based upon a variety

of convincing but not unequivocal indicators. In March 1984, G. Schutz et al. (Heidelberg, FRG) published a report describing cloning and analysis of the TAT gene; subsequent experimentation by Hargrove (Emory University) has established this identification conclusively. Since the restriction map of $cDNA^{33}$ does not match that of the gene it became clear that $cDNA^{33}$ must be cognate to another gene (henceforth, "gene 33"). The mRNA product of this gene is 3.4 kb and undergoes turnover in the liver at the same rate as $mRNA^{TAT}$ ($t1/2$ = 1 h). As yet we know little about the protein product save that it migrates as a 50 kDa band (like TAT) in denaturing gels when translation products are analyzed after hybridization-selection of the mRNA. Synthesis of $mRNA^{33}$ in the liver is hormonally and developmentally regulated much like TAT. These many similarities raised the possibility that the two genes are related in some fashion. However, after arranging a trade with Dr. Schutz of our respective cDNAs we have established that there is no cross reaction at either DNA or RNA levels. We are currently making a determined effort to characterize more fully the protein product of gene 33 in both structural and functional terms.

From a library of cloned fragments of rat genomic DNA (courtesy of J. Bonner, Cal Tech) we have isolated a 15.5 kbp fragment of rat DNA of which 14.9 kbp can now be definitively said to be from gene 33. This has been extensively mapped by restriction cutting and a number of fragments have been subcloned. Northern blotting demonstrated that the 5'-terminus of the gene is not present, demanding further screening of the library to find a contiguous fragment containing the 5'-end and flanking sequences. This has now been accomplished and characterization is underway by restriction mapping. Preliminary results of heteroduplex analyses in the EM confirm the expected high degree of complexity of this gene, containing many introns.

Several of our cDNA probes cognate to hormonally and developmentally regulated genes have now been cloned into the SP6 "riboprobe" vector. This construct permits transcriptional production of highly labeled cRNAs in large (μg) quantities that enhance the sensitivity of hybridization measurements by tenfold or more. Reversing the polarity of the cDNA insertion permits the production of the specific mRNA that can be used in a variety of experiments.

Hormonal Regulation. Earlier results had established that the mRNAs of both TAT and gene 33 are rapidly increased in adult liver following treatment with hydrocortisone, insulin or glucagon, the latter acting via cAMP. While each of these hormones is known to induce synthesis of several liver proteins, a similar metabolic response to the usually antagonistic hormones insulin and glucagon (cAMP) is most unusual and was hitherto thought to be unique to synthesis of TAT. Increased mRNA concentrations could be due to enhanced gene transcription or to changes in rates of processing or degradation of initial gene transcripts. To resolve this issue we analyzed rates of transcription of gene 33 in isolated nuclei taken after treatment with one or another of the inducing hormones. We found that each of them act by increasing the rate of transcription of the gene. We know from a variety of analyses that each hormone must accomplish

this by a discrete mechanism. Glucocorticoids, complexed to a receptor protein, generally interact with specific DNA sequences upstream from the transcriptional start site and thereby enhance promoter function. Virtually no information is as yet available regarding comparable enhancer sequences active in insulin- or cAMP-mediated inductions, or of the nature of cellular molecules interacting with them to effect enhanced transcription. As our analyses of gene structure proceed it will become possible to probe these phenomena in detail in this unusual system exhibiting multiple hormonal control of expression of a single gene.

Expression of both TAT and gene 33 is largely, but not entirely, silenced in the fetal liver throughout gestation. We asked whether these essentially inactive genes are responsive to hormonal stimulation in fetal liver, both for inherent interest and as one means of exploring the question of the potential role of hormonal regulators in differentation-associated activation of gene expression. Treatment of fetuses in utero with cAMP caused a highly significant increase in hybridizable transcripts of both genes. The induced $mRNA^{TAT}$ was active in translation assays and the enzyme was synthesized in commensurate amounts. Thus, the enhancer elements of both genes that are activated by cAMP are present and functional, despite the low level of gene activity. The response of fetal liver to glucocorticoids, however, proved to be gene-specific. Transcripts of gene 33 were increased in amount to an extent comparable to the adult response. Sucrose gradient fractionation of postmitochondrial supernatants, followed by analysis by cytoplasmic dot hybridizations, revealed that the induced $mRNA^{33}$ is present in the polysomes and is presumably being translated. However, TAT is not responsive to glucocorticoid treatment under these circumstances: $mRNA^{TAT}$ did not increase at all in some experiments and only to a barely detectable extent in others. Since the receptor component of the steroid induction is clearly operative, this result focuses attention on gene structure per se, suggesting that the glucocorticoid-responsive sequence of TAT has not yet "matured" while that of gene 33 has undergone the required change, perhaps one involving demethylation. These studies are currently being extended to include a comparative study of the response to insulin of these two genes and also the other glucocorticoid-responsive mRNAs for which we have cloned cDNAs.

Differentiation. Our explorations of the mechanisms underlying the programmed series of activation of gene expression that together culminate in the differentiated hepatocyte phenotype have continued to focus on DNA methylation/demethylation and on a comparative analysis of TAT and gene 33. In normal development these genes are programmed somewhat differently, expression of gene 33 being activated just before and at birth (i.e., biphasic), while full TAT expression (and responsiveness to steroid-mediated induction) is attained in the first 12 h after birth. In our most recent report we described the results of experimentation in which we treated fetuses 2 days prior to expected delivery with the hypomethylating agent, 5-azacytidine (5-azaC). We found truly dramatic changes in both hepatocyte morphology and biochemical properties; the latter include full activation of expression of both TAT and of gene 33. Interestingly, as in normal development, the drug-induced activation of expression of gene 33

precedes that of TAT by a few hours. Taken together the data indicate that 5-azaC accelerates hepatocyte maturation by several days; the changes observed are those normally taking place at birth or shortly thereafter.

With the aid of colleague L. Shugart, we have now measured the content of 5-methyldeoxycytidine ($d^{m5}C$) in the DNA of fetal livers in the perinatal period. The $d^{m5}C$ content in the DNA of fetal livers (0.76 ± 0.02 mole %) is appreciably below that of the adult (0.91 ± 0.04%) but is further reduced to 0.55 ± 0.05 after 5-azaC treatment, a reduction of about 40% in only 18 h. More surprising are the changes observed in normal development: at birth the content is below the fetal level (0.67 ± 0.01%), but rises to nearly the adult level by 24 h after delivery (0.84 ± 0.01%). These results clearly show that developmental activations of gene expression during the perinatal period are associated with large and rapid changes in the extent of DNA methylation. Experimental demethylation of the DNA in the fetal liver by 5-azaC appears to anticipate and augment these events well before their programmed occurrence.

The mechanism by which 5-azaC effects hypomethylation of DNA is thought to be due to inhibition of a DNA methylase and consequent inability of newly-replicated DNA to be methylated. Hepatocyte replication in fetal and perinatal liver is slow; the rapid and extensive shifts in content of $d^{m5}C$ that we observe cannot be reconciled with a mechanism requiring DNA replication. Instead, our data are consistent with a dynamic process involving both methylation and demethylation of existing DNA.

The demethylations associated with developmental activation of gene expression presumably are at key control regions of affected genes. We have initiated a study of the methylated sites in and near the DNAs of gene 33 and of TAT, by cutting genomic DNA with methylation-sensitive restriction enzymes followed by Southern blotting and probing with radioactive cDNAs or cloned fragments of genomic DNA. Preliminary results, limited to probing with $cDNA^{33}$ (3' terminal exon of the gene), are indicative of a site in the 3' region of the gene that is methylated in the DNA of fetal liver but not in that from adults.

Tissue Specific Expression. Studies of this aspect of differentiation have revealed significant differences in the regulation of expression of gene 33 and of TAT. The latter is truly liver-specific, as neither the protein gene product (assayed by an immunoblot procedure) nor the RNA gene products (dot or Northern blot hybridizations) are detected in any tissue examined except liver, even after treatment with hydrocortisone. But gene 33 is expressed in a number of tissues: the 3.4 kb mRNA is present in lung, kidney, brain and testis as well as in liver, but was not detected in heart. The extent of expression in nonhepatic tissues is low relative to that in the liver. Hydrocortisone treatment effects a very large induction of the mRNA in kidney, much larger than the response in liver. These analyses are being extended to include the other expressing organs and to determine their responses to insulin and cAMP as well as to the steroid hormone. The capacity of gene 33, but not TAT, to respond to the steroid inducer in the kidney is reminiscent of the similar situation in fetal

liver, perhaps indicating that the same structural features of these genes are involved.

Chromosomal Mapping. We have initiated experiments to map the chromosomal location of gene 33 and of the TAT gene in both mouse and human genomes. The human studies, done in collaboration with R. J. Preston (Biology Division), involve "in situ hybridization" of metaphase spreads from lymphocyte donors and analysis with very highly ^{3}H-labeled gene probes. Current results indicate that the cRNAs produced in the "riboprobe" system may be ideal for this purpose. Mapping in the mouse genome is being done with a battery of mouse:hamster hybrid cells provided by former colleague P. A. Lalley (Institute for Medical Research, Bennington, Vermont) and carrying a variable, but known, complement of specific mouse chromosomes. Our rat DNA probes react with both mouse and hamster DNAs from parental cell lines. After cutting with any of several restriction enzymes the patterns observed are typical of single copy genes and the species of origin can be readily distinguished. It will be of interest to determine if these two genes, regulated identically in some respects but not in others, should map in proximity to one another or to other genes responsive to regulation by hormones.

