

ORNL--6021

DEC 006 248

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**Contract No. W-7405-eng-26**

**BIOLOGY DIVISION  
PROGRESS REPORT**

**For Period of August 1, 1982 - September 30, 1983**

**Date Published: January 1984**

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operated by  
UNION CARBIDE CORPORATION  
for the  
DEPARTMENT OF ENERGY**

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## Introduction and Division Overview

R. A. GRIESEMER

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

The energy-related substances of interest are both physical 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 responses to low doses, the relative biological effectiveness of various forms of radiation, and comparisons with gamma radiation for which a considerable data base exists. Among the chemical agents, special emphasis is placed on problems associated with the emerging energy technologies, notably synthetic fuels of fossil origin. Since the energy-related substances to which people may be exposed tend to be complex mixtures of chemicals, the Division's activities concentrate on the adverse health effects from chemical-chemical or chemical-physical interactions and on methods to evaluate the effects of complex mixtures.

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

The resources available for the Division's activities during the report period included a staff of approximately 57 at the doctorate level, 149 support personnel, 43 doctoral students, 9 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 and toxicology and the Analytical Chemistry Division where collaboration in research has been especially fruitful.

A significant change in resources from the previous year was a planned reduction in the amount of laboratory space occupied by the Division. The Biology Division had experienced a 40% reduction in the size of its staff in the previous two years due to budget restrictions. To conserve operating costs, the remaining staff members and their programs were relocated this year so that portions of the Biology Division space could be made available for lease by other divisions or temporarily shut down.

Technical progress during this report period is described in the following sections but a few highlights of Division activities deserve special mention. Of the Division's 220 publications last year, more than a third were co-authored 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 advisors to federal agencies, and as members of editorial boards. Two senior members of the Division, Drs. Liane B. Russell and John B. Storer, received special honors. Both were named Corporate Fellows by Union Carbide Corporation in recognition of their outstanding, internationally recognized research accomplishments in mammalian genetics and radiation biology, respectively. Designation as a Corporate Fellow is a special distinction because there are only 18 Corporate Fellows in the Nuclear Division of the Union Carbide Corporation and only 47 Fellows in the entire Corporation.

The body of this report provides summaries of the aims, scope and progress of the research of groups of investigators in the Division during the period of August 1, 1982, through September 30, 1983. At the end of each summary is a list of publications covering the same period (published or accepted for publication). For convenience, the summaries are assembled under Sections in accordance with the current organizational structure of the Biology Division; each Section begins with an overview. It will be apparent, however, that currents run throughout the Division and that the various programs support and interact with each other.

In addition, this report includes an outline of educational activities, a listing of the members of the Advisory Committee, seminar programs in the Division, research conferences sponsored by the Division, extramural activities of staff members, abstracts for technical meetings by staff, and a table that summarizes funding and personnel levels for fiscal year 1983.

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# Research Activities

## Molecular Genetics Section

### SECTION OVERVIEW - F. C. HARTMAN

As part of the Department of Energy's life sciences program, our mission is to investigate basic aspects of adverse health effects of energy production. Since nuclear reactors and fossil fuels are our nation's major energy sources, interactions of radiation and chemicals derived from fossil fuels with biological systems are of primary concern. The more profound clinical manifestations of human exposure to these agents may include cancer, genetic damage, birth defects, and acute toxic poisoning, all of which represent perturbations of normal cellular processes. There is such a vast array of potentially damaging agents that to attempt to assess each of their consequences singly and in combinations is likely doomed to failure. The only hope 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 share common origins that entail modification of gene structure or alterations in nucleic acid enzymology. Major emphasis is therefore placed on gene structure and function. This central theme includes studies of the structure of DNA and chromatin, the interaction of nucleic acids with environmental agents, replication and transcription of DNA as well as their regulation, enzymology of repair of DNA damaged by chemicals or radiation, and molecular genetics. During the past year, notable accomplishments in these general areas of molecular biology include the following:

(1)  $^{32}$ -Ethylthymine is a potentially mutagenic and carcinogenic base. To evaluate its mutagenic properties and to characterize its repair pathway, isotopically labeled  $^{32}$ -ethylthymidine triphosphate has been synthesized and incorporated into synthetic DNA.

(2) Ordered nucleoprotein complex formation is a prerequisite for efficient yet specific transcription by enriching for essential protein components that interact with the template. These complexes contain chromosomal nonhistone and possibly nuclear matrix proteins.

(3) As a prerequisite to cloning and site-specific modification of the T5 DNA polymerase gene, the sensitivity of bacterial transformation by restriction fragments of T5 DNA has been greatly enhanced by devising improved experimental conditions.

(4) A variety of chemical mutagens are potent inhibitors of ribonucleotide biosynthesis and hence lead to an imbalance of nucleotide pools. Recent results suggest a correlation between these imbalances and the observed mutagenic and toxic effects of the chemicals.

(5) A number of variants of a complex, G+C-rich (63%) satellite DNA have been cloned and sequenced. Because of the arrangement of interspersed domains of simple sequences rich in guanine and cytosine, the satellite provides a model system for the study of the influence of primary structure on secondary and tertiary structure of DNA.

(6) A new ultrastructural technique, electron microscope tomography, has been developed which permits an accurate three-dimensional construction of electron-density distributions within a sectioned and stained biological specimen.

(7) In Escherichia coli radiation and chemicals which damage DNA are known to induce certain SOS functions, such as mutagenesis, which require the lexA<sup>+</sup> and recA<sup>+</sup> gene products. Another of the functions, induced shutdown of respiration, has now been shown to also require exonuclease V which is determined by the recB<sup>+</sup> and recC<sup>+</sup> genes.

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 repair pathways; in other cases, membrane components are rendered inactive by combination with exogenous agents and repair mechanisms must then be invoked. Current studies emphasize the dynamic aspects of cell surfaces, including their repair by turnover, protein sorting in the continual exchange of cell surface with a large intracellular membrane pool, and the controls on the differentiation of membrane function. Another aspect of cellular-environmental interactions and membrane integrity which receives emphasis is cryobiology. Recently, the long-held view that injury to cells subjected to slow freezing is due to osmotic dehydration has been challenged. New data suggest that survival is primarily dependent on the fraction of intracellular water remaining unfrozen rather than on the salt concentration therein.

Given the absolute dependence of life processes on catalysis and the adverse consequences of altering catalytic events, our long-standing interests in enzyme mechanisms continues. A major activity is the design of affinity labels for the characterization of catalytic sites. These studies also relate to toxicology in that acute toxic poisoning frequently reflects the specific interaction of a chemical with a key metabolic enzyme; e.g. the inactivation of acetylcholine esterase by nerve gases. Recent advances include (1) characterization of the CO<sub>2</sub>/Mg<sup>2+</sup>-induced activation of the CO<sub>2</sub>-fixation enzyme from Rhodospirillum rubrum, (2) design of new active-site specific reagents for the CO<sub>2</sub>-fixation enzyme, (3) development of an improved procedure for affinity chromatography of GTP-cyclohydrolase, (4) detection of an inducible

cadmium-binding protein in *Drosophila* whose concentration appears to reflect the degree of resistance to cadmium toxicity, and (5) purification of a  $\text{Ca}^{2+}$ -activated neutral protease important in the atrophy of muscle in molting crustaceans.

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.

## MECHANISMS OF MUTAGENESIS AND DNA REPAIR

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Chemical and physical factors associated with energy production have the potential to cause DNA damage that can contribute to human health problems. If meaningful judgments are to be made regarding the health risks associated with energy technologies, it is imperative that we understand the biochemical mechanisms used by the cell to cope with damage to its genetic material as well as the molecular mechanisms responsible for mutagenesis associated with DNA damage. The aim of this research program is to learn more about DNA repair pathways and to study factors that affect the fidelity of DNA replication and repair.

One aspect of our research deals with mechanisms of excision repair in Escherichia coli exposed to chemicals or ultraviolet (UV) radiation. During the past year we have focused our attention on the incision proteins that initiate removal of pyrimidine dimers, benzo[a]pyrene adducts, and other bulky lesions from DNA. The product of the E. coli uvrD gene was isolated and shown to be important in mediating closure of single strand DNA breaks promoted by the incision complex coded for by the uvrA, uvrB, and uvrC gene products (3). This suggests that the uvrD gene product (now known to be a helicase) is necessary either for dislodging the incision complex from the nicked DNA or for preparing a DNA primer-template configuration suitable for proper repair resynthesis. Studies with mutants deficient in DNA single strand binding protein (ssbA) also show an accumulation of unrepaired incision breaks (5). This is probably due to accumulation of double strand DNA breaks (perhaps caused by exonuclease V) in gapped DNA that is not protected by a coat of binding protein. Studies on one of the incision proteins (UvrA) have used site directed mutagenesis to generate an interesting new mutation, uvrA276, cloned on a multicopy plasmid and to partially characterize the effects of this mutant protein on excision repair (Lorenzen, E., Masker, W., Chase, J., submitted for

publication). These experiments have suggested that the UvrA protein has more than one active site, that mutant UvrA proteins altered in different active sites can complement one another, and that the UvrA, UvrB and UvrC proteins form a very stable complex.

Our studies on mutagenesis use a system in which bacteriophage T7 DNA is replicated in vitro and then encapsulated into phage heads in vitro so as to form viable T7 phage. The system has sufficient accuracy and efficiency to allow studies of in vitro mutagenesis and has, in fact, been instrumental in the first direct demonstration that  $O^6$ -methylguanine is a premutagenic lesion (1). Our recent work has improved the efficiency of the packaging system by optimizing the osmotic strength of the reaction mixture (4) and has demonstrated a role for the T7 gene 6 exonuclease in DNA packaging (Dodson, L., Masker, W. E., to be published). These results have improved the usefulness of our packaging system by raising its efficiency above 1% phage production per genome equivalent of input DNA and have provided clues regarding the mechanism by which viral DNA is packaged. We have also used the packaging system to demonstrate in vitro recombination of restriction fragments of T7 DNA into intact replicating T7 genomes. Further studies with analogues of normal DNA precursors have provided suggestive evidence that mismatch repair may figure prominently in the degree of mutagenesis detected with our system.

Our work on repair of damaged T7 DNA in host cells induced for the SOS response has shown that inducible repair mechanisms do operate on T7 but that these mechanisms are distinct in several ways from what is normally seen with other types of phage. Inducible reactivation of T7 is independent of both the host umuC gene product and the analogous pKM101 muc gene product (2). Since T7 DNA synthesis is independent of E. coli host protein, this result argues that the umuC protein may interact with one of the E. coli DNA replication proteins to cause increased survival at the expense of decreased fidelity of DNA synthesis as the DNA replication complex attempts to copy a damaged genome.

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## CONTROL OF TRANSCRIPTION IN EUKARYOTIC SYSTEMS AND STUDIES OF DNA-PROTEIN INTERACTION

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In order to understand the health effects of various agents associated with energy technologies, it is imperative to conduct fundamental studies aimed at elucidating the underlying mechanisms involving the genetic material. The control of transcription in eukaryotic systems is a key process at the genetic level. Investigations in our laboratory are specifically directed towards elucidating the molecular mechanisms of transcription in two animal viral systems, namely, simian virus (SV) 40, and adenovirus 2. We are particularly interested in understanding the mechanisms underlying the initiation of transcription as a key event in gene expression. Since transcription in vivo is known to occur at the level of "chromatin", we are also conducting studies of the transcriptionally active viral nucleoprotein in order to fully understand the regulatory roles of various proteins in viral transcription.

Transcription Studies. Recently, cell-free systems have been developed from eukaryotic, including human, cells that can be utilized to monitor correct initiation of transcription by eukaryotic RNA polymerase II (the enzyme responsible for synthesizing messenger RNA) on promoter-containing viral or cloned DNA templates. Besides measuring promoter strength, capping of the RNA product can be demonstrated in these systems. Low activities for splicing and chain termination can also be shown.

Previous studies from our laboratory have shown that the interaction of template DNA with crude cell-free transcription extracts from HeLa cells leads to the formation of ordered nucleoprotein complexes (NPCs) that have a number of structural similarities with eukaryotic chromatin. However, unlike nucleosomes, no histones could be detected in the purified complexes. Rather, the complexes are generated by the interaction of nonhistone proteins with DNA. Ordered NPCs containing pFLBH (an adenovirus 2 major late promoter-containing plasmid) DNA were purified and concentrated by velocity sedimentation onto 62% (w/w) sucrose cushions and used directly as templates for RNA polymerase II in the HeLa whole cell transcription extract. Transcription of these NPCs yielded an accurate and specific run-off RNA product as demonstrated by nuclease S1 analysis and by denaturation with glyoxal followed by size analysis in 2% agarose gels. Specific RNA synthesis is sensitive to  $\alpha$ -amanitin (1  $\mu$ g/ml). The specific product can be obtained either by prior digestion of the NPCs with restriction enzyme or by restriction in situ.

We have also found that the reaction parameters for the transcription of NPCs are different from those of naked DNA. In general, the salt ( $MgCl_2$  and  $KCl$ ) dependence curves display broader optima with the NPC templates as compared to naked DNA. Similarly, the rather stringent extract/template ratios needed with naked DNA are alleviated with NPC templates, thereby

eliminating the need for tedious assays to establish the optimal conditions for each extract prepared. It appears that the use of NPC's as templates leads to a more easily manageable assay system.

Current cell-free transcription systems are notoriously template inefficient. There is a bulk DNA dependence that can often be satisfied by the use of a synthetic DNA like poly(dI-dC), thereby conserving the precious template. We find that without the use of poly(dI-dC) a concentration of over 5  $\mu$ g/ml naked DNA is needed, whereas with NPC templates about 1  $\mu$ g/ml is sufficient to detect the specific RNA product. Thus, the use of NPC templates increases the template efficiency by about one order of magnitude and obviates the necessity of using poly(dI-dC).

Our results indicate that the NPC's represent authentic intermediates of eukaryotic transcription and suggest that their formation leads to an enrichment of protein components that are required for specific yet efficient transcription.

Isolation of HeLa Nuclear Matrix Proteins and their Binding to DNA.  
The nuclear matrix is the residual nuclear structure obtained after removal of the nuclear membrane and bulk chromatin with a mild nonionic detergent, high salt and nucleases. During the last few years, a number of important functions have been shown to occur in the nuclear matrix, for example, DNA replication, transcription and processing of RNA and its transport, steroid hormone action, etc. Most of the evidence has come from in vivo studies. Very few investigations have been reported on the biochemical properties of the matrix proteins that play regulatory and functional roles in the above processes. This is presumably because of the difficulty of isolating these proteins in their native state.

During the last year we have conducted studies to optimize the conditions for the isolation of HeLa nuclear matrix at low temperatures (0-4°C). We have found that pancreatic DNase I and Alu I, the restriction endonuclease, are effective in almost completely digesting the nuclear DNA within 60 min either at room temperature or 0°C. However, the restriction endonuclease, Eco RI, used by several investigators in matrix isolation is very inefficient even at high concentrations and either at room temperature or 37°C. Thus, the use of DNase I or Alu I has permitted us to conduct the isolation at 0-4°C.

Several detergents were tested for their efficiency in releasing proteins from the nuclear matrix as well as maintaining biological activity of the released proteins (as measured by their DNA binding properties and function during in vitro transcription). We find that sodium dodecyl sulfate at low concentrations (0.1-0.2%) and several zwitterionic detergents that are N-alkyl (either long chain aliphatic or cholamidopropyl) derivatives of sulfobetaines are quite effective in releasing proteins from the nuclear matrix. Nonionic detergents like NP40 and Triton-X-100, although gentle, are inefficient in releasing proteins from the matrix.

Incubation of the isolated proteins with SV40 DNA leads to the formation of ordered nucleoprotein complexes, as revealed by gel filtration, electron microscopy and analysis by micrococcal nuclease digestion. The beaded structures are sensitive to proteases and phenol extraction. These structures resemble the ordered nucleoprotein complexes formed when template DNA is incubated with HeLa cell-free transcription extracts, as described in the previous section. These DNA binding proteins are being tested for their possible roles in transcription.

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#### STRUCTURAL ASPECTS OF DNA IN ITS REPLICATION AND REPAIR

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Toxic byproducts that interact with cellular macromolecules, including DNA, are among the health hazards associated with energy production and utilization. The alterations of DNA as a result of reaction with the genotoxic chemicals may manifest in permanent changes like mutation and cancer. The research objective of this laboratory is to investigate the structure of DNA, the mechanism of DNA replication and its regulation, and the mechanism and role of repair of the altered DNA in the expression of heritable changes. This research has two broad aims, namely investigation of (a) the regulation of DNA replication in mammals, using parvovirus DNA as a model system and (b) the role of DNA repair in mutagenesis and carcinogenesis induced by simple alkylating mutagens.

Structure of DNA of Parvoviruses. Mammalian nondefective parvoviruses contain linear single-stranded (SS) DNA genomes of about 5000 bases. The 3' and 5' termini are present as somewhat imperfect hairpins of about 115 and 190 nucleotides respectively that are not homologous to each other. The *in vivo* DNA replication involves synthesis of linear, double-stranded (replicative form) DNA that either in a monomeric or oligomeric form serves as a precursor of progeny SS DNA, which is subsequently packaged into native virions. Parvoviruses provide a good model for studying regulation of mammalian DNA replication because they are absolutely dependent for replication on the S-phase and the replication machinery of the host cells. Various mechanisms proposed for the mode of viral DNA replication are based on Cavalier-Smith's model of hairpin transfer that involves the 3' hairpins

acting as primers for DNA synthesis without de novo initiation and then transfer of the hairpin to the complementary strand by action of a site-specific endonuclease.

It was shown that the intracellular RF has proteins covalently attached to the termini. We have confirmed the observation that both monomer and dimer RF DNAs have proteins attached at the 5' termini. This was shown by (1) resistance of RF DNA isolated from the infected cells but not synthesized in vitro to  $\lambda$  exonuclease (specific for 5' strand of duplex DNA), (2) sensitivity of both in vivo and in vitro RF to E. coli exonuclease III (specific for the 3' termini of duplex DNA), (3) inability of the in vivo RF (but not in vitro RF) to migrate in agarose during electrophoresis in the absence of sodium dodecyl sulfate, and (4) identical electrophoretic behavior of these RF DNAs after protease treatment. The presence of polypeptide covalently bound to in vivo RF was also shown by comigration of 35S from methionine and  $^3$ H-thymidine (used in labeling KRV-infected NRK cells) during banding of DNA in CsCl and agarose gel electrophoresis (in the presence of SDS). Attempts to label the RF with  $^{125}$ I and subsequent analysis of the protein (after digestion of DNA) by polyacrylamide gel electrophoresis showed several autoradiographic bands including 68K and 40K polypeptides. However, only the 40K band appeared after digestion of DNA. Experiments are in progress to identify the 40K polypeptide, to establish its cellular or viral origin, and to localize it on the DNA molecule.

Because the intracellular RF is present not only as monomers, but also as dimers and higher oligomers, we carried out pulse-chase experiments to establish the precursor of progeny viral DNA. This is an important point because various models have been proposed to explain the unique nucleotide sequence of the 3 terminuses but a "flip-flop" sequence of the 5' terminus of the viral SS DNA. These models invoke an oligomeric RF on the immediate precursor of progeny SS DNA. Our results strongly suggest that the dimeric RF is a dead end product of replication.

We are studying in collaboration with Dr. V. Ramakrishnan of Brookhaven National Laboratory, the architecture of the virion capsid and the condensation of DNA in it by neutron scattering and electron microscopy. The empty capsids purified from infected cells are unusually impermeable to uranyl acetate stain for electron microscopy, suggesting a very tight interaction between the protein subunits. Neutron scattering data are compatible with a 2-shell model for the capsid, where, apparently, the basic domain of the subunits may form the concentric inner shell and interact with the core DNA.

$^6$ -Methylguanine-DNA Methyltransferase in Bacteria and Eukaryotic Cells. We reported a quantitative assay method for the  $^6$ -methylguanine-DNA methyltransferase that acts stoichiometrically by transferring a methyl group to itself. We have worked out a new rapid assay for the enzyme based on this transfer of a methyl group. [ $^3$ H]methyl labeled-DNA (containing 58% of total label in  $^6$ -methylguanine) mixed with  $^{32}$ P-labeled DNA was incubated with E. coli extract containing the methyltransferase. Because

the  $^3\text{H}$ -methyl group was transferred to the enzyme, the reduction of the ratio  $^3\text{H}/^32\text{P}$  in the aqueous layer after extraction of the proteins with phenol is a measure of the methyltransferase. Using this assay we purified the methyltransferase from a methyltransferase constitutive *E. coli* mutant. The enzyme gave a single band after electrophoresis in SDS/polyacrylamide gel and has a molecular weight of 19,000, as determined by electrophoretic mobility, filtration in Sephadex G-75 and equilibrium centrifugation. This value is in agreement with that of Dempte *et al.* (J. Biol. Chem. 257: 13776, 1982), published recently. The methyltransferase tends to aggregate in high mercaptoethanol. We are currently purifying the methyltransferase from *Bacillus subtilis* in order to study the comparative properties of the enzyme from the two bacteria.

We extended our assay of the methyltransferase in different mammalian cell lines, and the results can be summarized as follows. (1) Hamster cell lines (CHO, V79 and derivatives of V79) have no detectable methyltransferase. Syrian hamster liver has about a third (~3500 molecules/cell) as much activity as rat liver. This is in agreement with the published *in vivo* data and is significant in view of the fact that CHO and V79 cells are routinely used as test systems for mutagenicity assays. (2) Contrary to the observation of Waldstein *et al.* (Nucleic Acids Res. 10: 4595, 1982), the methyltransferase is not inducible in several human and rodent cell lines following treatment of the cells with repeated subtoxic doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). (3) The level of the methyltransferase does depend, to some extent, on the cell cycle, the highest level being observed in the late S-phase. Our results also do not agree with the published experiments of Waldstein *et al.* (Proc. Nat. Acad. Sci. USA, 79: 5117, 1982) in that we could not detect any methyltransferase in several  $^0\text{G}$ -methylguanine repair-deficient ( $\text{Mex}^-$ ) cell lines, and *de novo* synthesis of the enzyme took nearly 40 hours rather than 6-12 hours in cells in which the preexisting enzyme was inactivated by exposure to MNNG. Yarosh *et al.* (Mutat. Res., in press) have recently observed that some  $\text{Mex}^-$  cells have an intermediate level of methyltransferase suggesting that  $\text{Mex}$  ( $\text{Mer}$ ) may be a regulatory locus. Our attempts to induce the enzyme by treatment of cells with 5-azacytidine (to inhibit methylation of DNA, which is known to affect gene expression in mammalian cells) have, so far, been unsuccessful.

Base-pairing Properties of  $^0\text{G}$ -Methylguanine. It has been proposed on theoretical grounds that the  $^0\text{G}$ -methylguanine ( $^0\text{G}$ )-thymine pair should be nearly as normal as the A-T and  $^0\text{G}$ -C pair should be unstable. On the other hand, earlier experiments suggested that  $^0\text{G}$  in the template DNA may pair with T about a third as frequently as with C. We investigated the base-pairing behavior of  $^0\text{G}$  present both in the template DNA and as a deoxynucleotide precursor during *in vitro* replication of synthetic DNA primer-template with purified T4 and T5 phage DNA polymerases and *E. coli* DNA polymerase I. Our results on base pairing of  $^0\text{G}$  in template DNA can be summarized as follows: (1) When poly(dT, $^0\text{G}$ ) or poly(dC, $^0\text{G}$ ) containing covalently attached oligo(dA) or oligo(dG) as a primer was used as a template,  $^0\text{G}$  preferred pairing with T six to twenty times over that with C, depending on the polymerase. This is based on an apparent

Michaelis constant ( $K_m^{app}$ ) of dTTP and dCTP during DNA synthesis. T4 DNA polymerase shows more discrimination towards the  $m^6G\cdot T$  pair as compared with the A·T pair than do the other two polymerases. The metal ion cofactor ( $Mg^{2+}$  or  $Mn^{2+}$ ) affects the relative incorporation of dTMP and dCMP directed by  $m^6G$ . (2)  $m^6G$  in the template also directs incorporation of dAMP although the  $K_m^{app}$  for dATP is quite high. While dTPP and dCTP are competitive inhibitors of each other, dAMP incorporation is not inhibited by dTTP. (3) The presence of  $m^6G$  causes inhibition of DNA synthesis. (4) The turnover of dTTP is higher with  $m^6G$  in the template than that of dATP with dT in the template. While these results support the theoretical prediction that the  $m^6G\cdot T$  pair is much more favorable than the  $m^6G\cdot C$  pair, they also indicate that  $m^6G\cdot T$  is hardly a normal A·T-like pair, at least insofar as the DNA polymerases are concerned.

It has recently been proposed that in vivo alkylation of deoxynucleotides in the DNA precursor pool, in addition to alkylation of DNA by alkylating mutagens, may be a major mutagenic event. Our results with incorporation of  $m^6dGMP$  into both synthetic and natural DNA templates indicate that (1)  $m^6dGMP$  pairs preferentially with T in the template, (2)  $m^6dGTP$  inhibits DNA synthesis, apparently acting as a chain terminator, (3) T4 DNA polymerase is much less efficient than T5 DNA polymerase or E. coli DNA polymerase I in accepting  $m^6dGTP$  as a substrate. While in the absence of dATP  $m^6dGMP$  cannot be incorporated (in the presence of dTTP) in the poly(dA·dT) template by any DNA polymerase, the T4 enzyme, unlike T5 and the E. coli enzyme, cannot utilize  $m^6dGTP$  in the presence of a low concentration of dATP. The turnover of  $m^6dGTP$  is much higher with the T4 enzyme than with the other two enzymes. Based on the relative  $K_m^{app}$  of  $m^6dGTP$  and  $K_m^{app}$  and  $K_m^{app}$  of dATP, it appears unlikely that  $m^6dGTP$  is significantly incorporated in vivo to act as a promutagenic precursor. Finally, Stankowski and Hsie (this Division) attempted to incorporate  $m^6dGTP$  in CHO cells. While the permeabilized cells did take up a significant amount of the nucleotide, no  $m^6G$  was detected in the cellular DNA and no mutation was observed.

Synthesis of  $O^4$ -Ethyl-dTTP and Incorporation of  $O^4$ -Ethylthymidine in DNA. In addition to  $O^6$ -alkylguanine,  $O^4$ -alkylthymine, also produced in DNA by simple alkylating agents, is believed to be a promutagenic lesion as well. We have initiated a program to study the repair and miscoding properties of  $O^4$ -ethylthymine ( $e^4T$ ) by utilizing an approach similar to that used for  $O^6$ -methylguanine. We have synthesized both unlabeled and [ $^3H\text{-CH}_3$ ]  $O^4$ -ethyl-dTTP. The unlabeled  $e^4dTTP$  was synthesized by ethylation of thymidine, isolation of  $e^4dT$ , and then stepwise conversion of the deoxynucleoside to the corresponding triphosphate. For synthesis of the labeled  $e^4dTTP$ , we started with 5-hydroxymethyldeoxyuridine. After synthesis and purification of  $O^4$ -ethyl-5-hydroxymethyl dUTP, we have reduced the triphosphate with  $H_2$  in the presence of Pt.  $O^4$ -ethyl-dTTP has been used as a substrate for calf thymus deoxynucleotidyl terminal transferase for the synthesis of poly(dT,  $e^4dT$ ), which was then annealed with poly(dA) to produce duplex DNA and containing  $e^4T$  as the only modified base. We are at present studying the mechanism of repair of the alkylated base.

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## PROCESSING OF RNA IN YEAST

Audrey Stevens

Following transcription of RNA molecules, these molecules undergo unique processing reactions to yield the final active RNA species. tRNA molecules are trimmed at both ends and intervening sequences are removed by cleavage and ligation reactions. The same is true of rRNA molecules. mRNA molecules are capped, polyadenylated, and intervening sequences removed in reactions which are still largely uncharacterized. Each mRNA molecule turns over in both the nucleus and the cytoplasm in order to preserve its optimal cytoplasmic concentration in the cell. Since cell survival is dependent on most of these many RNA molecules, any environmental agent that interferes with the reactions described may be deleterious. Studies of enzymes involved in RNA processing reactions continue in this laboratory.

A unique exoribonuclease of yeast which produces 5'-mononucleotides by a 5'+3' mode of hydrolysis has been investigated further in order to discern the possible function and also the potential use of the enzyme. It has been suggested that the exoribonuclease may have an important function in the cell in the degradation or turnover of mRNA after removal of the 5' cap structure by a decapping enzyme which is found closely associated with the enzyme. It is also possible that the enzyme could serve as a tool in the isolation of capped RNA species if their hydrolysis rate is considerably less than that of uncapped species such as rRNA. A study of the hydrolysis of mRNA has been carried out using cap-labeled mRNA, [<sup>3</sup>H]<sup>m</sup>Gppp-[<sup>14</sup>C]RNA-poly(A), and the rate of hydrolysis compared with that of rRNA. Products ([<sup>3</sup>H]<sup>m</sup>G and [<sup>3</sup>H]<sup>m</sup>GpppN) of the cleavage of the cap-labeled RNA have been separated by paper electrophoresis. The results show that the phosphodiester bonds of the cap structure and the terminal phosphodiester bond of the capped RNA are degraded at less than 10% (about 4%) of the rate of rRNA. Other studies show that all uncapped RNA species are hydrolyzed at very similar rates. The results suggest that the enzyme could play a role in vivo in mRNA turnover with the cap structure providing unique protection and could be important as an in vitro tool in the isolation of capped RNA species.

Studies of a yeast endoribonuclease which is found in the same protein fractions as the exoribonuclease have continued. The hydrolysis of yeast poly(A)-containing RNA by the enzyme is inhibited by ethidium bromide and proflavin, which intercalate in double-stranded structures. At low enzyme concentrations, the hydrolysis is stimulated several-fold by small nuclear RNAs U1 and U2 of Novikoff hepatoma cells. The stimulation appears to involve an interaction of the small nuclear RNA with the poly(A)-containing RNA. The enzyme does not cleave the synthetic polyribonucleotides-poly(U), poly(C), or poly(A). It does not cleave tRNA and hydrolyzes rRNA at about 25% of the rate of poly(A)-containing RNA. The specificity of cleavage of the poly(A)-containing RNA has been determined by degrading the products with alkali, venom phosphodiesterase and P<sub>1</sub> nuclease, and by phosphorylating the products with [ $\gamma$ <sup>32</sup>P]-ATP and polynucleotide kinase. The enzyme

shows a unique preferential cleavage (>90%) of UpA or CpA bonds. In 50% of the scissions, the nucleotide linked to the A is G. The products of the cleavage are U>p or C>p-terminated oligonucleotides complexed with the enzyme. The nature of the complex is under investigation. The cleavage specificity of the enzyme suggests that it may play a role in a key processing reaction. It is possible that it may function in an endonucleolytic inactivation of mRNA (in prokaryotes, the turnover of mRNA is precipitated by an endonucleolytic cleavage) or in splicing of pre-mRNA (UAG or CAG is part of the consensus sequence at the 3' splice junction of all introns).

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

R. K. Fujimura      B. C. Roop

Background. Phage T5 DNA is a linear duplex DNA with a molecular weight of about  $7.7 \times 10^7$  and consisting of more than 100 genes. In vivo analysis suggests that it is capable of initiating DNA replication from several sites with a primary site around a midregion of DNA. Thus we believe that the replication system is intermediate in complexity between that of small circular DNA and eukaryotic chromosomal DNA. It is our objective to characterize every process involved in DNA replication, particularly initiation, translocation, unwinding, and fidelity. We wish further to study effects of mutagenesis of genes, products of which are involved in these replication processes.

So far, two gene products coded by the phage T5 DNA are known to be absolutely essential for DNA replication. These are the DNA polymerase coded by gene D7-D9 and the DNA binding protein coded by gene D5 (gpD5). Both of these are routinely isolated to homogeneity in our laboratory. There are several other phage gene products known that 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 T5 DNA polymerase is a monomeric, multifunctional enzyme. Its most preferred primer template is single-stranded DNA with a primer. It preferentially binds to such DNA at a 3'-OH primer end rather than at an end of a template strand or a single-stranded region. However, it does bind to a nick of duplex DNA and utilizes a 3'-OH end of a nick as a primer and elongates it from such an end, displacing the strand ahead of it. With a

nicked circular duplex DNA 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 poly(dA) used as a template which was about 400 nucleotides long. It 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 may 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 control base analogue incorporation at a specific region thus producing mutation at a specific site.

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

The role of gpD5 in DNA replication is not clear. When nicked DNA is complexed to saturation with gpD5, both DNA polymerase and 3'→5' exonuclease activities are inhibited. Thus this protein may be used in control of fidelity of DNA replication in vitro.