TUMOR CELL SURFACE PROTEINS

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Tumor Antigen Characterization. Cell surface proteins mediate interaction between cells and their environment. Unique tumor cell surface proteins are being identified and quantified in several tumor systems to address the following questions: (1) How do tumor-specific proteins arise during cell transformation? (2) Can these proteins be used as markers of tumor cell distribution in vivo? (3) Can solid state radioimmunoassay of these proteins provide a means to quantify transformation frequencies?

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

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. One MoAb is bound to Sepharose beads and used to concentrate TSP-180 from a tumor or tissue homogenate while the other MoAb, radiolabelled with ^{125}I, is used to detect the amount bound. This assay allows quantitation of low levels 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.

Tumor Therapy with MoAb. Syngeneic monoclonal antibodies (MoAb) to Moloney sarcoma cell (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.

Monoclonal Antibody for Lung Toxicology. Analysis of toxic injury to lungs can be obtained chiefly through invasive procedures, such as biopsy

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

Study of Antigenic Markers in Development Neoplasia. Most malignant tumors in man arise from epithelia via a multistep process in which new cell populations with altered biological and morphological properties appear. The purpose of this research project is to define and quantify cellular markers that can identify chemically altered cell populations appearing in transformation of rat trachea epithelial cells, and to delineate their role in the development of neoplastic cell populations. Our approach has been to define early phenotypic changes in short-term cultures of rat tracheal epithelial cells exposed to chemical carcinogens. Altered phenotypes have been identified on the basis of an increase in nuclear DNA content as compared to non-altered cells and expression of antigen that is not detected on normal cells. These changes appear early, occurring in precursor cells that eventually give rise to neoplastic populations. The changes also correlate with the initial breakdown of those mechanisms which regulate normal growth and differentiation. Cloning experiments have been done using one tracheal cell line which changes from preneoplastic to neoplastic growth during cell culture in vitro. Analyses of the phenotypes of cloned progeny indicate that most or all cells in the preneoplastic phase of growth are preprogrammed to switch to neoplastic cells. The time of the change and the phenotype of the resulting neoplastic cells are nearly identical, indicating that information stored in the preneoplastic phase cells becomes activated at later times.

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

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

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CYTOMETRICS

R. C. Mann R. E. Hand, Jr.

The Cytometry Group concentrates its activities 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 very rapidly measure a set of features, e.g., DNA content, size and amount of antibody bound, extremely large numbers of cells, or other biological particles. 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 in a suspension through a highly focused laser beam. Particles with certain features of interest can be sorted from the original population for further analyses. FCM has become a powerful technique in basic

and applied biomedical research and is being used in many programs in the Biology Division. Consequently, we have been collaborating with several investigators. Out of these collaborative efforts we have been developing new FCM assays and methods of data analysis.

Together with the Legionella Testing Group at ORNL we have demonstrated the applicability of FCM to the detection of Legionella bacteria in environmental water samples. Legionella are a natural component of the aquatic microflora. Only when water becomes aerosolized and Legionella are inhaled do they represent a potentially fatal hazard to man. Among the main sources of Legionella in air are cooling towers at locations of energy production (power plants) and energy consumption facilities, e.g. large air-conditioning equipment installations. It is therefore important to have a rapid method for monitoring cooling tower water for the presence of infectious Legionella. Analyses currently in use involve concentrating the microbial populations and testing the concentrates for the presence of various Legionella species using specific fluorescein-bound antisera and fluorescent microscopy. Infectivity testing requires injection of samples into guinea pigs and eventually sacrificing the animals after 2 weeks.

We have shown that a two-color fluorescence FCM assay measuring the intensity of green light emitted by FITC-conjugated antiserum bound to, and the DNA content of Legionella as determined by the intensity of red light emitted by propidium iodide bound to DNA, is adequate to detect Legionella in water samples from cooling towers. Moreover, we have consistently observed differences in the data patterns from Legionella samples of low and high virulence. Further research to substantiate these preliminary observations is under way.

In collaboration with the Chemical Carcinogenesis Group we have been using the cell sorter to separate live cells from populations based on their ability to clear polycyclic hydrocarbons, e.g. benzo(a)pyrene, from the cytoplasm after different terms of incubation. Cell separation is accomplished using the 353 nm and 360 nm lines of an argon ion laser to excite the hydrocarbons directly. The intensity of the fluorescent light emitted by the cells indicates to what extent the benzo(a)pyrene has been metabolized. The sorted cells are being propagated to compare their transforming ability and their complete metabolic profiles.

FCM has been applied to *in vivo* studies of the toxicity and mutagenicity of ethylene oxide in mice. In these studies the effects of different schedules of exposure on different cell populations in the bone marrow of mice was assayed using FCM. Special FCM measurements of the intensity of light scattered by the cells allows one to distinguish between uniformly circular-shaped cells with little internal structure (i.e. uniform refractive index), such as lymphocytes, and cells with more complex internal structure, such as granulocytes. In addition, specific cell surface markers (fluorescein-labeled antibodies) and DNA content were measured. Using combinations of these parameters we were able to demonstrate considerable persistent perturbations in bone marrow cell subpopulations of mice during exposure to ethylene oxide. The analysis of

data from this study made it necessary to devise a method for data dimensionality reduction for the purpose of visualizing multi-parameter FCM data in three dimensions. This assay is now being optimized for application to rat bone marrow cell samples and further use in toxicity and mutagenicity testing of chemical substances.

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METABOLIC ACTIVATION AND CARCINOGEN METABOLISM

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The burgeoning data base for metabolism of polycyclic aromatic carcinogens continues to exhibit a marked similarity in the profile of metabolites formed by all eukaryotic species. The identity of the overall chemical processing pathway for carcinogens is also seen in macromolecular binding studies that show the activated electrophilic species of the carcinogen binding to the same nitrogen bases in DNA and in relatively the same proportions. Paradoxically, there is a broad spectrum of relative susceptibility to these carcinogens, suggesting there are relative perturbations in the metabolic pathway between susceptible and resistant cells, tissues, and species. It is therefore necessary to probe more deeply into the cellular processing of the chemical carcinogen as it passes through its activation and detoxification schemes in order to focus on those steps that are most critical to determining whether that cell will undergo malignant transformation, mutation, or continue in its normal phenotypic state. The metabolic products of benzo(a)pyrene are well documented in numerous biological systems, and it is necessary to extend these studies to B(a)P congeners to understand which regions of the molecule are metabolically active and therefore potentially involved in the transformation process.

We have been studying 6-nitrobenzo(a)pyrene, which is present as a pollutant in a variety of sources, including automobile exhaust, urban air, cigarette smoke, and shale oil fractions. This substance can be formed from B(a)P in the air by trace amounts of nitric acid and nitrogen dioxide. It has been shown to be a direct acting mutagen in the Salmonella typhimurium assay and has the ability to be further activated by tissue enzymes into an even more potent mutagen. While the major form of activation in bacteria is reduction of the nitro group to an amine and on to an hydroxylamine, it was important to know if this molecule was attacked by mammalian drug metabolizing system in a similar fashion, or whether it was ring-hydroxylated as is the case with nonsubstituted polyaromatic hydrocarbons. If mammalian systems did not activate through the nitro position, it would suggest that 6-nitrobenzo(a)pyrene would probably be less hazardous to mammals than the bacterial assays would indicate. We incubated radio-labeled 6-nitrobenzo(a)pyrene with hamster embryonic fibroblasts as a model for human cell metabolism and isolated the metabolic products for analysis by high-pressure liquid chromatography. Unlike the large number of major metabolites seen with benzo(a)pyrene, two products were observed, one chromatographing in the dihydrodiol region and a second in the phenol region. Inhibition of epoxide hydrase confirmed the presence of a dihydrodiol and UV analysis confirmed the other major peak to be phenolic in character. Most of the phenolic products are conjugated to inactive forms in intact cells. Therefore the positive mutagenic action exhibited by these metabolites and bacterial systems may not be as important in an in vivo situation. However, macromolecular binding studies showed that 6-nitro-benzo(a)pyrene bound to DNA and RNA with similar specific activities as benzo(a)pyrene. This is interesting in light of the skin-painting studies that show 6-nitro-B(a)P to be far less tumorigenic on mouse skin than benzo(a)pyrene. While the exact chemical structure of the dihydrodiol of 6-nitro-B(a)P is presently unknown, it is important to know if the presence of a nitro group at the 6 position of the benzo(a)pyrene molecule would shift metabolism toward the carcinogenically important bay region or away to the relatively inactive K region on the benzo(a)pyrene molecule. This knowledge would help us to understand the effect of the relative position of the dihydrodiol with regard to mutagenesis and carcinogenesis in susceptible species.

Separation of Water-soluble Metabolites of Benzo(a)pyrene by High-pressure Liquid Chromatography. While in vitro metabolism studies in cell-free systems are excellent models for determining activation pathways, in vivo metabolism of carcinogens ultimately forms a water-soluble conjugated product that is excreted as a nontoxic waste derivative. This conjugation process is accomplished through cytoplasmic and microsomal transferases that bind chemically reactive sites on the molecule to endogenous substrates. Therefore it is probable that the relative activity of the conjugation within the process helps determine susceptibility and resistance of a given species, and the rate of conjugation will help dictate the half-life of the reactive molecular species. We have begun studies to extract these conjugated moieties from cells and develop HPLC methodologies for accurately isolating and characterizing these products. The major conjugation forms in most eukaryotic species are glucuronides,

sulfates, and glutathiones, all of which have markedly different structures and functional groups. This results in dissimilar polarities and solubilities since, at physiological pHs, glucuronides, sulfates, and glutathione conjugates exist as anions. We have been successful in separating glutathiones from glucuronides using a water/methanol gradient with acid. Varying the concentration of acetic acid in pH 2.75-3.05 or trifluoroacetic acid pH 2.10-2.65 has resulted in base-line separation of glucuronides from glutathiones. Although sulfates have a broad band, and coelute with the glucuronide, this does not present a problem biologically since benzo(a)pyrene sulfates are extractable in organic solvent and would not be present in the water-soluble metabolite fraction. Our early results have shown the possibility of a family of glutathione conjugates that may represent a vastly more complex metabolic pathway regarding conjugation of reactive metabolites than previously envisioned. The relative selectivity of the various oxygenated benzo(a)pyrene intermediates may be an important factor determining how much of the reactive electrophile will be available for interaction with critical target sites for malignant transformation.

Metabolism of Benzo(a)pyrene in Variant Mouse Hepatoma Cells. One of the critical questions to be answered before fully understanding susceptibility and resistance to malignant transformation from chemical carcinogens is whether tumor origin involves cell subpopulations and is truly clonal in origin or whether all cells in a tissue are equally at risk. It is important to determine if there are subpopulations in mass cultures of cells that are predisposed to malignant transformation. We have studied three cell variants from the mouse tumor line Hepa1c1c7. These were separated in a fluorescence-activated cell sorter, based on the amount of benzo(a)pyrene fluorescence within the cell after short-pulse incubations. These cells are defined as variants, not mutants, since they are all taken from a parent clone and, by current definition, remixing would reconstitute the intact parent cell line. We were anxious to know whether these subpopulations of cells had identical metabolic capability for metabolism of benzo(a)pyrene. Our results showed that subpopulations of cells separated from the parent HEPA clone had markedly different metabolic ability. One variant (MuL12) had similar or slightly better metabolism than the parent HEPA clone, but a slower doubling time and a much higher modal chromosome number (100 for MuL12 versus 58 for Hepa1c1c7). Another variant, BP^{rc1}, had a similar doubling time and modal chromosome number, but showed almost total inability to metabolize benzo(a)pyrene. These results have shown the possibility that in a so-called homogeneous cell population there can be a number of subpopulations of cells with vastly different metabolic competencies — competencies that would be masked when studying metabolism in mass culture. It also raises the possibility that there can be cells within the parent population that may have greater susceptibility to malignant transformation.

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RADIATION CARCINOGENESIS

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

The program in ultraviolet radiation carcinogenesis (UVR) is concerned with the following studies: (1) development of model systems, methods and background information that is necessary for designing quantitative UVR carciogenesis experiments; (2) the role of interactions of UVR and chemicals; and (3) interactions between ionizing and ultraviolet radiation in skin carcinogenesis.

Neutron Carcinogenesis. An unresolved question that is of considerable interest is the shape of the dose response for tumor induction after low dose neutron exposures. Two alternative models have been suggested, but present 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 recently begun using fractionated exposures which should 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 development of myeloid leukemia in RFM mice following neutron irradiation is also of current interest. Coupled with a study of the neutron dose response, the possible role of specific chromosome aberrations in myeloid leukemogenesis after neutron exposures is being examined in collaboration with R. J. Preston (Biology Division). In another collaborative study, W. K. Yang of this Division is examining the possible role of transposition of genetic elements in the development of myelogenous leukemia.

Previous observations in this laboratory have shown that protraction of neutron exposures enhances the mammary tumorigenic effect of neutrons. This effect has been postulated to be a result of either enhanced transformation rates or a result of influences on tumor expression similar to tumor promotion. We have begun experiments which will examine the sequential 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 will allow the separation of initial transformational effects of neutron irradiation from those effects which influence tumor expression. These studies have only recently become possible with the development of new model systems for the study of mammary tumor development. Results thus far suggest that low dose-rate neutron exposures enhance the progression of initiated cells.

Another question that has arisen from previous carcinogenesis studies is whether there are qualitative differences, i.e., the tumorigenic effects of neutrons compared to gamma rays. Specifically, it has been suggested that neutron irradiation produces more malignant tumors than does gamma irradiation. This question is being addressed using the 10 T1/2 transformation system. We are currently carrying out experiments designed to

determine whether type III transformants induced in 10 T1/2 cell by different qualities of radiation (neutrons, X-rays) and chemicals are systematically different from each other in terms of "malignancy." Malignancy here is defined in terms of the number of cells required to yield tumors in 50% of the sites inoculated. To date we have several clones from each exposure group and are in the process of doing tumor-cell-dose studies.

Mammary Carcinogenesis: Development of an Experimental Model. Over the last few years we have been attempting to develop a model to study mammary carcinogenesis which allows the study of the sequence of events which leads to mammary tumor development after exposure to radiation and chemical carcinogens. This includes the identification and characterization of altered cell populations which emerge, and the identification of factors which influence their progression to neoplasia. The approach taken utilizes the mammary cell dissociation (CD) system originally developed by DeOme and his coworkers (Cancer Res. 38: 2103-2111, 1978) and the epithelial focus assay system developed by Terzaghi and Nettesheim (Cancer Res. 39: 4003-4010, 1979).

Based on studies thus far, the expression and persistence of the ductal dysplasias observed in the cell dissociation derived mammary outgrowths appears to be a complex process which is influenced by the developmental state of the outgrowth as well as by the time after carcinogen treatment at which the cells were assayed. When mammary cells were transplanted 24 h after carcinogen treatment, the outgrowths exhibited ductal dysplasias when the tissues were actually growing but not when the ducts had reached the edges of the fat pads and stopped growing. Thus, it appeared that these lesions regressed or remodeled when the growth stimulus was absent. Although some cells had acquired an alteration in their growth potential, they still responded to the regulatory signals that halt ductal growth. This interpretation is supported by the observation that when apparently normal 16-week outgrowths were redissociated and transplanted into gland-free fat pads, the resultant second generation outgrowths again expressed ductal lesions when actively growing (8 weeks) but not when growth had ceased (16 weeks).

The acquisition of the ability of the ductal dysplasias to persist in full outgrowths, i.e., to gain some autonomy from tissue regulatory mechanisms, appeared to be a separate event that occurred with increased frequencies at longer times after carcinogen treatment. This suggestion comes from the finding that when longer times were allowed to elapse between exposure and transplantation of the cells, the lesion frequencies increased and a substantial fraction of the lesions did persist within the full outgrowths.

The epithelial focus (EF) assay was used to isolate and quantitate *in vitro* mammary epithelial cells altered by exposure *in vivo* to DMBA and/or gamma radiation. The EF assay was performed as originally described with only one major modification necessary to accomodate the *in vitro* growth characteristics of mammary cells. Based upon experience with an EF

assay for rat tracheal epithelium, we anticipated being able to quantitate foci in vitro 4 weeks after dissociation and plating. We found, however, that most mammary cells have a considerably longer lag time until onset of in vitro proliferation as well as a slower doubling time than do rat tracheal cells. Only after 6-8 weeks when nonproliferative single cells finally underwent metabolic death and peeled off the dish did altered cells begin to proliferate and form distinct epithelial foci. The average time in vitro for development of a 1-2 mm focus was 10-12 weeks. An even longer timespan for development of in vitro growth of normal mammary cells from mid-pregnant BALB/c mice (6 months) has been reported by Danielson et al. (Proc. Natl. Acad. Sci. USA 81: 3756-3760, 1984). In our assay, each focus contained 10-20,000 cells and many of the foci spontaneously formed "domes" characteristic of epithelia capable of forming tight (occluding) junctions. The percentage of dishes with an epithelial focus for all treatment groups was highest when cells were dissociated and plated after 4 weeks in situ following treatment and declined at later times.

Each primary epithelial focus reaching at least 1 cm in diameter by 12-16 weeks in vitro was removed from its dish and the percentage of epithelial foci which could be subcultured at least three times (EFs) was determind. Cells derived from EF of mice exposed to DMBA exhibited a higher frequency and earlier appearance of EFs than did those derived from EF from mice exposed to radiation. Interestingly, however, EFs frequency from all treatment groups did not peak at 4 weeks in situ following treatment, but continued to increase with time.

The EF assay has shown that mammary epithelial cells exposed in vivo to low doses of gamma radiation and/or DMBA exhibit altered in vitro growth potential as early as 24 h following treatment. The data suggest that the epithelial focus assay can detect both time and dose dependent differences in altered growth potential in a quantitative manner. Moreover the evidence that EFs increases with time even though the absolute number of altered cells apparently decreases, suggests that this assay may also be used to detect qualitative differences (e.g. subculturability) in carcinogen altered mammary epithelial cells.

Time-dose Relationships. Because of the many factors involved in tumorigenesis, 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. To develop the needed information we have begun a series of studies designed to examine time-dose relationships for radiation carcinogenesis. 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. For the second and third objectives, we are conducting experiments to examine early, radiation-induced alterations in growth potential (transformation) of mammary epithelial cells and their progression to neoplasia using the methods discussed above.

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 are currently evaluating (1) the influence of intracellular communications (requiring direct cell contact) or intercellular interactions (e.g., diffusible factors) between normal and carcinogen-altered cells 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 tracheal epithelial cells 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. The extent of inhibition on growth of altered cells is dependent on both the proportion of normal cells and the target cell population. The greater the proportion of normal cells the greater the inhibition. Neoplastic cells appear to be inhibited less than preneoplastic cells. These effects are observed in vivo and

in vitro. The effect does not appear to be mediated through a factor released into the medium since medium conditioned by normal cells does not markedly inhibit growth of carcinogen-altered cell populations.