#### Experiments in Progress:

a. Interaction of gpD5 with duplex DNA and its effect on DNA polymerase functions. Interactions of duplex DNA with gpD5 were characterized by velocity sedimentation of the complex to apparent equilibrium in a metrizamide gradient (1,3). The studies showed that duplex DNA saturated with gpD5 has a buoyant density of 1.17 g/cm<sup>3</sup>. The binding of gpD5 to duplex DNA is cooperative in a buffer of low ionic strength, but apparently noncooperative in the presence of 10 mM MgCl<sub>2</sub>. Calculations based on these data showed that at saturation 40 base pairs are covered per gpD5 molecule. When nicked DNA was complexed with T5 DNA polymerase and then complexed with gpD5 to saturation, both the polymerase and its associated 3'→5' exonuclease activities were inhibited (2). E. coli exo III was also inhibited. These findings suggested that enzymes that require properly H-bonded 3' OH 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. For quantitative determination of cooperativity, better techniques are being developed. One possibility

is direct measurement of cluster size by electron microscopy. This is under investigation in collaboration with David Allison.

b. Correlation of structure and function of T5 DNA polymerase. According to genetic analysis, the structural gene for T5 DNA polymerase is located at loci D7-D9 of the "D" segment of T5 DNA. (On the basis of recombination frequencies among various conditional lethal mutants of phage T5, T5 DNA is made up of four "segments;" D is the largest and occupies the region from the middle to the right part of T5 DNA, consisting of genes that are transcribed from the early to the late period of infection.) There is no vigorous work correlating gene loci to physical loci on DNA. However, by transfection of E. coli with restriction enzyme digests that were fractionated according to size and by use of such transfected cells as hosts for "marker rescue" experiments with amber mutants of the T5 DNA polymerase gene, we were able to identify the DNA fragment with the T5 DNA polymerase gene. With recent improvement in the sensitivity of marker rescue experiments we are about to identify a minimal DNA segment with the gene. We have isolated DNA fragments containing a fraction of the gene, and it is being cloned. Meanwhile we have learned to purify large quantities of the restriction fragments of T5 DNA. These were used to isolate some ts mutants of T5 DNA polymerase. We are planning to isolate more, and we hope that these mutants will consist of T5 DNA polymerase with altered properties on fidelity of replication, processiveness of translocation along the template and strand displacement. Essential structural properties of the polymerase for these processes will be studied.

Future Goals. Our objective is to learn to modify specific regions of the polymerase by site directed mutagenesis of its gene or by direct modification of functional domains of the enzyme. By these means we hope to make polymerase that is more efficient as replicative polymerase, as repair polymerase, or as mutator polymerase. We may be able to make conditional mutator polymerase that will alter genes cloned into a variety of vectors with different requirements. Such a mutator polymerase may be used to study effects of agents associated with energy technologies.

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## NUCLEIC ACID BIOCHEMISTRY OF CELLS

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One of the major programs of the Biology Division is directed toward determining the mechanisms by which a variety of energy-related products may cause pathological conditions (mutations, cancer, toxicity, etc). Our particular approach has been to investigate the effect of these agents on the nucleotide pools - the proximal precursors in the synthesis of DNA and RNA.

We have shown that nucleotide biosynthesis in Novikoff hepatoma and CHO cells is markedly altered by a variety of chemical mutagens (N-methyl-N'-nitro-N-nitrosoguanidine, 4-nitroquinoline 2-oxide, 9-aminoacridine, and mitomycin C). Concentrations of these compounds that cause some inhibition of RNA and DNA synthesis bring about a large increase in the pool levels of all four nucleoside triphosphates. At the same time, the biosynthetic pathways leading to the synthesis of CTP from exogenous uridine and GTP and ATP from exogenous hypoxanthine are severely inhibited. The mutagens cause similar inhibitions in the *de novo* purine and pyrimidine nucleotide synthesis routes, as demonstrated with experiments with labeled formate and aspartate. Thus, a widely divergent reduction in the labeling of RNA-CMP versus RNA-UMP and of RNA-GMP versus RNA-AMP is created, mostly a result of these agents causing large differences in the specific activities of the respective triphosphate precursors. These observations suggest that, in addition to the reactions with DNA, nucleotide biosynthesis is another important biochemical target of chemical mutagens.

More recent experiments using an excess of deoxyribonucleosides such as thymidine in the growth medium show that these compounds not only alter DNA synthesis, but also have a profound effect on the ribonucleotide pool and RNA synthesis itself. Taken together, these experiments reinforce the hypothesis invoked by many laboratories that severe imbalance of nucleotide pools, particularly as brought about by mutagenic agents, may be a major cause of mutations and/or toxicity.

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## CHROMOSOME CHEMISTRY

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The major goal of this laboratory is to analyze and understand the macromolecular structure of eukaryotic chromosomes. This macromolecular structure is intimately involved in the three major functions of chromosomes: DNA packaging, transcription, and replication. Any of these functions can be damaged by exposure to abnormal physical or chemical agents. Our laboratory employs a wide range of biophysical, biochemical and ultrastructural techniques to work towards detailed macromolecular models. During the past year our laboratory has concentrated its efforts in two major directions: (1) chromatin structure in the hypotrichous ciliated protozoa, and (2) 3-D reconstruction of a transcriptionally-active gene by electron microscope tomography (EMT).

1. Chromatin Structure in the Hypotrichous Ciliated Protozoa. All ciliated protozoa exhibit nuclear dualism, i.e., the existence of transcriptionally-active macronuclei in the same cytoplasm with inactive micronuclei. The hypotrichous ciliated protozoa possess two distinct nuclear features that distinguish them from other ciliates: (1) macronuclei consist of a "bag" of high polyploid (ca. 10<sup>4</sup> fold), short (ca. 2-3 kbp), linear DNA molecules of low sequence complexity - each fragment probably corresponding to an individual structural gene and its regulating flanking sequences; and (2) macronuclear DNA replication is localized exclusively in a Replication Band (RB) that migrates along the nucleus during S phase. Both of these features are unique in biology and offer considerable advantage compared to typical eukaryotic nuclei. In order to capitalize upon these advantages we have spent considerable time with the following projects: (1) large-scale cultivation and harvesting of protozoa, especially *Oxytricha* and *Euplotes*; (2) lysis of cells and isolation of macronuclei, with minimum nucleolytic and proteolytic degradation; (3) preparation of macronuclear DNA fragments, gel electrophoresis and blot-hybridization with specific gene probes; (4) preparation of soluble chromatin, with characterization of nucleosome repeat lengths and companion biophysical parameters (i.e., thermal stability and circular dichroic spectra); (5) analysis of various nuclear histones and non-histones; (6) development of unique cytochemical reactions for the Replication b d (7) isolation and characterization of the chromatin properties of RB; an (8) ultrastructural analysis of the patterns of replicating DNA in the RB. Current experiments are focusing upon: (1) the subfractionation of *Euplotes* macronuclear chromatin into transcriptionally-active and inactive species, and (2) the development of specific immunochemical probes for replicational machinery components.

2. Three-dimensional Reconstruction by EMT. We have deve oped 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. In the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. Using EMT, we have analyzed the 3-D arrangement *in situ* of these RNP around the chromatin, as well as the patterns of folding of the nascent RNP. We are currently pursuing EMT on a number of other chromosomal structures including the 25 nm higher-order arrangement of nucleosomes and the Replication Band of hypotrichous ciliates. Furthermore, we are developing a 3-D display viewer that would permit user-interactive manipulation, editing and model building of the reconstructed images.

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## NUCLEOSOME AND CHROMATIN BIOPHYSICS

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The major goal of this laboratory 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) small-angle neutron scattering studies of nucleosomes, HMG-nucleosome complexes, and nucleosome oligomers; (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<sub>1</sub> 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.

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. 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. SANS has been used to evaluate the conformational effects of binding HMG 14 to mononucleosomes in a stoichiometric 2:1 complex. This study provided important insights as to how HMG 14 may stabilize an unfolded nucleosome. An additional study involving HMG 17 is planned.

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.

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#### X-RAY DIFFRACTION

C. H. Wei

One of the simplest  $Pt^{2+}$  complexes, cis-diamminedichloroplatinum(II) (cis-DDP), is known to be a clinically effective antitumor drug. The binding of cis-DDP to DNA by intercalation is presumably responsible for its selective cytotoxicity. The toxic mechanism of this compound is being intensively studied. Molecular configurations of cis-DDP-like complexes involving nucleoside are therefore useful information in our understanding of structure-function relationships.

A compound of presumed formula  $Pt(Ado\cdot Cl_2)$  was prepared in 1978 by J. D. Hoeschele (Health and Safety Research Division) by the reaction of  $K_2PtCl_4$  with adenosine. Repeated attempts to crystallize the brownish powder product finally yielded yellow crystals from a mixed solution of pyridine and DMSO. Useful untwinned crystals (I) were obtained from a solution of 1,2-dichloroethane. When complex I was further treated with pyridine and DMSO (volume ratio 9:1) in the presence of a small amount of water over a steam bath, large colorless crystals were obtained (II).

Subsequent structural analyses by X-ray diffraction have shown complex I to be *trans*-dichloro(dipyridine)platinum(II) and complex II to be tetrapyridineplatinum(II) dichloride trihydrate. As expected, the  $\text{Pt}^{2+}$  ion possesses a typical square-planar configuration in each case with  $\text{Pt}^{2+}$  coordinated to two  $\text{Cl}^-$  and two N atoms of pyridine ligands in complex I, and to four N atoms of pyridine molecules in complex II.

As part of our efforts to synthesize metal complexes with moieties of DNA,  $\text{K}_2\text{PtCl}_4$  was reacted with guanosine some years ago. From the greenish amorphous product greyish chunky crystals were grown from an aqueous solution. Prior to the X-ray investigation, it was thought that the crystals were those of a complex form of platinum and guanosine and therefore merited structural analysis. Results of the X-ray analysis, however, revealed that the crystal was that of bulbocapnine (an aporphine alkaloid) methiodide, structure of which had previously been determined by S. Basu and J. R. Einstein some five years ago (has not been published). Although the compound was not a platinum complex of guanosine, as anticipated, this example serves to demonstrate the powerfulness of the X-ray characterization method in analyzing a solid compound to unravel its ultimate structure. The advantage of our determination over that of Basu-Einstein is that more data were collected by the use of  $\text{Mo K}\alpha$  radiation from a larger crystal, thereby the apparent high precision of the molecular parameters was made even more credible.

The structural investigations of the above-mentioned three compounds were accomplished in collaboration with B. E. Hingerty of the Health and Safety Research Division. The X-ray counter data were collected in the laboratory of G. M. Brown and W. R. Busing of the Chemistry Division.

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

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We have cloned into three different restriction enzyme sites of pBR322 repeat units or subunits of a G+C-rich (63% G+C) satellite that accounts for 3% of the genome of the Bermuda land crab, Gecarcinus lateralis. The satellite, which is very much more complex than any other satellite characterized to date with the exception of a minor component of very highly repeated DNA scattered throughout the genome of humans, has an average  $2.07 \pm 0.1$  kb repeat unit. A series of variants of the satellite contain transitions, transversions, amplifications, insertions and/or deletions from specific regions of as little as a few and as many as some hundreds of base pairs. The cloned variants of the satellite offer a unique opportunity to determine structure-function interactions.

We have sequenced three of the cloned satellite variants. One variant is close to the average size of the repeat unit of the satellite (RU, 2.089 kb); another is truncated (TRU, 1.639 kb) by the presence of an extra Eco RI site near its 3' end; a third is extended (EXT, 2.639 kb). EXT has a 142 base pair segment that has undergone a fivefold amplification to yield six tandem copies of the segment. The amplified sequence is bounded by a tetranucleotide inverted repeat; the upstream arm of the inverted repeat is missing from the RU and TRU. The latter variants contain only one copy of a sequence that is closely related to the amplified sequence. By contrast to the amplification seen here, in several satellite DNAs of other organisms, extra DNA is inserted. These three variants have been stable through multiple rounds of replication in HB101 and therefore seem unlikely to have been derived from cloning accidents.

Comparisons among the sequences of the three variants are informative. There are highly conserved domains (from 84 to 96% homology); these are interspersed with other domains of marked sequence divergence. The conserved domains can be as long as 594 bp; they contain "garden variety" DNA. The divergent domains contain repeated sequences, such as homopolymers [ $n = 7+23$ ] of  $C_n$  or  $G_n$  but not  $A_n$  or  $T_n$ ; or homopolymers [ $n = 4+17$ ] of  $(CCT)_n$ ,  $(CA)_n$ ,  $(AGGG)_n$ , or  $(AG)_n$  but not  $(AT)_n$ . Other regions adjacent to major sequence variations are characterized by long

stretches of alternating purines and pyrimidines (pu/py). That the latter adopt a Z conformation appears likely, since at least one stretch of pu/py of complex sequences which should not permit strand slippage exhibits  $S_1$  sensitivity. Thus, the satellite variants provide a model system for the study of Z-DNA in naturally occurring sequences.

In contrast to the overall similarity among restriction maps of RU, EXT, and TRU,  $S_1$  cleavage patterns are quite distinctive. This lends strong support to our proposal that divergences in simple repeated sequences can markedly affect the secondary and tertiary structure of DNA molecules with overall high homology.

Beyond the obvious homoco- and homopolymers, five tandem (CGCAC)<sub>8</sub>, and other variations on that pentamer, or the fivefold amplification in EXT, computer searches have failed to reveal any large blocks of simple repeating sequences and their variants. The complexity of some domains of this crab satellite appears to be real.

Given such complexity, is the satellite transcribed and translated? The three possible reading frames of the sequence of RU have been searched for initiation and termination codons. In the three reading frames on one strand, there are 6, 5, and 5 initiation codons and 9, 18, and 23 termination codons. The longest possible protein coded for in each frame is 163, 97, and 49 amino acids. A similar analysis, reading in from the 5'-end of the opposite strand, yields longest possible peptides of 84, 77, and 23 amino acids. The microheterogeneity of the satellite does not make transcription any the less likely, since mutations in the wobble base may yield such microheterogeneity without significantly altering the ultimate peptide readout.

Other research has been on:

a. Isolation of a  $Ca^{2+}$ -dependent neutral proteinase. The claw closer muscle of Gecarcinus undergoes a sequential atrophy and restoration during each molt cycle. We have purified and characterized a  $Ca^{2+}$ -dependent proteinase involved in the turnover of myofibrillar protein in normal anecdysial (intermolt) and atrophying proecdysial (pre molt) claw muscle. Crab  $Ca^{2+}$ -dependent proteinase degrades the myofibrillar proteins actin, myosin heavy and light chains, paramyosin, tropomyosin, and troponin-T and -I. The enzyme is a sarcoplasmic cysteine proteinase that shows maximal activation at 1 mM  $Ca^{2+}$  at neutral pH. Hydrolysis of <sup>125</sup>I-myosin occurs in two phases, both  $Ca^{2+}$ -dependent. Large fragments produced in the first phase are hydrolyzed to acid-soluble material in the second. Although radioiodinated native hemolymph proteins are not susceptible to the  $Ca^{2+}$ -dependent proteinase, those denatured by carboxymethylation are degraded. These data suggest that crab  $Ca^{2+}$ -dependent proteinase is involved in turnover of myofibrillar protein in normal muscle and muscle undergoing proecdysial atrophy.

b. Ecdysteroid titers in blue crabs. While ecdysteroid titers in other crabs reach a peak just prior to ecdysis, those of the edible blue crab were claimed to reach their maxima after ecdysis. Our recent data challenge these findings. We have measured ecdysteroids in hemolymph, ovaries, and whole animal extracts of blue crabs using a RIA. In hemolymph and whole animals, ecdysteroid levels rose during proecdysis to a maximum at stage D<sub>3</sub>. Ecdysteroids declined rapidly from late proecdysis through metecdysis, then increased slightly, and returned to low levels where they remained during anecdysis.

c. Partial characterization of a crustacean mono-oxygenase. We have partially characterized the enzyme in Gecarcinus that converts  $\alpha$ -ecdysone to 20-hydroxyecdysone, the active form of the molting hormone. Of the tissues examined, midgut gland was most active in metabolizing  $\alpha$ -ecdysone to 20-OH-ecdysone, which was the only metabolite. The enzyme is localized in a mitochondria-enriched cell fraction, has a  $K_m$  of 1  $\mu$ M and a temperature optimum of 30°C, similar to the mono-oxygenases in other arthropods. It is, however, exceptional in that it requires a high pH (pH 8.3-8.8 optimum) and does not have an absolute requirement for NADPH. The hydroxylase is fully active during anecdysis and proecdysis but not at the time of ecdysis. This finding indicates that the rise of 20-OH-ecdysone levels is not reflected in hydroxylase activity.

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

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E. B. Wright                           J. Ferre<sup>3</sup>

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

During the past year our studies have shown that (1) the defective step for the purple mutant involves the synthesis of ramipterin, (2) ramipterin is an unusual pteridine whose properties are similar to those of 5,6-dihydropteridine, (3) ramipterin loses one or more hydrogens on conversion to a closely related metabolite called  $X_1$ , (4)  $X_1$  would be an appropriate precursor for 6-acetylpyrimidodiazepine whose structure we established recently, (5) 6-acetylpyrimidodiazepine has the ability to replace riboflavin for the growth of Lactobacillus casei, and (6) 6-acetylpyrimidodiazepine is quite similar to a chromophore that is isolated from numerous lymphoid cells. Also we found that an improved procedure for isolation of GTP cyclohydrolase was possible thus making a contribution to the technique in the study of pteridine biosynthesis. One of our immediate goals is to study ramipterin synthase and we have devised a convenient assay procedure for the assay of this enzyme activity. At this time progress continues in unraveling steps in pteridine biosynthesis and we are better prepared to study ramipterin synthase.

In previous studies a role of tRNA<sup>Tyr</sup> had been suggested in the mechanism of suppression by su(s)<sup>2</sup>. At that time we predicted a difference in the minor base composition between normal and su(s)<sup>2</sup> tRNA<sup>Tyr</sup>. Recently we isolated these tRNAs from the usual laboratory strains of normal and su(s)<sup>2</sup> *Drosophila* and demonstrated two minor base changes. However, we also constructed two strains of *Drosophila* su(s)<sup>+</sup> and su(s)<sup>2</sup>, that are genetically identical except for the <1% of their genome that accompanies the su(s) locus. These tRNA<sup>Tyr</sup> were isolated and found not to have a difference in base composition. This is not consistent with our earlier hypothesis regarding the involvement of tRNA<sup>Tyr</sup> in the mechanism of suppression. Henceforth we plan to turn our attention elsewhere in the study of su(s)<sup>2</sup>.

Toxic Mechanisms. The physical chemical properties of metal ions have been measured in many ways, but our primary goal is to determine which of these many parameters are most relevant to the biological damage 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).

Earlier studies had identified cadmium as the most toxic divalent ion for Drosophila melanogaster. Using <sup>109</sup>Cd the rate of uptake and release of Cd<sup>2+</sup> was measured for two strains of *Drosophila* that are most resistant and most sensitive to this metal ion. No difference between the strains was found for the rate of cadmium uptake but they do differ in the rate of excretion. The more resistant strain retained the cadmium for markedly longer times than the sensitive strain; we had predicted the opposite behavior. This indicated that the resistant strain could immobilize the cadmium so we looked for a cadmium-binding-substance that could chelate the toxic ion. Indeed such a substance does appear when flies are fed cadmium chloride and the levels in the resistant strain exceed those in the sensitive strain. Currently we are preparing to isolate and characterize this naturally occurring, inducible chelator of cadmium. The mechanism by which this organism can complex and immobilize toxic metal ions may be of general application and help explain how resistance is achieved.

To explore the effects of metal ions on nucleic acids the study of Zn<sup>2+</sup> on transfer RNA is continuing. To facilitate the use of physical chemical techniques we now use highly purified tRNA<sup>Phe</sup> of yeast. As in previous chromatographic studies with other tRNAs, tRNA<sup>Phe</sup> adsorbs to the RPC-5 column more strongly in the presence of Zn<sup>2+</sup> and in proportion to the concentration of Zn<sup>2+</sup>. Neither Mg<sup>2+</sup> nor Cd<sup>2+</sup> cause this effect. Detailed studies on the effects of Zn<sup>2+</sup> showed that isocratic elution as well as gradient elution gave similar results and that the effect of Zn<sup>2+</sup> could be countered by Cd<sup>2+</sup> or Mg<sup>2+</sup>; the former is much more effective, surprisingly. High resolution nuclear magnetic resonance spectra allow each hydrogen-bonded base pair and base triple to be observed. Zn<sup>2+</sup>, Cd<sup>2+</sup> and Mg<sup>2+</sup> each causes alterations in this secondary and tertiary structure; again Cd<sup>2+</sup> and Mg<sup>2+</sup> exhibit effects that are similar to each other and different from

those caused by  $Zn^{2+}$ . These new studies are incomplete but very promising. Using small angle neutron scattering we are starting to evaluate the interaction of  $Zn^{2+}$  and tRNA<sup>Phe</sup>. Similarly a detailed study of the melting behavior of tRNA<sup>Phe</sup> is being initiated to evaluate how metal ions perturb the tRNA structure. The studies on tRNA are interesting in themselves and may also be considered model studies that would find application in evaluating the effects of metal ions on DNA and other RNAs.

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## DNA DOSIMETRY APPLIED TO PROBLEMS IN GENETIC TOXICOLOGY

R. O. Rahn      Helen Sellin

Our major goals are to devise methods for quantifying low levels of DNA damage, to analyze the chemical nature of DNA base damage, and to understand the mechanisms by which chemical and physical agents cause DNA damage. There is a need to understand the relationship between genetic damage and biological response, i.e., how much of a given type of damage contributes to the possibility of a cell being transformed. There is also a need to know what normal background levels are for a given form of damage. Finally, there is a need to know how much damage to DNA results when a cell is exposed to a given insult from the environment. Studies have been conducted using UV, metal ions, and polycyclic aromatic hydrocarbons as DNA damaging agents. The UV studies have focused recently on the substitution of halogenated bases in DNA as a means of introducing a specific type of photo damage into the DNA. The metal ion studies have centered on platinum binding to DNA because of its role in treating cancer and because of the availability at Oak Ridge of the radioisotope  $^{195}\text{Pt}$ . Finally, studies have been done to quantify very low levels of binding to DNA of benzo(a)pyrene, a chemical carcinogen, which is produced in the synthesis of synthetic fuels.

A method has been devised for removing Pt-base adducts from DNA and for separating these adducts chromatographically. This method has been applied to DNA isolated from tissue culture cells treated with cisplatin. The results indicate that a significant (~35%) portion of the cisplatin binds in a form which is the same as that found in DNA treated in vitro. This adduct consists of two guanine molecules connected by a platinum atom. It remains to be determined how the rest of the platinum binds in vivo, but one likelihood could be a crosslink with protein. The significance of our results at present is that a methodology now exists for examining the binding under in vivo conditions. From such information a better understanding of how cisplatin functions as an antitumor agent can be derived. In turn, more efficient means of using antitumor agents can be obtained.

A very useful tool in photobiological research has been the substitution of BrdUrd for Thd in DNA. Following radiation, debromination and damage to the sugar phosphate backbone results. Also uracil is formed. However, the actual chemical event responsible for the observed enhanced cell killing is not known. Neither single strand chain breaks nor uracil formation is considered important. Our attempts to answer this question have employed the use of IdUrd instead of BrdUrd, because of certain spectroscopic and chemical advantages. The information gained with IdUrd should apply to BrdUrd. Current research deals with the mechanisms by which the uracil radical formed upon dehalogenation reacts with its environment to pick up a hydrogen atom and form uracil. There are many interesting questions one can ask and a great deal of relevance to research conducted by others involving the reaction of allyl radicals in aqueous media. Considerable information has also been gathered concerning the

origin and fate of the iodide and/or iodine atoms formed upon photolysis. From such information we can understand the mechanism by which the Hoechst dye 33258 can sensitize dehalogenation of DNA using 350 nm radiation. In particular, is there an electron ejection mechanism involved resulting in iodide but not iodine release.

Progress on the refinement of our fluorescent-HPLC assay for detecting benzo(a)pyrene binding to DNA has not proceeded as quickly as one would like because of the funding cutback. In fact, little progress has been made in adapting the laser on hand (Cd·He,  $\lambda_{ex} = 325$  nm) to this purpose. After three moves, the equipment is finally located in a place where experiments can be run, and it is expected that in the near future data will start being collected.

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#### RESPIRATION SHUTOFF IN ESCHERICHIA COLI AFTER FAR-UV IRRADIATION

Paul A. Swenson      I. L. Norton

Damage to DNA of Escherichia coli by UV, ionizing radiation and chemicals causes a number of responses that require the recA<sup>+</sup> and lexA<sup>+</sup> gene products. The responses include error prone repair (as indicated by mutagenesis), filamentation and induction of prophage lambda. We study another important rec/lex response, shutoff of respiration, which occurs 60 min after exposure to UV. Our objective is to understand the genetic and biochemical bases of the shutoff process and its control.

Most of our work on UV induced shutoff of respiration has been done with Escherichia coli B/r and Salmonella typhimurium strains. Work was not previously done with E. coli K12 strains because the shutoff response was not seen by us and by others. K12 strains are more resistant to UV killing than B/r and we found that at higher UV fluences (125 J/m<sup>2</sup> vs 52 J/m<sup>2</sup> for B/r) a good shutoff response was obtained. The UV induced response in E. coli K12 strains W3110 and AB1157 is eliminated in both strains by 5

fluorouracil as in E. coli B/r. On the other hand, the response was amplified by cAMP in W3110 but not in AB1157. Mitomycin C treatment caused respiration shutoff in both strains.

One of the advantages of being able to study UV induced respiration shutoff in E. coli K12 strain AB1157 is that there is a wealth of mutant strains available. As expected, the recA and lexA derivatives of this strain did not show UV inducible shutoff of respiration. Two other rec mutants F and J did shut their respiration off; both are deficient in DNA repair and recombination functions. Both umuC and uvrD mutants showed normal UV induced respiration shutoff responses. The umuC<sup>+</sup> gene is required for mutagenesis and umuC mutants are only slightly UV sensitive; the uvrD<sup>+</sup> gene codes for a DNA helicase and the mutant is UV sensitive.

No rec/lex responses to UV damage to DNA have been reported to be affected in recB or recC mutants. We found, however, that UV induced respiration did not take place in them. The recB<sup>+</sup> and recC<sup>+</sup> genes each code for a subunit of exonuclease V which degrades DNA in irradiated cells. recB and recC mutant strains are UV sensitive and show about 99% loss of recombination ability.

The sbcA and sbcB mutant genes each suppress the radiation sensitivity and recombination deficiency effects in a recB recC mutant strain but they do not affect the lack of ability to shut off respiration in this strain. The sbcA<sup>+</sup> gene controls the production of exonuclease VIII and the sbcB<sup>+</sup> gene codes for exonuclease I. The sbcA and sbcB mutants each have normal UV sensitivity and showed normal UV induced shutoff of respiration. The recB recC sbcA and recB recC sbcB strains are the only strains known to us that show normal UV sensitivity to killing but which do not show the respiration shutoff response.

Graham Walker and Cynthia Kenyon of the Massachusetts Institute of Technology have isolated a group of din mutants of E. coli K12. These DNA damaged inducible mutations were formed by transducing a lac<sup>+</sup> gene via  $\mu$  phage into promoter control regions of E. coli. They selected for transductants that formed  $\beta$ -galactosidase when their DNA was damaged by UV radiations. We tested six of these din mutants for their ability to shut off their respiration after UV irradiation. Four of them showed a normal respiration shutoff response, but two of them did not shut off their respiration. It is possible that one of these two strains contains a mutation in a gene which controls UV inducible respiration shutoff.

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## REGULATION OF TRANSPORT SYSTEMS IN CULTURED MAMMALIAN CELLS

J. S. Cook                    C. J. Shaffer  
 J. B. Fishman<sup>1</sup>            E. R. Weiss<sup>1</sup>

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

Biosynthesis and Turnover of Na,K-ATPase. This membrane-bound transport enzyme is the fundamental agent in regulating the salt content of cells. It plays a major role in controlling other transport systems to which it is coupled as well as in the maintenance of the cell membrane potential, cell volume, and an appropriate ionic milieu for the activity of cellular enzymes. This essential transport enzyme is itself maintained at appropriate levels and in a state of repair by constant turnover and replacement of the surface protein. The regulation of turnover is to some degree specific: in normal growth conditions Na,K-ATPase turns over 6-10 times faster than the bulk of membrane proteins. When the cells are K-starved its turnover is reduced with no change in the biosynthetic rate; this results in a compensatory increase in surface activity.

The enzyme is composed of a catalytic subunit ( $M_r = 94,000$ ) and a glycosylated subunit ( $M_r = 45-50,000$ ) of unknown function. Other laboratories working on the biogenesis of the enzyme have suggested that the catalytic subunit is synthesized on free cytosolic ribosomes while the glycosylated subunit is synthesized on ribosomes bound to the endoplasmic reticulum. In rat tumor cells, HTC, we find no evidence for free catalytic subunit; it is always associated with a membrane compartment. With an antibody method we can detect the onset of its synthesis 2 min after the addition of tracer. Over the next 5 hr it moves through a series of membrane compartments until it enters a compartment that includes, but may not be exclusively composed of, the cell surface. Turnover takes place from this compartment at a rate of one set of surface molecules per half generation.

Recycling of Cell Surface Sialoglycoproteins. We reported last year that after the general labeling of HeLa cell surface sialoglycoproteins they could be observed to exchange with an internal pool twice the size of

the cell surface. From this pool the glycoproteins were removed slowly ( $t_{1/2} = 100$  hr) and degraded in turnover. By fractionation studies we have followed the pathway for the internalization side of recycling. The surface material moves into endosomes and is transferred into the lysosomal compartment. Most of the label is not degraded in the lysosomes but is further transferred into the Golgi compartment. This step is blocked if the acidification of the endosomes and lysosomes is inhibited with chloroquine or  $\text{NH}_4\text{Cl}$ . The label in the Golgi finally accumulates in a compartment distinct from either lysosomes or Golgi but with the properties of GERL (Golgi-endoplasmic reticulum-lysosome complex). Presumably most of the recycling back to the surface takes place from this complex, although some membrane in the earlier steps on the pathway may circumvent the later steps and return directly to the surface. Analysis of the proteins at each point shows that a few of them do not recycle but are degraded after the first internalization. The sorting appears to occur at the endosome/lysosome step. This dynamic behavior of the surface has profound implications for the conceptual view of what constitutes the cell surface and how its activities are regulated.

Differentiation of the Concentrative Hexose Transporter In Vitro in Cultured Kidney Cells, LLC-PK<sub>1</sub>. This transport enzyme is the expression of a highly differentiated function of the renal proximal tubule, and is the transporter responsible for the reabsorption of filtered glucose from the urine. In cultured pig kidney cells LLC-PK<sub>1</sub>, which we have re-cloned, the activity is not observable during the growth phase of the cells, but develops slowly (days) after confluence. By radioautographic methods we have demonstrated that the cells require 72 hr in G<sub>0</sub> to develop the function. The methods are being refined to determine the kinetics on a cell-by-cell basis.

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## THEORETICAL AND APPLIED CRYOBIOLOGY

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 and cattle 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.

Research during the last year emphasized two major problems. The first concerns the mechanism of slow freezing injury. The second concerns the osmotic response of embryos to the cryoprotectants glycerol and DMSO.

Mechanisms of Slow Freezing Injury. As aqueous solutions freeze, the electrolytes in the external solution progressively concentrate, and if cooling is slow, the cells undergo progressive osmotic dehydration. It has been 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. The phase rule dictates that solute concentration and fraction unfrozen are reciprocally related and ordinarily inseparable. One can separate them, however, by freezing cells in ternary solutions of nonelectrolyte, electrolyte, and water which differ in total solute concentration but maintain a fixed ratio of nonelectrolyte to electrolyte. We have performed these experiments with human red cells in solutions of glycerol-NaCl-water. Samples were frozen to various subzero temperatures which were chosen to produce various molalities of NaCl (0.24 to 3.30) while holding the fraction unfrozen constant, or conversely to produce various fractions unfrozen (0.03 to 0.5) while holding the molality of salt constant. Damage became extensive when more than 90% of the solution became frozen regardless of whether the salt concentration in that unfrozen fraction was as low as 1 molal or as high as 2.4 molal.

Investigators have reported, and we have confirmed this past year, that the hemolysis of red cells frozen to given subzero temperatures rises

sharply when the cell hematocrit is above 40%. Thinking that this rise might be a consequence of effects of cell concentration on the unfrozen fraction, we have repeated the experiments referred to above with red cell hematocrits of 2%, 8%, 40%, and 60%. At hematocrits of 40% and 60%, the cells themselves occupy a considerable percentage of the unfrozen portion of the suspension. Or put differently, at the higher hematocrits the fraction of the suspension that remains unfrozen at a given temperature is considerably smaller than the fraction of the solution that remains unfrozen. When we plot survival vs the fraction of the suspension that remains unfrozen, most of the effects of hematocrit disappear.

If these findings o the significance of unfrozen fraction turn out to be applicable to cells other than erythrocytes, 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.

As a first step in determining their generality, we are studying the survival of mouse embryos as a function of unfrozen fraction and the concentration of salt in that unfrozen fraction. To date some 3300 mouse zygotes have been subjected to subzero temperatures producing a range of unfrozen fractions and a range of concentrations of NaCl in that unfrozen fraction. Also included in the study are various weight percent ratios of salt to glycerol. Definitive conclusions will have to await our completing the development of computer programs to analyze the data but it appears tentatively that the results are similar to those for red cells.

Osmometry of Mouse and Bovine Embryos and Their Permeability to Glycerol and DMSO. Embryos like most cells require the presence and permeation of cryoprotectants like glycerol and DMSO to survive freezing and thawing. However, the abrupt addition of these additives causes transient osmotic shrinkage, and their abrupt removal by dilution produces considerable osmotic swelling. If one knows the tolerated limits of shrinking and swelling, if in that range the embryos behave like ideal osmometers (i.e., plots of cell vol. vs 1/osmolality are linear), and if one knows the permeability coefficient of the additive, one can design optimum procedures for the addition and removal of the additives using sets of permeability equations developed several years ago in this laboratory. This past year we have been obtaining the necessary information for mouse and bovine embryos. We find that mouse and bovine blastocysts behave like ideal osmometers over hypo- and hyper osmolalities varying from 0.08 to 1.7. They survive 30-min exposure at 23°C over a range of 0.1 to 1.1 osmolal, but are damaged outside that range.

We have also determined the permeability coefficients of day-7 bovine blastocysts to DMSO and glycerol by an indirect method involving measurements of the volume changes of embryos from serial photographs as a function of time in the cryoprotectant. Then curves of relative volume vs time were plotted and compared with curves generated by the computer for several permeability coefficients to determine which coefficient yielded

the best fit. At 23°C the permeability coefficient is estimated to be  $1.5 - 1.8 \cdot 10^{-3}$  cm/min for DMSO and  $2 - 2.5 \cdot 10^{-3}$  cm/min for glycerol.

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

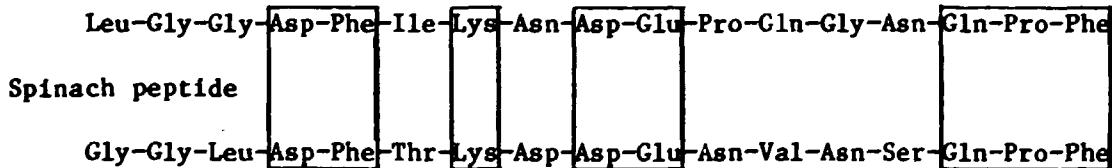
Many of our recent investigations have centered on ribulosebis-phosphate carboxylase, the plant enzyme essential for the photosynthetic assimilation of CO<sub>2</sub>. This enzyme also possesses inherent oxygenase

activity which accounts for photorespiration, a nonessential, energy-wasteful process that reduces net  $\text{CO}_2$  fixation. There is general agreement that preferential abolition of the oxygenase activity would elevate by 50% the yields of C3 plants (plants in which ribulosebisphosphate is the initial acceptor of atmospheric  $\text{CO}_2$  in contrast to C4 plants in which phosphoenolpyruvate is the initial acceptor). Thus, an understanding of the in vivo modulation of the carboxylase/oxygenase ratio and a determination of whether this ratio can be systematically manipulated by external means are of major significance to agriculture and production of biomass.

In the absence of  $\text{CO}_2$  and  $\text{Mg}^{2+}$ , ribulosebisphosphate carboxylase is devoid of both catalytic activities. The activation process entails the reaction of  $\text{CO}_2$  with a specific lysyl  $\epsilon$ -amino group to form a carbamate which requires  $\text{Mg}^{2+}$  for stabilization. Activator  $\text{CO}_2$  is distinct from substrate  $\text{CO}_2$ ; hence, characterization of the activator site as well as the catalytic site are pertinent to elucidation of mechanism of action and mode of regulation. During the past year, we have made substantial progress in mapping both sites.

The activation process for the enzyme from the purple, nonsulfur photosynthetic bacterium Rhodospirillum rubrum has been carefully examined by chemical and physical methods. Stabilization of the carbamate by esterification with diazomethane permitted the isolation of a chymotryptic peptide containing the lysyl residue that is reactive toward  $\text{CO}_2$ . Considerable sequence homology is seen between this region and the corresponding one in the spinach enzyme:

R. rubrum peptide



In collaboration with V. Ramakrishnan of the National Center for Small Angle Scattering Research at ORNL, we have compared the conformations in solution of deactivated and activated ribulosebisphosphate carboxylase (both spinach and R. rubrum enzymes) by use of neutron scattering. We did not detect any major conformational change accompanying the activation process despite a suggestion in the literature to the contrary. With the spinach enzyme, the scattering data closely fit that expected for a hollow sphere with an outer radius of 56.4 Å and inner radius of 14.3 Å. This model agrees nicely with that determined by X-ray crystallography for the deactivated tobacco enzyme.

With respect to the catalytic site, we have designed a series of potential affinity labels in which a chemically reactive side-chain is placed at the position corresponding to C2 of ribulosebisphosphate. The rationale for this series of compounds is twofold: 1) to probe that

portion of the active site where substrate  $\text{CO}_2$  binds and 2) to attempt to map the distance between the catalytic and regulatory sites. One of these new reagents, 2(4-bromoacetamido)anilino-2-deoxypentitol 1,5-bisphosphate, fulfills all experimental criteria for affinity labeling and permits the identification of His-44 as an active-site residue in the R. rubrum enzyme.

We have virtually completed the determination of the complete primary structure of the carboxylase from R. rubrum. This project was undertaken because this organism is one of the more primitive that contains the photosynthetic apparatus and also its carboxylase is a dimer of identical subunits whereas the corresponding enzyme from higher plants and most other photosynthetic bacteria consists of eight pairs of nonidentical subunits. Hence, because of evolutionary diversity and gross differences in quaternary structure, we felt that comparisons of primary structure between the R. rubrum and higher plant enzyme would be very revealing with respect to regions required for function. This has indeed been the case. Although the total sequence homology is <30%, every residue that we have implicated at the active site with affinity labels (Lys-175, Lys-334, and His-44) is located within a segment of striking sequence invariance. Furthermore, because of lack of conservation of any cysteinyl residues, we were able to dispel the long-standing notion that sulfhydryls are required for catalysis.

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## Comparative Genetics Section

### SECTION OVERVIEW - J. L. EPLER

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

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

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

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

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

(teratogenesis and embryo toxicity), mammalian cells in vitro (mutagenesis, chromosome aberrations, DNA repair, and sister chromatid exchanges), and mammalian cells in vivo (chromosome aberrations, mutagenesis, and sister chromatid exchanges). The individual research summaries provide details of these various research programs. The point to be emphasized here is that information from short-term and longer-term assays using a wide variety of different systems provides a basis for extrapolation to potential effects in man.

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

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

Other mechanistic approaches involve the study of metabolic activation whereby nonmutagenic compounds are metabolized into mutagenic forms, and the study of the relationship between the structure and mutagenic potential of related compounds, e.g., nitrosamines, polycyclic aromatic hydrocarbons, and aromatic amines. An end-product of mutagenesis research is information from which regulatory guidelines can be established. Several projects directly impinge upon regulatory guidelines, including interdivisional projects concerning the applicability of short-term tests and chemistry. Much of the developmental work on the use of short-term assays in the evaluation of health effects of coal conversion and shale oil technologies has been carried out within the Division under Interagency Agreements between the EPA and DOE. These contributions stand not only as examples of how our long experience in fundamental research can be applied to the problems of modern technology but also as examples of cooperation between teams of investigators in various divisions of ORNL.

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

### COMPARATIVE MUTAGENESIS

|                 |                 |
|-----------------|-----------------|
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| F. W. Larimer   | S. I. Simms     |
| C. E. Nix       | R. D. Wilkerson |
| L. C. Waters    | W. Winton       |
| A. A. Hardigree |                 |

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) biodirected 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 more confident estimates of risk from energy-related materials, the correlation of biochemical data with biological endpoints, and 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

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.

Bacterial Mutagenesis. Mutagenesis studies using the *Salmonella* histidine reversion assay developed by B. N. Ames were applied in the following areas: (1) isolation and identification of mutagenic agents in synfuels (with Analytical Chemistry Division); (2) comparative studies of mutagenic compounds with various assays (with Analytical Chemistry Division); and (3) determination of relationships between chemical structure and mutagenic activity.

a. Isolation and identification of mutagenic agents from synfuels. Separation by chemical class coupled with biological assays using *Salmonella* histidine reversion assays was used to isolate and identify mutagenic agents from synfuels. Earlier studies using acid/base separation demonstrated that mutagenic substances are present in the basic and neutral fractions. The mutagenic constituents have been concentrated into a single, chemically defined fraction of very high mutagenic activity. The organic constituents of the basic fraction are suspected to be mainly primary aromatic amines. Mutagenesis studies with a large number of suspect compounds revealed higher molecular weight (fused ring) aromatic amines, azaarenes and nitrogen heterocyclics to be potent mutagens.

Significant mutagenic activities were also noted in the neutral fraction. The neutral fraction was subjected to further separation using Sephadex LH-20 column chromatography and the individual fractions were assayed for mutagenic activity. Mutagenicity studies in a series of reference samples representing coal liquefaction (SRC-II; H-Coal), coal gasification (UMD tars) and shale oil have indicated that the aliphatic constituents though contributing to the bulk of the neutral fraction were not mutagenic. The mutagenic activity was distributed in the polycyclic aromatic hydrocarbon (PAH) fraction, the nitrogen containing PAH (N-PAH) fraction and the polar fraction. The polar fraction contained most of the mutagenicity associated with the neutral fraction (possible chemical classes: quinones and other oxygen-containing PAH's). However, in parallel with work at other laboratories, the key threats have been established as: basics - aminoanthracene(s) and aminophenanthrene(s); neutral - benzo(a)pyrene(s), fluoranthene(s), chrysene(s), and cyclopentylpyrene(s).

b. Relationship between mutagenesis and carcinogenesis. The key study on the relationship between microbial mutagenesis and skin carcinogenesis was carried out in collaboration with J. M. Holland. A qualitative and quantitative comparison of biological activity of fractionated neutral materials from both H-Coal and SRC-II showed similar results. The *Salmonella* histidine reversion assay and skin may agree qualitatively on the potency of a crude material but are responding quantitatively to different classes of molecules. For example, the most potent carcinogenic fraction in the neutral materials was the PAH fraction while the most potent mutagenic fraction was a "polar" neutral fraction which was low in cancer producing activity. This was not unexpected since

it was known that specific activities, especially of nitro- and amino-substituted derivatives, measure extremely high in the Ames system.

c. Structure-activity relationships. A coordinated study on nitrosamines involving the collaboration between the staff of the Biology Division, W. Lijinsky (Frederick Cancer Research Center, Frederick, Maryland) and T. K. Rao (Environmental Health Research and Testing, Inc., Research Triangle Park, North Carolina) was brought to a conclusion. A compilation, guest-edited by the investigators, will appear in the near future.

Within the last year we shifted our emphasis within this task away from microbial screening. The next sections illustrate our move to a consideration of the comparative approach from a more fundamental and experimental view. Only a selected group of comparative compounds are under consideration.

## II. Yeast Mutagenesis

The yeast Saccharomyces cerevisiae is an ideal organism for the study of mutagenesis and DNA repair in eucaryotes. A principal advantage of this model eucaryote, not shared by mammalian systems, is an extensive body of genetic analysis concerned with the control of mutagenesis and the repair of damage to DNA. Nearly 100 mutations that affect sensitivity to radiation and chemical mutagenesis have been described. Three major epistasis groups that control 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 responsible for a form of error-prone repair for nearly all induced mutagenesis. Since a fundamental understanding of the molecular nature of mutagenesis is necessary for interpretation, extrapolation and development of the health effects program, we have sought to exploit the exquisite degree of genetic control afforded by this system. The program addresses chemical mutagenesis in four basic areas: mutagenesis by alkylating agents, and attendant repair processes; mutagenesis by intercalating agents and their interplay with repair; isolation, identification and characterization of repair genes and their gene products, and the study of the interaction of repair functions; and an analysis of the role that the DNA sequence plays in the targeting of mutations.

Alkylation Mutagenesis. Work this year has been directed toward characterizing the repair of alkylated nucleotide bases. Activities have been identified in crude extracts which liberate N-7-methylguanine and N-3 methyladenine from alkylated DNA. There is no evidence of either the release or demethylation of the miscoding base O-6-methylguanine. In vivo labeling experiments indicate that N-3-methyladenine is rapidly removed from DNA. These studies are being extended to include members of the RAD6 epistasis group.

Frameshift Mutagenesis. The REV-dependence study has been extended to include five agents: quinacrine mustard (QM), 4-nitroquinoline-N-oxide (4NQO), the quinacridine mustard ICR170, methylnitrosourea (MNU), and UV. RAD6 function is required for frameshift induction with all five agents. The two quinacridine mustards require all REV functions, while MNU and UV are largely REV-independent. A striking dichotomy was observed with 4NQO; although REV2-REV3 dependent, i.e. hypomutable in rev2 and rev3 mutants, rev1 strains were hypermutable, suggesting that 4NQO lesions are rather specifically accommodated by REV1.

Repair Genes and Products. A new yeast DNA library has been prepared from 10-20 kbp fragments inserted into the minichromosome vector YCp50. This library is being screened for the REV1 and REV2 loci, with the ultimate intent of identifying and characterizing their gene products.

It was recently reported that the umuC and D genes of E. coli have mutagenic repair activity when transformed into yeast. We are constructing yeast and mammalian expression vectors containing umuC and D to study their effect in different host repair backgrounds.

Sequence Specificity. A 400 bp segment of the ARO1 gene, comprising the 5' non-coding region (indicated by mRNA/DNA mapping) and the initial coding region, has been sequenced. A sequence related to the ATATGACTC box common to the coordinately regulated genes of the general amino acid control system has been identified. Deletion mapping of the coding region has located several of the mutant alleles. New mutants have been constructed by in vitro mutagenesis. These new mutants will be utilized in analysis of sequence specificity of reversion.

Sequence analysis of the his4-38 frameshift indicates the possible involvement of quasi-palindromic regions forming unusual secondary structures responsible for localizing error-prone repair events. Sequencing revertants may indicate the nature of these events. A plasmid vector for recovering revertant sequences has been constructed.

New Initiatives. Several mammalian shuttle vectors have been constructed utilizing the phosphotransferase gene from Tn5. SV40 based and HSV-tk based vectors have been designed. These vectors are intended for gene marking and plasmid rescue or cosmid rescue with G418 aminoglycoside selection. Target genes are the Gardners proto-oncogene and tumor-specific gene (collaboration with Regan).

A study of the mutagenic and toxic effects of oxygen have been initiated with Adler. Anaerobic yeast show unusual oxygen sensitivity for killing. Anaerobic stationary cultures appear to lose oxygen resistance. The genetic basis of oxygen resistance is being investigated.

A preliminary investigation of the effect of iodo-cytidine in DNA on the recognition and cleavage by restriction endonucleases was initiated in collaboration with Rahn. Iodo-C blocks cleavage by BamHI, HindIII and EcoRII.

### III. Insect Mutagenesis

Genotypic Variation and Alkylation Induced Mutagenesis in Drosophila Males. Two obvious ways in which the genotype may play a major role in controlling the frequency of induced (or spontaneous) mutations are through metabolism of promutagens and DNA repair. While much is known about the mechanisms of alkylation-induced mutations in prokaryotes the situation in eukaryotes is less well understood and may, in fact, be quite different from that described in *E. coli* or other prokaryotes. Although most of the investigation of alkylation induced DNA repair in eukaryotes has been with *Saccharomyces cerevisiae*, recently several mutagen sensitive mutants that are also defective in DNA repair have been characterized in Drosophila. Use of these mutants in in vivo mutagenicity studies, coupled with biochemical analysis, should shed more light on the mechanisms of alkylation-induced mutagenesis in Drosophila.

As a starting point we have been testing the effects of DNA repair mutants on alkylation-induced mutagenesis in males. Highlights of our results to this point are as follows:

- (1) In males containing the mei-9 mutation, an excision repair defect, the methylating agents dimethylnitrosamine (DMN) and methylnitrosourea (MNU) are hypomutagenic (in early spermatids and late spermatocytes) to the wildtype strains — Oregon-R, Canton-S, Hikone-R, and Berlin-K. The corresponding ethylating agents diethylnitrosamine (DEN) and ethyl-nitrosourea (ENU) are equally mutagenic in mei-9 and wild-type males.
- (2) The reduction seen with DMN is not due to differences in metabolism.
- (3) Early spermatids and late spermatocytes are 2- to 3-fold more sensitive than late spermatids and mature sperm to mutation induction by MNU and ENU. This is in contrast to ethylmethanesulfonate (EMS) and methylmethane-sulfonate (MMS) where there is little or no specificity between postmeiotic germ-cell stages. We interpret this as: (a) differences in the level of initial alkylations or (b) the existence of a mutagenic repair process in early spermatids and spermatocytes. Experiments to distinguish between the two are in progress.
- (4) The alkylating agents, DMN, DEN, MNU, and ENU, produce mutations equally well in postreplication repair deficient, mei-41, and wild-type males.

Preliminary experiments suggest that the expression of DNA repair defects, in relation to mutagenesis, is different in males and females. This is not unexpected, however, as in Drosophila meiotic recombination is very different in the two sexes. Many of the repair defective mutants are also deficient in meiotic recombination. Future plans include experiments designed to explore these differences in more detail. In our efforts to understand the role of DNA repair in mutagenesis of Drosophila, we will attempt to define the specific biochemical lesions of mei-9 and its temporal expression in spermatogenesis.

A second major way the genotype may influence the frequency of mutations is by controlling the levels of mixed-function oxidases (MFOs). We have surveyed a number of wild-type strains for differences in induced mutation frequency in response to the simple alkylating agents, DMN, DEN, and methylethylnitrosamine (MEN). A number of differences that were strain, as well as concentration, dependent were found. When MNU or ENU were tested these differences were no longer observed, suggesting that the variation reflects differences in MFO activity. In order to gain further insight into these problems we began a collaborative effort with Larry Waters.

#### IV. Metabolism of Xenobiotics

Metabolites of xenobiotics, and not the parent chemical, are most often responsible for their adverse effects to health. Primary metabolism is via the cytochrome P-450-containing mixed-function oxidase (MFO) system. The sensitivity of a particular organism to the deleterious effects of a particular chemical is therefore directly related to the metabolic capabilities of its MFO system. In order to learn more about the relevant metabolic processes and about how the expression of the relevant enzymes is regulated, we are studying the MFO system in *Drosophila*. Advantages of *Drosophila* over mammalian systems for these studies include: (1) *Drosophila* is susceptible to the toxic and mutagenic effects of a wide variety of chemicals; (2) metabolism and biological effect occur within the same organism and within a short enough period of time to make relevant correlations between the two events; (3) the MFO system in *Drosophila* is amenable to biochemical characterization, e.g., microsomes prepared from extracts of whole flies contain fully one-half the content of cytochrome P-450 of those prepared from uninduced rat livers; (4) genetic variation exists in the constitutive levels of MFOs, as well as in their inducibility, in *Drosophila*. These genetic differences can be further manipulated and exploited for studying the system.

The period covered by this report has been an exciting and productive time for this research. Highlights of our progress are summarized as follows:

(a)  $\alpha$ -Hydroxylation via dimethylnitrosamine demethylase (DMN-d) is probably not the pathway for metabolic activation of DMN to a mutagen. *Drosophila* strains that are negative for this activity are as sensitive to mutation by DMN as positive strains. These conclusions are based on activities in whole body extracts; the possibility of equal DMN-d activity in the gonads of both positive and negative strains has not been eliminated. The system can be used to determine the relevant pathway for DMN activation. These findings are very relevant to mammalian studies where DMN-d has been implicated, but not proven, in DMN activation. Although DMN-d does not appear to correlate with DMN-induced mutagenesis, it might relate to the toxicity of DMN.

(b) Expression of MFOs is dependent on genotype and age. Some activities appear to be coordinately expressed, some not. The finding of large differences in MFO activities among strains of the same species suggests that similar studies should be made in mouse strains, particularly those being used in tests for mutagens. The unique patterns of MFO expression in *Drosophila* can be exploited by using genetic manipulation and molecular probes (see d) to elucidate the mechanisms by which MFO expression is regulated.

(c) The structural gene for DMN-d has been localized on chromosome II, with evidence for an "enhancer" of this activity located elsewhere, probably on chromosome III. It will be of interest to more precisely locate the DMN-d gene on chromosome II with particular attention to its position relative to insecticide resistance genes that are known to exist on that chromosome. We find a positive correlation between DMN-d expression and resistance to phenylurea among the strains tested. The notion of an "enhancer" of an MFO that is located on a separate chromosome is novel and should be further studied. Mapping the chromosomal location of those MFOs that appear to be coordinately expressed versus those that aren't should yield considerable insight into the overall question of how MFO expression is regulated. *Drosophila* is probably the only organism in which it is feasible to do these studies.

(d) DMN-d activity (and probably other MFOs) is positively correlated with a unique protein band as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis. There is a possible correlation between the presence of this protein(s) and insecticide resistance. This unique protein band undoubtedly contains MFO proteins (certainly DMN-d) that are expressed in some strains and not in others. It provides a convenient handle that can be used in the cloning of specific MFO genes. These clones can be used to study the mechanism of MFO expression at the molecular level.

We believe that the techniques and concepts developed from this research are basic and necessary for understanding the complex metabolic processes that result in adverse health effects. Many of the methods and concepts that develop from this study are applicable to other programs in the Division. In this regard, we have initiated a collaborative study with Dr. J. N. Dumont to assess the role of MFOs in the production of teratogenic effects in the frog. Future plans include similar studies of the MFO system in mouse skin.

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## MAMMALIAN CELL GENETIC TOXICOLOGY

|                             |                               |
|-----------------------------|-------------------------------|
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Our long-range goal is to study the mechanisms of mammalian gene mutations and to determine the mutagenic effects of environmental agents associated with energy technologies. We have used the following multi-phasic mammalian gene mutational systems at the molecular, cellular and animal levels to achieve this goal.

**1. Quantitative Mutagenesis with CHO/HGPRT Assay: Studies of Mechanism of Mutagenesis and Environmental Mutagenesis.**

**A. Molecular dosimetry of nitrosourea mutagenesis.** We have used the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase (CHO/HGPRT) assay to quantify cytotoxicity and mutation at the *hprt* locus induced by various chemicals. Earlier, we found that this assay is useful to study structure-mutagenicity relationships as exemplified by a study with ten direct-acting alkylating chemicals. Within each structurally related group (nitrosamidines, nitrosamides, alkyl alkanesulfonates and alkylsulfates), chemical reactivity (s value) decreases with the size of the alkyl group with the methyl agent being more mutagenic and cytotoxic than the corresponding ethylating chemical on an equimolar basis but not equitoxic level. Both the toxic nature and the DNA lesions induced by a chemical affect the determination of mutagen potency. For example, MNU is 5 times more mutagenic than ENU on a molar basis, however, MNU is only one-half 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, ethylation of DNA appears to produce more mutants than the corresponding methylation. This is consistent with our recent observation that a higher proportion of  $O^6$ -ethyl-guanine was produced after ENU treatment as compared with  $O^6$ -methyl-guanine formed from MNU and the longer persistence of the ethylated than the methylated DNA adducts.

**B.  $O^6$ -Methyl dGTP and mutagenesis in the CHO/HGPRT assay.** The deoxy-nucleotide pool is a target of alkylating agents and alkylated nucleotides are incorporated into DNA during *in vitro* replication. Incorporation of alkylated nucleotides may be an important mutagenic mechanism, and in fact incorporation of  $O^6$ -methyldeoxyguanosine triphosphate ( $m^6$ dGTP) during *in vitro* replication of phage T7 DNA is mutagenic. We have analyzed the fate of  $m^6$ dGTP and found it to lack mutagenicity in mammalian cells *in vivo*. Using a calcium phosphate-mediated CHO/HGPRT assay, significant amounts of  $m^6$ dGTP can be introduced into CHO cells. Using  $m^6$ [ $^3$ H]dGTP, we found that intracellular uptake of  $m^6$ dGTP is concentration dependent; 1.5% of the added  $m^6$ dGTP is taken up by  $10^6$  cells during a 5 hr treatment, with 3% of that being converted into an ethanol insoluble form. Analysis of the

ethanol insoluble fraction shows that 25% of the label is incorporated into DNA (released as deoxyguanosine), while 75% is found in RNA (released as guanosine); no label is released from DNA as  $0^6$ -methyldeoxyguanosine ( $m^6$ dG). Since CHO cells have no detectable methyltransferase activity, it appears that  $m^6$ dGTP enters the nucleotide pool where it is converted to  $m^6$ dG and then demethylated by adenosine deaminase. In separate experiments, concentrations of unlabeled  $m^6$ dGTP up to  $5 \times 10^9$  molecules of  $m^6$ dGTP into each cell without appreciable cytotoxicity or mutation induction. It thus appears that this alkylated nucleotide is not incorporated into cellular DNA and is therefore not mutagenic.

**C.  $0^6$ -Methylguanine-DNA methyltransferase in mammalian cells.**  $0^6$ -Methylguanine-DNA methyltransferase activity was assayed in extracts of mammalian cells which are proficient (Mer<sup>+</sup>) or deficient (Mer<sup>-</sup>) in the repair of DNA methylation damage. The Mer<sup>+</sup> HeLa CCL2 cell strain was found to contain approximately 100,000 molecules/cell of methyltransferase, assuming that each molecule can demethylate only one  $m^6$ G residue, whereas a Mer<sup>-</sup> HeLa S3 strain contained no detectable activity. Constitutive methyltransferase activity was also lacking in extracts of CHO and V79 cells. No induction of methyltransferase activity was observed in CHO cells which had been treated with N-methyl-N'-nitrosoguanidine (MNNG) according to a protocol reported to render the cells resistant to alkylation-induced sister-chromatid exchanges. Preliminary experiments indicate that treatment of HeLa CCL2 cells with subtoxic doses of MNNG results in reduced levels of methyltransferase for up to 24 hr following treatment, rather than an adaptive increase in activity.

**D. Mutagenicity of crude and neutral fractions of coal-liquified oils in the *Salmonella* and CHO/HGPRT assays.** The neutral fraction of coal-liquified oils accounts for over 50% of the crude sample and contributes a large portion of the mutagenic activity as determined by the *Salmonella* histidine reversion assay. To determine what classes of compounds are responsible for the mutagenic activity of the neutral fraction, we have subfractionated this fraction by means of Sephadex LH-20 gel chromatography and tested the subfraction for mutagenicity in strain TA98 of *Salmonella* and in the CHO/HGPRT assay. We tested the crude, neutral fraction, and the following subfractions: aliphatic, polar aromatic, and a mixture composed of two subfractions — one enriched in polycyclic aromatic hydrocarbons (PAHs) and one enriched in neutral nitrogen heterocyclics (N-PAH). Also, a liquified blend of light and heavy fuel oils was tested before and after hydrogenation. The results in *Salmonella* in the presence of Aroclor-1254-induced rat liver S9 show that polar aromatic compounds account for most of the mutagenic activity of the neutral fraction. The mixture containing the PAH and N-PAH subfractions was weakly mutagenic, and the aliphatic subfraction was not mutagenic. The liquified blend before hydrogenation was mutagenic, but this activity was eliminated after hydrogenation. All seven mixtures were highly cytotoxic to CHO cells when the cells were treated for 5 hr at 37° in a serum-free medium. Also, 3-4 times higher doses of the mixtures were needed in the presence of S9 in order to obtain cytotoxic responses similar to those

found in the absence of S9. However, none of the seven mixtures induced a clear mutagenic response in the CHO/HGPRT assay.

E. Mutagenicity and cytotoxicity of five antitumor ellipticines in the CHO/HGPRT assay and their structure-activity relationships in Salmonella. The mutagenicity and cytotoxicity of five antitumor compounds (ellipticines) were investigated in the CHO/HGPRT assay and in six strains of Salmonella. All five compounds (ellipticine, 9-methoxyellipticine, 9-hydroxyellipticine, 9-aminoellipticine, and 2-methyl-9-hydroxyellipticine) were cytotoxic and mutagenic in the CHO/HGPRT assay in the presence or absence of Aroclor 1254-induced rat liver S9, and all except the last compound were mutagenic in Salmonella. Based on the reversion pattern obtained in various frame-shift and DNA repair-proficient ( $uvrB^+$ ) or -deficient ( $uvrB$ ) strains of Salmonella in the presence or absence of S9, the first three compounds appear to cause frame-shift mutations by both intercalation and covalent bonding with DNA; thus, these are classified as reactive intercalators. However, 9-aminoellipticine intercalates only weakly and may instead exert its mutagenic activity primarily (or exclusively) by forming a covalent adduct with DNA. Compared to the published antitumor data obtained in mice, the results in Salmonella and CHO cells suggest that the ability of ellipticine, 9-methoxyellipticine, and 9-hydroxyellipticine to intercalate with DNA, induce frame-shift mutations, and cause cell killing is associated with and may be the basis for their antitumor activity. The observation that the ellipticines are mutagenic in mammalian cells suggests that these antitumor agents may be carcinogenic.

## 2. Cytogenetic Studies with CHO Cells.

Methylphosphonic dichloride as a potent clastogen. Using the CHO multiplex system which measures cytotoxicity, gene mutation, chromosome aberrations and sister-chromatid exchanges showed that methylphosphonic dichloride (MPOD), manufactured by the Ethyl Corporation, is an efficient clastogenic agent with little or no activity to induce mutation at the hgprt locus. MPOD is an organophosphorous halide that reacts readily with metals and organic compounds. As a result of hydrolysis, hydrogen chloride and methyl phosphonic acid are liberated. To counteract the acidic nature of MPOD, treatment is conducted with 20 mM Hepes buffer (pH 7.4) in serum-free F12 medium. MPOD is quite cytotoxic with no cell survival above 120  $\mu$ g/ml. At a concentration of 48  $\mu$ g/ml, which reduces survival to 20% but does not induce mutations, there are 76 SCE's per cell (control value 12) and 38% of the mitotic figures were aberrant with 3 chromosome aberrations per cell of the chromatid type (control gave no aberrations). At a concentration of 72  $\mu$ g/ml which rendered a 17% survival without inducing mutations, 76% of the cells exhibited aberrant metaphases with 3 chromosome aberrations per cell of the chromatid type. The comparison of MPOD with known clastogenic, mutagenic and carcinogenic agents for the induction of SCE's and chromosome aberrations should be of interest in regards to the relationship of clastogenesis to mutagenesis and carcinogenesis.

### 3. Molecular Analysis of Mammalian Cell Gene Mutation.

An analysis of gene mutation in DNA-transformed CHO cells. We have isolated a CHO cell line which carries a single, stable, functional copy of the *E. coli* xanthine-guanine phosphoribosyl transferase (XGPRT) gene (*gpt*). This line was obtained after introduction of the plasmid vector pSV2gpt into a HGPRT<sup>-</sup>, presumptive deletion mutant CHO cell line. The transformant expresses the bacterial purine salvage enzyme, XGPRT. *gpt*<sup>-</sup> mutants can be recovered by selection for 6-thioguanine (10  $\mu$ M) activity. UV-light and X-ray irradiation show a dose dependent induction to TG<sup>r</sup>. This *gpt*<sup>+</sup> cell line is hypersensitive to killing by UV-, but not X-ray irradiation (versus wild type CHO cells) and there is a 2-20 fold increase in mutation induction at the *gpt* locus versus the *hprt* locus depending upon the dose and type of radiation. Southern blot hybridization detected deletions through *gpt* structural gene sequences in the two TG<sup>r</sup> mutants analyzed: a deletion of 1.0 kb in a spontaneous mutant and of 3.1 kb in an X-ray induced mutant. Additional TG<sup>r</sup> clones are being similarly analyzed. This *gpt*<sup>+</sup> CHO cell line appears to be useful for quantitative and molecular analysis of gene mutation in mammalian cells.

### 4. Development of a Chinese Hamster Model System for Induction of Mutations In Vivo.

Detection of 6-thioguanine resistant spleenocytes from Chinese hamsters. An assay for the detection of specific locus mutant lymphocytes arising *in vivo* in the Chinese hamster is being developed. The method is based on the autoradiographic detection of TG<sup>r</sup> spleenocytes following *in vitro* mitogenic stimulation for 42 hr in the presence or absence of TG. After removal of spleens from Chinese hamsters,  $1 \times 10^6$  cells/ml suspensions are prepared. Dose-response relationships for two mitogens, Phytohemagglutinin (PHA) and Concanavalin A (Con-A), indicate that a maximal response occurred between 1.8 and 2.7  $\mu$ g of Con-A per ml. Using a concentration of 2.1  $\mu$ g/ml of Con-A, five concentrations of TG were used for cytotoxicity determination with non-cryopreserved lymphocytes. These data indicate that a concentration of  $2 \times 10^{-4}$  TG resulted in an average variant frequency of  $3.85 \times 10^{-6}$  ( $n=4$ ).

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## MAMMALIAN CYTOGENETICS

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We have continued our studies of the mechanisms of induction of chromosome aberrations and sister chromatid exchanges (SCE) by chemical and physical agents, and the application of this knowledge to improve the basis for estimating the genetic and carcinogenic risk to man from exposure to such agents. We are also using these mechanistic approaches for the determination of the possible reasons for differences in sensitivity to the induction of chromosome aberrations and SCE in the same cell types of different species and different cell types within a species. We have also applied our information for conducting human population cytogenetic monitoring studies and interpreting the data.