Ultraviolet Radiation Carcinogenesis. The studies in this program are designed to investigate mechanisms of ultraviolet radiation (UVR) carcinogenesis and the interactions of UVR with chemical agents, X rays and psoralen plus UVA (PUVA) and to develop animal model systems suitable for the study of mechanisms.

We have continued our comparative study of UVR-induced tumorigenesis in three stocks of hairless mice: SKH:hr-1, HRS/J/Anl and SKH-SENCAR. It is now clear that susceptibility to the induction of sarcomas does not go hand in hand with susceptibility to the induction of squamous cell carcinomas. In fact, there appear to be separate genetic factors that determine susceptibility to papillomas, carcinomas and sarcomas. SKH-SENCAR are markedly more susceptible to papilloma induction but less susceptible to sarcomagenesis than HRS/J/Anl.

It has been suggested that UVR-induced skin carcinogenesis involves not only the initiation of epidermal cells but a suppression of the immune mechanisms that are thought to be an important aspect of the defense against tumor growth (Kripke, Adv. Cancer Res. 34: 69-106, 1981). Much of the evidence that has been used in support of this hypothesis comes from work with sarcomas. Kripke noted that UVR-induced sarcomas could be transplanted to syngeneic mice that had been UV irradiated but not to unirradiated mice.

In order to investigate the effects of UVR on sarcomagenesis itself and not rejection of transplanted sarcoma cells, we chose to compare plastic disc-induced sarcomagenesis in HRS/J/Anl mice with and without UVR. UVR at dose levels that induced epidermal carcinomas was started after the insertion of the discs. In the group of the mice with discs alone, 83% had sarcomas by 85 weeks. In mice with discs and 1250 J/m^2 3/week, 56 exposures, the incidence of sarcomas was 100% in 74 weeks. At 74 weeks the incidence of sarcomas in mice without UVR was 64%.

These results suggest that UVR-induced suppression of the immune system may have influenced the time of appearance of the sarcomas. These results are of interest first, because it is a demonstration of an autochthonous tumor, and second, it has been suggested that plastic disc tumors are not as highly antigenic as chemical carcinogen - or virus-induced sarcomas. Therefore we may surmise that the degree of UVR-induced immunosuppression in our system may not have been as great as with highly antigenic sarcomas. In the case of epidermal tumors, De Gruijl and van der Leun (Photochem. Photobiol. 35: 379-383, 1982) have found that pre-irradiation with UVR has a systemic effect that influences subsequent UVR tumorigenesis. We decided to test whether UVR induced a systemic effect sufficient to reveal the carcinogenic potential of 12-O-tetradecanoylphorbol-13-acetate (TPA) which has been shown to be a skin carcinogen in some strains of mice (Iversen and Iversen, Virchows Arch. B

Cell Path. 30: 33-42, 1979) but not in any of our stocks of mice. We chose to determine whether UVR would increase the susceptibility of SKH-SENCAR mice to the induction of tumors by TPA. The dorsal skin was exposed to 500 J/m^2 UVR, 36 exposures, and the ventral skin to multiple treatment of TPA. No tumors occurred with the TPA treatments. It appears that any potential carcinogenicity of TPA cannot be revealed by whatever changes in immune response the UVR induced. Other experiments to probe the significance of UVR-induced systemic effects are under way.

It is probable that UVR-induced effects on the immune system are mediated by the Langerhans cell the most peripheral cell of the complex that constitutes the afferent limb of some immune responses. We are comparing the effects of different UVR spectrums and soft X rays on the Langerhans cell populations.

Although it is clear that the strain-dependent differences in susceptibility to UVR-induction of skin cancer involve differences in expression, we have not been able to indict the immune system as the cause of the differences. Thus, it seems reasonable to examine other systems, for example, the steroid hormones, that play a role in skin carcinogenesis. Glucocorticoids and antiandrogens inhibit DNA synthesis and cell proliferation in the epidermis and the promotion stage of two-stage skin carcinogenesis. Androgens counteract the inhibitory activity of antiandrogens.

Preliminary studies have been carried out to study the influence of androgens and antiandrogens on skin carcinogenesis induced by UVR. These preliminary experiments involved an analysis of changes in epidermal DNA synthesis in HRS hairless mice exposed to UVR and the androgens, testosterone propionate (TP) and 5-dihydrotestosterone (DHT) and the antiandrogen, cyproterone acetate (CPA). The mice were exposed to 1250 J/m^2 with or without steroid. It was expected that UVR and TP or DHT would increase DNA synthetic activity in the epidermis. However, the increases found were small and not significant. It is of interest that the treatment of the HRS/J/Anl mice with antiandrogen had an opposite effect of that found in SENCAR mice.

We intend to investigate what strain-dependent differences exist. For example, are there differences in glucocorticoid and androgen receptor content and in sensitivity to glucocorticoid, androgen, and antiandrogens? The growth curves for the three stocks of mice under study are quite different and it may be that these curves reflect a number of hormonal differences, one or more of which plays a role in the expression and growth of skin tumors.

Two areas of co-carcinogenesis are under study. First, we are investigating the initiation of potential cancer cells in the epidermis by X-irradiation and whether expression of such initiated cells is influenced by TPA, UVR and psoralen-UVA (PUVA) treatments. Regimens of X-irradiation that alone produce no carcinomas do so when multiple TPA, UVR or PUVA treatments are given after the end of the X-ray regimen. Questions of

concern are whether or not there are common features in the effects of these treatments and whether or not they involve the immune system.

In another series of experiments the co-carcinogenic effect of UVR and exposure to shale oil has, so far, proven to be less than additive.

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7. Fry, R. J. M. and R. L. Ullrich. Combined effects of radiation and other agents. In: Radiation Carcinogenesis, ed. by A. C. Upton. Elsevier/North Holland Biomedical Press, Amsterdam, in press.
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Educational Activities

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences

W. E. BARNETT - DIRECTOR

The University of Tennesee-Oak Ridge Graduate School of Biomedical Sciences is located within the Biology Division of Oak Ridge National Laboratory. The program is primarily designed for training leading to the Ph.D. degree, although there are a few Master's degree candidates. Ph.D. students are supported by the University of Tennessee in the form of research assistantships or by federal training grants awarded to the School by the National Cancer Institute and by the National Institutes of Health. The School currently has 39 students working toward the Ph.D. degree and 3 in the Master's program. As of October 1984, 97 students have been awarded the Ph.D. degree.

The fall quarter of 1984 marks the beginning of the School's nineteenth 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.

Postdoctoral Training Programs

Postdoctoral training is an important feature of Division activities, providing benefits both to the trainees and to the Division. Support for these training activities is derived from a variety of sources and is administered by the University of Tennessee. Two major 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). As of September 30, 1984, there were 11 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.

Undergraduate Training Programs

The Biology Division participates in three undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Southern Colleges University Union Science Semester (SCUU), and (iii) Oak Ridge Associated Universities Summer Student Trainee (ORAU). Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors are offered opportunities for independent research in the life sciences. In the past 12 months, there were 16 students, possessing the educational qualifications and the potential for a successful scientific career, who spent 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) performing research under the guidance of Biology Division staff members.

Although the principal purpose of the programs is to provide a training experience for the students, it often allows division staff members an opportunity to broaden their areas of research. Upon completion of their research activities in the laboratory, students prepare a formal scientific paper and present a talk on their work. The programs, in which over 500 students have participated during the past 20 years, have received the enthusiastic endorsement of the students, their colleges, and the members of the Biology Division.

Appendices

Advisory Committee - FY 1985

Dr. Robert Barker	Provost, Cornell University, Ithaca, New York
Dr. Verne M. Chapman	Chairman, Department of Molecular Biology, 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

Seminar Programs

INTERNAL SEMINARS AND JOURNAL CLUBS

Biochemistry Journal Club	Semiweekly
Cancer Research Seminar	Weekly
Flow Cytometry Journal Club	Biweekly
Genetics Seminar	Weekly
Histopathology Slide Seminar	Weekly
Staff Seminar	Weekly

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, 1983 through September 30, 1984.

Speaker	Affiliation	Subject
Ch'ang, Lan-yang	Vanderbilt University Nashville, Tennessee	Specific immunity induced in mice inoculated with irradiated Schistosoma manosoni
Chakrabarty, A.	University of Illinois Medical Center Chicago, Illinois	Genetic approaches to the problem of toxic chemical pollution
Charles, Daniel J.	Institute for Genetics Munich, West Germany	Enzyme-charge and -activity mutants in mice
Chase, John W.	Albert Einstein College of Medicine Bronx, New York	The role of E. coli single-strand binding protein in DNA replication, repair, and recombination
Copeland, Neal G.	University of Cincinnati College of Medicine Cincinnati, Ohio	Endogenous murine retroviruses: Molecular probes for dissecting the mammalian genome
Grdina, David J.	Argonne National Laboratory Argonne, Illinois	Tumor heterogeneity and radiation biology: The search for a prognostic indicator of tumor response
Jenkins, Nancy A.	University of Cincinnati College of Medicine Cincinnati, Ohio	Retroviruses as insertional mutagens of mice

Lewis, Barbara A.	Massachusetts Institute of Technology Cambridge, Massachusetts	Solid state NMR studies of molecular dynamics in bacterial rhodopsin
Morgan, William F.	University of California San Francisco, California	Do inhibitor studies indicate a role for poly(ADP-ribose) polymerase in DNA repair?
Mural, Richard	Frederick Cancer Research Facility Frederick, Maryland	Homologous recombination in mammalian cells following gene transfer
O'Neill, J. P.	University of Vermont Burlington, Vermont	Use of human T-lymphocytes for studies of mutation induction in vivo and in vitro
Paoletti, C. A.	Stanford University Palo Alto, California	Mechanisms of action and clinical trials of antitumor ellipticines
Paterson, Malcolm C.	Chalk River Nuclear Laboratories Chalk River, Ontario Canada	Nature-nurture interaction in the causation of human cancer
Pfleidener, Wolfgang	University of Konstanz Konstanz, West Germany	The p-nitrophenylethyl group — a universal blocking group for nucleic acid chemistry
Schröder, Claus	University of Michigan at Ann Arbor, and German Cancer Research Center Heidelberg, West Germany	Defective interferring herpes simplex genome
Schutz, Gunther	German Cancer Research Center Heidelberg, West Germany	DNA sequences controlling expression of the lysozyme gene