1. Induction of Chromosome Aberrations by Fission Neutrons and X rays. In a series of experiments (Preston, R. J., *Mutat. Res.* 69: 71, 1980; Preston, R. J., *Cytogenet. Cell Genet.* 33: 20, 1982) we have shown that cytosine arabinoside (ara-C) inhibits the excision repair process at the repair replication stage, thereby causing the accumulation of single strand gaps in the DNA. On reversing this inhibitory effect with deoxycytidine, the accumulated gaps can interact to produce chromosome aberrations. If  $G_0$  human lymphocytes are X-irradiated, incubated with ara-C for 1, 2 or 3 hr, and then stimulated with phytohemagglutinin, with the addition of deoxycytidine, there is a considerable increase in the frequency of chromosome-type aberrations compared to the frequency in cells that are irradiated but are not incubated with ara-C. There is an approximately linear increase with increasing ara-C incubation times. These results indicate that X-ray-induced chromosome aberrations are the result of the interaction of coincidentally repairing base damages. Further, it was shown that the increased frequency of X-ray-induced chromosome aberrations in  $G_0$  lymphocytes of Down syndrome individuals is the result of a more rapid repair of base damage in lymphocytes of these individuals than in normal lymphocytes (Preston, R. J., *Environ. Mutagen.* 3: 85, 1981). The more rapid repair will increase the probability of there being coincidentally repairing base damage regions able to interact to produce aberrations.

When  $G_0$  human lymphocytes were irradiated with fission neutrons and incubated with ara-C for 1-5 hr, no increase in aberrations was observed compared to cells irradiated with neutrons alone. The RBE for the fission neutrons compared to X rays was about 5. Since we have shown that ara-C does not inhibit the repair of directly induced DNA strand breaks (Hiss, E. A., Preston, R. J., *Biochim. Biophys. Acta* 478: 1, 1977), it appears that the chromosome aberrations induced by neutrons result from the interaction of directly-induced DNA strand breaks, in contrast to their

induction by X rays. This observation has several important implications, and additional evidence suggests that it is indeed tenable.

It was suggested above that the increased frequency of X-ray-induced chromosome aberrations in  $G_0$  Down lymphocytes was the consequence of a more rapid repair of base damage in these cells than in normal lymphocytes. If neutrons induce aberrations by the interaction of directly induced double strand breaks, then there should be no difference in the neutron-induced aberration frequency between normal and Down lymphocytes, since the DNA contents of the two cell types are about identical. Experiments reported by DuFrain *et al.* (Radiation Research Society Meeting, San Antonio, March, 1983, Abstract Cd3) show that this is indeed the case.

Recent experiments in our laboratory (Heartlein, M. W., Preston, R. J., unpublished) have shown that the differences in sensitivity to X-ray-induced chromosome aberrations in the lymphocytes of different species (human, marmoset, pig and rabbit) are the result of differences in the rate of repair of ara-C inhibitable damage (base damage). However, since these species have similar DNA contents they should not show the same differences in sensitivity to neutron-induced aberrations as shown for X-ray-induced aberrations, i.e., the RBE should be different for different species. Initial studies comparing X-ray- and neutron-induced aberration frequencies in human and pig lymphocytes have confirmed this hypothesis. The frequency of dicentrics induced by X rays in pig lymphocytes is about one-half that for human lymphocytes, whereas the frequencies are, as predicted from the similarity in DNA contents, about the same following neutron irradiation. The RBE for the human cells is about 5 whereas it is about 8 for the pig. Thus in any particular cell type the RBE for chromosome aberrations will be proportional to the ratio of misrepaired double-strand breaks to misrepaired base damage, and can clearly vary from cell type to cell type or from species to species for a particular radiation quality.

It has also been shown that the rate and extent of repair of ara-C inhibitable damage, as indicated by increases in the frequency of chromosome aberrations in cells X-irradiated and incubated with ara-C, is dose dependent. The rate of repair is decreased as the X-ray dose is decreased, and the overall increase in aberrations in the presence of ara-C is lower at lower X-ray doses. At a dose of 50 rad there is no increase in aberration frequency in the presence of ara-C. These results indicate that at low X-ray doses (<50 rad) the aberrations probably result from the interaction of directly-induced double strand breaks, which is perhaps predictable since the probability of aberrations resulting from the interaction of repairing base damages will be low because of the low frequency of induction of base damages. It can also be argued that the linear quadratic dose response for chromosome aberrations induced by X rays is a consequence of the induction of the initial damage by one or two ionization tracks, and also the fact that as the dose is increased the induced base damages are repaired at a faster rate, and there will be a greater probability of interaction of repairing damage that can result in aberrations.

The aberrations induced by fission neutrons result from the interaction of directly-induced double-strand breaks, with perhaps some contribution at high doses (>40 rads) due to the interaction of repairing base damages, when the induced frequency of base damage could be sufficiently high that its involvement in aberration induction could be observed over and above the large contribution of double-strand breaks. This latter point is being checked. The aberrations induced by X rays are the result of the interaction of directly-induced double-strand breaks at doses of 50 rad or lower, but at higher doses the major mechanism of induction is from the interaction of repairing base damages. The RBE will be a reflection of this.

Two other conclusions can be drawn from these experiments. Firstly, the target size for x-ray-induced aberration at doses above 50 rads can be large since the base damages can be induced some distance apart - the only requirement is that they be close together (within the rejoining distance) at the time of repair, and that they can interact. The target size for neutrons is likely to be much smaller because the interacting double-strand breaks are induced by a single ionization track, and are repaired much more rapidly and consequently will be induced in close proximity. Secondly, a limiting RBE can be calculated for fission neutrons compared to X rays, because at low X-ray doses the damage resulting in aberrations is directly-induced double-strand breaks, as is the case with fission neutrons, and so a calculation of RBE for chromosome aberrations at these low X-ray doses will reflect a comparison of aberration frequencies where the aberrations result from the same mechanism of induction. This assumption does not hold true at higher X-ray doses (>50 rad).

These studies are continuing, and hopefully will give a much better understanding of the mechanism of induction of chromosome aberrations over a range of radiation qualities, and a means whereby RBE can be calculated or estimated with regard to the mechanisms of induction of aberrations.

2. Mechanism of Induction of Sister Chromatid Exchanges and Specific Locus Mutations. In order to be able to obtain chromosome differentiation for analysis of SCE in cells, it is necessary to grow cells in a medium containing a thymidine analog, usually bromodeoxyuridine (BrdU). It has been shown that the incorporation of BrdU or its replication causes SCE, and the SCE frequency is higher at higher extracellular BrdU concentrations. We found that we could use chlorodeoxyuridine (CldU) instead of BrdU, and that differentially stained chromosomes could be obtained after two rounds of replication in CldU. However, it was observed for Chinese hamster ovary cells that the frequency of SCE when CldU was used was about 7 times that when BrdU was used, for equimolar extracellular concentrations. The frequency of mutations at the hprt locus (thioguanine sensitivity to thioguanine resistance) following growth for two rounds of replication in either CldU or BrdU was approximately the same.

It was important to determine if this difference in the frequency of induction of SCE was due to the difference in incorporation into replicating DNA for CldU and BrdU, even though the extracellular concentrations

were the same. We used HPLC to determine the incorporation of the two analogs over a concentration range in the medium of 1.5 to 20  $\mu$ M. The two analogs were incorporated to the same extent over this concentration range. The frequency of SCE was linearly proportional to substitution for thymidine by CldU and BrdU. Based on a linear extrapolation, a spontaneous level of approximately 6 SCE per cell was estimated.

The fact that CldU induces about 7 times as many SCE as BrdU or equal substitution for dT in Chinese hamster ovary cells in culture allowed us the opportunity to study the mechanism of induction of SCE by these two analogs. Cultures were incubated with either BrdU or CldU for one cell cycle, followed by incubation in the presence of dT alone or BrdU or CldU for the second cell cycle, and the SCE frequency was determined in M2 cells. The results showed that the induction of SCE is dependent only on the replication of the analog-substituted DNA, during the second cell cycle. Additional studies used cultures grown in the presence of BrdU or CldU for 7 days to obtain mainly bifilarly-substituted DNA, followed by two rounds of replication in the presence of dT alone. The SCE frequencies were approximately twice those found in cultures which had undergone the usual two rounds of replication in the presence of the analog. This is consistent with the hypothesis that SCE result from errors of replication of analog-substituted DNA.

The mutant frequency at the hgprt locus from long-term growth of cells in BrdU or CldU was dependent on the concentration of the analog in the medium. Also the frequency of mutants in cells grown in CldU was about 7 times that for cells grown in BrdU - a similar ratio to that found per SCE. This is interpreted as being a consequence of the induction of specific locus mutation by errors of replication of the analog-substituted DNA, with more errors resulting from the replication of CldU-containing DNA. In addition, only at high concentrations of BrdU or CldU ( $>50 \mu$ M) are mutants induced from the two rounds of replication. At concentrations of 50  $\mu$ M and above there is a 100% substitution of thymidine by BrdU and CldU. Thus the mutant frequency increases over a concentration range where there is no increase in substitution of BrdU or CldU for thymidine. The mutants induced appear to be a consequence of misincorporation of BrdU or CldU into replicating DNA, quite possibly in place of cytidine, since the addition of dC results in a decrease in the mutant frequency.

These results are not only important as regards the mechanism of induction of SCE and specific locus mutations, but also because they appear to provide a way by which the sensitivity of the assays for the two end-points can be considerably increased. This could prove to be of significant benefit particularly in human population monitoring studies.

3. Human Population Monitoring Studies. In conjunction with the Centers for Disease Control (Atlanta) and Dr. M. A Bender of the Brookhaven National Laboratory we conducted a cytogenetic monitoring study of persons who had resided in the area of the Love Canal and a matched control group in nearby Niagara Falls. The study was designed to determine

if there were any demonstrable differences in the frequencies of chromosome aberrations or SCE in the two groups.

Frequencies of chromosomal aberrations and/or sister chromatid exchanges (SCE) were measured in peripheral blood specimens obtained between December 1981 and February 1982 from 46 persons. Blind analyses were performed with 44 matched control specimens from persons living in another part of Niagara Falls. Two sets of Love Canal participants were included. The first group consisted of 29 persons who in 1978 lived in 7 of 12 homes directly adjoining the canal, in which air, water, and soil testing showed elevated levels of chemicals spreading from the canal. The second group included 17 persons in whom cytogenetic analyses had been performed in 1980 as part of a pilot investigation supported by the U.S. Environmental Protection Agency.

In neither group did frequencies of SCE or chromosomal aberrations (such as gaps, breaks, fragments, or "supernumerary acentrics") differ significantly from control levels. Karyotypes were normal in all specimens. In assessing chromosome damage, several factors of interest - sex, cigarette smoking, history of playing on the canal site, and history of attending an elementary school that adjoined the site - were examined as possible causes of cytogenetic variation. History of current cigarette smoking was significantly associated with increased SCE frequency, a result observed independently in studies elsewhere. Other factors, alone or in combination, were not associated with any significant increase in chromosome damage.

In May 1980 results of an earlier cytogenetic study led to concern that chromosome damage might be increased among residents of the Love Canal area. Results of the present study do not support that conclusion and indicate instead that the chromosome alteration frequencies are the same in Love Canal residents as in residents elsewhere in Niagara Falls. Interpretation of these findings are limited by (1) considerable passage of time since 1978 when homes adjoining the canal were evacuated and corrective drainage work began at the site, and (2) lack of objective measurements for canal-related chemical exposures in individual residents. Although cytogenetic changes in peripheral blood lymphocytes are known to persist for years after exposure to ionizing radiation, similar persistence after chemical exposures may not necessarily occur.

Cytogenetic studies may prove useful in the future in assessing subclinical toxic damage in situations where tests can be done at the time of exposure or soon after, and where individual exposure can be readily measured. Present experience, however, suggests that while cytogenetic measurements of this sort may provide good correlations with doses of radiation or toxin, their predictive value for future individual health is quite uncertain.

Also in conjunction with Dr. Bender (BNL) we are analyzing the frequencies of SCE and chromosome aberrations in about 400 persons employed at BNL. This project is funded by the National Toxicology Program. The

selected sample group has been established such that approximately equal numbers of individuals are included in each age decade, that males and females are approximately equally represented, and that different races are represented (although not necessarily equally). A medical record can be obtained at a later date. The purpose of the study is to obtain a value for the background frequency of SCE and chromosome aberrations, with particular emphasis on the variations in these frequencies, and possible intrinsic or extrinsic factors that could be responsible for any variations. To date samples from some two hundred individuals have been analyzed. The results will not be completely assessed until all samples have been analyzed so that there will be no opportunity for bias in interpretation based upon a partial sample.

4. Studies on the Induction of Chromosome Aberrations in RFM Mouse Bone Marrow, and their Possible Relationship to Myelogenous Leukemia. In order to understand the clastogenic and carcinogenic potential of neutron irradiation, we have initiated a project in collaboration with Dr. R. L. Ullrich to study the dose-response relationship for the induction of chromosome aberrations and of myeloid leukemia in RFM mice by fission neutrons.

It was felt that in order to study induction of aberrations in bone marrow cells it was necessary to determine the specific cell stage in which the cells analyzed were at the time of irradiation, because different cell stages have different sensitivities to aberration induction. In addition, different types of aberrations are induced in different cell stages, and their consequences can be quite different, particularly with regard to probability of transmission through many cell divisions. For this type of analysis we decided that the proliferation kinetics of these cells needed to be elucidated since the bone marrow is composed of heterogeneous cell types. Our recent study of the cell cycle kinetics of normal mouse bone marrow cells shows that the average cell cycle time for different populations ranges from 5.75 to 30 hr. If these phenomena are not taken into consideration in the analysis of chromosome aberrations, the aberrations induced in different cells at different phases of the cell cycle will be indiscriminately considered together. In order to overcome such difficulties we have been developing a program to combine the analysis of cell proliferation and cell cycle specific induction of aberrations in the bone marrow cells. This requires the in vivo incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA and subsequent staining of the chromosomes to reveal the amount of cell progression. The cell that has progressed from the non-S- or the S-phase through one, two or three cell cycles can be readily identified. If the first post-treatment metaphase cells are analyzed, the aberrations induced in the non-S phase (presumably G<sub>1</sub> phase) and in the S phase can be quantified separately.

We have initiated the application of this protocol to study the induction of aberrations by neutron radiation in the mouse. Mice were individually housed in a rotating chamber, exposed to 40 rads of fission neutrons, implanted with 35 mg BrdU tablets, and bone marrow cells were harvested at 3 to 24 hr after irradiation. Non-irradiated control mice

were similarly housed and harvested. The stained cytological preparations were analyzed and metaphases were categorized according to their staining patterns, which indicate the cell cycle progression. These metaphases are given a score in order to calculate a proliferation index for comparison of the kinetics from one mouse to another. If a cell has progressed through one complete cell cycle, the score is one. If a cell has progressed from the early S phase to metaphase, this cell is considered to have progressed through half a cell cycle and the score is one half. If a cell has progressed from the very late S phase when the Y is the only chromosome to be replicated, the score is one quarter. Thus, a proliferation index can be calculated that reflects the progression of the bone marrow cell. Our data show that the 40 rads neutron irradiation caused a consistent delay in cell progression. The average delay was approximately 2 hr with this dose of radiation. Additional experiments are in progress to study the dose-response relationship in neutron induction of cell cycle perturbation and of cell-cycle phase specific aberrations.

5. Analysis of Human and Mouse Myeloid Leukemia. We have recently developed a transplantable mouse myeloid leukemia model in which the leukemic cells can be unequivocally distinguished from the co-existing normal cells in the bone marrow of the host mice. The leukemic cells can be readily identified due to the presence of a large marker chromosome identified as an isochromosome 8 by chromosome banding analysis. In addition, the cells also contain a deleted chromosome 2 which is characteristic of all mouse myeloid leukemic cells analyzed by different investigators. The availability of this cell line will permit us to study the various responses of the leukemic cells to insult in vitro as well as the differential effects of normal and leukemic cells in the same animal.

The human counterpart of the mouse myeloid leukemic cells is most likely the human promyelocytic leukemia cell line HL60. This line was identified by chromosome banding to contain a translocation between chromosomes 10 and 13, monosomy in chromosomes 5, 10, 14 and 17, deletions in chromosomes 9 and 13 as well as the addition of three marker chromosomes. This line is susceptible to induction of differentiation upon exposure to phorbol-12-myristate-13-acetate. Sublines with various degrees of resistance to differentiation have been isolated and their resistance is associated with chromosomal alterations including gains or losses of chromosomes 5, 8, 11, 13, 16 and 17. It is suggested that acquisition of resistance to induction of differentiation may involve one or more of the above chromosomal changes.

The existence of both human and mouse myeloid leukemic cell lines will allow us to investigate whether specific responses are myeloid leukemia specific or species specific.

The studies described in the sections above are all continuing, and from them we hope to obtain information that we can eventually use to provide estimates of the genetic and carcinogenic risks to man from exposure to radiation and chemical agents. We are also developing new approaches, particularly with regard to the involvement of specific

chromosome translocations and the relocation of oncogene sequences, to the induction of specific tumors.

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#### DNA REPAIR IN HUMAN CELLS

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Our primary objective is to elucidate the molecular events in human cells when cellular macromolecules such as DNA are damaged by radiation or chemical agents. We study and characterize (i) the sequence of DNA repair events, (ii) the various modalities of repair, (iii) the genetic inhibition of repair due to mutation, (iv) the physiological inhibition of repair due to mutation, (v) the physiological inhibition of repair due to biochemical inhibitors, and (vi) the genetic basis of repair. Our ultimate goals are

to (i) isolate and analyze the repair component of the mutagenic and/or carcinogenic event in human cells, and (ii) elucidate the magnitude and significance of this repair component as it impinges on the practical problems of human irradiation or exposure to actual or potential chemical mutagens and carcinogens.

The significance of these studies lies in (1) the ubiquitousness of repair (most organisms, including man, have several complex repair systems), (ii) the belief that mutagenic and carcinogenic events may arise only from residual (nonrepaired) lesions or that error-prone repair systems may be the major induction mechanisms of the mutagenic or carcinogenic event, and (iii) the clear association of repair defects and highly carcinogenic disease states in man [xeroderma pigmentosum (XP)].

Ultraviolet light induces dimer formation between adjacent pyrimidines in DNA of cells resulting in a variety of deleterious effects. We have studied the UV-light induction and excision of pyrimidine dimers in the DNA of mammalian cells. The number and kinds of dimers formed in DNA is related to the wavelength of UV-light, pyrimidine content, and the total dose. Also, the number of dimers produced in cells growing in a monolayer may differ by a factor of 2 and is attributable to cell shape. In human cells, a low dose of 254 nm light produces cytosine-cytosine, cytosine-thymidine and thymidine-thymidine dimers at a ratio of 16:24:60 while a low dose of simulated sunlight produces dimers at a ratio of 20:40:40. The number of cytosine-cytosine dimers saturates at  $50 \text{ Jm}^{-2}$  of 254 nm light and never exceeds 0.05% of total pyrimidines independent of the wavelength used for irradiation. Normal human cells are able to excise  $10^6$  dimers from their DNA in a day following irradiation with 254 nm light or simulated sunlight. Mouse cells in culture are deficient in their ability to excise dimers as measured by bromodeoxyuridine (dBrU) photolysis, UV-endonuclease sensitive sites and dimer chromatography. Inhibitors of DNA synthesis [hydroxyurea (HU) and cytosine arabinoside (ara-C)] also have an effect on the excision repair of pyrimidine dimers. Repair processes for UV-induced DNA damage may provide a model for the study of DNA damage by chemical and by other physical agents which result in mutagenesis and carcinogenesis in mammalian cells.

HU is most often used for the selective suppression of replicative DNA synthesis. Repair synthesis, elicited by the excision of DNA damage, is more easily detected under these conditions. However, HU in some instances interferes with the excision repair process. We have compared the effects of HU on the excision of ultraviolet light induced pyrimidine dimers in unsynchronized human skin populations of growing cells and in cells outside of S phase (quiescent populations). HU at low concentrations has little or no effect on the number of pyrimidine dimers excised from the DNA of normal human skin fibroblasts in a proliferating phase. The effect of HU on cells in a quiescent stage is quite different. HU at 2 mM has a marked effect on the number of dimers excised while 10 mM reduces excision by as much as 80%.

It is thought that the different response of these cells is due to the existence of different precursor pools. Cells undergoing excision repair in S phase may have a greater access to nucleotide pools than cells in non-S phase. Clearly, the growth phase must be taken into consideration in the evaluation of the effect of HU on excision repair and its use in the measurement of repair replication.

We have evidence of the induction and repair of a UV-induced lesion in human cells which is apparently not a pyrimidine dimer. This observation was made by measuring strand breaks induced by 313 nm light in the DNA following UV (254 nm) irradiation. Immediately following irradiation ( $20 \text{ J/m}^2$ , 254 nm) of either normal, XP or XP-variant cells, about 2 breaks/ $10^9$  daltons DNA can be induced by photolysis with 313 nm light. These breaks are not demonstrable 5 hr after irradiation in normal cells; however, in XP-variant cells, the breaks are inducible for up to 24 hr after irradiation. XP complementation group A cells in the same 5 hr period show an increase in the number of strand breaks seen with 313 nm light photolysis to about 4 breaks/ $10^9$  daltons DNA which can be induced for up to 24 hr. These data indicate that in normal cells the lesion responsible for this effect is rapidly repaired or altered, whereas in XP-variant cells it seems to remain unchanged. Some change apparently occurs in the DNA of XP group A cells which results in an increase in photolability. These data may, thusly, indicate a deficiency in DNA repair of XP-variant cells as well as in XP group A cells. Pyrimidine dimers are probably not responsible since our assays indicate the XP-variant cells repair these lesions in their DNA as well as normal cells.

In addition to dimer chromatography and dBrU photolysis, we have developed other assays of repair which aid in the study of repair kinetics in different cell lines. One useful assay involves the inhibition of repair by ara-C and the combination of ara-C and HU (ara-C/HU). This assay was used for comparative studies of UV-induced DNA repair and its inhibition by these compounds in log phase and confluent cell cultures of normal and XP-variant human skin fibroblasts. Repair inhibition was determined by measuring the accumulation of DNA single-strand breaks following cell culture exposure to ara-C or ara-C/HU in a series of 3 hr pulses up to 24 hr after UV insult. Normal and XP-variant cells showed a wide range of sensitivity to both ara-C or ara-C/HU in log phase cultures. The amount of repair inhibition observed was inversely proportional to the generation time of the cells. In confluent cultures, maximum repair inhibition was observed with ara-C/HU in both normal and XP-variant cultures. The maximal level of repair inhibition was observed in normal cells after receiving ara-C alone after UV, but was 62-68% lower in XP-variant cells receiving ara-C vs ara-C/HU. Repair arrest was more rapidly reversed by increasing concentrations of exogenous deoxycytidine 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 to a lower degree in the XP-variant when compared to cells exposed to ara-C alone. However, the same addition of HU had relatively little effect on dCyd competition in normal cells. Assay of dNTP pools by HPLC in the above cell lines suggested an elevated level of dCTP

in the XP-variant lines during log phase, which is a possible explanation for lowered sensitivity to DNA repair inhibition by ara-C in the cells. The overall increase in dNTP pools in the XP-variant may be the result of defective DNA polymerase(s) which ultimately leads to error-prone repair.

The dBrU photolysis assay has proven to be useful in monitoring the effects of products of coal conversion (liquefaction and gasification) on DNA in human cells. This repair assay is capable of detecting, in addition to damage-induced DNA repair, agent induced photolysis which produces DNA strand breaks and DNA cross-linking. Results such as these are of interest since they indicate unrepaired DNA damage.

An array of products from steps in the H-coal liquefaction process was assayed for ability to induce DNA damage. These samples included crude samples taken at different steps of successive hydrogenation (hydrotreatment or HDT) and samples resulting from fractionation of a crude fuel oil sample (provided by the Analytical Chemistry Division, ORNL), including aliphatic, mono/diaromatic and polycyclic aromatic fractions. No significant level of DNA repair was detected in any of the samples tested. However, the presence of photosensitive lesions was noted in almost every sample assayed. Successive hydrogenation steps in the HDT series of samples resulted in fewer DNA strand breaks/ $10^8$  daltons DNA induced by 313 nm light - suggestive of less DNA damage. The aliphatic fraction of crude oil caused significant photolytic damage (1.09 strand breaks/ $10^8$  daltons DNA), while the polycyclic aromatic fraction caused extensive DNA cross-linking to occur. The overall results point out potential hazards to personnel who deal directly with coal liquefaction products and further suggests that successive hydrotreatment steps significantly reduce the mutagenic/carcinogenic potential of the final products of H-coal liquefaction.

Benzo[a]pyrene a ubiquitous environmental pollutant, when metabolized to its active metabolite, benzo[a]pyrene diol epoxide (BPDE), has been shown to be highly mutagenic and carcinogenic. Several stereoisomeric forms of BPDE with varying amounts of biological activity are produced during the activation process. Recently we have studied the induction of DNA damage and its repair in human cells by two diastereomeric forms of BPDE, namely the potently carcinogenic anti- and non-carcinogenic syn-BPDE. Interestingly enough, we have found the anti-isomer to induce two-fold more DNA adducts, yet induce similar amounts or slightly less repair than the syn-isomer. These strand breaks occur at a much reduced frequency in XP cells, which carry a genetic defect rendering them incapable of DNA excision repair. These differences in repair may correlate with the mutagenic and carcinogenic properties of these two isomeric forms of BPDE. In addition we have examined the size of the repair patch produced during the repair of these two DNA-adducts, and have found both to induce a long patch repair with approximately 40 nucleotides being inserted per repaired lesion.

Several classes of compounds exist which inhibit individual steps or the entire process of DNA excision repair. Using specific acting inhibitors we can dissect out individual molecular events induced in the

DNA excision pathway. To this end we have recently examined the effects of aphidicolin, a specific competitive inhibitor with deoxycytidine triphosphate for DNA polymerase alpha, UV-induced DNA excision repair. Three major DNA polymerase species exist in the human cell, with DNA polymerase alpha being in highest concentration. We have found aphidicolin to inhibit both the resynthesis step and the excision step following UV-induced repair, thus implicating a major role for DNA polymerase alpha in human DNA excision repair. This inhibition is dependent on the metabolic state of the cells. Cells which are undergoing rapid growth and division are relatively resistant to aphidicolin, while cells which are in a quiescent monolayer are quite sensitive to the inhibitory action of aphidicolin. We have found it takes a concentration of aphidicolin 100-fold higher to inhibit repair in rapidly proliferating cells to the same extent as in nondividing cultures. These differences in inhibition are due to at least two factors, the size of the deoxynucleotide pools and the levels of DNA polymerase alpha in human cells. Rapidly proliferating cells have elevated levels of deoxynucleotides and DNA polymerase alpha thus rendering them relatively insensitive to the drug. Using specific acting inhibitors like aphidicolin we have shown how various metabolic states of the cell can modulate the repair response to DNA damage.

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

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

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## GENETIC ANALYSIS OF DNA REPAIR IN MAN WITH CELL HYBRIDS

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

The mechanisms by which cells repair induced DNA damage play a central role in carcinogenesis such that deficient or inaccurate repair could result in carcinogenesis. The most direct evidence for this is derived from the existence of the recessively inherited human disorder xeroderma pigmentosum (XP) in which there has been a direct linkage between deficient repair of UV-induced DNA damage and indication of cancer. The genetic heterogeneity in XP, as reflected in the existence of at least seven complementation groups, indicated that a greater understanding of the genetic structure of the DNA repair system is required if xeroderma pigmentosum is to be fully utilized as a model system for studying radiation induced carcinogenesis. To analyze the genetic structure of excision repair in man, human  $\times$  mouse somatic cell hybrids were employed. The preferential segregation of human chromosomes in proliferating hybrid cells and the ability to specifically identify each chromosome make it possible to dissect this complex polygenic system by isolating its component parts and to determine the chromosomal assignment of genes required for the repair of UV-induced DNA damage.

Human  $\times$  mouse somatic cell hybrid clones, isolated from five separate fusion experiments employing human cells derived from unrelated individuals normal for repair of UV-DNA damage, were analyzed for repair ability employing the bromodeoxyuridine photolysis assay. Mouse cells repair UV-induced DNA damage at 5-10% of the magnitude of human cells. Therefore, it was possible to distinguish quantitatively human and mouse DNA repair components in cell hybrids. The repair ability of hybrid clones falls into one of three categories: (1) those having human-like repair; (2) those having mouse-like repair; and (3) hybrids intermediate between the two. Data derived from experiments which measure the induction of M. luteus edonuclease sensitive sites following UV irradiation indicate that the number of dimers/10<sup>8</sup> daltons induced in human, mouse, and human  $\times$  mouse

cell hybrids is the same. Therefore, the difference in repair capabilities observed in hybrid cells by dBrU photolysis is due to retention or loss of specific human genes due to human chromosome segregation by the hybrid cells.

The man  $\times$  mouse somatic cell hybrids were analyzed for the presence or absence of each human chromosome. When the segregation of the ability to repair UV-induced DNA damage was compared to the segregation of human chromosomes in three sets of hybrids made between human fibroblasts and mouse cells there was a strong correlation between the presence of human chromosome 3 and the ability to repair UV-induced DNA damage and the loss of chromosome 3 and the loss of excision repair capacity. These data suggest that a gene or genes required for DNA repair is located on chromosome 3. In these same clones there was a correlation between chromosome 14 and excision repair capacity. It is interesting to note in this regard that aberrations of chromosomes 3 and 14 have been associated with specific human malignancies, suggesting a possible association between disturbances of the repair system and carcinogenesis.

#### GENETIC DISSECTION OF DNA REPAIR IN XERODERMA PIGMENTOSUM CELLS

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J. D. Regan

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

gene-chromosome assignments, to dissect this complex polygenic system by isolating its component parts and to investigate the genetic basis for complementation of defective repair in XP cells. To carry out these studies, several sets of hybrids were generated employing human parental cells derived from each of five XP complementation groups, namely Groups A-E. These human  $\times$  mouse cell hybrids were formed such that they either segregated human chromosomes and retained mouse chromosomes or segregated mouse chromosomes and retained human chromosomes. Seven sets of hybrids were isolated from these fusion experiments.

When the XP  $\times$  mouse hybrid cells were analyzed for repair ability, it was found that mouse cells complemented the defective repair in four XP complementation groups, namely Groups A, B, C, and D, but not in Group E, suggesting that the genetic defect in Group E is different from that found in the other groups. Segregation analysis of the cell hybrids demonstrated that when XP  $\times$  mouse somatic cell hybrids segregated certain human chromosomes, they lost the ability to be complemented by the mouse cell. Presumably, these hybrids have lost the gene(s) coding for the repair step that is rate limiting in the mouse. On the other hand, the mouse supplies the repair step that is defective in the XP cell. In XP-A  $\times$  mouse hybrids, there is a strong correlation between the presence of chromosome 14 and the ability to be complemented and the loss of complementation and the loss of chromosome 14. No such correlation was seen with chromosome 3. In XP-B  $\times$  mouse hybrids there is a correlation between the presence of chromosome 3 and complementation and absence of chromosome 3 and the loss of complementation. Chromosome 14 may also be associated with complementation since only one clone in the XP-B  $\times$  mouse hybrids was discordant for complementation and chromosome 14.

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

The complementation of defective repair of UV induced DNA damage in XP cells by mouse genetic material was shown to be biologically significant. This was done by testing hybrid clones for resistance to UV irradiation. XP cells are highly sensitive to UV irradiation. Using this approach viable XP  $\times$  mouse hybrid clones were isolated following UV irradiation, demonstrating that the XP defect had been complemented by mouse DNA both enzymatically and biologically. These findings provide the means for cloning mammalian repair genes and for studying DNA repair at the molecular level.

## GENETIC BASIS OF MUTAGENESIS AND CARCINOGENESIS

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|-----------------------------|------------------------------------|
| P. A. Lalley<br>B. E. Allen | P. Braden<br>K. Moore <sup>1</sup> |
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The primary objectives of this project are (1) to investigate the genes important for susceptibility to mutagens and carcinogens whether they be physical, chemical or biological agents; (2) to identify, characterize and determine the chromosomal assignment of these genes; and (3) to investigate the linkage relationships of homologous genes in man, mouse and other species. The data derived from these studies are important since an elucidation of the genetic basis of these systems is essential for the ultimate understanding of how genetic and environmental factors interact in the process of mutagenesis and carcinogenesis. Comparative mapping data are important for the extrapolation of biological and biomedical data from laboratory animals to man. These studies are being pursued utilizing somatic cell hybrids as our experimental system, which allows for the genetic dissection of complex polygenic traits by isolating their component parts, and the determination of gene-chromosomal assignments due to preferential chromosome segregation.