Smith, Lewis L.	ICI Limited Central Toxicology Laboratory Macclesfield, Cheshire, England	Toxicology and biochemistry of acute paraquat poisoning
Sobels, F. H.	State University of Leiden Leiden, The Netherlands	Studies of the mutation process in Drosophila through the use of repair deficient mutants
Sweeney, Terry	Harvard School of Public Health Boston, Massachusetts	Does chronic pulmonary disease alter the deposition of inhaled particles in the lungs?
Wise, J. G.	University of Rochester Medical Center Rochester, New York	The defective H^+-transporting ATP-synthase of *uncA* mutants of *E. coli*
Woodcock, C. L. F.	University of Massachusetts Boston, Massachusetts	Order and disorders in the cell nucleus
Würgler, F. E.	Swiss Federal Institute of Technology, and University of Zurich Zurich, Switzerland	Response *in vivo* of Drosophila somatic cells to mutagens
Zeman, Elaine	Colorado State University Fort Collins, Colorado	Changes in early and late radiation effects with dose per fraction — an *in vitro* perspective

Research Conferences

A DOE Model Compound Selections Meeting was organized and hosted by the Biology Division, with assistance from the Analytical Chemistry and Environmental Science Divisions of ORNL, on September 17, 1984. Chemists, toxicologists, and environmental scientists from five collaborating DOE national laboratories and from DOE itself met to select chemical substances for programmatic DOE research on adverse health effects. This interlaboratory coordinative effort represented the major first step in implementing the DOE/OHER complex chemical mixtures research plan.

Biology Division staff took the lead in forming the Southeastern Regional Chapter of the Society of Toxicology. At the organizational meeting on June 28, 1983, Drs. Hanspeter Witschi and Simon Lock of the Biology Division were elected President and Secretary-Treasurer, respectively. The purpose of the regional chapter is to provide a forum by which groups with diversified toxicological interests might exchange information. Since the organizational meeting, membership has increased to 73 persons drawn from the Biology Division as well as other divisions of ORNL, The University of Tennessee, East Tennessee State University, and from local industrial concerns and institutions (such as The Center for Disease Control) in Georgia and South Carolina. Since the formation of the regional chapter, two scientific meetings have been held. The first, entitled Toxicology and Other Life Sciences in Our Region, was organized by the executive committee with the assistance of Dr. John Kao (Biology Division) as program chairman. The meeting, held December 9-10, 1983, comprised a series of 7 invited papers describing some diversified regional toxicological resources and 16 "free" papers presented by members of the regional chapter. The second meeting of the chapter was held at the Quiller Dishner College of Medicine in Johnson City, Tennessee, June 1-2, 1984.

Extramural Activities

1. Officer of Society

D. Billen	- Executive Committee, Radiation Research Society, 1979-present
J. L. Epler	- Councilor, Environmental Mutagen Society, 1979-1983
S. Lock	- Secretary-Treasurer, Southeastern Regional Chapter of the Society of Toxicology, 1983-1985
P. Mazur	- Board of Governors, Society for Cryobiology, 1981-1985
A. L. Olins	- Councilor, American Society for Cell Biology, 1984-1986
R. J. Preston	- Councilor, Environmental Mutagen Society, 1983-present
R. O. Rahn	- Councilor, American Society for Photobiology, 1980-1983
L. B. Russell	- President-Elect, Environmental Mutagen Society 1983-1984 President, Environmental Mutagen Society, 1984-1985
D. M. Skinner	- Chairperson, Section G, Biological Sciences, American Association for the Advancement of Science, 1984
H. R. Witschi	- President, Southeastern Regional Chapter, Society of Toxicology, 1983-1984 President-Elect, Society of Toxicology, Inhalation Specialty Section, 1984

2. Society Committees

H. I. Adler	- Education and Training Committee, Radiation Research Society, 1980-1984
D. Billen	- Finance Committee, Radiation Research Society, 1979-present

J. S. Cook - Publications Committee, Society of General Physiologists, 1982-1986
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

J. L. Epler - Membership Committee (Chairman), Environmental Mutagen Society, 1982-1983

R. J. M. Fry - Awards Monitoring Committee (Chairman), Radiation Research Society, 1982-present
History Committee, Radiation Research Society, 1982-present

W. M. Generoso - Committee on Workshops and Training, Environmental Mutagen Society, 1982-present

S. Lock - Information Handling Committee, Society of Toxicology, 1983-1984

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

R. J. Preston - Parliamentarian, Environmental Mutagen Society, 1983-present

R. O. Rahn - Representative to the American Institute of Biological Sciences and Committee on Public Affairs, American Society for Photobiology, 1984-

L. B. Russell - Program Committee (Chairman), Environmental Mutagen Society, 1983-1984
Executive Committee (Chairman), Environmental Mutagen Society, 1984-1985

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

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
Ad hoc member, Board of the Tennessee Center for Biotechnology (representing ORNL), 1983-present

D. 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

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
Oversight Committee for Radioepidemiological Tables, National Academy of Sciences, 1983-

W. M. Generoso - Panel on Cholinesterase Reactivators, Committee on Toxicology, National Research Council, 1982-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-present
Third Task Force for Research Planning for the National Institute for Environmental Health Sciences, 1983-1985

Chemical Selection Committee, U.S. Department of Energy, 1984-
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-

A. W. Hsie - Member of Scientific Directorate, Coordinating Council for Cancer Research, Villejuif, France, 1978-present
Member, Special Review Committee, National Institutes of Health, 1983-1984

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-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 Population Monitoring, World Health Organization, 1983-present
Committee on Aneuploidy (Chairman), Environmental Protection Agency, 1984

L. B. Russell - Committee I, International Commission for Protection Against Environmental Mutagens and Carcinogens, 1977-1983
International Committee on Standardized Genetic Nomenclature for Mice, 1977-present
Coordinating Committee of Gene-Tox Task, Environmental Protection Agency, 1980-present
Committee on Risk Assessment of Gene-Tox Task (Chairman), Environmental Protection Agency, 1980-present
Science Advisory Panel, Litton Bionetics, 1980-present
Board on Toxicology and Environmental Health Hazards, National Academy of Sciences, 1981-1984
Distinguished Scientist Committee, University of Tennessee-Oak Ridge National Laboratory, 1983-

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

P. B. Selby - Scientific Adviser to U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation, 1984
Task Group XI of National Council on Radiation Protection Committee 57, Genetic Risk from Internal Emitters, 1984

J. K. Selkirk - Biochemistry Study Section, American Cancer Society, 1983-1988
Metabolic Pathology Advisory Committee, NIH, 1984
Working Group on Polyaromatic Carcinogens, International Agency for Research on Cancer, Lyon, 1983

D. M. Skinner - Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-present
External Advisory Committee to The Biology Department, Georgetown University, 1983

A. S. 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-present

R. L. Ullrich - Scientific Committee 40 on the Biological Aspects of Radiation Protection Criteria, National Council on Radiation Protection and Measurements, 1977-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
Committee for the Characterization of the Status of Toxicity Data Elements for a Select Universe of Compounds, National Research Council, 1980-1983
Ozone Program Project Committee, Primate Center, University of California-Davis, 1983

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
Cell and General Physiology, American Physiological Society Handbook Series, 1982-1984
Current Topics in Membranes and Transport (Advisory Board), 1983-present

J. L. Epler - Mutation Research, 1977-1983
Environmental Mutagen Society Newsletter, 1980-1983

W. M. Generoso - Teratogenesis, Carcinogenesis, and Mutagenesis, 1979-present

F. C. Hartman - BioScience, 1980-1986
Journal of Protein Chemistry, 1982-1987
Journal of Biological Chemistry, 1983-1988

A. W. Hsie - Fundamental and Applied Toxicology, 1983-
Teratogenesis, Carcinogenesis, and Mutagenesis, 1983-

S. Lock - Journal of Toxicology and Environmental Health, 1984-1987

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
Environmental Mutagenesis, 1978-1983

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

D. M. Skinner - Growth, 1979-1986
Biological Bulletin, 1981-1984

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

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

5. Awards, National

H. I. Adler - Industrial Research-100 Award, 1984

R. O. Rahn - Congressional Fellowship (AAAS), Representing Biophysical Society and American Society for Photobiology, 1983-1984

6. Awards, International

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

S. Mitra - B. C. Guha Memorial Award, Calcutta University, 1983

J. D. Regan - Japanese Government Research Award for Foreign Specialists, 1984

International Activities

Australia and England

Dr. Paul Selby exchanges specimens and research data and provides consultation on skeletal mutations with the University of Sydney, Australia, and the Medical Research Council, Harwell, England.

Chili and Antartica

Dr. H. I. Adler has hosted Dr. Alicia Carrasco from the University of Chile on two occasions for visits of nine and three months to study anaerobic bacteria.

Dr. J. K. Selkirk has obtained a grant from the National Science Foundation to collaborate with the University of Chile, Santiago, on a study of carcinogen metabolism in species in the Antarctic region.