We have been investigating the genetics of the induction of the aryl hydrocarbon hydroxylase system (AHH) in the mouse, a system of major importance in chemical carcinogenesis. The Ah locus encodes the receptor that controls the induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC 1.14.14.1) by polycyclic hydrocarbons such as a benzo[a]-anthracene or 3-methylcholanthrene. The hydroxylase inducibility by benzo[a]anthracene was studied in 29 somatic cell hybrid clones. In 28 of the 29 clones, the presence or absence of inducibility is associated with the presence or absence of mouse chromosome 17. Liver microsomal aryl hydrocarbon hydroxylase induction by 3-methylcholanthrene or benzo[a]-anthracene was also assessed in appropriate backcrosses with the Mus musculus molossinus, Mus musculus castaneus, MOR/Cv, PL/J, SM/J and DBA/2J inbred strains and in 13 NX8 recombinant inbred lines. It was concluded from the studies that the presence of aryl hydrocarbon hydroxylase inducibility, i.e., the Ah locus encoding the receptor, is localized to the distal end of mouse chromosome 17, a chromosome which carries several genes (e.g. H-2 complex) which control the host response to foreign substances.

Treatment of mice with polycyclic aromatic hydrocarbons results in the induction of cytochrome P<sub>1</sub>-450 and P<sub>3</sub>-450. The genes for both cytochromes have recently been cloned and shown to be coordinately regulated by the Ah locus. The mouse analogue of P<sub>1</sub>-450 and P<sub>3</sub>-450 can be distinguished from its hamster counterparts by Southern blot analysis with Kpn I restricted DNAs. DNA from hamster-mouse somatic cell hybrids segregating mouse chromosomes were used in Southern blots to map the location of the two mouse genes. Chromosome segregation analysis of 12 hybrid clones demonstrated that the structural genes for both P<sub>1</sub>-450 and P<sub>3</sub>-450 are linked and can be assigned to mouse chromosome 9 and therefore are not linked to the gene which controls their expression, the Ah receptor locus on chromosome 17. These data are aiding in our understanding of the

control, expression and organization of the AHH system which is directly associated with chemical carcinogenesis.

The chromosomal locations of genes involved in murine viral carcinogenesis have been investigated utilizing restriction enzyme analysis with cDNA probes and somatic cell hybrids. In collaboration with Dr. W. K. Yang and R. W. Tennant we have investigated the genetics of the RF virus, an inducible murine leukemia virus from the RFM mouse. RFM/Un strain mice possess a single host range (N-tropic) class of endogenous retrovirus, which can be induced from virus negative cells in culture. We assayed virus induction in interspecific hybrid clones of RFM spleen or bone marrow cells and Chinese hamster (E36) cells that segregated mouse chromosomes. The data demonstrated that inducibility was controlled by a gene on chromosome 5. Independently, the hybrid clones were tested for endogenous murine retroviral sequences by restriction endonuclease digestion and electrophoresis-hybridization with a molecular cloned probe specific for ecotropic retroviruses. Virus inducibility was concordant with the presence of a 20 Kbp band that was present only in clones retaining chromosome 5 indicating that the RF virus sequences are integrated on chromosome 5. These results make it possible to identify novel rearrangements of this endogenous virus in cells with different neoplastic phenotypes. We believe that the data derived from these studies will help to make the RFM/Un mouse strain a valuable model system in which to study the relationships between radiogenic myeloid leukemia, specific chromosomal rearrangements and retrovirus genes.

Man, mouse and all mammalian species studied possess normal cellular genes (proto-oncogenes) homologous with acutely transforming retrovirus oncogenes. Recently altered forms of several proto-oncogenes have been identified in malignant cells. These data have fostered speculation of possible roles of proto-oncogenes in malignant transformation of cells and prompted the investigation of their chromosomal assignments. Since consistent numerical and structural chromosome abnormalities have been associated with a number of different cancers, it is possible that proto-oncogenes might reside at chromosome breakpoints associated with specific tumors. We have pursued these studies by screening somatic cell hybrids with  $^{32}P$ -labeled viral oncogene probes. A molecular clone of viral myc(v-myc), the oncogene of avian myelocytomatisis virus MC29 detected homologous human, mouse, and Chinese hamster cellular myc(c-myc) sequences by Southern filter hybridization. Using human  $\times$  mouse somatic cell hybrids we demonstrated that the human c-myc is located on human chromosome 8 close to the breakpoint on chromosome 8(q24) involved in the t(8;14) translocation found in Burkitt's lymphoma. The mouse c-myc gene was shown to be located on mouse chromosome 15 in a region involved in the t(15;12) translocation associated with mouse plasmacytomas. In addition, in collaboration with Leroy Hood and colleagues at the California Institute of Technology, it was demonstrated that this region of mouse chromosome 15 contains a DNA sequence termed NIRD (non-immunoglobulin rearranged DNA) which is also rearranged in mouse plasmacytomas. In both the t(8;14) in man and the t(15;12) in the mouse the myc gene is translocated into a chromosomal region involved in immunoglobulin production. These gene assignments are noteworthy because the myc gene is located in homologous

chromosomal regions in both man and mouse which are involved in structural abnormalities associated with homologous B-cell neoplasms. Following the same approach we have assigned five additional mouse proto-oncogenes using DNAs of 15 mouse-Chinese hamster somatic cell hybrids which were screened with  $^{32}P$ -labeled viral oncogene probes. We assigned a mouse c-src gene to chromosome 2, c-Ki-ras to 6, c-Ha-ras to 7, c-myb to 10, and c-erb-a to 11. Mouse c-src, c-Ki-ras, and c-Ha-ras appear to reside in conserved linkage groups in mouse and man; for example, c-src is on human chromosome 20 (with ADA and ITP homologous to mouse chromosome 2), c-Ki-ras2 is on 12p (with TPI and GAPD homologous to mouse chromosome 6), and c-Ha-ras is on 11p (with INS, HBB, and LDHA homologous to mouse chromosome 7). It has been reported that a deletion commonly encompassing bands C-D of mouse chromosome 2 is found in 94% of myeloid leukemias. Whether these deletions affect the c-src gene or the c-abl gene, which are both located on chromosome 2, is not presently known. In man the c-abl gene is located in a region on chromosome 9 involved in a reciprocal t(9;22) translocation associated with chronic myeloid leukemia. Rearrangements of mouse c-myb and altered c-myb mRNA transcripts have been observed by others in mouse plasmacytoid lymphosarcomas. Whether the alterations in c-myb arise by intra- or inter-chromosomal events is not known. These assignments should be useful in assessing the significance of chromosome rearrangements in murine neoplasms.

We have continued to combine the use of somatic cell hybrids with recombinant DNA techniques to map genes coding for proteins which are not expressed in tissue culture cells.

The interferons have been classified as  $\alpha$ ,  $\beta$ , and  $\gamma$  (or leukocyte fibroblasts and immune) on the basis of biological, biochemical, and antigenic properties. The human interferons have been cloned and characterized quite extensively genetically. Recently, a cDNA clone specific for mouse immune interferon (IFN- $\gamma$ ) was isolated. We used this clone containing a 638 bp insert to detect mouse interferon sequences in mouse-Chinese hamster hybrids segregating mouse chromosomes. Mouse DNA digested with EcoRI and probed with mouse IFN- $\gamma$  cDNA in a Southern filter analysis contains a single 18 kb HindIII Fragment which anneals with the probe. In contrast, Chinese hamster DNA does not hybridize to the cDNA. This 18 kb fragment segregated in 15 mouse-Chinese hamster hybrids depending on the mouse chromosome composition. This segregation pattern indicated that the mouse immune interferon gene (Ifg) is located on chromosome 10. It is interesting to note that the human immune interferon maps to chromosome 12 (p12 $\rightarrow$ qter). This region of chromosome 12 also contains the genes for peptidase-2 (B) and acetate synthetase. The homologous genes in mouse are also located on chromosome 10, suggesting that this is a conserved linkage group between man and mouse.

Nerve growth factor (NGF, also referred to as the  $\beta$ NGF) is a protein essential for neuron development. High levels of NGF activity are expressed in the adult male mouse submaxillary gland. A 1.1 kb cDNA from this tissue encoding the mouse NGF precursor (prepro NGF) was used to chromosomally assign the mouse Ngf gene by the methodologies described above. The cDNA probe hybridized to 15 kb and 7 kb fragments of BALB/c

mouse DNA cleaved with HindIII, whereas HindIII cleaved Chinese hamster DNA yielded 14 kb and 5.5 kb hybridizing fragments. DNAs from 15 mouse-Chinese hamster cell hybrids were isolated and hybridized to the NGF probe. Mouse Ngf segregated concordantly only with mouse chromosome 3. This segregation pattern was identical to that of mouse amylase when these cell hybrid DNAs were annealed with a rat amylase probe. Mouse amylase (Amy-1, Amy-2) is known to be on mouse chromosome 3. The data suggest that mouse Amy-1, Amy-2, and Ngf reside in a conserved linkage group, as the human AMY1, AMY2, and NGF are all located on the short arm of chromosome 1.

The genes for the serine endopeptidase chromtrypsinogen B (Ctrb), elastase-1 (Ela-1), and trypsin-1 (Trp-1), and the exopeptidase carboxypeptidase A (Cpa), have been isolated from a rat cDNA library. In order to map these genes in the mouse, DNA from mouse  $\times$  Chinese hamster hybrids was cleaved with HindIII or Bam HI and hybridized to Ctrb, Ela-1, Trp-1, or Cpa cDNA probes. Mouse Ctrb gene sequences, detected on 5.3 kb and 3.1 kb fragments, segregated only with chromosome 8. Mouse Ela-1 gene sequences detected on 10 kb and 4.2 kb fragments, segregated only with chromosome 15. Trp-1 gene sequences, detected on 12.8 kb, 9.2 kb, and 6.56 kb fragments, and Cpa gene sequences, detected on 5.2 kb and 3.7 kb fragments, both segregated only with chromosome 6. These data identify several conserved linkage groups. The human homolog of Ctrb maps to human chromosome (HSA) 16 and therefore comprises part of the region of homology previously observed between mouse chromosome (MMU) 8 and HSA16. We have mapped the human homologs of both Trp-1 and Cpa to HSA 7q22+pter and thus a region of homology exists between MMU6 and HSA7q.

The chromosomal locations of the genes for the common  $\alpha$  subunit of the glycoprotein hormones and the  $\alpha$  subunit of chorionic gonadotropin in mice and humans have been determined by restriction enzyme analysis of DNA isolated from somatic cell hybrids. The CG  $\alpha$  gene (CGA), detected as a 15 kb BamHI fragment in human DNA hybridization to CG $\alpha$ cDNA, segregated with the chromosome 6 enzyme markers MEI (Malic enzyme, soluble) and SOD2 (superoxide dismutase, mitochondrial) and an intact chromosome 6 in human-rodent hybrids. Cell hybrids containing portions of chromosome 6 allowed for the localization of CGA to the q12-q21 region. The 30 and 6.5 kb BamHI CGB fragments hybridizing to human CG $\beta$  cDNA segregated concordantly with the human chromosome 19 marker enzymes PEPD (peptidase D) and GPI (glucose phosphate isomerase) and a normal chromosome 19 in karyotyped hybrids. A KpnI-HindIII digest of cell hybrid DNAs indicated that the multiple copies of the CGB gene are all located on human chromosome 19. In the mouse, the  $\alpha$  subunit gene, detected by a mouse thyrotropin (TSH)  $\alpha$  subunit probe, and the CGB-LHB gene, detected by the human CG $\beta$  cDNA probe, are on chromosomes 4 and 7, respectively. Lhb appears as though it may lie in a conserved region since chromosome 7 also carries the genes for glucose phosphate isomerase and peptidase-4 whose homologous genes as well as the LHB gene are on human chromosome 19.

Humans show a single insulin protein and gene while mice and rats possess two distinct insulins. To determine the chromosomal location of the mouse insulin genes we have examined Chinese hamster  $\times$  mouse somatic cell hybrids segregating mouse chromosomes by restriction enzyme analysis

with a mouse specific cDNA probe for the insulin gene. The data demonstrate that the mouse carries a total of three insulins located on three different chromosomes: 6, 7 and 15. The insulin gene located on chromosome 7 has been partially sequenced and these data indicate that this gene codes for insulin II protein and appears to be homologous to the human and rat II insulin genes. In man the homologous insulin gene (INS) has been assigned to chromosome 11p and is syntenic with LDHA and HBB. In the mouse the homologous genes are also located on chromosome 7 indicating that a region of human chromosome 7 is homologous to a region of human chromosome 11p. The functions of the other two insulin genes on chromosome 6 and 15 are not known. However, one should code for insulin I while the other may be a pseudo-gene. These data demonstrate that evolution of the insulin proteins has proceeded differently in rodents and man. This has implications for extrapolation of data from laboratory animals to man when dealing with systems that have genetic components.

To further our understanding of the evolution of the mammalian genome we have initiated gene mapping experiments in nonhuman primates. To determine which linkage groups have been conserved between man and the more distantly related primates, namely the Cercopithecoidea, somatic cell hybrids were formed between mouse cells and baboon (Papio papio) lung fibroblasts derived from two unrelated baboons. Thirty-two primary clones segregating baboon chromosomes were analyzed for the expression of 25 gene markers. In particular, genes assigned to human chromosomes 4, 6, 14, 15, and 20 were studied. Data derived from these studies demonstrated that the genes coding for phosphoglucomutase 2 (PGM2) and peptidase S (PEPS) are syntenic. These data indicate that the pter $\rightarrow$ q12 region of human chromosome 4 (HSA4), is homologous to a region of baboon chromosome 5 (PPA5). Our data further demonstrated that the gene coding for the mitochondrial form of superoxide dismutase (SOD2) segregated concordantly with malic enzyme (MEI) and glyoxylase (GLO) indicating that the linkage group of MEI-GLO-SOD2 is conserved between man and baboon. The concordant segregation of inosine triphosphatase (ITP) and adenosine deaminase (ADA) in these hybrids demonstrates that ITP and ADA are syntenic, indicating that PPA10 is homologous to HSA20. In addition, data derived from these studies confirm that the genes coding for mannosephosphate isomerase (MPI), pyruvate kinase 3 (PK3) and nucleoside phosphorylase (NP) are syntenic in the baboon, indicating that regions of PPA 7 are homologous to HSA 14 and 15.

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#### MAMMALIAN BIOCHEMICAL GENETICS

R. A. Popp                    D. M. Popp

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. The studies can be divided into the four general areas of research discussed below.

Mechanisms of Mutations Induced in Germinal Cells by Irradiation and Chemicals. We have continued to study the expression of hemoglobin genes in progeny of mice exposed to mutagens. Last year we reported on an ethylnitrosourea induced A → T transversion at the codon for position 89 (CAC → CTC) of an alpha-globin gene, which resulted in an amino acid substitution (His → Leu). This mutation has not been observed previously in humans, mice or other mammals, and its novel occurrence may be indicative of other unusual mutational events that do not ordinarily occur in the absence of specific mutagen exposure.

We are presently analyzing an ethylnitrosourea induced mutation at the compound beta globin (Hbb) locus. The mutation changes the electrophoretic mobility of the majority of the beta-globin polypeptide controlled by the Hbb<sup>B</sup> allele. The specific nature of this mutation is under investigation but it is likely that the electrophoretic mobility change is caused by the substitution of a single amino acid resulting from an ethylnitrosourea induced substitution of a single base in the DNA. Definition of the precise base substitution involved will add to our data base of specific mutations induced by chemicals such as ethylnitrosourea. As additional induced mutations are characterized, comparisons between newly induced and naturally occurring mutations in the mouse may provide insight into potential genetic hazards to which man may be subjected from exposure to mutagens in the environment.

The proband bearing the newly discovered mutation of the Hbbs allele was a female. Upon electrophoresis her hemoglobin gave an unusual four-banded pattern and the quantitative analysis of the four hemoglobins in the proband was different from that in her progeny that also exhibited four electrophoretically distinguishable hemoglobins. Genetic studies established that the proband was a germinal mosaic; her ova contained either the normal or the mutant beta-globin genes. The unusual quantitative analysis of the individual hemoglobins in the proband can be explained on the basis that she is also a somatic cell mosaic and her red cells express either the normal or the mutant beta-globin genes. It has been possible to establish a colony of mice with this ethylnitrosourea induced mutation because the mutation was expressed in some of the germinal cells. We had previously found an X-ray-induced beta-globin mutation that was expressed only in somatic cells (3695) but we were not able to establish a colony of mice with that X-ray-induced mutation.

In July of 1982, Dr. Susan Lewis (Research Triangle Institute) discovered a DBA/2J mouse in which the ratio of beta-major/beta-minor hemoglobin, usually 80/20, was shifted to 55/45. Homozygotes for this mutation produced only the beta-minor polypeptide — no beta-major polypeptide was produced. The molecular basis for the failure of the beta-major polypeptide synthesis was revealed through Southern blot analysis of DNA, which showed that a 3.3 kilobase segment of DNA, including the beta-major gene, was missing in homozygotes. This mutation arose spontaneously and the exact nucleotide sequences surrounding both ends of the deletion are being analyzed. Mice homozygous for this spontaneous deletion exhibit symptoms of beta-thalassemia, which is a severe genetic disease in man. The line of mice established from this mutant represents the only animal model of this disabling disease.

Genetic Regulation of Gene Expression and the Organization of Genetic Information. The studies mentioned above indicate that a fundamental understanding of the structure, function, organization, and regulation of genes and their products was essential to interpret the mechanisms of the spontaneous beta-thalassemia mutation and the X-ray- and ethylnitrosourea-induced mutants at the alpha- and beta-globin genes. We reported last year that transcription of the alpha-globin genes was elevated to 75% of normal and partially compensated for the loss of one-half of the alpha-globin genes in alpha-thalassemic mice. A similar but more dramatic compensation appears to occur in beta-thalassemic mice. The Hbb<sup>d</sup> beta-minor globin gene controls the synthesis of 3 picograms of beta globin per erythrocyte in normal mice but 10 picograms of beta globin are produced in erythrocytes of beta-thalassemic mice. Our previous studies in mice have shown that the synthesis of alpha- and beta-globins exactly reflects the quantities of alpha- and beta-globin mRNAs. Thus, it would appear that transcription of the beta-minor globin gene in beta-thalassemic mice is three-fold that in normal mice. Possible mechanisms for this three-fold increase in the level of beta-minor globin gene transcription are being investigated. Elucidation of such mechanisms should help us to understand better how specific genes are regulated during development and cell differentiation and, by inference, what may go wrong during abnormal growth and differentiation of tumor cells.

The newly found, ethylnitrosourea induced mutation at the compound Hbb locus is being used in our studies on the genetic regulation of hemoglobin gene expression. Mice of the Hbb<sup>s</sup>/Hbb<sup>s</sup> genotype produce only one kind of beta-globin polypeptide even though they possess two beta-globin genes, called beta<sup>s</sup> and beta<sup>t</sup>. The beta<sup>s</sup> and beta<sup>t</sup> genes can be recognized by using Southern blotting techniques but their gene products — mRNAs and beta-globin polypeptides — cannot be distinguished by mRNA/cDNA hybridization and amino acid sequencing. Thus, it was not known whether or not both beta<sup>s</sup> and beta<sup>t</sup> genes were functional and, if both functioned, whether the levels of expression of these two genes differed in a manner comparable to the differential expression (80/20) of the beta-major/beta-minor globin genes, respectively, of the Hbb<sup>d</sup> allele. Mice homozygous for the ethylnitrosourea induced mutation of the Hbb<sup>s</sup> allele produce two electrophoretically distinguishable beta-globin polypeptides; the product of the mutant's Hbb<sup>s</sup> gene represents about 75 percent of the total beta-globin. Thus, we now know that both beta-globin genes of mice of the Hbb<sup>s</sup>/Hbb<sup>s</sup> genotype are active and that the level of expression of one gene is higher than the other. Studies are in progress to determine whether beta<sup>s</sup> and beta<sup>t</sup> of the Hbb<sup>s</sup> allele are homologous to the beta-diffuse-major and the beta-diffuse-minor genes of the Hbb<sup>d</sup> allele as regards both the organization and the structure of these genes on chromosome 7 of mice.

Pathophysiological Effects of Induced Mutations. We have previously reported on the clinical symptoms (anemia, splenomegaly and iron overloading) in mice heterozygous for alpha-thalassemia, and on the embryonic lethality in mice homozygous for alpha-thalassemia. In humans the clinical symptoms of beta-thalassemia are severe; humans homozygous for beta-thalassemia die unless supportive blood transfusions are given regularly. In contrast, mice homozygous for beta-thalassemia survive even though they are very anemic at birth and are easily recognized by their paler appearance. Adult beta-thalassemic mice have lower than normal hematocrit and hemoglobin values and elevated reticulocyte counts; their red cells are microcytic and exhibit anisocytosis. The synthesis of alpha- and beta-globin polypeptides has been measured using <sup>3</sup>H-leu incorporation; the synthesis of beta-globin is about 70% of normal. In spite of these severe clinical symptoms, both male and female homozygotes are able to breed and females raise their young. A few homozygous beta-thalassemic mice have been sacrificed for gross inspection. Splenomegaly (four- to ten-fold normal size) is obvious. The population of mice is being enlarged to initiate thorough studies on the pathophysiological effects of this mutation. These mice are the only animal models of beta-thalassemia; until now beta-thalassemia has been exclusively a human disease. These mice will be valuable experimental animals for studies on the progression of symptoms associated with human beta-thalassemia and in our attempts to do gene therapy to correct this genetic defect.

In Vivo Studies on the Toxicity and Mutagenicity of Ethylene Oxide. We explained last year why we use the hematopoietic system to study the in vivo toxic and mutagenic effects of chemicals, drugs and environmental pollutants. Briefly, the number of pluripotent stem cells can be quantified (CFU-S), the effects on undifferentiated stem cells and more differentiated cell populations can be assayed, changes in cell cycle

parameters can be analyzed by measuring the DNA content of cells by flow cytometry, and the number of 6-thioguanine resistant (putative mutant) CFU-S induced by a chemical can be quantified. In collaboration with R. Mann and R. Hand (Biology Division) flow cytometric analysis on the Ortho 50H has been modified to characterize specific cell types in the heterogeneous population of cells in bone marrow. Forward and 90° light scattering are being used to identify cells of different sizes and morphology, respectively, and fluorescence is being used to identify cells with specific cell surface markers (fluorescein-labeled anti- $\mu$  heavy chain) and to quantify changes in DNA content (propidium iodide). By using a combination of these techniques, we have been able to identify and enumerate separate cell populations in normal bone marrow. The above techniques and other tests developed in our laboratory and described last year have been used to study the effects of ethylene oxide on the hematopoietic system of mice. Mice were exposed by inhalation to 255 ppm of ethylene oxide for 6 hr per day for 2, 4, 6, 8 and 10 consecutive days in a preliminary study done with R. B. Cumming (Biology Division) and for 1, 2, 4, 8, 10 and 14 consecutive days or for 4, 6, 8 and 10 weeks (5 days per week, 6 hr per day) in the second experiment done with Simon Lock (Biology Division). Analysis of peripheral blood and bone marrow showed that marked perturbations occurred beginning after the first 6 hours of exposure to ethylene oxide. Red cell, white cell, bone marrow cellularity and CFU-S values were all depressed. The red blood cell counts and hematocrits were slightly but continuously depressed throughout the period of exposure. The white blood counts showed periodic shifts toward normal beginning at four days but were as low as 35% of normal after 8 weeks of exposure to ethylene oxide. The peripheral leukocytes showed the greatest perturbation. A marked granulocytosis (200% of normal) and a lymphopenia (25% of normal) were seen through day 4. The neutrophilia and lymphopenia continued over the 10-week exposure to ethylene oxide. The highest granulocyte and lowest lymphocyte differentials occurred in mice with the lowest peripheral white blood cell counts. The numbers of monocytes and eosinophils also changed.

The bone marrow also showed severe perturbations after 1 day of exposure to ethylene oxide when the number of granulocytes was 76% of normal and the number of lymphocytes was 128% of normal. These values shifted even more after two days after exposure to ethylene oxide before an attempt at recovery of homeostasis. However, the number of lymphocytes was then depressed below normal and the number of granulocytes fluctuated. The number of CFU-S remained below control values throughout the exposure to ethylene oxide; at 6 and 10 weeks the CFU-S in bone marrow was 70% of normal. The number of 6-thioguanine resistant CFU-S in the bone marrow of ethylene oxide exposed mice was not elevated above the control incidence; however, the bone marrow of the exposed mice was more sensitive to the 6-thioguanine used during selection for mutant cells.

The results of this study show that ethylene oxide was not highly toxic and it was not mutagenic under the conditions tested in vivo. However, ethylene oxide did cause a perturbation in bone marrow subpopulations and a homeostatic imbalance was continuous as long as the

mice were exposed. The possible health effects of such a continuous homeostatic imbalance in the bone marrow has not been investigated.

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#### DROSOPHILA CYTOLOGY AND GENETICS

R. F. Grell                    E. E. Generoso

Meiosis occupies the central position in eukaryotic genetics. It provides for the precise partitioning of the genetic material from the diploid to the haploid amount. Errors which occur in the process are nearly always deleterious and frequently lethal to the progeny. For this reason an understanding of the normal sequence of events is a prerequisite for evaluating the ways that chemical and physical agents can induce errors in the process. The principal meiotic events are generally categorized as synapsis, recombination and segregation. The mechanisms of the former two, despite the critical role that they play in meiosis, remain obscure and controversial.

Studies completed last year demonstrated unequivocally that synapsis, in the case of the *Drosophila* oocyte, is incorrectly placed according to the conventional model of meioses. Rather than occurring during prophase, synapsis has begun early in premeiotic interphase and homologues are paired throughout the premeiotic-S phase. During the current year we have attempted to learn how great the requirement is for strict homology in the synaptic process. The presence of multiple inversions in one of a pair of homologues has long been recognized as an effective tool for eliminating crossover products. Such inverted chromosomes, called "balancers" are assumed to reduce exchange by causing pairing difficulties so that homologues are unable to align themselves properly for exchange. Cytological confirmation of pairing failure at the ultrastructural level, however, has not been demonstrated. We are using the length of the synaptonemal complex as a measure of the amount of homologous synapsis.

Our study utilizes females which carry multiple inversions in each of the five chromosomal arms so that recombinant progeny are virtually eliminated. The lengths of the synaptonemal complex in meiocytes with and without the heterozygous inversions, have been measured at four sequential timepoints during development using serially sectioned nuclei. Results show that the presence of the inversions causes a reduction in complex length of 45%, 50%, 48% and 45% for early pro-oocytes in a middle germarial cyst and for Stages 1, 2 and 3 oocytes, respectively. These reductions are far short of expectation if synapsis were impaired throughout the genome to the extent exchange results indicate. It may be that synaptic pairing is sufficiently flexible to permit recognition of sequences located in nonidentical positions on homologues, as recently suggested by Goldberg *et al.* (Proc. Natl. Acad. Sci. USA 80: 5017-5021, 1983) based on genetic grounds.

An examination of the first temperature-sensitive (ts) recombination mutant known in *Drosophila* and its two temperature-insensitive alleles has been completed. One method for characterizing mutants is through dosage studies. A leaky mutant is expected to cause gene expression to be more extreme with one dose than two, whereas a null mutant shows unaltered expression regardless of dose. The ts mutant, rec-1<sup>26</sup> behaves like a leaky mutant and shows 85% of normal expression in the hemizygote whereas its two alleles show no response to dose suggesting that they are null mutants. Examination of thermal response of ts rec-1<sup>26</sup> over a temperature range from 17° to 31°C has revealed a straight line response with a slight negative slope between 17° and 28°. A sharp drop off occurs between 29° and 31°. A sharp drop within a narrow temperature range is typical of a denaturation curve. The results are interpreted as temperature denaturation of an enzyme or other protein produced by rec-1<sup>26</sup>, which is involved in the recombination process.

The ability to uniformly or nonuniformly reduce recombination along a chromosome arm has been used to partition meiotic mutants into two functional types, the former involved directly with exchange and the latter with "preconditions" for exchange. The rec-1<sup>26</sup> mutant which shows a proximal directed polarity at 17°, reverses direction at 25° and abolishes polarity at 31°. The ability of small changes in temperature, acting on the same genotype, to reverse or eliminate polarity, suggests that it is not a sufficiently fundamental property on which to base a functional dichotomy.

P factors are chromosomally located mobile genetic elements which when present in combination with an M type cytoplasm induce hybrid dysgenesis. This is manifested as male recombination, sterility, increased mutation frequency and enhanced female recombination. Preliminary studies have begun to measure the ability of P elements to restore more nearly normal recombination in rec-1<sup>6</sup>/rec-1<sup>6</sup> females carrying M cytoplasm. Crosses were made at 17°, 25° and 28°. Complete sterility occurred at 17° and fertility was greatly reduced at the higher temperatures. Recombination was measured at 2.8% at 25° which is similar to that found in the absence of P elements. At 28° recombination was increased to 7%, indicating a temperature-dependent phenomenon.

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#### MICROBIAL MUTAGENESIS AND CELL DIVISION

H. I. Adler                    R. Machanoff  
 W. D. Crow                    A. Sozer<sup>1</sup>

During this reporting period we have completed the development of a rapid, inexpensive microbial technique for the detection of mutagens. This technique is based on the induction of filamentation in "lon" mutants of the bacterium *Escherichia coli*. The newly developed test has been compared with the Ames *Salmonella* assay and the results have been published (2).

Most of our effort has been directed toward the development and use of new methods for growing and manipulating anaerobic bacteria. Anaerobes are of interest because some are clinically significant, others are involved in the degradation of environmental contaminants, and still others can be useful in the production of potential fuels such as methane, ethanol, acetone and butanol from renewable resources. Anaerobes should also be useful in understanding the mechanism by which oxygen causes inactivation and mutation. We hope that our novel methods will eventually allow us to apply the modern techniques of genetic recombination to anaerobic bacteria.

The new methods for cultivating anaerobic bacteria are based on the use of a sterile membrane fraction from *Escherichia coli*. The fraction efficiently reduces oxygen to water over a broad range of temperature and pH in both liquid and solid media. Twenty anaerobes representing ten genera have been grown in liquid media containing the membrane fraction. If bacteria and the fraction are incorporated into pour plates overlayed with a thin layer of agar, colonies develop without the use of anaerobic containers. Bacteria and the fraction may also be spread on agar surfaces and incubated in anaerobic containers. Both techniques have high plating efficiencies. For many kinds of experiments, bacteria must be diluted into a nongrowth environment and held for considerable periods of time. We can now accomplish this for strict anaerobes without the use of anaerobic containers. Bacteria are diluted into a solution containing bovine serum albumin, sodium lactate and the membrane fraction in distilled water. Under these conditions, the organisms so far tested remain viable for at least 1 hour.

Before doing more sophisticated genetic manipulation, it is necessary to establish some basic parameters of the organism to be manipulated. Using our newly developed techniques, we have obtained growth curves,

ultraviolet and X-ray inactivation curves, and estimates of spontaneous mutation frequencies for two anaerobes of interest, Clostridium butyricum and Clostridium acetobutylicum. Both organisms grow rapidly in liquid cultures supplemented with the membrane fraction. Generation times are approximately 36 minutes. The ultraviolet and X-ray inactivation curves are similar to those previously obtained for Bacillus subtilis and spontaneous mutation frequencies for drug-resistant markers seem to be similar to those obtained in organisms such as Escherichia coli (1).

We are now also able to produce quantitative data for the inactivation of Cl. acetobutylicum exposed to air. The experiments have been performed under a variety of conditions and the inactivation curves obtained appear to be exponential without a significant initial threshold. These results suggest that oxygen may be reacting with a cellular component that is present in only one or a small number of copies per cell. We have also observed that Cl. acetobutylicum cells when added to nutrient media efficiently remove dissolved oxygen. This removal of oxygen does not take place if the cells are suspended in distilled water. The removal of oxygen from nutrient broth does not seem to be an effective protective mechanism for Cl. acetobutylicum because it is accompanied by significant loss of viability as judged by cloning efficiency.

A search for oxygen tolerant mutants of Cl. acetobutylicum has been undertaken. Such organisms, if they can be found, would be useful in studies of the mechanism of oxygen toxicity and an evaluation of the role played by oxygen in determining the "spontaneous mutation rate". In order to establish selective conditions for an oxygen tolerant mutant, we have developed a system in which nutrient agar plates are spread (approximately  $10^7$  organisms/plate) and incubated in chambers containing various amounts of oxygen added to a nitrogen-CO<sub>2</sub> mixture. We have established that, under these conditions, our strain of Cl. acetobutylicum is inactivated by oxygen concentration of approximately 1.5%. To date, no oxygen tolerant mutant has been obtained. Such mutants may occur only rarely but, because they would be extremely useful and we have now developed efficient techniques for their detection, additional experiments will be performed.

In contrast to other techniques, the membrane fraction provides a rapid and efficient means of maintaining oxygen free conditions without producing a toxic environment. In practice, many anaerobes grow more rapidly and to higher titer in media containing the membrane fraction than in media made anaerobic using nonspecific reducing agents such as sodium thioglycollate or cysteine. These observations suggest that it may be possible to use membrane based techniques to isolate unusual anaerobes from natural environments. We are testing this idea by using our techniques to isolate butanol and acetone producing anaerobes in soil samples from a variety of locations. We have obtained at least 12 such organisms. These isolates are being examined for the presence of plasmids that may be useful in gene transfer experiments.

One of them produces slightly higher concentrations of butanol and acetone than our best laboratory strain of Cl. acetobutylicum.

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### GENETICS OF REPAIR OF RADIATION DAMAGE TO DNA IN BACTERIA

D. Billen<sup>3</sup>      C. T. Hadden<sup>3</sup>

In our studies we have attempted to determine how environmental agents, and especially radiation, interact with DNA and how the cells respond to the damage resulting from those interactions. We have been concerned primarily with three questions: what is the spectrum of damage generated in DNA, what kinds of damage can be repaired, and what are the mechanisms of repair? We have studied the effects of radioprotective agents and certain sensitizing conditions on breakage of DNA in bacterial cells by ionizing radiation, and mechanisms of enzymatic excision of pyrimidine dimers and other DNA adducts.

(a) The production of strand breaks by X rays in cellular DNA can result from direct action of the radiation on DNA or from indirect action. Indirect action may depend on OH radicals, hydrogen atoms, or solvated electrons formed by the radiolysis of intracellular water. From studies on the rates of reaction of chemical scavengers for the OH radical and their protective activities, it has been concluded that the OH radical is the primary cause of indirect DNA strand breakage in cells.

Recently it has been proposed that certain protective organic compounds may function in a more complex way than by simple OH radical removal. Ewing (Radiat. Res. 68: 459-468, 1976; Int. J. Rad. Biol. 41: 203-208, 1982), using lethality as an end-point, has found that under certain conditions of irradiation there is a correlation between radiation protection and the formation of a secondary  $\alpha$ -hydroxyl radical. For example, he has shown that *t*-butanol and *t*-amyl alcohol, both excellent OH radical scavengers that are not converted to  $\alpha$ -hydroxyl radicals, also do not protect Escherichia coli B/r cells if they are X-irradiated in air or 100% N<sub>2</sub> but will protect if they are irradiated in 1% O<sub>2</sub>.

The purpose of this study was to determine whether chemical protection against single-strand breaks observed in toluene-treated E. coli (AB3063) subjected to X irradiation in air was due to the removal of OH radicals, or resulted from the production of secondary radicals as proposed by Ewing. In toluene-treated cells DNA strand-break production can be measured without the complication of strand ligation during or immediately following X-ray exposure since such cells are deficient in DNA ligase activity.

The seven chemicals used in this study and their rate constants [ $k(M^{-1}s^{-1})$ ] with OH radical were: sodium formate, ( $2.5 \times 10^9$ ), glycerol ( $1.9 \times 10^9$ ), cysteamine ( $1.5 \times 10^{10}$ ), n-butanol ( $4.0 \times 10^9$ ), t-butanol ( $5.2 \times 10^8$ ), t-amyl alcohol ( $1.8 \times 10^9$ ), and potassium nitrite ( $5 \times 10^9$ ). According to Ewing, glycerol and formate are converted to  $\alpha$ -hydroxy radicals by OH radicals but t-butanol and t-amyl alcohol are not.

The data obtained show that the production of DNA strand breaks by 20 krad of X rays can be markedly reduced by all seven chemicals in a concentration-dependent manner when they are present during X ray exposure.

As a convenient but arbitrary way of visualizing the relative effectiveness of the compounds in reducing DNA breaks relative to their OH radical scavenging capacity, the reciprocal of concentration of each chemical producing a 50% reduction in strand breaks was plotted against the rate constant for reaction with OH radicals. Protection by the agents against X-ray-induced DNA strand breaks was directly related to their OH radical scavenging capacity with the exception of cysteamine. Cysteamine seems to be more effective than would be predicted from its OH radical scavenging ability and is likely to protect by several mechanisms including oxygen removal, hydrogen donation, and OH radical removal.

It is difficult to envision that the protection is brought about by the secondary reducing radicals as proposed by Ewing since the formation of  $\alpha$ -hydroxy radicals is not necessary for protection against DNA strand rupture in our system. If their formation were necessary t-butanol and t-amyl alcohol should have been unable to protect the cells against DNA breaks.

Similarly, purified DNA from Bacillus subtilis was irradiated in the presence of various concentrations of protective agents and the extent of loss of biological activity was measured by transformation. In this in vitro system there are no cellular materials to interact with the DNA, radioprotective agents, or water radiolysis products. The results indicated that much lower concentrations of radioprotective agents were required to reduce the DNA damage than in the toluene-treated E. coli cells. The maximal level of protection conferred by n-butanol was much greater than that of KI, but the concentration required to achieve 50% protection was again related to the rate constant for reaction with OH radical.

In addition, Ewing's hypothesis predicts that the protective hydroxy  $\alpha$ -radicals are produced through hydrogen extraction by OH radicals, and thus that radical scavengers should reduce the formation of protective  $\alpha$ -hydroxy radicals. In a competition experiment, KI did not change the protective effect of either n-butanol, which can make  $\alpha$ -hydroxy radical, or t-butanol, which cannot.

We have no ready explanation for these differences between laboratories with E. coli X irradiated in air. Ewing used colony-forming ability of E. coli as an endpoint, and we have studied DNA breaks in toluene-treated E. coli. The measurement of DNA strand breaks under alkaline

conditions reflects X-ray induced frank strand breaks and breaks resulting from apurinic site production. The biological consequences of these lesions is not fully understood.

(b) Investigations on the mechanisms of repair of DNA damage have included studies of the adaptive response by B. subtilis to alkylating agents. In collaboration with S. Mitra and R. S. Foote, we previously demonstrated that B. subtilis cells contain a high level of  $O^6$ -methyl-guanine-DNA methyltransferase, and that this level is increased by exposing the cells to low concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This adaptive response reduces both killing and mutagenesis by a challenge with a high concentration of MNNG. We have now shown that the adaptive response is induced by some alkylating agents, but not by others. MNNG pretreatment induces protection against the lethal effects of methyl- and ethylnitrosourea (MNU and ENU) and ethyl-nitrosoguanidine (ENNG) but not methanesulfonates (MMS and EMS); it actually increases mutagenesis by MNU. Pretreatments with MNU and ENU are adaptive to killing and mutagenesis by MNNG but ENNG is adaptive only to killing. MMS and EMS are not protective against MNNG; ENNG increases mutagenesis by MNNG. In general, the methanesulfonates react predominantly with nitrogen in DNA, whereas the nitroso compounds react mainly with oxygen. It appears that the nitrogen adducts, although lethal, are not adaptive.

(c) In a collaborative effort with H. I. Adler we have begun an investigation of DNA repair and mutagenesis in anaerobes. By using an E. coli cytoplasmic membrane preparation to remove oxygen from liquid and solid media, we can carry out routine treatment of anaerobic bacteria without the need for anaerobic chambers or toxic reducing agents. We have shown that the dose response of Clostridium butyricum to killing by ultraviolet radiation is essentially the same as that of B. subtilis. These two organisms are also similar in their response to ionizing radiation in the presence of air. Comparison of dose response of Clostridium butyricum in aerobic and anaerobic conditions shows an oxygen enhancement ratio of about 3, as is typical for aerobic and facultative bacteria. These experiments show that studies of DNA damage, repair, and mutagenesis of anaerobes should be feasible. Information gained from these studies should facilitate the improvement of industrially useful strains of anaerobes.

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ANALYSIS OF TERATOGENS AND POTENTIAL TERATOGENS  
USING FETAX (FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS)

James N. Dumont

Rhonda G. Epler

The development of abnormal embryos and fetuses has been of concern since the time of Aristotle. In more recent times, especially since the thalidomide episode in the 1960's, there has been a resurgence of interest in the factors that influence both normal and abnormal embryonic and fetal development. Even more recently, with the advent of the revolution in the synthesis, manufacture, and application of new pharmaceuticals and chemicals and of new forms of energy production which may produce streams of complex mixtures of many compounds, potential birth effects resulting from the use of and/or exposure to these compounds or mixtures has become a major health and environmental concern. This concern has generated an intense interest in the development of biological assay systems with which potential embryonic and fetal damage might be detected in a rapid and cost effective manner. The FETAX system was developed in response to this need. The assay is rapid and cost effective and uses the developing embryos of the amphibian, Xenopus laevis. Embryos are exposed for 96 hours beginning at the mid to late blastula stage of development. The end-points of the assay are easily scored and provide information on embryotoxicity and teratogenicity, and, in addition, data on the relative toxic or teratogenic potential. These relative potencies are used to suggest priorities for further testing in more advanced and expensive systems.

In addition to providing guidance for more costly testing, the FETAX system can be useful for studying effects of teratogens on specific organ systems as well as providing a model to examine teratogenic mechanisms. Not all teratogenic effects are produced by genetic damage. Some teratogens affect cytoplasmic or cell membrane components and thus interfere with normal cell sorting and organization in the embryo. Other teratogens act by killing cell populations and in this way prevent normal development of organs and organ systems. Since FETAX is a whole embryo system, it is useful for studying these effects and can provide fundamental information on the mechanisms of aberrant developmental phenomena. Similarities between mammalian (or human) and amphibian responses make detailed study of the biological effects of teratogens in embryos much easier to implement. Thus, FETAX provides a rapid and cost effective means of evaluating the teratogenic potency of compounds (or complex mixtures), and a system with which fundamental information on underlying mechanisms can be obtained.

The attributes of FETAX which make it appealing as a model include the facts that it is a whole embryo system, that large numbers of embryos are available, that teratogenic and embryotoxic effects can be differentiated, and that quantitative dose-response relationships are displayed. In addition the assay is adaptable to a variety of testing protocols and it can be used to study developmental abnormalities in both the axial and appendicular skeletons.

FETAX has been validated with 40 known mammalian teratogens and nonteratogens. Those used for validation during this year include pesticides (Carbaryl, Disulfiram, Captan, Lindane and Griseofluvin), an

inhibitor of oxidative phosphorylation (2,4-dinitrophenol), a compound used as a solubility vehicle (propylene glycol), a cation chelator (EDTA), and two pharmaceuticals, an antihistamine-antinausiant (meclizine) and antimitotic and antineoplastic agents (Velban, vinblastine sulfate, cyclophosphamide, and methotrexate). One of the pesticides, Captan, gave a false negative result but the response of FETAX for the remaining compounds gave results consistent with those obtained in mammals. The false negative rate for all compounds tested is ~10% and the false positive rate is ~5%. These rates compare very favorably with those obtained by other teratogen in vitro assays and with the reliability of the now classical Ames test for mutagens. Conclusions about teratogenicity are based on the TERATOGENIC INDEX (TI), a number that is generated from the ratio of the LC<sub>50</sub> and the EC<sub>50</sub> (the concentration of teratogen that produces 50% abnormalities among the survivors at 96 hours). TI's of 1.0 or less indicate that the material is embryotoxic while TI's of 1.0 or more indicate that the test material is teratogenic. The larger the value of the TI the greater the teratogenic risk. It is on the basis of the TI that priorities for longer term mammalian tests are recommended.

Work is ongoing to examine specific cellular and tissue damage as a result of teratogen exposure. More detailed analyses of abnormal development include quantitative analyses of tissue and organ abnormalities using computer-assisted morphometric methods. We have, for example, found that methotrexate (a folic acid antagonist and abortifacient as well as an antineoplastic agent) causes severe hydrocephalia in developing embryos, a syndrome common to human fetuses and neonates whose mothers have used the drug. Our morphometric analyses have shown a 20-30% reduction in the area of the brain occupied by cell mass. While the cross-sectional area of the brain is relatively unaffected, the area of the ventricle is greatly increased. The increase is dose dependent but at a concentration of 150 mg/L ventricular area is increased by as much as 38 times over that of the control. These results demonstrate that similar responses occur in both amphibian and mammalian embryos. Although the mechanisms involved in the induction of hydrocephaly are not specifically known, one suspects that the choroid plexus that secretes cerebrospinal fluid might be defective.

Lathyrogens, compounds that induce skeletal and connective tissue defects in mammals, induce similar defects in amphibian embryos. Electron microscopic studies of the notochords of lathyrone-treated embryos reveal that the collagenous sheath that surrounds the notochord is highly disarrayed and attenuated. As a result the notochord ruptures through its sheath and the long axis of the embryo becomes distorted. Cross-sectional area of the notochord may increase by as much as 5 times that of controls. Later premetamorphic embryos (i.e., those with well developed embryonic limbs) exposed to lathyrogens exhibit, after metamorphosis, abnormalities in their appendicular and axial skeletons. Histologic examination of skeletal joints reveals abnormal joint capsule development. As a result the embryos are unable to swim normally.

It is well known that some compounds require activation or metabolism to become teratogenic. Cyclophosphamide is such a compound. Although it yields positive results in FETAX, it also causes abnormal development of limbs and digits in older embryos (as in the mammal, shortened limbs and

loss of digits). These effects are enhanced by prior treatment of the embryos with phenobarbital, a classic inducer of bioactivating enzymes. In addition to providing a model with which to study limb and digit abnormalities, this observation also demonstrates (1) that later stage embryos possess activating enzyme systems and (2) that these systems can be enhanced by classic inducers. The fact that limb development can be affected broadens the application of FETAX to include later stage embryos that are actively undergoing limb development.

Validation of the assay with a broader base of teratogens and non-teratogens will allow rapid and cost-effective establishment of priorities for other chemicals for further testing as well as provide insights into the mechanisms by which materials affect embryonic and fetal development. The assay has been applied to a host of potential environmentally hazardous materials, many of which were derived from emerging energy technologies. These investigations contribute to and support a prevailing interest of the Division and Laboratory with regard to potential health and environmental hazards that are associated with the development of alternate energy technologies.

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## Mammalian Genetics and Reproduction Section

### SECTION OVERVIEW - LIANE B. RUSSELL

The unifying theme of the Section's research is the mammalian germ-line. More specifically, the work deals with both the direct and transmissible effects of environmental agents on mammalian germ cells, and with basic information on the structures and processes in which these effects occur.

Five subthemes may be identified. (1) The frequency of induced heritable damage. It includes the detection of inherited lesions representative of various endpoints, and the study of their relative frequencies depending 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 inherited sterility and morphological anomalies. (4) 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. (5) 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.

Intimate, and often reciprocal, relationships exist among several of these major themes. Thus, mutagenesis experiments produce altered genes and chromosomes, and thereby supply tools for basic studies; in turn, detailed information on the structure and behavior of specific genomic regions helps in understanding what type of mutagenic agent induces what type of genetic lesion. Certain gross phenotypes can also be related to certain basic genetic lesions, e.g., inherited male sterility to specific types of chromosomal conditions. A detailed understanding of normal germ-line development and studies on direct germ-cell effects (e.g., cell killing) are essential in illuminating secondary factors in mutagenesis (such as cell selection or repair) that can reduce transmission of mutations, and in making the all-important correlations between exposed germ-cell stage and quantity and/or quality of genetic lesions transmitted to offspring. Finally, chemical dosimetry studies in the gonad are a prerequisite to the interpretation of mutagenesis data.

As in the past, method development and validation have this year been carried out in a diversity of areas. Some examples follow.

- Non-breeding-test (NBT) indices for more rapid estimation of dominant skeletal effects have been shown to be scorable in a genetically random population; NBT experiments can, as a result, be piggy-backed on specific-locus studies.
- The use of long-term inhalation exposures in mutagenesis studies was perfected in an investigation of ethylene oxide (EtO) effects. This permitted coordinated experiments on EtO induction of a number of different genetic endpoints, as well as molecular dosimetry studies with this inhaled agent.
- Alkaline-elution procedures were developed to measure DNA strand breaks in spermatozoa. In, so far, limited tests the magnitude of elution values parallels the frequency of dominant lethals. If further correlations can be demonstrated, the method may become useful in the monitoring of human sperm for DNA breakage.
- Modifications in embryo freezing and transfer were worked out which will permit improvements in the cryopreservation of our genetic stocks. Embryo transfer also permitted a study on the causes of spontaneous early embryonic death.
- A computer analysis was devised that permits the reliable detection of even small excesses in early mortality of first-generation offspring of mutagenized animals. Worked out for radiation studies, this type of analysis will be applicable to experiments with chemicals and will provide yet another measure of induced dominant damage.
- High-resolution chromosome banding has been successfully worked out for mouse chromosomes and will permit a greatly superior cytological localization of certain genetic lesions.
- A high-dose fractionation regimen for ENU was worked out that further increases the effectiveness of this supermutagen. Our discoveries of efficient methods for producing new mutations are receiving worldwide application in laboratories desiring to produce mouse models for human genetic disorders.
- A critical examination of methodologies that have been used to detect induction of chromosomal nondisjunction in meiosis led to the identification of the most promising approaches as well as of meiotic stages at which mutagen should be applied to maximize the chance of inducing nondisjunction.

Although testing of chemicals is not a major objective of this Section, a number of substances were 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 genetic and/or reproductive

effects were ethylene oxide, dibromochloropropane (DBCP), hexamethylphosphoramide (HMPA), nitrosoethylcarbamate, ethanol, mercury (as methylmercuric hydroxide), various seed oils and mineral oil, and  $^{239}$ plutonium. The related compounds, ethylnitrosourea (ENU) and methylnitrosourea (MNU), were used for in-depth studies to explore the biological variables that affect mutagenicity in mammalian germ cells.

This last group of experiments, as well as a number of others, provided some important findings on the properties of the biological system and about the nature of its interaction with environmental agents. Some examples follow.

- Germ-cell-stage-related sensitivity was found to differ greatly for different chemicals, even in the case of chemicals as closely related as ENU and MNU. While MNU has little mutagenic effect in stem cell spermatogonia, in contrast to the supermutagenicity of ENU in these cells, it is extremely mutagenic (and probably more so than ENU) in a very short post-stem cell stage.
- The array of genetic changes that causes sterility in offspring of irradiated males is very different depending on whether spermatogonia or postgonial stages are irradiated.
- The frequency of spontaneous early embryonic death is controlled largely by the genotype of the embryo, which determines development rates, and little, if at all, by the genotype of the uterus within which the embryo grows up. The high frequencies of deciduomata that characterize certain strains thus do not result from newly-occurring dominant-lethal mutations.
- Spermatogonial stemcells of the mouse testis do not exhibit a clear circadian rhythm in mitotic activity, nor do cell-cycle properties of these spermatogonia change with age of the males from 3 weeks on. Stemcell numbers, however, decline with age.
- Chemicals that react by an  $S_N1$ -type mechanism interact much less with protamine than do chemicals that react by an  $S_N2$  mechanism.
- The relative frequencies of various alkylation products formed after ENU injection change with exposure level. A decrease in the  $O^6$ -EG/ $N^7$ -EG ratio as exposure is lowered parallels a significant drop below linearity in mutation rate. The results are consistent with evidence from other systems that  $O^6$ -EG is an important mutagenic lesion, and one that can be repaired.
- An attempt to saturate the  $O^6$ -G alkyl transferase in a spot-test experiment was successful with regard to teratogenic effects but unsuccessful with respect to mutations in pigment cells.
- Comparisons of dominant-lethal and heritable-translocation induction by various alkylating agents suggest that these two types of lesions may

arise along different pathways. Translocations are associated with the formation of unstable N<sup>7</sup> alkylguanine, which produces intermediate lesions that are converted into chromosome exchanges following sperm entry into the egg. Dominant-lethals may result from stable alkylations at oxygen positions, which, during pronuclear chromosome replication, lead to chromatid-type aberrations.

From the beginning, a major thrust of the mammalian genetics program has been the assessment of genetic risk. It should be obvious that several of the studies here summarized in other contexts have at least some bearing on genetic risk assessment. Several additional highlights may be listed.

- The most extensive and reliable study on the frequency of heritable translocations induced by radiation in spermatogonia was completed this year. Spermatogonial stemcells are of prime importance in risk estimation.
- An analysis made possible by a new measure of pre-adult deaths from all causes, including aneuploidies, strengthens the conclusion that, for radiation, there is less genetic risk from chromosomal aberrations than from gene mutations.
- An attempt was made to evaluate the degree of reliability with which various types of mutagenesis data from tests other than mammalian germ-line tests can identify possible genetic hazard, and can be used to establish priorities for choosing chemicals on which to proceed to risk assessment.
- Since heritable translocations pose a much greater risk to human populations than do dominant lethals, the finding that these two types of chromosomal endpoints may result from different basic types of DNA lesions has considerable importance in evaluating risk from specific chemicals.
- Among chemicals studied this year are several to which human beings are exposed, e.g., corn oil and other seed oils, ethanol, ethylene oxide, and organic mercury.

Our work in basic genetics and cytogenetics continues to make good use of chromosomal aberrations and point mutations that are generated in the course of mutagenesis studies.

- The continuing electron-microscope analysis of synaptonemal complexes of X-autosome translocations has provided evidence against homology in X-Y synapsis.
- Cytological studies on fertility-impaired offspring in heritable-translocation experiments have led us to the conclusion that all Y-autosome translocations may be sterile.

- Autosomal reciprocal translocations that do not sterilize were used in a study on the relation between length of exchanged segments and frequency of aneuploid progeny.
- Detailed analysis of mutations recovered in specific-locus experiments has been extended to a new locus, p, at which a major deficiency has been identified.
- There is evidence that ENU-induced mutations are probably smaller genetic lesions than are radiation-induced mutations.

Since we lost our teratologist, Ron Filler, in September 1982 during the reduction in force, teratology efforts were put on hold this year. However, work in other areas of reproductive biology continued at full force. In addition to conducting studies (some of which are summarized above) on germ-cell histology and on the interaction of chemicals with germ-cell DNA, we are working at several of the interfaces between genetics (or mutagenesis) and reproductive biology.

- Effects of chemicals or radiations on lifetime fertility of exposed animals are analyzed as an ancillary result of specific-locus studies in males. Such effects are studied in the total-reproductive-capacity test on females, along with clastogenic effects. Fertility impairments in offspring (as opposed to those in exposed animals) are among the dominant genetic effects under study.
- Fertilization and early cleavage are stages that enter into studies of various induced and spontaneous events. Among these are the induction of nondisjunction, the occurrence of chromosome exchanges or losses resulting from lesions induced earlier (in germcells), the repair of such lesions in the fertilized egg, the possible induction of abnormal fertilization (e.g., sperm entry in the polar body), and the genetic control of mortality of early embryos.
- Embryo-manipulation techniques acquired and perfected as part of the stock-freezing program are being utilized for other studies.

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

|                            |                     |
|----------------------------|---------------------|
| W. L. Russell <sup>7</sup> | J. W. Bangham       |
| P. R. Hunsicker            | G. M. Guinn         |
| D. A. Carpenter            | M. H. Steele        |
| E. L. Phipps               | H. M. Thompson, Jr. |
| C. S. Montgomery           |                     |

Our discovery in 1979 that ENU is by far the most effective of all mutagens so far tested in the mouse made it feasible, for the first time,

to perform extensive studies of the chemical and biological factors, such as dose, dose fractionation, sex, cell stage, etc., that can affect gene mutation rate in mouse germ cells. We had determined the effect of these factors in radiation mutagenesis, but very little research of this kind had been possible in chemical mutagenesis, mainly because of the relative ineffectiveness of chemical mutagens on mammalian germ cells, and the large numbers of animals required to measure low mutation rates. Prior to the discovery of the effect of ENU, the most effective chemical mutagen known in the mouse was only one-third as effective as acute X rays. In contrast, a single dose of ENU is 8 times as effective as the most effective single dose of X rays. As a result, rapid progress is being made in exploring how ENU-induced mutagenesis is affected by the factors mentioned above.

Dose-response curve for ENU-induced specific-locus mutations in spermatogonia. Last year we reported that in the lower portion of the dose-response curve, below a dose of 100 mg/kg, the data fell statistically significantly below a maximum-likelihood fit to a straight line. Results at the 50 mg/kg dose point have been increased to explore the magnitude of this reduction. The total mutation frequency at this dose is now 26 mutations in 35,956 offspring. This continues to be statistically highly significantly below a linear response, and the induced mutation frequency is only 45% of that expected on a linear interpolation between the frequencies at 0 and 100 mg/kg.

Effect of sex and cell stage. For the estimation of genetic risk, it is important to know the relative mutational sensitivities of the various germ-cell stages. Last year we reported that ENU-induced mutation frequency in postspermatogonial stages is very low compared with that in spermatogonial stemcells. However, we pointed out that even with the lowest dose used, 100 mg/kg, no offspring were recovered from matings made during the sixth and seventh weeks post-injection, owing to the lethal effect of this dose of ENU on differentiating spermatogonia and early spermatocytes. It was important to test the mutational sensitivity of these stages to ENU, because we had discovered that they are extremely sensitive to mutation induction by methylnitrosourea (MNU). Accordingly, we have now performed six replications of an experiment with a 50 mg/kg dose of ENU which does allow some survival of cells in the stages of interest. In matings made in the period between 32 and 42 days after injection (the period that gives the high mutation rate with MNU), we have obtained 4 specific-locus mutations in 5,576 offspring. This is a markedly different mutation rate from 1 mutation in 20,836 offspring obtained from matings made in the period before 32 days after injection. The difference is statistically significant ( $P = 0.008$ ). Thus, as with MNU, differentiating spermatogonia or early spermatocytes are mutationally sensitive to ENU, and the currently estimated mutation frequency with ENU is similar to that induced by the same dose in stemcell spermatogonia.

In a new experiment with female mice injected with 100 mg/kg of ENU, the animals were not mated until two weeks after treatment, thereby increasing our sample size of oocytes exposed in an intermediate stage of the maturation process. In a total of 1,226 offspring from matings made in

the third to sixth week after injection, no mutations were obtained. Matings made after the sixth week in this experiment, and other earlier experiments continued through this year, produced 7,749 offspring from cells exposed as arrested oocytes. This group also yielded no mutations, thereby continuing to show the ineffectiveness of ENU in inducing mutations in female mice, despite its powerful effect in stemcell spermatogonia.

Mutagenic effect of sequential exposure to X rays and ENU. The rationale for this experiment was based on our finding that splitting an X-ray dose into two fractions separated by 24 hours greatly augments the mutation rate in spermatogonia, presumably because germ-cell death from the first exposure results in a synchronization of cells such that the second exposure finds them in a stage of high sensitivity. This experiment was conducted to test whether a dose of X rays would have a similar augmenting effect on a dose of ENU given 24 hours later. In three experiments using 100, 300, or 500 R of X rays, followed 24 hours later by an injection of 100 mg/kg of ENU, a total of 13 specific-locus mutations was obtained in 4,394 offspring. On the basis of past single-exposure radiation and ENU experiments, the number of mutations expected in the 4,394 offspring would be 2.6 from X rays, 13 from ENU, and 0.2 spontaneous, or a total of 15.8 mutations. The observed frequency of 13 mutations is not significantly different from this and, therefore, so far shows no departure from an additive effect of the two mutagens, and no suggestion of any augmenting effect.

Relative mutation frequencies at the seven specific loci. Allelism tests are now complete for the mutations in the dose-response experiments, with the exception of the additional experiment at the 50 mg/kg dose. The distribution of mutations among the loci is similar at all doses where the numbers are large enough for useful comparison. These results do not support the claim by Ehling (in de Serres, F. J., Sheridan, W (eds.): Utilization of Mammalian Specific Locus Studies in Hazard Evaluation and Estimation of Genetic Risk, Plenum Press, New York, 1983, pp. 169-190) that the mutation spectrum changes with dose, a conclusion he reached from the data he reported at two doses, 160 and 250 mg/kg. However, by our calculation, the distributions he obtained at these two doses did not differ significantly from each other ( $0.5 > P > 0.3$ ). Picking out, after the event, as Ehling did, the two loci that happened to show the largest difference in such a comparison, where there are 21 combinations of two loci to choose from, can lead to false statistical impressions.

Use of large-dose fractionation to augment mutation rate in spermatogonia. Results obtained this year show that the mutation frequency can be increased above that produced with 250 mg/kg by giving 4 fractions of 100 mg/kg spaced at weekly intervals. In our specific-locus mutation test, this dose regimen has produced 17 mutations in 1700 offspring. This is  $1.6 \times$  the mutation frequency of 104 mutations in 16,693 offspring obtained in our total published results from 250 mg/kg single-dose experiments. The length of the temporary sterile period was not increased significantly over that induced by a single dose of 250 mg/kg. Shorter fractionation intervals (1 day, 2 days, and 4 days) were tried, but the mortality and

fertility effects rendered these undesirable for a standard experimental procedure.

Widespread use of ENU in mammalian mutagenesis. In our 1979 publication announcing the discovery that ENU is a supermutagen in the mouse, we stated that "ENU would now seem to be the mutagen of choice for the production of new mutations in the mouse whenever gene mutations of any of various kinds might be needed." This has already happened internationally, and to such an extent that Professor William F. Dove of the University of Wisconsin has started a monthly "Mouse Mutagenesis Memo" to circulate and "exchange technical information and mutant production rapidly, rather than through publications" in order "to optimize a new thrust in mouse genetics to explicitly produce NEW mutations of critical value." It is gratifying to know that ORNL has made this useful contribution to the scientific community, a contribution which, through the production of mutant mouse models of human disease, is likely to have important medical benefits.

USE OF METHYLNITROSOUREA (MNU) AS A MODEL MUTAGEN TO EXPLORE BIOLOGICAL  
VARIABLES AFFECTING MUTAGENICITY IN MOUSE GERM CELLS

W. L. Russell<sup>7</sup> K. F. Stelzner  
P. R. Hunsicker S. C. Maddux

In last year's report we presented doubly surprising results with MNU. In the first place, MNU proved to have little mutagenic effect in stemcell spermatogonia, in contrast to the supermutagenicity of ENU in these cells. Secondly, MNU turned out to be an extremely potent mutagen in post-stemcell stages, but only in one narrow window of these stages — namely, in the offspring of matings made between 32 and 42 days after injection, which correspond to cells that were exposed either as differentiating spermatogonia or as preleptotene spermatocytes. The dose used in the experiment reported last year was 75 mg/kg. We have now repeated the study with a dose of 44 mg/kg of MNU. The experiment was designed partly to check the response by augmenting the data, partly to measure dose response, and partly to compare the results with those from ENU experiments summarized in the preceding report (44 mg/kg of MNU being equimolar with 50 mg/kg of ENU).

In the matings made in the 32-42 day period after injection, 21 specific-locus mutations were obtained in 10,027 offspring. In all other matings involving post-stemcell stages, (matings made on days 1-31 and 43-49 after injection) only 3 mutations were obtained in 25,656 offspring. The difference is highly significant ( $P < 1 \times 10^{-8}$ ), confirming the results reported last year. The mutation frequency is slightly, but not significantly, below what would be expected on a linear interpolation between the frequencies at 0 and 75 mg/kg. MNU appears to be more effective than ENU in these mutationally sensitive germ-cell stages, but even at 50 mg/kg of

ENU there is clearly more killing of these cells than there is with 44 mg/kg of MNU, and this may obscure the true mutation rate with ENU.

These surprising results on the supersensitivity of certain germ-cell stages should provide useful clues on the mechanisms of mutagenesis in mammalian germ cells.

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#### USE OF MNU-ENU DOUBLE EXPOSURE IN THE SPOT TEST TO EXPLORE THE IN VIVO ACTION OF AN $O^6$ ALKYL TRANSFERASE

Liane B. Russell      C. S. Montgomery

Alkylations at the  $O^6$  position of guanine are considered to be more highly mutagenic than alkylations at certain other sites in DNA, possibly explaining, in part, the high mutagenicity of ENU (ethylnitrosourea) found by us in mouse spermatogonia. Experiments in the Biology Divisions of ORNL (Mitra) and Brookhaven (Setlow) have identified and studied a transferase that de-alkylates the  $O^6$  position in bacterial and mammalian in vitro systems, leading to repair of the genetic lesions. There is some evidence that this enzyme may be more effective in removing methyl than ethyl groups. The enzyme is thought to undergo irreversible ("suicidal") changes on accepting the alkyl groups in a stoichiometrical fashion.

To explore the action of this transferase in mammalian cells in vivo, we carried out a mouse spot test in which exposure to MNU (methyl nitrosourea) was followed by ENU-exposure. If, in fact, most of the available transferase is tied up (and thus inactivated) by removing the  $O^6G$  methyl groups resulting from the first exposure, little or no active enzyme should remain to "repair" the subsequent ENU-caused ethylations, and the mutation rate should be greater than additive.

Pregnant females at 10-1/4 days of gestation were injected with 3 mg/kg MNU, followed an hour later by 25 mg/kg ENU. Other groups of females received one or the other chemical alone, or phosphate buffer only. With respect to recessive mutations (RS-type spots) and cytotoxicity of melanocytes (WMVS), the treatments appeared to be additive. However, the teratogenic effects of ENU (digital anomalies) were greatly enhanced by the pre-treatment with MNU.

It may be that the transferase is not present in pigment precursor cells, or that its action there is not suicidal. In the appendicular

primordia, on the other hand, the transferase may act in line with predictions from other systems.

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#### SPECIFIC LOCUS STUDIES WITH DBCP AND HMPA

|                  |                     |
|------------------|---------------------|
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| P. R. Hunsicker  | M. H. Steele        |
| G. M. Guinn      | K. F. Stelzner      |
| S. C. Maddux     | H. M. Thompson, Jr. |

Under contract with the National Toxicology Program, we are enlarging the data base for chemicals investigated in the specific-locus test. Two chemicals were studied this year.