Federal Republic of Germany (West Germany)

International cooperation is much in evidence in Dr. Peter Mazur's cryobiology laboratory as interest from various parts of the world continues in the form of direct communications, reprint material, requests for seminars, and direct consultations. Particular interest has been shown by the Federal Republic of Germany (FRG) by sending two scientists to study with Dr. Mazur. Dr. Ullrich Schneider, School of Veterinary Medicine, Hannover, FRG, has been sponsored by the German Research Council for nearly two years to pursue research on permeability of embryos to freezing-protective substances. Dr. Winfred K. Berger, Professor at the Physiologisches Institut, Universität des Saarlandes, FRG, is here for at least six months to work on cryopreservation of electrically coupled tissues.

Another West German scientist, Dr. Rheinhold C. Mann, came to the Biology Division in 1981 as a Humboldt Foundation Scholar to work with Dr. P. F. Mullaney (deceased) on flow cytometry and cell sorting problems in relation to pattern recognition techniques. Dr. Mann was employed by the Laboratory on a temporary appointment in 1983 and 1984 to continue this fruitful, pioneering effort in automated data acquisition and analysis of flow cytometry.

India

Dr. Sankar Mitra received a National Science Foundation U.S./India Exchange of Scientists Travel Award for the purpose of visiting several laboratories in India to deliver lectures on his work and confer with local scientists. Dr. Mitra also is collaborating with G. Padmanabhan of the Indian Institute of Science, Bangalore, on site-directed mutagenesis of cloned P-450 genes and with D. K. Dube, Institute of Microbial Genetics, Chandigarh, on recombinant DNA techniques for biotechnology.

Japan

Dr. R. K. Fujimura has been appointed by the Commerce Department to spend two years in Japan as a member of the U.S. Ambassador's staff to study and interact with Japanese industry on biological opportunities of mutual interest to both countries.

Dr. Ann C. Marchok hosted for a two-year period Dr. M. Shiba, a surgeon from the Institute for Pulmonary Research, Chiba University, Chiba, Japan.

Japan indicated its interest in Biology Division scientists by inviting Dr. James D. Regan for a five-month appointment to Mie State School of Medicine, Tsu, Japan, to collaborate in studies on DNA repair in normal and malignant human colon cells. In July of this year, the Japan Ministry of Science awarded Dr. Regan a Research Award for Foreign Specialists to study and teach DNA repair and carcinogenesis. Much of his time was spent at the Biology Division, National Institute for Radiological Sciences, Chiba, Japan.

Korea

Dr. K. Bruce Jacobson received a National Science Foundation U.S./East Asia Cooperative Science Grant to continue collaboration with Dr. John Yim (The National University of Korea) on gene suppression in Drosophila. Dr. Yim visited for a month in Dr. Jacobson's laboratory this past summer.

Spain

Valencia Univerisity in Spain sent Juan Ferre here for 14 months to study and conduct research with Dr. K. Bruce Jacobson. Dr. Jacobson attended the thesis defense of Juan Ferré in Spain while collaborating with Dr. Ménsua on a joint research project involving biochemical mechanisms of pteridine biosynthesis (funded by the National Science Foundation).

Taiwan

Dr. C. H. Wei has a collaborative study under way with staff at the Chemistry Department, National Taiwan University.

Other

Dr. R. A. Griesemer and J. K. Selkirk have served on Working Groups for the International Agency for Research on Cancer (IARC), Lyon, France. The IARC Working Groups review and evaluate published reports on chemical carcinogenicity. The resulting monographs have served as the single standard for regulatory and informational purposes throughout the world.

Dr. E. H. Grell supplies Drosophila stocks, containing mutations and chromosomal rearrangements, throughout the world; requests have been received from Australia, Canada, Columbia, France, The Federal Republic of Germany, Great Britain, Hungary, India, Japan, Korea, The Netherlands, Spain, Switzerland, Romania, and the USSR.

Dr. R. J. Preston regularly consults as expert cytogeneticist to the World Health Organization on population monitoring and to the International Atomic Energy Agency on biological dosimetry.

Dr. Liane B. Russell serves on two international committees: Committee I, International Commission for Protection Against Environmental Mutagens and Carcinogens; and the International Committee on Standardized Genetic Nomenclature for Mice.

Drs. W. L. Russell and P. B. Selby have served as Scientific Adviser to the U.S. Delegation, United Nations Scientific Committee on the Effects of Atomic Radiation.

ABSTRACTS FOR TECHNICAL MEETINGS HELD
OCTOBER 1, 1983 — SEPTEMBER 30, 1984

Aardema, M. J., W. W. Au, and R. J. Preston. X ray induced cell cycle delay and chromosome aberrations in myeloid leukemia cells and normal bone marrow cells. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Adams, L. M. and R. L. Ullrich. Changes in growth characteristics with time of cells from carcinogen treated mammary tissue of virgin female BALB/c mice. American Association for Cancer Research, Toronto, Canada, May 9-12, 1984.

Adler, H., W. Crow, R. Machanoff, S. Haney, and A. Sozer. New methods for growing anaerobes. 84th Annual Meeting, American Society for Microbiology, St. Louis, Missouri, March 4-9, 1984.

Allen, R. L., D. E. Olins, E. Wilkinson-Singley, J. Harp, and A. L. Olins. Isolation and characterization of the replication band from the macronucleus of Euplotes eurystomus. American Society for Cell Biology, San Antonio, Texas, November 29-December 3, 1983.

Au, William W., Ti Ho, and R. Julian Preston. Neutron radiation induced cell cycle delay and chromosome aberrations in bone marrow cells of RFM mice. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Ayer, S. P., D. P. Allison, and S. K. Niyogi. DNA binding properties of proteins isolated from the nuclear matrix of HeLa cells. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Balogh, Lisa A., Kai-Lin Lee, and Francis T. Kenney. Tissue-specific expression of tyrosine aminotransferase. UCLA Symposia on Molecular and Cellular Biology, Steamboat Springs, Colorado, March 31-April 7, 1984.

Balogh, Lisa A., Kai-Lin Lee, and Francis T. Kenney. Tissue-specific expression of tyrosine aminotransferase. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Bingham, G. A. Incomplete effectiveness of trichlorfon against Aspicularis tetraptera in mice and adverse effects. American Association for Laboratory Animal Science, San Antonio, Texas, November 6-11, 1983.

Bunick, Gerard J., Edward C. Uberbacher, and Venkatraman Ramakrishnan. Structural analysis of HMG 17-nucleosomes by small-angle neutron scattering. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Carrier, W. L., A. Castellani, and J. D. Regan. Excision repair of UV-light induced damage to DNA of human cells treated with 1-β-D-arabinofuranosylcytosine (ara-C) and hydroxyurea (HU). Ninth International Congress on Photobiology and Twelfth Annual Meeting of the American Society for Photobiology, Philadelphia, Pennsylvania, July 1-6, 1984.

Carrier, W. L., and J. D. Regan. The effect of DNA synthesis inhibitors on excision repair in human cells. 8th International Biophysics Congress, Bristol, England, July 29-August 4, 1984.

Charp, P. A., and J. D. Regan. Inhibition of DNA repair by trifluoperazine is manifest by an inhibition of dNTP precursor uptake. Ninth International Congress on Photobiology and Twelfth Annual Meeting of the American Society for Photobiology, Philadelphia, Pennsylvania, July 1-6, 1984.

Cook, J. S., and J. B. Fishman. Sorting of surface sialoglycoproteins in HeLa cells during internalization and recycling. 1984 UCLA Symposia on Membrane Receptors and Cellular Recognition, Park City, Utah, March 25-30, 1984.

Dumont, James N. The frog embryo teratogenesis assay: Xenopus (FETAX). Fourth Annual Meeting of the American College of Toxicology, Arlington, Virginia, November 30-December 2, 1983.

Dumont, J. N. The frog embryo teratogenesis assay: Xenopus (FETAX) — A short-term screen for detecting and ranking potential teratogens. 1st Annual Meeting of the Southeastern Regional Chapter, Society of Toxicology, Oak Ridge, Tennessee, December 9-10, 1983.

Dumont, J. N., and R. G. Epler. Validation studies on the FETAX teratogenesis assay (frog embryos). Teratology Society, Boca Raton, Florida, June 3-7, 1984.

Dumont, J. N., and T. W. Schultz. Teratogenesis of retinoic acid in Xenopus embryos. American Microscopical Society, Philadelphia, Pennsylvania, December 27-30, 1983.

Fishman, J. B., and J. S. Cook. The sequential transfer of internalized surface material through the lysosomes and Golgi in HeLa cells. American Society for Cell Biology, San Antonio, Texas, November 29-December 3, 1983.

Flanagan, J. F., and K. B. Jacobson. Zn^{2+} causes unique alterations of $tRNA^{Phe}$ conformation. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Flint, Kimberly B., and J. P. O'Neill. X irradiation and cell cycle specificity. Association of Southeastern Biologists, Memphis, Tennessee, April 11-14, 1984.

Flint, K. B., and R. J. Preston. SCE induction by combined X-ray/EMS treatments — A consideration of mechanism. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Fowler, R. F., V. Bonnewell, and D. M. Skinner. Region of sequence divergence in a complex (G+C)-rich satellite is associated with specific effects on DNA structure. American Society for Cell Biology, San Antonio, Texas, November 29-December 3, 1983.

Fry, R. J. M. Approaches to radiation guidelines for space travel. Committee on Space Research (COSPAR), Graz, Austria, June 25-July 7, 1984.

Fry, R. J. M., R. D. Ley, E. Perkins, and L. Triplett. Cocarcinogenesis: ionizing radiation and other agents. Radiation Research Society, Orlando, Florida, March 25-29, 1984.