1. 1,2-dibromo-3-chloropropane (DBCP). This pesticide has shown mutagenic (or related) activity in various systems and is suspected of causing fertility impairments in men, but had not been tested for induction of transmissible effects, except for dominant lethals (positive, rat; negative, mouse). We have carried out a large specific-locus study, preceded by a toxicity test (9 exposure levels) and a fertility experiment. The major portion of the specific-locus test (SLT) was carried out with males that received five daily i.p. injections of 80 mg/kg each (total exposure, 400 mg/kg) of DBCP in corn oil. These males showed no fertility disturbance at any interval after treatment. A histological study of germ-cell survival in the testis also showed no effect. Because these results seemed at variance with some earlier reports, a second batch of DBCP was obtained from the NTP chemical repository partway through the study. Like the first batch, it produced no fertility disturbances.

In the SLT, 34,451 and 5,940 offspring were classified from 400 mg/kg-exposed stemcell or poststemcell stages, respectively. About 50% of the former group came from fathers exposed to batch A, the remainder from fathers exposed to batch B of DBCP. Only one mutant was found; it derived from spermatogonial stemcells exposed to batch A. The minimum multiple of the control rate that is ruled out (at the 5% probability level) by these

results is 1.6 for spermatogonial stemcells, and 8.4 for poststemcell stages. Since, in earlier studies (Lee, I. P., Suzuki, K., Mutat. Res. 68: 169-173, 1979), DBCP was shown to induce UDS in mouse spermatocytes, it is unlikely that the absence of induced mutations in spermatogonial stemcells found in our experiment results from a failure of the chemical (or its metabolites) to reach the testis.

2. Hexamethylphosphoramide (HMPA). This chemical induces various mutational endpoints in *Drosophila*, but has yielded conflicting results in mammalian *in vitro* tests. We carried out a specific-locus study at the MTD of 2000 mg/kg (dissolved in HBSS and administered by i.p. injection). No fertility disturbances were noted at any interval after exposure. In the SLT, the spermatogonial stemcell result (no mutations in 20,998 offspring classified) was clearly negative and rules out (at the 5% probability level) an induced rate 1.7 times the control rate. The sample derived from exposed poststemcell stages, 2901 offspring with no mutations, yielded an inconclusive result, ruling out only 18.2 times the control rate. It is not known whether HMPA (which is highly reactive at point of entry) reached the testis.

#### THE EFFECTS OF INHALED ETHYLENE OXIDE ON INDUCTION OF HERITABLE GENE MUTATIONS

|                  |                 |
|------------------|-----------------|
| Liane B. Russell | J. W. Bangham   |
| R. B. Curting    | D. A. Carpenter |
| P. R. Hunsicker  | E. L. Phipps    |

Because of the industrial importance and widespread human exposure to ethylene oxide, EtO, this chemical poses a potential risk to future generations, in addition to any carcinogenic risk it may hold for this present generation. The induction by EtO of several types of genetic changes has been demonstrated in some non-mammalian systems and in mammalian *in vitro* assays and somatic tissues. In the mammalian germline, EtO can induce DNA lesions, clastogenic effects, and direct damage in meiotic and postmeiotic cells. However, none of the prior studies has addressed the possible induction of gene mutations in the germline; nor has it involved spermatogonial stemcells, the cell stage of greatest importance in assessing genetic risk to men.

In a large-scale inhalation study, we exposed male (101 x C3H)F<sub>1</sub> mice to EtO in "workday" periods (6 hours/day, Mondays-Fridays), mostly at the level of 255 ppm. Total exposures were ~100,000 ppm·h or ~150,000 ppm·h. Although males were exposed up to 22 weeks, survival was good except in one group that received the first part of the treatment at 300 ppm. Males were mated to multiple-recessive T-stock females shortly after the end of the inhalation period, and (following an initial 2-weeks during which dominant-lethal effects depressed the litter size) bred normally for the remainder of the study.

Altogether, 71,387 offspring were classified in the experiment and 5 mutations observed, including a cluster of two. In the ~100,000 ppm·h group, the results ruled out (at the 5% significance level) an induced mutation frequency that is 0.97 times the historical control frequency. For the ~150,000 ppm·h group, the corresponding multiple ruled out was 5.5; and for the two exposure groups combined, it was 1.75. Et0 thus appears to present a very much smaller risk for induction of gene mutations in stemcell spermatogonia than it does for other genetic endpoints induced in other types of cells.

#### HERITABLE TRANSLOCATION AND DOMINANT-LETHAL MUTATION DOSE-RESPONSE STUDIES WITH INHALED ETHYLENE OXIDE

W. M. Generoso                    J. B. Hoskins  
K. T. Cain                        J. R. Inman

Currently, there is considerable interest in the determination of the magnitude of the health hazard posed by inhaled ethylene oxide (Et0). Et0 had earlier been shown to be capable of inducing chromosome damage in certain male germ cells. Short-term inhalation exposure to high concentrations of Et0, as well as i.p. injections of Et0, were found to induce high levels of dominant lethals in postmeiotic male germ cells of both rats and mice (Embree, J. W., Toxicol. Appl. Pharmacol. 40: 261-267, 1977; Cumming, R. B., Michaud, T. A., Environ. Mutagen. 1: 166-167, 1979; Generoso, W. M., et al., Mutat. Res. 73: 133-142, 1980). Even more important for its risk implication was our finding that i.p.-injected Et0 induces heritable reciprocal translocations in mice (Generoso, W. M., et al., Mutat. Res. 73: 133-142, 1980).

During the past year we completed portions of a study to determine the shape of the dose-effect curves for heritable translocations and dominant-lethal mutations induced by inhaled Et0. This study will provide data for long-term exposures at 300, 250, 204, and 165 ppm. Male 10- to 12-week old (101 x C3H)F<sub>1</sub> mice are being exposed 6 hours per day to Et0 for a total of 11 weeks. For the first 8 weeks the exposures are being given on the 5 working days, and then daily for the last three weeks. Exposed males are mated during the 11th week of exposure (by being caged with females in the evening and exposed to Et0 during the daytime) and for one to two weeks after the end of exposure.

Exposure of parental mice, production of F<sub>1</sub> progeny, and dominant-lethal experiments have already been completed for 250, 204, and 165 ppm concentrations. Testing of F<sub>1</sub> progeny for translocation heterozygosity is currently underway. Results available to date come from the dominant-lethal studies. In matings that took place during the first week post-treatment, the incidences of dominant-lethal mutations were 18%, 22%, and 31% for 165, 204, and 250 ppm concentrations, respectively. This result indicates that dominant-lethal mutations were induced at the three Et0

concentrations and that the responses appear not to deviate from a straight line.

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**NON-BREEDING-TEST METHODS FOR STUDYING INDUCTION OF DOMINANT SKELETAL MUTATIONS ARE EASILY APPLIED TO STRAIN USED IN SPECIFIC-LOCUS EXPERIMENTS**

P. B. Selby                    S. L. Niemann<sup>1</sup>

In earlier non-breeding-test (NBT) experiments, the skeletons of (C3H × 101)F<sub>1</sub> mice were examined. These mice were the offspring of unexposed 101 males, or of 101 males that had been exposed to chemicals or radiation. A large-scale experiment of this type in which the exposure was to <sup>239</sup>plutonium is nearing completion.

Because the NBT methods depend strongly on an understanding of non-mutational and mutational variation in (C3H × 101)F<sub>1</sub> mice, it was uncertain whether these methods could be applied easily to another strain, specifically the mice used in specific-locus experiments. Nonetheless, it seemed especially worthwhile to test this possibility because of the large numbers of offspring that are always being produced in our Section in specific-locus experiments.

The animals examined using NBT methods were drawn from the sample of mice collected by Hitotsumachi *et al.* at our laboratory in a specific-locus experiment in which (101 × C3H)F<sub>1</sub> males were injected with either 300 or 400 mg/kg of ethylnitrosurea administered in 100 mg/kg fractions separated by a week. Only offspring derived from treated stem-cell spermatogonia were studied. The frequencies of presumed dominant skeletal mutations following 0, 3 × 100 mg/kg, and 4 × 100 mg/kg of ENU were 2/374, 10/243, and 10/180, respectively. At both exposure levels, there is a highly statistically significant increase over the control frequency. The induced mutation frequencies at the two exposure levels are 3.5 and 5.0%. At the higher exposure, the induced frequency of presumed mutations of a type likely to be of clinical importance is 4.5%. The "indices of mutation" were 0% in the control, 11.5% in the 3 × 100 mg/kg group, and 12.4% in the 400 mg/kg group. In each case, the increase over the control is statistically significant.

These results are especially important to our Section because they show that the NBT methods can easily be piggy-backed onto specific-locus experiments in order to greatly increase the yield of data useful in estimating genetic risk that can be obtained from a single experimental group. A combined experiment of this type will yield three independent measures of the frequency of mutations being induced, namely the specific-locus mutation frequency, the frequency of presumed dominant skeletal mutations, and the "index of mutation." The data obtained could be used for estimating genetic risk both by indirect (specific-locus data) and direct (skeletal data) methods.

Earlier, we reported the induction of dominant skeletal mutations by a single exposure to 150 mg/kg of ENU. In that experiment we used the strain on which we have done most of our skeletal research. Comparison of the results to those in the present experiment shows that the extent of induction in the two strains fits the expected dose response well. This is especially interesting in view of several important differences between the mice used in the two experiments. Not only were the injected males derived from different strains, but they differed drastically as to the extent of heterozygosity, with those in the former experiment being inbred and those in the present experiment being  $F_1$  hybrids between two inbred strains (accordingly, still genetically uniform). Thus, either the strain difference or the extreme difference in degree of heterozygosity might have caused them to have dissimilar mutational responses.

The  $F_1$  offspring whose skeletons were examined also differed greatly. In the former experiment, they were hybrids between two inbred strains and thus genetically uniform (unless mutations were present). In the present experiment, they had vastly more genetic variability than that caused by mutations, because of the heterozygosity of their fathers and because their mothers were from a strain of mice that is not inbred.

The comparison of these experiments represents an important first step in the detailed investigation that is needed of how well one can extrapolate the extent of induced genetic damage from one strain to another or, more importantly, from species to species. At least it is reassuring that this first comparison of different mouse strains by our methods shows good agreement in the yield of mutations.

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BREEDING-TEST EXPERIMENT SHOWS TRANSMISSION  
OF MANY ENU-INDUCED MUTATIONSP. B. Selby  
G. D. RaymerB. J. M. Whitt<sup>8</sup>  
T. W. McKinley, Jr.

Following the finding of the dramatically high mutation frequencies reported in the preceding section, we decided to perform a small experiment using the much more time-consuming breeding-test method in order to see if we could demonstrate the transmissibility of many of the presumed mutations scored in the NBT (non-breeding test) studies. Altogether 141 F<sub>1</sub> males were collected and given a chance to sire offspring before they were killed and prepared for skeletal examination. Of these, 114 came from the 4 x 100 mg/kg sample of Hitotsumachi *et al.* and 27 from the concurrent control.

Five of the 114 experimental males were presumed mutants according to the criteria used in the NBT methods. Two of these failed to sire any offspring even though they were paired with many females for many weeks. Thus, there was no possibility of confirming the transmissibility of their traits. A third male was shown to transmit a mutation having low (probably extremely low) penetrance for a very severe effect. This male had scoliosis and fusions in the lumbar region and a grossly deformed left hind leg, missing a fibula, most of the tarsals, and two of the digits. One of his daughters was similarly, but more severely, affected. Thus far, no more of his descendants have manifested the effect. Even with just 1 affected offspring, the frequency of the severe malformation was statistically significantly higher among his total progeny than in the control, with the result that he is confirmed to be a mutant. The remaining two presumed mutants are still being tested. If they transmit any abnormalities, it seems apparent that the penetrance of these mutations will be incomplete. Based on our earlier work, it is likely that the skull fusions exhibited by both of these presumed mutants are caused by mutations with incomplete penetrance.

An increase in the "index of mutation" is expected only if dominant mutations are being induced that cause index anomalies. Many of the index anomalies occur occasionally as nonmutant variants. (The "index of mutation" in the control is zero because the control frequency of index anomalies is subtracted in calculating index values.) Twenty-three of the 114 experimental males had at least one index anomaly, but none of the 27 control offspring had any. So far, 13 of the males having one or more index anomaly have been shown to transmit their effect, thus demonstrating its mutational basis.

In addition, several anomalies that were not exhibited by presumed mutants, or that were not index anomalies, have been shown to be caused by mutations. Such mutations can be found by the breeding-test method because all unusual anomalies are tested for transmission even though the syndrome present is not sufficiently unusual to meet either the sensitive-indicator or multiple-anomaly-inferential criteria.

When we finally complete this experiment, it is likely that the combined frequency of known mutant and untestable presumed mutant mice will be in the neighborhood of 18-20%. In contrast, the estimate of the induced frequency of presumed dominant skeletal mutations of a type likely to have clinical importance was found to be only 4.5% by NBT methods. The excess of mutations found in the breeding-test experiment consists largely of mutations that are unlikely to adversely affect health.

It will be important to conduct much larger breeding-test experiments on mice injected with ENU in order to further our understanding of the nature of the damage induced by this chemical. Little can be concluded about the numbers of sterile, fully penetrant, and only slightly penetrant mutations from such a small sample of presumed mutations, but should the relative numbers of each type come anywhere close to what was found, the contrast with radiation would be large, and the ratio of mutation rates estimated from NBT and breeding test methods would be different for the two mutagens.

A major advantage of NBT methods over the breeding-test method is that they are so much faster. More important, the need for having methods that can identify serious mutations with low penetrance or mutations that cause sterility is also illustrated by this experiment. A method for studying the induction of dominant mutations that requires a test of transmission, such as the cataract test, would obviously overlook many important mutations if a large number of those induced are similar to the presumed mutations found in this small breeding-test experiment. Much of the burden of genetic disorders present in the human population is irregularly inherited, and NBT methods are essential if we are to make estimates of risk that fully account for irregularly inherited disorders.

#### DETAILED ANALYSIS OF RADIATION-INDUCED LITTER-SIZE REDUCTION IN SPECIFIC-LOCUS EXPERIMENTS

P. B. Selby                    W. L. Russell<sup>7</sup>

A detailed analysis of the records from many past specific-locus experiments is in progress now that these data have been entered into ORNL's PDP-10 computer. Many interesting facets of these data can now be explored that would have been much too time-consuming to examine earlier.

Our analysis of the extent of the litter-size reduction induced by radiation has been completed. The BEIR committee, in its 1980 report, recognized the importance of information on the litter-size reduction. They used the crude estimate then available to demonstrate that the amount of damage from gross chromosomal mutations is much less than that from gene mutations.

Our recently completed analysis of 14 radiation experiments involved approximately 900,000 litters. Comparisons were made between experimental groups and their concurrent controls in an attempt to cancel out seasonal differences and differences that might occur in the stocks over the years. The most important difference between the experimental and control groups was in the age of the mothers when the litters were born. Maternal age greatly influences litter size, and it was unclear how much error crept into the earlier crude estimate because of some differences in experimental and control groups regarding the age of the dams. Accordingly, the age of the mother at the time of birth for each litter was calculated for all of the approximately 900,000 litters. These ages were grouped into 50-day intervals, and comparisons of average litter size at weaning between experiments and controls were made only between groups within the same interval.

It now appears that the litter-size reduction at 600 R is 4.12% instead of our earlier crude estimate of 2.96%, which was used by the BEIR Committee. Although an adjustment for this new estimate would raise the BEIR estimate of the number of individuals having a serious handicap because of radiation-induced segmental aneuploidy from 0.35 to 0.49 per million for 1 R of low-LET radiation, this analysis strengthens the view that these data clearly indicate that the risk from such induced gross chromosomal damage among liveborn individuals is slight.

The relative litter-size reductions at various doses and dose rates in these early specific-locus experiments agree remarkably well both with relative specific-locus and with reciprocal-translocation mutation frequencies. Thus, there is a humped-dose-response curve for 90 R/min radiation and there is a pronounced dose-rate effect. The valuable new measure of damage now made available with this analysis includes the total induced mortality before three weeks of age from all causes (segmental aneuploidy, trisomy, monosomy, gene mutation, and so on, as well as dominant subvital mutations). These data strengthen the conclusion for radiation that there is much less genetic risk from chromosomal aberrations than from gene mutation. They also show that at low dose rates there is very little dominant lethality induced by low-LET ionizing radiation.

#### CYTOGENETIC ANALYSIS OF STERILE MALE PROGENY FROM MUTAGEN-EXPOSED PARENTS

N. L. A. Cacheiro<sup>3</sup>      C. V. Cornett

Approximately one-third of transmitted reciprocal translocations induced by ionizing radiations or alkylating chemicals in male postmeiotic stages cause complete sterility in male progeny. The sterility is associated with blockage in spermatogenesis or spermogenesis. Through detailed karyotype analysis we found that reciprocal translocations in sterile males often involved at least one breakpoint located close to a centromeric or telomeric region. (Cacheiro, N. L. A., *et al.*, *Genetics* 76: 73-91, 1974).

In the past few years, we have been performing similar analyses on sterile male progeny produced in various mutagenesis studies.

Effects of treatment of spermatogonia. One group of steriles was derived from irradiated spermatogonia. To date, we have analyzed a total of 54 sterile male progeny from this group. Out of these, 9 were carriers of reciprocal translocations, one was XXY, 10 were XYY, one was XY/XYY mosaic, and one was XO/XY/XYY mosaic. No chromosomal abnormalities were detected among the remaining 32 steriles. This result shows that, while practically all sterile male progeny derived from exposure of male postmeiotic germ cells are carriers of reciprocal translocations, less than one-tenth of the steriles derived from exposed spermatogonia were found to carry reciprocal translocations. Furthermore, sex-chromosome aneuploidy and sex-chromosome mosaicism contributed significantly to the cause of sterility in the spermatogonia group.

Effects of methylmercuric hydroxide. This experiment was conducted because of several reports that indicated the ability of methylmercuric hydroxide to induce chromosomal nondisjunction in *Drosophila* germ cells, and in plant and animal somatic cells. The present mouse study was designed to measure any increase in the incidence of sex-chromosome aneuploids (XXY and XYY conditions are sterile) as a result of induced sex-chromosome nondisjunction in male or female germ cells. About 10-week old (SEC C57BL)F<sub>1</sub> females and (101 C3H)F<sub>1</sub> males were randomized into experimental and control groups. In order to maximize the chance of detecting any mercury-induced effect, the maximum tolerated dose was used (4 ppm in drinking water), and both males and females were exposed continuously, beginning at least two months prior to mating and until the progeny were weaned. This ensured presence of mercury in premeiotic, meiotic, and postmeiotic germ cells, and in the progeny from conception to weaning. At sexual maturity, male offspring were tested for fertility, and sterile ones were analyzed cytologically.

In the mercury-exposed group, there were 17 steriles out of 1267 progeny tested (1.34%), while in the control group there were three steriles out of 1262 tested (0.24%). The difference is highly significant. Analysis of the 17 steriles from the mercury group revealed one XYY and 16 with no detectable chromosomal abnormalities. The three steriles from the control group had no detectable chromosomal abnormalities. These data suggest that the mercury treatment was not effective in inducing sex-chromosome nondisjunction in male and female meiocytes. The significant increase in mercury-induced sterility among male progeny could be due to types of genetic damage that are not detectable by the cytological techniques employed, or to nongenetic effects of mercury during the gestation and early postnatal periods.

RESPONSE OF MOUSE SPERMATOGONIAL STEM CELLS TO X-RAY INDUCTION  
OF HERITABLE RECIPROCAL TRANSLOCATIONS

W. M. Generoso  
K. T. Cain  
N. L. A. Cacheiro<sup>3</sup>

C. V. Cornett  
D. G. Gosslee<sup>9</sup>

In mice, heritable translocations are an important endpoint with respect to evaluation of hazards from radiation-induced chromosomal aberrations. It is, therefore, unfortunate that there has been a paucity of data on the rates at which heritable translocations are induced by ionizing radiations in spermatogonial stemcells, the stage of greatest importance in risk estimation. Before the present study was completed, virtually no data were available for single doses up to 600 R; and for fractionated exposures, a wide range of values for the number of translocations per rad per gamete had been published ( $0.5$  to  $5.6 \cdot 10^{-5}$  in the 1977 UNSCEAR Report), partly as a result of the small numbers of offspring tested in most studies.

The primary objectives of the present study were to obtain an accurate estimate of the number of heritable translocations expected per rad per gamete, and to determine how the rate of induction is affected by conditions of radiation exposure such as dose, dose-rate, and dose-fractionation. Furthermore, it was obviously important to obtain a fairly good estimate of the spontaneous frequency. All these objectives (except for dose-rate studies) have been fulfilled. First, we have obtained a good estimate of the shape of the heritable-translocation dose-effect curve for spermatogonial stemcells. Doses of 150, 300, 600, or 1200 R of acute X rays were used. The ascending portion of the "humped" dose-effect curve fits the linear model very closely. Second, the frequencies of heritable translocations observed in the 600 R 2 and 500 R 4 fractionation experiments appear to be within reasonable bounds of what might be expected from additivity. And thirdly, we have obtained the most reliable figure to date for the spontaneous frequency of heritable translocation; based on 5433 tested offspring, this frequency was  $1.8 \cdot 10^{-4}$ .

The linearity of the ascending portion of the dose-effect curve, the additivity of effects observed in the dose-fractionation experiments, and the reliable control rate permit a direct calculation of the rates of induction of heritable translocations per gamete per rad of acute exposure. At 1200 R single exposure, the rate is clearly lower than at other doses; this point lies at the tail end of the descending portion of the humped dose-effect curve. All other values, on the other hand, are very close to one another, ranging from  $3.07 \cdot 10^{-5}$  to  $4.44 \cdot 10^{-5}$ , with no significant differences among them. On the basis of this linear portion of the curve, the average number of translocations per rad per gamete, weighted by the reciprocal of the variances, is estimated to be  $3.89 \cdot 0.25 \cdot 10^{-5}$ .

In another radiation study on spermatogonia, we are attempting to provide information that should clear up some of the confusion on the

relation between the frequency of reciprocal translocations scored cytologically in meiocytes of exposed males and the heritable translocation frequency scored in offspring. The latter is, on the average, only half of what is expected on the basis of the former, and there is a lack of consistency in the ratio of cytologically to genetically scored translocations. It has been suggested that the discrepancy could be attributable to selection during postmeiotic stages against translocation-carrying gametes, or to metaphase-I delay (i.e., spermatocytes carrying translocations might take longer to pass through metaphase I) that results in a spuriously elevated cytological frequency. We are estimating the actual proportion of unbalanced segregants by conducting a sizable dominant-lethal experiment simultaneously with a cytological study of meiocytes of the males that are used as sires. The optimum dose for this combined study,  $4 \times 500$  R, was chosen on the basis of the results of the translocation experiment described above. With this treatment to spermatogonia we expect a dominant-lethal-type effect of about 20% from unbalanced segregants of reciprocal translocation.

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#### DNA TARGET SITES ASSOCIATED WITH CHEMICAL INDUCTION OF DOMINANT-LETHAL MUTATIONS AND HERITABLE TRANSLOCATIONS IN MICE

W. M. Generoso                    J. B. Hoskins  
K. T. Cain

Chromosomal aberrations that lead to early lethality among conceptuses do not contribute to the population's genetic burden. On the other hand, aberrations that permit survival and reproduction can have untoward effects on carriers, as well as on some of their immediate descendants. How these two classes of aberrations are produced in mammalian germ cells has been the objective of our long-term study.

In mice, dominant-lethal mutations and heritable translocations best exemplify the nonviable and viable endpoints of chromosome breakage induced in germ cells. In males, induction of these endpoints is stage dependent, and the sensitive stages vary from one mutagen to another. Both endpoints are induced by certain alkylating chemicals in male meiotic and postmeiotic germ cells, but the relative rates at which they are induced is dependent upon the alkylating chemical. The recent completion of the ethyl nitrosourea (ENU) and methyl nitrosourea (MNU) studies enabled us to relate certain DNA adducts with the production of dominant-lethal mutations and heritable translocations.

Among compounds that have been studied in our laboratory, isopropyl methanesulfonate (IMS) and ENU were found to be effective in inducing dominant-lethal mutations, but these chemicals induce very few heritable translocations in the same germ-cell stages. By contrast, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), triethylenemelamine (TEM), ethylene oxide (EtO), MNU, and cyclophosphamide are all effective in inducing both endpoints. This suggests that IMS- or ENU-induced primary lesions that result in dominant-lethal mutations are different from the lesions that result in heritable translocations. In other words, the simple interpretation that the same mechanism is responsible for the random production of dominant-lethal mutations or heritable translocations is not always correct.

We have postulated that the production of dominant-lethal mutations and heritable translocations is determined by the stability of alkylation products in the chromosomes. If these products are intact in the male chromosomes at the time of sperm entry, they may be repaired in fertilized eggs. If repair is not effected and the alkylation products persist to the time of pronuclear chromosome replication, they lead to chromatid-type aberrations and eventually to dominant-lethality. The production of heritable translocations, on the other hand, requires the transformation of unstable alkylation products into suitable intermediate lesions. These lesions are then converted into chromosome exchange (both symmetrical and asymmetrical) after sperm enters the egg but prior to the time of pronuclear chromosome replication (i.e., chromosome-type). Thus, dominant-lethal mutations result from both chromatid- and chromosome-type aberrations, while heritable translocations result primarily from the latter type.

Our findings with ENU and MNU not only strengthen the above conclusions, but they also contribute to the following interpretation regarding the DNA adducts involved. The chemicals that are effective in inducing heritable translocations (EMS, MMS, MNU, and EtO) alkylate primarily the N-7 position of guanine. The chemicals that are ineffective in inducing heritable translocations but are effective in inducing dominant-lethal mutations (IMS and ENU) alkylate primarily the oxygen of the phosphate backbone (forming phosphotriesters) and of bases, e.g., the O-6 position in guanine; very little alkylation occurs at nitrogen positions. Consistent with the stability interpretation, N-7 alkyl guanine adducts are not stable and are lost via hydrolysis, resulting in the formation of apurinic sites. Alkylation products with oxygen of bases and phosphotriesters, on the other hand, are highly stable. Thus, heritable translocations are associated with unstable N-7 alkylguanine and, possibly, with N-3 alkyladenine as well, since the latter product is also highly unstable.

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**EARLY EMBRYONIC LETHALITY INDUCED BY SEED OILS IN FEMALE MICE  
TREATED PRIOR TO INSEMINATION**

W. M. Generoso                    K. T. Cain  
J. B. Hoskins                    W. J. Washington<sup>10</sup>

In animal carcinogenicity and toxicity tests, corn oil is often used as the carrier of test substances that are insoluble in water but soluble in various fat solvents such as ether, chloroform, or benzene. We have been using corn oil as the carrier of coal-derived mixtures in short-term dominant-lethal and long-term total-reproductive-capacity tests. In the course of one dominant-lethal study on females injected intraperitoneally with a coal mixture dissolved in 0.4 ml corn oil, we noticed an unusually high incidence of deciduomata in the control group (which had received 0.4 ml of corn oil). Because the strain of females used, (SEC x C57BL)F<sub>1</sub>, has in the past shown consistently low levels of deciduomata in control groups treated with Hanks' Balanced Salt Solution, HBSS, we decided to test corn oil for induction of early embryonic lethality in treated female mice.

Virgin females 10-12 weeks old, from strains (SEC x C57BL)F<sub>1</sub>, (C3H x C57BL)F<sub>1</sub> and (C3H x 101)F<sub>1</sub>, were given a single i.p. injection of 0.4 ml corn oil (cold pressed). Control mice were injected with 0.4 ml of HBSS. All females were mated during the 6 days following injection and killed for uterine analysis 12 to 15 days after mating. The corn oil-treated groups had up to 30% deciduomata (all three strains were affected), while none of the control groups had more than 5%.

To determine whether other oils might produce a similar effect, we tested 0.4 ml single i.p. injections of peanut oil, sesame oil, olive oil, or mineral oil in (SEC x C57BL)F<sub>1</sub> females. All three plant oils were found to increase the incidence of deciduomata. Mineral oil, on the other hand, had no effect whatsoever.

To determine whether the corn-oil action was affected by route of administration, we compared the effects of 0.4 ml or 0.8 ml corn oil given orally with those of 0.4 ml corn oil i.p., or 0.8 ml HBSS given orally. Results clearly showed that the oral administration of corn oil did not increase the incidence of deciduomata above control levels.

Thus, i.p. injection of corn oil, but not oral administration, markedly increases the incidence of deciduomata, and a similar effect is observed with three other seed oils, but not with mineral oil. This reproductive response to seed oils was surprising in view of the general belief that these substances are nontoxic. It will be of considerable interest to determine the basis for this response. Because females were treated, early embryonic lethality may be due to direct effects on oocytes or embryos, or to maternal-environment effects associated with alterations in female physiology. We are following up on this problem.

It should be noted that seed oils, as well as mineral oil, are metabolized and eliminated from the peritoneal cavity very slowly. Traces

of the oils in the peritoneal cavity can be detected 10 days or more after injection. The most promising lead, at this point, is the observation that in a good number of corn oil-treated females that were killed for uterine and ovarian analyses, the number of implantation sites was clearly higher than the number of corpora lutea. In other words, there were more embryos implanted than could be accounted for by the number of ovulation sites. This observation seems to suggest that corn oil, or a metabolite, interferes with the normal processes of polar-body extrusion and fertilization (e.g., a polar body may be fertilized) and/or with early cleavage stages. The ovaries and oviducts, which are surrounded by fatty tissues, are located in the peritoneal cavity. It is likely that the slow elimination of corn oil from the peritoneal cavity allows the exposure of oocytes and eggs to the oil and its metabolites during divisions. Such exposure is not possible when oil is administered orally.

Investigation of the effects of corn oil on meiotic and early cleavage divisions is in progress. The almost universal human use of seed oils warrants a thorough investigation of their biological effects. Because of the problems in getting the oil (which cannot be delivered in aqueous media) to target cells, such studies are difficult in in vitro and many in vivo systems.

#### A RESTUDY OF THE EFFECTIVENESS OF INGESTED ETHYL ALCOHOL TO CAUSE NONDISJUNCTION IN MOUSE OOCYTES

W. J. Washington<sup>1,0</sup>  
N. L. A. Cacheiro<sup>3</sup>

W. M. Generoso

Trisomies, which result from nondisjunction, play a major role in human spontaneous abortions and genetic disorders (e.g., Down's Syndrome). A recent report by M. H. Kaufman (Nature 302: 258-260, 1983) on the induction of chromosomal nondisjunction by ethanol present at conception in mice thus attracted considerable attention. Since all autosomal monosomies and several trisomies are lethal by day 12 in the mouse, we anticipated that Kaufman's treatment, which is reported to have caused about 20% aneuploidy (monosomy + trisomy), should result in a detectable amount of embryonic death. Accordingly, we investigated the dominant-lethal response of female mice given ethyl alcohol orally shortly after mating. We also conducted a corresponding cytological analysis of the first postfertilization metaphase prior to first cleavage division.

In 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 hours after the end of mating period. (We had found in an earlier study that sperm entry into the egg starts about 1.75 hours 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 hours 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 hours after HCG (i.e., about 1.5 - 2.5 hours after the expected time of ovulation). Since sperm entry starts about 1.75 hours 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 hours after HCG.) Mated females were injected intraperitoneally with 2.5 mg/kg of colchicine 9-10 hours after ethanol administration and were killed the following morning (about 15 hours 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.

In the dominant-lethal study, the frequency of embryonic death in the ethanol-treated group was not significantly higher than that in the control group. Similarly, the cytological findings on superovulated eggs did not indicate a significant ethanol-related increase in aneuploidy. Thus, our results to date do not suggest an induced nondisjunctional response. Close comparison with Kaufman's results is, however, not possible because of strain differences and other factors. Most important, our dominant-lethal study must be expanded to include a later age at sacrifice, since several mouse trisomies cause death after the 12th day of gestation, the stage for which the present results were obtained. Such a study is in progress.

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#### DEVELOPMENT OF TESTS THAT DETECT INDUCTION OF MEIOTIC NONDISJUNCTION IN MAMMALS

Liane B. Russell

Commonly used mammalian germline mutagenesis tests do not detect numerical chromosome anomalies per se (that is, aneuploidy caused by mis-segregation as opposed to chromosome breakage and rearrangement). Because

trisomic offspring can bring considerable grief to human families (e.g., Ts21 which causes Down's syndrome), tests that detect nondisjunction inducers should be developed. A literature search was carried out for the purposes of surveying and comparing techniques that have been used to detect meiotic nondisjunction in experimental mammals, and of designing the potentially most informative studies.

The discovery in recent years of mouse populations carrying Robertsonian translocations that, in the aggregate, involve all chromosomes has provided tools for the systematic study of the phenotype of animals trisomic or monosomic for any given chromosome. The method, which involves a cross in which one parent carries two Robertsonian translocations having one common arm [e.g., Rb(1.15) and Rb(3.15)], has shown that (a) all trisomics in the mouse die after midgestation, (b) none of the autosomal trisomies regularly survives birth (though some do so occasionally), and (c) some have morphological phenotypes that are externally recognizable during fetal stages. All monosomics, except XO (which survives to adulthood), die preimplantation or shortly postimplantation.