Gardner, M. J., A. Estival, R. H. Bassin, and W. K. Yang. Chemically transformed NIH 3T3 cells selected from soft agar do not contain activated cellular ras oncogenes. Symposium on RNA Tumor Virus, Cold Spring Harbor, New York, May 22-27, 1984.

Goad, M. E. P., H. Witschi, and A. F. Tryka. Delayed onset of acute respiratory distress syndrome. American Thoracic Society, Miami Beach, Florida, May 20-23, 1984.

Goldberg, S. Z., L. Skow, S. Lewis, R. Popp, and W. F. Anderson. A mouse model of β-thalassemia. American Society of Hematology, San Francisco, California, December 3-6, 1983.

Hadden, Charles T. Studies of differentiation of Clostridium butyricum. Wind River Conference on Genetic Exchange, Saugerties, New York, June 11-14, 1984.

Hand, Russell, Jr., Reinhold Mann, Cynthia Evans, Rebecca Jernigan, and Richard Tyndall. Flow cytometric analysis of Legionella. International Conference on Analytical Cytology, Pacific Grove, California, June 3-8, 1984.

Hand, Russell, Jr., Raymond Popp, Diana Popp, and Reinhold Mann. Flow cytometric analysis of bone marrow from mice exposed to ethylene oxide. International Conference on Analytical Cytology, Pacific Grove, California, June 3-8, 1984.

Hartman, Fred C., and Eva H. Lee. Reactivity of an active-site lysine in ribulosebisphosphate carboxylase/oxygenase. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Heartlein, M. W., and R. J. Preston. 3-Aminobenzamide increases chromosome aberrations after irradiation in G_1, but not in G_0. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Hellwig, R. J., S. P. Ayer, and S. K. Niyogi. Interaction of HeLa cell transcription factors with nucleoprotein complexes and DNA containing the adenovirus 2 major late promoter. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Hsie, A. W., P. A. Brimer, R. L. Schenley, C. Q. Lee, R. S. Foote, and S. Mitra. A nitrosoguanidine-resistant CHO mutant. Third International Congress on Cell Biology, Tokyo, Japan, August 26-31, 1984.

Jacobson, K. Bruce, M. W. Williams, and Nelwyn T. Christie. Genetic basis of cadmium toxicity in Drosophila melanogaster. 1st Annual Meeting of the Southeastern Regional Chapter, Society of Toxicology, Oak Ridge, Tennessee, December 9-10, 1983.

Jacobson, K. Bruce, M. W. Williams, and Nelwyn T. Christie. Genetic basis of cadmium toxicity in Drosophila melanogaster. Meeting on High Affinity Metal-Binding Proteins in Non-Mammalian Species, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, September 19-21, 1984.

Johnson, Alfred C., Robin Rothrock, Kenneth R. Isham, Kai-Lin Lee, and Francis T. Kenney. Translational control of tyrosine aminotransferase; induction by hydrocortisone in fetal rat liver. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Kao, J. In vitro skin penetration and cutaneous metabolism of topically applied chemicals. International Union of Pharmacology 9th International Congress of Pharmacology, London, England, July 29-August 3, 1984.

Kao, J., and J. Whitaker. Skin penetration of chemicals in vitro: metabolic viability and species differences. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Lewis, S. E., F. M. Johnson, L. B. Barnett, L. C. Skow, and R. A. Popp. Mosaic mutants may be induced in postgonial germ cell stages of the mouse. Genetic Society of America, Vancouver, British Columbia, Canada, August 12-16, 1984.

Lewis, Susan E., F. M. Johnson, L. C. Skow, Lois B. Barnett, and R. A. Popp. Mosaic mutations among the progeny of parents treated with ethylnitrosourea. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Lindenschmidt, R., and H. P. Witschi. Propranolol-induced elevation in collagen. Federation of American Societies for Experimental Biology, St. Louis, Missouri, April 1-6, 1984.

Lindenschmidt, R. C., and H. P. Witschi. Attenuation of pulmonary fibrosis by aminophylline (AM). American Society for Pharmacology and Experimental Therapeutics, Indianapolis, Indiana, August 19-23, 1984.

Lock, S. Effects of multiple exposure to aerosolized diesel fuel. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Lock, S. The inhalation toxicology of a smoke screen derived from diesel fuel. Southeastern Regional Chapter, Society of Toxicology, Johnson City, Tennessee, June 1-2, 1984.

Mann, Reinhold C. The use of spline functions for the analysis of flow cytometric histograms. International Conference on Analytical Cytology, Pacific Grove, California, June 3-8, 1984.

Mann, Reinhold C., and Russell E. Hand, Jr. The analysis of multivariate flow cytometric data. International Conference on Analytical Cytology, Pacific Grove, California, June 3-8, 1984.

Mazur, P., and U. Schneider. Does the magnitude of the unfrozen fraction affect the survival of slowly frozen mouse 8-cell embryos? I. Theory. Society for Cryobiology, La Jolla, California, August 20-24, 1984.

Mitra, S., E. T. Snow, A. Bhattacharyya, L. A. Dodson, W. Masker, B. C. Pal, and R. S. Foote. Mutagenic properties of O-alkylated bases in DNA in vivo and in vitro. Symposium on "Mutagenesis-Cellular Processes," XV International Congress of Genetics, New Delhi, India, December 12-21, 1983.

Mykles, D. L., and D. M. Skinner. Purification and characterization of crab calcium-dependent proteinase that degrades myofibrillar proteins. American Society for Cell Biology, San Antonio, Texas, November 29-December 3, 1983.

Mykles, D. L., and D. M. Skinner. Role of calcium-dependent proteinase in proecdysial claw muscle atrophy. International Union of Biological Sciences, Congress on Comparative Physiology and Biochemistry, Liège, Belgium, August 27-31, 1984.

Olins, D. E. Electron microscope tomography (EMT) of chromosomal structures. International Conference on Analytical Cytology, Pacific Grove, California, June 3-8, 1984.

Olins, D. E., R. L. Allen, C. L. Cadilla, and A. L. Olins. Macronuclear and chromatin structure in Euplotes eurystomus. First International Ciliate Molecular Genetics Meeting, Cold Spring Harbor, New York, May 2-6, 1984.

O'Neill, J. P., and K. B. Flint. Mammalian cells in the G_0/G_1 phase of the cell cycle are most sensitive to mutation induction by X-rays. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Ou, C. Y., C. K. Koh, L. R. Boone, R. Callahan, and W. K. Yang. An apparent novel family of retroviral sequences in the mouse genome. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 22-27, 1984.

Ou, C. Y., C. K. Koh, L. R. Boone, and W. K. Yang. An apparent novel family of retroviral sequences in the mouse. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Pal, B. C., C. K. Ghosh, and J. A. McCloskey. Reaction of 5-halodeoxycytidine with cysteine. 35th Southeast Regional American Chemical Society, Charlotte, North Carolina, November 9-11, 1983.

Perkins, E. H., and P. L. Glover. Increasing immune competence and mean life-span of aging mice by multiple sequential thymus grafting. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Popp, D. M., S. Lock, and R. A. Popp. Hemotoxicity of ethylene oxide (EO). Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Popp, D. M., J. A. Otten, and R. A. Popp. Genetic regulation of lifespan immunoglobulin levels and retrovirus activation. Genetics Society of America, Vancouver, British Columbia, Canada, August 12-16, 1984.

Popp, R., S. Lock, D. Popp, R. Mann, and R. Hand, Jr. Use of flow cytometry (FCM) to analyze bone marrow perturbations induced by ethylene oxide (EO). Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Popp, R. A., C. J. Wawrzyniak, E. J. Smith, S. H. Murphy, and D. M. Popp. Hemoglobin synthesis in alpha- and beta-thalassemic mice. Genetics Society of America, Vancouver, British Columbia, Canada, August 12-16, 1984.

Preston, R. Julian. Chromosome rearrangements in cancer. NCI Symposium on Comparison of Mechanisms of Carcinogenesis by Radiation and Chemical Agents, Gaithersburg, Maryland, December 6-7, 1983.

Rao, T. K., and J. L. Epler. Precautions in interpreting test results with coal derived fuels. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Recio, L., and A. W. Hsie. Glucuronide conjugation of benzo(a)pyrene in the CHO/HGPRT assay: differential effects of cytotoxicity and mutagenicity. 1st Annual Meeting of the Southeastern Regional Chapter, Society of Toxicology, Oak Ridge, Tennessee, December 9-10, 1983.

Recio, L., and A. W. Hsie. Glucuronide conjugation of benzo(a)pyrene reduces cytotoxicity but not mutagenicity in the CHO/HGPRT system. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Recio, L., and A. W. Hsie. The effects of glucuronide conjugation on benzo(a)pyrene-induced cytotoxicity and mutagenicity in the CHO/HGPRT assay. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Regan, J. D. Inhibitors in the study of human DNA repair. Radiation Research Society of Japan, Tokyo, Japan, September 23-26, 1984.

Regan, J. D. Photoreactivation and dark repair in the vertebrates. Radiation Research Society of Japan, Tokyo, Japan, September 23-26, 1984.

Regan, James D., and William L. Carrier. The use of inhibitors in the study of DNA excision repair in human cells. Radiation Research Society, Orlando, Florida, March 25-29, 1984.

Rose, I. A., A. Jaworowski, and F. C. Hartman. Reaction intermediates of ribulose-bisphosphate carboxylase of R. rubrum. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Russell, Liane B., and C. S. Montgomery. Use of MNU-ENU double exposure in the mouse spot test to explore the in vivo action of an O^6-alkyl transferase. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Russell, W. L. Dose response, repair, and the threshold problem in mammalian germ-cell mutagenesis. Symposium on "Dose-response relationship for genetic effects of environmental chemicals with special regard to the problem of threshold," Tokyo, Japan, May 7-9, 1984.

Russell, W. L., and P. R. Hunsicker. Mutagenic effect of ethylnitrosourea (ENU) on post-stemcell stages in male mice. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Schneider, U., P. Mazur, and K. Cole. Does the magnitude of the unfrozen fraction affect the survival of slowly frozen mouse 8-cell embryos? II. Experimental. Society for Cryobiology, La Jolla, California, August 20-24, 1984.

Selby, P. B., and W. L. Russell. F_1 litter-size reduction following irradiation of stem-cell spermatogonia in mice. Genetics Society of America, Vancouver, British Columbia, Canada, August 12-16, 1984.

Selby, P. B., B. J. M. Whitt, G. D. Raymer, and T. W. McKinley, Jr. Breeding-test experiment demonstrates transmissibility of many dominant skeletal mutations induced by ethylnitrosourea. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Selkirk, James K., and Ann Nikbakht. Metabolism and macromolecular binding of benzo(a)pyrene to normal mammary epithelial cells and human mammary tumor lines T47D, MCF-7 and HBL-100. American Association for Cancer Research, Toronto, Canada, May 9-12, 1984.

Sheu, C. W., G. Sega, and J. G. Owens. Effect of mode of administration on the extent of unscheduled DNA synthesis induced in the germ cells of male mice. Fourth Annual Meeting of the American College of Toxicology, Arlington, Virginia, November 30-December 2, 1983.

Shugart, L. Covalent binding of benzo[a]pyrene diol epoxide to DNA of mouse skin: persistence of adduct formation. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Shugart, Lee, Jerry Hall, and John Kao. Alkylation of hemoglobin in mouse after exposure to benzo[a]pyrene. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Shugart, Lee, and John Kao. Examination of adduct formation in vivo in the mouse between benzo[a]pyrene and DNA of skin and hemoglobin of red blood cells. Conference on DNA Adducts, Research Triangle Park, North Carolina, September 24-26, 1984.

Skinner, D. M., and R. F. Fowler. A complex satellite DNA contains sequences characteristic of RNA splicing. UCLA Symposia on Molecular and Cellular Biology, Steamboat Springs, Colorado, April 7-13, 1984.

Smith, L. H., H. P. Witschi, and R. N. Maronpot. Studies on the mouse lung tumor assay as a screen for carcinogens. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Sozer, A. C., H. I. Adler, R. Machanoff, and S. Haney. Sensitivity of Clostridium acetobutylicum to oxygen and ionizing radiation. Radiation Research Society, Orlando, Florida, March 25-29, 1984.

Stankowski, L. F., Jr., and A. W. Hsie. Quantitative and molecular analysis of mutation in an Ecogpt-transformed CHO cell line. XV International Congress of Genetics Satellite Symposium on Mutagenesis, Darbhanga, India, December 22-23, 1983.

Stankowski, L. F., Jr., and A. W. Hsie. Quantitative and molecular analyses of mutation in pSV2gpt transformed CHO cells. Environmental Mutagen Society, Montreal, Canada, February 19-23, 1984.

Stankowski, L. F., Jr., and A. W. Hsie. Quantitative and molecular analyses of radiation-induced mutation in pSV2gpt transformed CHO cells. Radiation Research Society, Orlando, Florida, March 25-29, 1984.

Stankowski, L. F., Jr., and A. W. Hsie. Analysis of mutation in pSVgpt transformed CHO cells. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Stankowski, L. F., Jr., D. S. Katz, and A. W. Hsie. Molecular analysis of gene mutations in CHO cells. Third International Congress on Cell Biology, Tokyo, Japan, August 26-31, 1984.

Stringfellow, Leslie A., Kai-Lin Lee, and Francis T. Kenney. Cyclic AMP induction of tyrosine aminotransferase: transcriptional or translational regulation. UCLA Symposia on Molecular and Cellular Biology, Steamboat Springs, Colorado, March 31-April 7, 1984.

Swenson, P. A., and I. L. Norton. Far UV induced respiration shutoff in Escherichia coli requires recBc nuclease activity. Ninth International Congress on Photobiology and Twelfth Annual Meeting of the American Society for Photobiology, Philadelphia, Pennsylvania, July 1-6, 1984.

Tindall, K. R., L. F. Stankowski, Jr., R. Machanoff, and A. W. Hsie. Analysis of mutation in DNA-transformed mammalian cells. XV International Congress of Genetics, New Delhi, India, December 12-21, 1983.

Turner, J. E., M. W. Williams, and K. B. Jacobson. Correlations of acute toxicity of metals and the covalent/ionic character of their bonds. Symposium on Quantitative Structure Activity Relationships in Toxicology and Xenobiochemistry, Prague, Czechoslovakia, September 12-14, 1984.

Uberbacher, E. C., V. Ramakrishnan, and G. J. Bunick. Structural analysis of HMG 17-nucleosomes by small-angle neutron scattering. Biophysical Society Meeting, San Antonio, Texas, February 19-23, 1984.

Ullrich, R. L. The role of animal experiments in risk estimates. American Nuclear Society, New Orleans, Louisiana, June 3-8, 1984.

Van Houten, B., W. E. Masker, W. L. Carrier, and J. D. Regan. Measuring carcinogen-induced DNA damage and repair in human cells with the UVR ABC endonuclease from Escherichia coli. American Association for Cancer Research, Toronto, Canada, May 9-12, 1984.

Van Houten, B., and James D. Regan. Initial rates of incision of UV-light induced DNA damage in growth arrested human fibroblasts. Ninth International Congress on Photobiology and Twelfth Annual Meeting of the American Society for Photobiology, Philadelphia, Pennsylvania, July 1-6, 1984.

Wasilenko, W., and A. Marchok. Utilization of ^{14}C-metabolic substrates by 7,12-dimethylbenz(a)anthracene-altered rat trachel epithelial cells that grow without pyruvate. American Association for Cancer Research, Toronto, Canada, May 9-12, 1984.

Waters, Larry C., and Carroll E. Nix. Studies on the genetic regulation of mixed-function oxidase (MFO) activities in Drosophila. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Wawrzyniak, C. J., and R. A. Popp. Quantitation of the $\underline{Hbb}^{sm}$ haplotype products during mouse development. Developmental Biology Meeting, Cincinnati, Ohio, May 17-19, 1984.

Wei, Chin Hsuan, and B. E. Hingerty. The structure of dichloro(tetrapyridine)platinum(II)trihydrate, $[Pt(C_5H_5N)_4]^{2+}.2Cl^-.3H_2O$. XIIIth International Congress and General Assembly International Union of Crystallography, Hamburg, Federal Republic of Germany, August 9-18, 1984.

Williams, M. W., J. E. Turner, T. L. Hayden, K. Bruce Jacobson, and E. H. Lee. Correlations of metal-ion toxicity with physical and biological parameters. American Physical Society, Detroit, Michigan, March 26-30, 1984.

Witschi, Hanspeter. The role of interactions in chemical carcinogenesis. 2nd International Meeting on Chemical Carcinogenesis: Xenobiotics and Biotransformation, Alghero, Italy, October 12-15, 1983.

Witschi, H. P. Enhancement of lung tumor formation in mice. Symposium on Tumor Promotion and Enhancement in the Etiology of Human and Experimental Respiratory Tract Carcinogenesis, Williamsburg, Virginia, June 17-20, 1984.

Witschi, H. P., and D. G. Doherty. Modification of lung tumor development in mice by butylated hydroxyanisole. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Witschi, H. P., and R. C. Lindenschmidt. The pathogenesis of acute and chronic lung damage. Symposium of The National Academy of Clinical Biochemistry, Washington, D.C., July 27-28, 1984.

Witschi, H. P., and C. C. Morse. Cell kinetics in mouse lung initiated with urethan and promoted by butylated hydroxytoluene. Society of Toxicology, Atlanta, Georgia, March 12-16, 1984.

Wobbe, C. R., and S. Mitra. Characterization of proteins tightly associated with replicative form DNA of Kilham rat virus. American Society of Biological Chemists/American Association of Immunologists, St. Louis, Missouri, June 3-7, 1984.

Wobbe, C. R., and S. Mitra. Isolation of proteins covalently associated with replicative form DNA of Kilham rat virus. American Society of Virology, Madison, Wisconsin, July 22-26, 1984.

Yang, W. K., L. R. Boone, C. Y. Ou, D. M. Yang, F. E. Myer, and R. W. Tennant. A distinct viral $p30^{gag}$-directed host restriction of endogenous marine type C retroviruses. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 22-27, 1984.

Financial Summary and Personnel Distribution

FY 1984

Funding Source	Funding in thousands	% of total budget	Person-years
Department of Energy	13,862	79.0	152.9
National Cancer Institute	1,775	10.1	21.3
National Institute of Environmental Health Sciences	1,098	6.3	13.6
National Institute of Child Health and Human Development	239	1.4	2.9
Environmental Protection Agency	215	1.2	1.5
Miscellaneous	163	0.9	1.3
National Institute of General Medical Sciences	56	0.3	0.9
Department of Agriculture	48	0.3	0.1
National Institute of Allergy and Infectious Diseases	45	0.3	0.4
Department of Defense	35	0.2	0.3
TOTAL	17,536		195.2

ORNL-6119
Distribution Category UC-48

INTERNAL DISTRIBUTION

1. T. D. Anderson
2. S. I. Auerbach
3. W. E. Barnett
4. R. J. M. Fry
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