The methods that have been used for the detection of trisomics in experimental mammals differ in the developmental stages examined, the type of analysis, and the signal for trisomy. (a) Chromosomal analysis at the first meiotic metaphase (M I) (signal: low chiasma frequency; univalents) is presumed to predict the occurrence of nondisjunction (ND) in M I. (b) Chromosomal analysis in M II (signal: extra dyads) can detect ND that occurred in M I. (c) Fluorescence studies of spermatids or sperm (signal: extra fluorescent bodies presumed to be sex chromosomes) could detect ND from M I and/or M II. (d) Chromosomal analysis in the zygote (first cleavage) can show extra chromosome(s) in the maternal or paternal complements, which can often be distinguished (origin: ND in M I and/or M II of either parent). (e) Chromosomal analysis of several cells of morulae, blastocysts, or midgestation embryos, if it shows consistent hyperploidy, can reveal ND in meiosis of either parent. Similar studies on newborns are now seen to be largely useless in view of the generally prenatal death of trisomics. (f) Postnatal analysis using genetic markers. This may consist of the numerical sex-chromosome anomaly test (developed by us some time ago); or, to detect autosomal ND, a method in which complementing ND segregants from translocation parents are recognizable. (g) Postnatal analysis using a preliminary fertility test, followed by cytological analysis of steriles to determine whether some are XXY or XYY. This method is a -product of the usual Heritable Translocation Test.

All of these methods have certain drawbacks and certain advantages, which were enumerated during the study. It should be noted that trisomics detected by methods (e) through (g) could, at times, be the result not of meiotic ND, but of mitotic ND in the embryo. The complementary monosomic cell line presumably died at an early stage. Chance cell sampling, or, alternatively, chance assortment of the trisomic line into inner-cell-mass (as opposed to trophoblast) of the blastocyst could lead to failure to detect original mosaicism.

One method that has not yet been used, and which we hope to explore, involves analysis during fetal stages, at which time most trisomics are now known to die. The signals would be fetal growth retardation and morphological anomalies, to be verified by cytological analysis.

The stage at which an agent is most likely to induce ND depends, in our opinion, on the mechanism by which ND is thought to occur. If the underlying cause of ND is a reduction in genetic recombination, the agent should be applied during premeiotic DNA synthesis (34-36 days pre-ejaculation in the male; half-way during intra-uterine life in the female). If, on the other hand, the agent acts on spindle formation or function, the sensitive stage is probably M I and M II (22-23 days pre-ejaculation in the male; ca. one-half day preovulation in the female). Agents acting on the kinetochore may be active throughout meiotic prophase, metaphase, and anaphase.

Among chemicals that appear to have produced trisomy by meiotic ND in mammals are agents that act on DNA (EMS, MMS, 6-Mercaptourine, and possibly cyclophosphamide), agents that act on the spindle (colchicine), and agents with largely unknown action (cadmium chloride, halothane, certain hormones). It is noteworthy that agents of the first group gave positive results when applied to preleptotene stages of spermatogenesis.

#### EVALUATION OF EXISTING MUTAGENICITY BIOASSAY FOR PURPOSES OF GENETIC RISK ASSESSMENT

Liane B. Russell

It must be assumed that there is a high probability of genetic hazard to human beings if mutation induction can be demonstrated in the germline of experimental mammals. However, for a large percentage of chemicals, mammalian germline (MG) mutation data do not yet exist, and efforts to identify possible genetic hazard must be based on results from other tests. An attempt was made to evaluate the degree of reliability with which various types of data may accomplish this task. Two approaches were used. (1) A weighting scale was devised, based on major parameters by which the conditions of an assay resemble or differ from those encountered in the induction of transmitted genetic damage in mammals. (2) Findings from each of two standard MG tests were compared with those from each of the other assays in turn, and correlations calculated.

The first approach was complicated by the circumstances that (a) for over 85% of the 2367 chemicals in the Gene-Tox data base results are available in only one or two test categories, and (b) MG tests have in the past been performed preferentially on chemicals that had already been shown to be mutagenic in other systems. In comparisons with two separate MG tests -- specific-locus test (SLT) and heritable-translocation test (HTT) -- no positive MG results were found for chemicals that scored low with

regard to weighted results for non-MG tests. On the other hand, some negative MG results were found for high-scoring chemicals.

The second approach was complicated by the circumstance that there was only a limited number of cases in which the particular MG and non-MG tests being compared had enough chemicals in common to make the comparison meaningful. A cut-off point as low as ten common chemicals had to be chosen. Each observed MG-non-MG concordance was compared with a random assortment of outcomes, calculated on the null hypothesis, for the same pair of tests. The observed outcome was found to be most elevated over random expectation when MG results were compared with some of the mammalian in vivo tests (e.g., unscheduled DNA synthesis in the testis, mouse spot test); but, because of the low numbers of shared chemicals, none of the differences was statistically significant.

The data base as it stands can be utilized to identify genetic hazard if a positive MG result is available, or if the non-MG score (using the weighting system) is high. It cannot be used to assess risk, but it can help to establish priorities for choosing chemicals on which to proceed to various lengths along the path to risk assessment. Such priorities must be based in part on the expected magnitude of genetically significant exposure. A decision matrix for this purpose was devised.

[This analysis was performed in the context of evaluating the Gene-Tox data base. The assistance of EMIC in this endeavor is gratefully acknowledged.]

## CHEMICAL DOSIMETRY STUDIES IN MAMMALIAN GERM CELLS

Gary A. Sega James G. Owens  
Amy E. Jetton<sup>3</sup> Susan C. Hansler<sup>12</sup>  
Helen R. Harvey<sup>11</sup>

Chemical dosimetry using radactively labeled chemical mutagens provides 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 at exposure levels that are orders of magnitude lower than those needed to produce a statistically significant genetic effect. As our dosimetry data are combined with other genetic and cytogenetic data using the same chemicals, we shall learn much 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.

Following the finding by W. L. Russell that ethylnitrosourea (ENU) is a very powerful mutagen in mammalian germ cells, we initiated studies of the biochemical interactions of this chemical with germ cells in the male mouse. After an i.p. injection of 50 mg/kg of  $^3\text{H}$ -labeled ENU, spermatozoa

have been recovered from the *vasa deferentia* of exposed males over a 28-day time period. The alkylation pattern observed in the developing sperm showed about a two-fold greater level of sperm-head ethylation 8-14 days after treatment than at earlier or later time points. This indicated that late spermatid to early spermatozoal stages were being alkylated somewhat more than the other meiotic and postmeiotic germ-cell stages. However, the increase in alkylation of late spermatid to early spermatozoal stages by ENU is very small compared to the increase seen in the same stages when methyl methanesulfonate (MMS) or ethyl methanesulfonate (EMS) are used. With these chemicals there is an eight- to ten-fold increase in the alkylation of late spermatids to early spermatozoa, due to increased alkylation of sperm protamine in those stages. It appears that chemicals like ENU and its homolog, methylnitrosourea (MNU), which react by an  $S_N1$ -type mechanism, interact much less with sperm protamine than do chemicals like MMS and EMS that react by an  $S_N2$ -type mechanism.

In studying the presence of DNA alkylation products in the testes after different exposures to ENU, we found that the ratio of  $0^6$ -ethylguanine ( $0^6$ -EG) to N-7 ethylguanine (N-7 EG) changes with exposure level. Thus, after a 100 mg/kg ip injection of [ $^3$ H]ENU, the  $0^6$ -EG/N-7 EG ratio in testicular DNA is around 1.1, but as the exposure is lowered to 10 mg/kg, the  $0^6$ -EG/N-7EG ratio reaches a value of 0.7. Since  $0^6$ -EG is believed to be an important mutagenic lesion in DNA, the changing proportion of this lesion measured in testis DNA as a function of chemical exposure level can be an important point to consider when interpreting genetic tests. Mutation frequencies obtained at high chemical exposure levels may not extrapolate linearly as chemical exposures are lowered.

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## DNA REPAIR STUDIES IN MAMMALIAN GERM CELLS

**Gary A. Sega**      **James G. Owens**

In submammalian test systems, nitrosocarbamates are 100-fold more mutagenic than are their corresponding nitrosourea homologues. Since ethyl nitrosourea proved to be such a powerful mutagen in the mouse, W. L. Russell has performed a specific-locus experiment using nitrosoethyl carbamate (NEC). While NEC has shown moderate to high mutagenic activity in the mouse, it is considerably less potent than ENU.

To learn more about this chemical's interaction with germ-cell DNA in the mouse testis, (101 x C3H)F<sub>1</sub> males (the same stock being used in the genetic experiments) were given i.p. injections of NEC. The exposures were

100 and 300 mg/kg. Testicular injections of [<sup>3</sup>H]dThd (18  $\mu$ Ci/testis) were given along with the NEC. In an additional experiment, the NEC was injected directly into the testes along with the [<sup>3</sup>H]dThd. The concentration of NEC was such that, for an average testis weight of 100 mg, the dose was 300 mg/kg. Sixteen days after treatment, sperm were recovered from the caudal epididymides and assayed for an unscheduled-DNA-synthesis (UDS) response using liquid scintillation counting. (The sperm sampled at this time were primarily in early spermatid stages at the time of treatment.)

Historical control values have averaged 10 disintegrations per minute (DPM)/10<sup>6</sup> sperm. With the 100 and 300 mg/kg i.p. injections of NEC, we obtained an average of 11.4 and 17.1 DPM/10<sup>6</sup> sperm. The direct testicular injection of NEC resulted in 24.5 DPM/10<sup>6</sup> sperm. The UDS response is marginal at best. The somewhat larger response from direct testicular injection of NEC may be attributable to failure of blood esterases to inactivate all of the NEC before it had an opportunity to alkylate some germ-cell DNA. We have now studied the ability of about 20 different agents to induce a UDS response in germ cells of the male mouse. There has been a very good correlation between those agents which give a positive UDS response and those which give a positive specific-locus test in the same germ-cell stage. The only exception may be hycanthone methanesulfonate, for which we obtained a weakly positive UDS effect (2 to 3 times control values) but which was negative in the specific-locus test. Other agents showing a positive UDS effect in mouse germ cells include methyl-, ethyl-, propyl-, and isopropyl methanesulfonate, cyclophosphamide, mitomycin, ethylene oxide, methylnitrosourea, ethylnitrosourea, triethylenemelamine, and X-rays. Agents that have been negative in the UDS test include ethylene dibromide, dimethyl nitrosamine, diethyl nitrosamine, benzo[a]pyrene, caffeine, methyl nitrosoguanidine, and mitomycin C.

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## DNA DAMAGE IN MAMMALIAN SPERM ASSAYED BY ALKALINE ELUTION

Gary A. Sega  
Estela Generoso

James G. Owens

To further assess the damage produced in mammalian germ cells by model chemicals, we are continuing to develop an alkaline-elution procedure to measure DNA strand breaks in spermatozoa. The procedure involves the lysis of the germ cells after treatment with the test chemical, followed by separation of the DNA double helix using a strong base. Any small pieces of single-stranded DNA resulting from breakage by the test chemical will rapidly pass through a filter, while normal-sized DNA will take much longer to pass through the same filter. This procedure may prove especially useful in looking for the accumulation and/or persistence of DNA strand breaks in mature sperm stages where no UDS (DNA repair) is observed after mutagen treatment. It is hoped that this method of detecting DNA damage in mouse sperm may eventually prove to be a useful means for monitoring DNA damage in human sperm.

The germ-cell DNA of a group of (C3H x 101)F<sub>1</sub> hybrid males was labeled with <sup>3</sup>H-thymidine (<sup>3</sup>HDdT), and the males were subsequently given a 50 mg/kg i.p. injection of MMS. (Our original studies had used 100 mg MMS/kg, but to avoid any possible toxic effects to the germ cells, we decided to lower the MMS exposure to 50 mg/kg.) A second set of (C3H x 101)F<sub>1</sub> males had their germ cell DNA pre-labeled with <sup>14</sup>CdT and served as controls.

At daily intervals through 21 days after MMS treatment, spermatozoa from the vasa deferentia of treated and control animals were placed together on polycarbonate filters and lysed DNA was eluted through the filter overnight, using an alkaline buffer at pH 12.2. The amounts of treated and control DNA eluted through the filter were determined using liquid scintillation counting techniques. In the first week following MMS injection the amount of sperm DNA that eluted increased gradually. Maximum DNA elution was found for sperm collected 7 to 14 days after exposure. In this time period the amount of elution was 20-25% greater from treated than from control DNA.

Genetic tests with MMS in the mouse have shown that sperm sampled in the second week following treatment (early spermatozoa to late spermatid stages at the time of treatment) show the highest frequency of dominant lethals and specific-locus mutations. There is, moreover, good agreement between the pattern of genetic effects produced by MMS in the developing sperm and the pattern of DNA elution that we have measured.

Similar work is in progress with ethylene oxide (EtO), a widely used industrial chemical and sterilant. After a 100 mg/kg i.p. injection of EtO the same sperm DNA elution pattern was observed as when MMS was used. (MMS and EtO have nearly identical Swain Scott  $s$  values, which means that both chemicals should attack the same nucleophilic sites in DNA and proteins.)

The genetic pattern of induced dominant lethality produced by EtO in developing mouse sperm shows a peak effect in sperm sampled in the second week posttreatment. This is correlated with our highest elution values for the EtO-exposed sperm DNA. There is overall good agreement between the pattern of genetic effects produced by EtO in developing sperm and the pattern of DNA elution that we have measured.

Possibly the most important finding in our work to date is that we can use alkaline elution procedures to measure DNA damage in mammalian sperm with at least some classes of chemicals. In the case of MMS and EtO, there is a good correlation between the pattern of DNA elution in the developing sperm of treated mice and the pattern of induced genetic damage. The possibility, therefore, exists for using the alkaline elution procedures we have developed in the mouse to monitor human sperm for DNA breakage that may be correlated with genetic damage.

## ANALYSES OF SPECIFIC REGIONS OF THE MOUSE GENOME

Liane B. Russell Jane Mendel<sup>1</sup>  
C. S. Montgomery Lorraine Albritton<sup>1</sup>

In a continuation of our detailed genetic analysis of specific chromosomal regions of the mouse, we have initiated a study of mutations involving the p (pink-eye) locus. Like the c (albino) locus analyzed by us earlier, p is on Chromosome 7, about 14 cM proximal to c. Over 170 p-locus mutants have been analyzed as to heterozygous and homozygous phenotype and mode of origin (type of mutagen and germ-cell stage of induction). A complementation experiment has been initiated with about 50 of the mutations. (In the case of our c-locus study, complementation analysis allowed us to identify 12 complementation groups that are probably overlapping small deficiencies involving the marker locus.) Although still in early stages, the p-locus study has already revealed some complementations for lethal effects. We are presently developing a computer program that will facilitate analysis of weaning weights and longevity in offspring of the many crosses of the matrix.

About 40 p-locus mutants are in process of being tested for possible deficiency for ru-2, a marker that is 2-3 cM proximal to p. One such deficiency has been found. A number of p locus lethals have also been screened for lactate-dehydrogenase-A expression by gel electrophoresis, followed by measures of enzyme activity. The p mutant that is deficient for ru-2 was found to be deficient for Ldh A as well. This mutant was, however, not deficient for Xld (xylose dehydrogenase), which is probably proximal to ru-2. Work has begun on cloning mouse Ldh-A. The clone will be useful in determining the coding sequence of the gene and in studying the regulation of the gene's expression (LDH-A is regulated both developmentally and in a tissue-specific manner). The genomic clone may also become useful as an

entry point into the region surrounding the p locus (defined by p-lethal deficiencies).

In earlier years we had genetically analyzed the d-se region in Chromosome 9, using a large series of radiation-induced d, se, and d se mutations. A recent enlargement of this study indicates that, in contrast to the findings for the c-locus region, many mutations in the d region cannot be fitted into any linear order. We have initiated a collaborative study with Nancy Jenkins and Neal Copeland, who recently found that the original spontaneous mutation to d (>50 years ago) resulted from integration of an ecotropic murine leukemia virus into the DNA of Chromosome 9, presumably at the d locus. We are supplying spleens from a large number of genetically analyzed d-se-region mutants for restriction analyses, the results of which we shall jointly attempt to correlate with the genetic findings.

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#### SYNAPTONEMAL COMPLEX STUDY OF NONHOMOLOGOUS SYNAPSIS IN X-AUTOSOME TRANSLOCATIONS SHEDS LIGHT ON X-Y DISJUNCTION MECHANISMS

T. Ashley<sup>3</sup>      L. B. Russell

Electron microscopy of pachytene nuclei of male mice heterozygous for either of two reciprocal translocations, T(X;7)5R1 and T(X;7)6R1 (abbreviated R5 and R6, respectively), revealed a high frequency of heteromorphic bivalents involving the translocated chromosomes. In both translocations, the breaks are in the proximal third of the 7 and the distal third of the X, but the R5 breaks are closer to the 7 centromere and X telomere than the R6 breaks.

In both translocations, the 7 frequently synapsed nonhomologously with the X7. In R5, the part of the X to which the 7 synapsed may include a region that synapses with the Y in normal mice. However, in R6, the 7 synapsed with a portion of the X that never synapses with the Y, synapsis involving the "differentiated" X region. In both translocations, the Y synapsed maximally with the X portion of the 7<sup>X</sup> in those nuclei in which there was nonhomologous synapsis of the 7 with the X<sup>7</sup>. The Y occasionally synapsed nonhomologously with the 7 portion of the 7<sup>X</sup>. The behavior of the

bivalents suggests that the autosomal portions of the  $7^X$  and  $X^7$  may alter the behavior of the sex-chromosome portions.

During pachytene of normal mice, the X and Y chromosomes have a region of synapsis (involving up to 35% of the distal portion of the X), as well as unsynapsed, or "differentiated" regions (the proximal two-thirds of the X and the proximal end of the Y). It has often been assumed that X-Y synapsis is homologous, entailing chiasmata that lead to normal X-Y disjunction. Our finding that the X — both in its "differentiated" and in its (normally) Y-pairing portions — is capable of nonhomologous synapsis with an autosome casts doubt on the homology of X-Y synapsis. The early desynapsis of the X and Y in normal mice (and other mammals) may have evolved to decrease the chance of X-Y exchange, which would produce a translocation (rather than crossing over, as would occur with homologous chromosomes). Normal X-Y disjunction may be achieved through an achiasmatic end association.

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#### INVOLVEMENT OF THE Y CHROMOSOME IN TRANSLOCATIONS

N. L. A. Cacheiro<sup>3</sup>

Although it is well known that reciprocal translocations involving the X chromosome cause complete sterility in male carriers in the balanced state, effects of translocations involving the Y chromosome are not well understood. In the course of our studies on sterility in male progeny from mutagen treated male parents we found 34 translocations involving the Y chromosome, which represents about 30% of all male-sterile translocations. Each autosome has been involved at least once, except chromosomes 1 and 11. A mapping of Y-chromosome breakpoints in banded preparations shows the distribution to be more or less random, with a slight concentration in the middle of the chromosome. By contrast, in autosome-autosome translocations that cause sterility (rather than semi-sterility) at least one of the

breaks is very close to a chromosomal end. Because all semisterile translocations found so far have been T(A;A), we can conclude that all T(Y;A)'s may be sterile.

#### MEIOTIC SEGREGATION IN PARTIALLY STERILE MALE TRANSLOCATION HETEROZYGOTES

C. V. Cornett  
N. L. A. Cacheiro<sup>3</sup>

Shinya Hitotsumachi<sup>3</sup>

During several years of studying of radiation- or chemically-induced heritable translocations we have observed that the ratio of partially sterile to sterile male translocation heterozygotes is stage dependent. When spermatogonia were mutagenized, virtually all (221 of 230) translocation carriers were partially sterile, but when postmeiotic stages were exposed, about one-third of the translocation carriers (among several hundred analyzed) were fully sterile. Full sterility results from blockage in spermatogenesis. Partially sterile males, on the other hand, produce functional sperm, the subsequent small litter size resulting from early death of embryos that received an unbalanced chromosome complement from their fathers. Such unbalanced complements result from adjacent-1, adjacent-2, or 3-1 segregations at meiosis in translocation-bearing individuals. We have found that, on the average, the proportion of living embryos is only 44% of normal, the average percentage of chromosomally unbalanced sperm in the ejaculate thus being 56%. However, different translocation lines are characterized by degrees of partial sterility that are either substantially lower or substantially higher than 50%.

Whether, and what type of, multivalent chromosome associations are found in translocation carriers is assumed to depend primarily on the size of translocated chromosome segments, which, in turn, influences the probability of chiasma formation between the translocated segments and their homologous segments in the intact chromosomes. From a study of partially-sterile translocations induced by TEM in male meiotic and postmeiotic stages, Generoso *et al.* (Mutat. Res. 81: 177-186, 1981) observed a positive correlation between the degree of partial sterility and the frequency at which multivalent chromosomes were observed in diakinesis-metaphase I spermatocytes. In the present study we are determining whether a similar correlation holds true for translocations derived from exposed spermatogonial stem cells.

Two groups of X-ray-induced partially-sterile translocations are included in this study: one group (128 animals) derived from treated spermatogonial stem cells, the other (72 animals) from exposed postmeiotic stages. All animals came from translocation experiments of W. M. Generoso. Translocation carriers were confirmed by genetic and extensive cytological analyses. As a rule, 25 diakinesis - metaphase I cells are scored from each pair of testes prepared by the air-drying technique. The index of

partial sterility is calculated from the average percentage of dead implantations found at midpregnancy in 6 females mated to each partially sterile male.

Results for both the gonial and the postmeiotic groups again indicate a positive correlation between degree of partial sterility and incidence of multiple chromosome associations. The groups appear to differ in the frequency of confirmed translocation carriers that show no multivalent chromosomes in any of the 25 cells scored per male: there were only 3% (2/72) of such animals in the group derived from postmeiotically-irradiated germ cells, but 13% (16/128) in the group derived from irradiated spermatogonial stemcells. This difference is not surprising if, indeed, short translocated segments favor the production of balanced gametes.

A detailed karyotype analysis of translocation stocks that have either very high or very low degrees of partial sterility is currently underway. This analysis should provide a direct proof for the role of the size of translocated chromosome segments in the meiotic segregation of balanced and unbalanced gametes. To date we have isolated 7 low-degree partially sterile translocation stocks (between 20 and 30% dead implantations) and 7 high-degree partially sterile stocks (between 65 and 80% dead implantations).

#### HIGH-RESOLUTION BANDING OF MOUSE PROMETAPHASE-PROPHASE CHROMOSOMES

N. L. A. Cacheiro<sup>3</sup>

Recent technical advances in the high-resolution banding of human chromosomes from lymphocyte cultures have provided a valuable tool for increasing the accuracy in the analysis of breakpoints, and for a better definition of chromosomal rearrangements. We have been successful in obtaining a high-resolution banding pattern in mouse chromosomes from kidney-derived primary tissue cultures, using actinomycin D and a short treatment with colchicine.

The technique has been used by us in conjunction with BrdU in the study of inactivation patterns of a number of our X-autosome translocations that, in previous experiments using <sup>3</sup>H thymidine and regular mitotic chromosome spreads, gave somewhat inconclusive results because of the poor resolution. At present we are also using the technique for the determination and cytological mapping of breakpoints in a number of the c-locus deletions in chromosome 7, and d se deletions in chromosome 9.

The examination of late-prophase chromosomes in the study of gene mapping will facilitate the localization of genes producing certain phenotypes to specific minute chromosome segments, extending our understanding of the organization and function of the mouse genome.

## MECHANISM OF GENETIC SUPPRESSION

E. H. Grell      K. B. Jacobson

The term, suppressor, is used to denote a mutation that reverses the effects of a mutation in a gene located elsewhere. The suppressor causes the phenotype of the suppressed mutation to appear completely or partially wild-type. This might be due to a variety of mechanisms. The type of suppressor that is of most concern to us is the suppressor that partially or completely suppresses several non-allelic mutations. The suppressor locus that we have most intensively examined is su(s). It suppresses vermillion, sable, speck, and purple alleles. It suppresses one or more spontaneous mutations at these loci but it will not suppress mutations induced by x-rays or chemical mutagens. On the other hand, mutations of su(s) are easily recovered after treatment with mutagens. The different alleles that are recovered are not equally effective as suppressors. They range from slightly suppressive to very effective suppressors. The relative suppressiveness is consistent. Suppressor alleles that are strong suppressors of one mutation are strong suppressors of other suppressible mutations.

We have concentrated on the suppression of mutations of the pr locus (purple). The pr<sup>+</sup> allele specifies the structure of a component of sepiapterin synthase. This enzyme can be measured. The enzyme catalyzes a reaction that is in the synthetic pathway of the red pteridine pigments of *Drosophila* eyes. The effect on eye color is an indicator of changes in the enzyme activity. The two suppressible alleles of pr are both spontaneous and leaky mutations. They are viable as homozygotes. Mutagen-induced mutations of pr are not leaky, not suppressible, and homozygous lethal. Additionally, variegated position effects affecting the pr locus have been recovered.

The suppression by su(s) mutations appears to be associated with a reduction in the amount of su(s)<sup>+</sup> substance. Our hypothesis is that the su(s)<sup>+</sup> substance is an inhibitor of the mutant sepiapterin synthase. A mutant of su(s) reduces the amount of the inhibitor and therefore such flies have more enzyme activity than those that are su(s)<sup>+</sup>. Extra doses of su(s)<sup>+</sup> have been added to the genome as small centric fragments attached to the X chromosome or attached to an autosomal chromosome. The extra doses of su(s)<sup>+</sup> have the effect of reducing the activity of sepiapterin synthase in pr flies. The amount of a heat-labile inhibitor of mutant sepiapterin synthase is dependent on the number of doses of su(s)<sup>+</sup>.

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### CIRCADIAN RHYTHMS OF SPERMATOGONIAL STEM CELLS

E. F. Oakberg      C. Crosthwait Cummings

It has repeatedly been suggested that spermatogonial stem cells surviving radiation undergo accelerated division in order to replenish the seminiferous epithelium. This more rapid division rate has been invoked to explain the effect of fractionated radiation exposures on chromosome breakage, translocation, and fertility. However, <sup>3</sup>H-TdR labeling, followed by acute radiation exposure, suggests that the cell-cycle properties of surviving stem cells are not altered by radiation. Insufficient attention has been given to cell synchronization through selective killing of sensitive cells and/or retardation of other stages of the cell cycle. Such an effect would give rise to an oscillating pattern of mitotic activity with time after exposure, with the observed response dependent upon the predominant cell-cycle stage present at given intervals sampled.

Another possible complication in these studies would arise if stem cells had a circadian rhythm. Recently, circadian rhythms have been shown to be a significant factor in post-irradiation mitotic activity of the corneal epithelium (Rubin, Radiat. Res. 89: 65-76, 1982) and bone marrow (Nečas, Cell Tissue Kinet. 75: 667-672, 1982). These results also suggest that the radiation had no effect on mitotic activity of the stem cells in these tissues. Absence of a circadian rhythm in spermatogonia was reported by Bullough (Proc. Roy. Soc. B, 135: 212-233, 1948), and we later confirmed his observations, but neither study identified the stem cell. It was essential to determine if the stem cells have a circadian rhythm before meaningful data on the effect of fractionated irradiation on cell-cycle kinetics of stem spermatogonia could be undertaken.

Mice were killed hourly from 8:00 a.m. to 4:00 p.m., and at 6 and 9 p.m., 12 midnight, and 3 and 6 a.m. Whole mounts of seminiferous tubules were prepared, and mitotic activity of A<sub>8</sub> spermatogonia determined. The highest mitotic rate, about 2%, was observed at 10 and 11 a.m., but owing to high variability among mice, there was no significant difference attributable to time of day.

In order to examine possible differences in DNA synthetic activity with time of day, mice were given a single intraperitoneal injection of 25  $\mu$ Ci <sup>3</sup>H-TdR, irradiated with 300 R 24 hrs later, and killed 207 hrs after labeling. This protocol restricts labeling to the long-cycling A<sub>8</sub> spermatogonia that are presumed to be the true stem cells of the testis. There was a significant effect of time of day, with low values at 9 a.m. and

12 midnight. This is not a typical circadian rhythm, and the experiment is being repeated in order to determine if the result was due to sampling error.

Since different groups of mice given  $3\text{H-TdR}$  were exposed to 300 R at 3-hour intervals throughout the day, a set of slides for scoring cell survival was prepared. There was no effect of time of day on number of stem cells observed 183 hrs after 300 R.

Stage of the cycle of the seminiferous epithelium was a significant variable, with the mitotic index at stage 5 (2.7%) being significantly higher than that at stages 1, 2, 3, 4, and 6. Percentage of labeled cells at stage 1 was significantly lower than that at stages 2-6. Therefore, the common practice of selecting a single stage of the cycle for study may lead to biased data on response to noxious agents.

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#### THE EFFECT OF AGE ON THE SPERMATOGONIAL STEM CELL POPULATION OF THE MOUSE

E. F. Oakberg

C. Crosthwait Cummings

Spermatogonial stem cells can first be identified about three days after birth. They have a morphology characteristic of stem cells in the adult, and have a cell-cycle time much longer than that of differentiating spermatogonia. The division rate of stem cells from 3 to 19 days of age is more rapid than that in the adult. On the basis of long-term  $3\text{H-TdR}$  labeling, cell-cycle properties characteristic of the adult are present at 21 days of age, and do not change thereafter.

Stem-cell numbers, however, decline between 12 weeks and 10 months. The new level is then maintained at 17 and 23 months of age. Some consistent and as yet unexplained changes in the numbers of differentiating spermatogonia ( $A_1$  through  $B$ ) and preleptotene spermatocytes occur with increasing age. The  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ , In, and  $B$  spermatogonia do not show the drop in numbers at 10 and 17 months that is shown by the  $A_5$  (A-stem) spermatogonia, but they do show a decline from 17 to 23 months. At present, cell counts of tubule whole mounts at 12 weeks, 11 months, and 23 months are being made to check the above data that were based on cell counts in sections. The whole mounts offer increased precision in the

identification of the  $A_g$ ,  $A_{pr}$  (A-paired), and  $A_{al}$  (A-aligned) spermatogonia, and will aid greatly in interpreting changes in the stem-cell population with age.

#### LACK OF EFFECT OF DBCP ON THE MOUSE TESTIS

E. F. Oakberg      C. Crosthwait Cummings

A reduction in sperm count in men exposed to DBCP (1,2-dibromo-3-chloro propane) has been indicated in several publications, and testicular atrophy and dominant lethals have been demonstrated in rats (Whorton and Foliart, *Mutat. Res.* 123: 13-30, 1983). In mice, however, Generoso (personal communication) observed neither dominant-lethal nor fertility effects after intraperitoneal administration of DBCP in corn oil. The possibilities existed that only a specific stage of spermatogenesis was sensitive, or that a fertility effect might not be observed in mice owing to the more rapid progress of spermatogenesis than in other species.

To investigate these possibilities, (101 x C3H) $F_1$  mice were randomized into three groups. Group 1 received no treatment; group 2 received an intraperitoneal injection of corn oil, and group 3 received 110 mg/kg of DBCP dissolved in corn oil. Mice that had received corn oil were killed at 5 and 8 days, and those that had received DBCP in corn oil were killed 3, 5, and 8 days after injection. Untreated controls were killed at 3, 5, and 8 days. Tissues were fixed in Zenker-formol, 5  $\mu$  paraffin sections were prepared and stained with periodic-acid-Schiff and hematoxylin, and 100 tubule cross sections per mouse were scored for numbers of spermatogonia and preleptotene spermatocytes.

Statistical analysis revealed no effect of corn oil or of DBCP on the spermatogonial and primary-spermatocyte populations. It therefore appears that there may be large species differences in the response to DBCP. Though there could be strain differences as well, on the basis of present data the mouse appears to be a poor model for studying the alleged fertility effects of DBCP in men.

#### ANALYSIS OF SPONTANEOUS EARLY EMBRYONIC LETHALITY IN MICE

Martha M. Larsen      W. M. Generoso

In the evaluation of dominant-lethal effects, the most important comparison made between experimental and control groups is based on the incidence of dead implantations, also called decidiuomata. However, from the controls of a series of dominant-lethal experiments conducted in our laboratory over many years, it became clear that while the normal incidence

of deciduomata in uteri of pregnant females does not seem to be affected by the strain of the male parent, it varies considerably with the strain of the female. For example, the normal incidence of deciduomata in pregnant 10- to 12-week-old T-stock females averages about 25%, but it is only about 5% in (SEC  $\times$  C57BL)F<sub>1</sub> or (C3H  $\times$  C57BL)F<sub>1</sub> females of the same age. It is not known whether the differences in early embryonic mortality are attributable to differences in uterine environment or to properties of the conceptus. The answer to this question has a bearing on the interpretation of dominant-lethal data, and specifically on whether strain differences in the normal incidence of dead implantations reflect differences in the spontaneous frequency of dominant-lethal mutations.

In order to analyze the factors influencing the incidence of spontaneous deciduomata we carried out an embryo transfer experiment and two short-term in vitro culture studies. The mice used were T-stock and (C3H  $\times$  C57BL)F<sub>1</sub> females and T-stock males.

The objective of the embryo-transfer study was to compare the incidence of deciduomata when normal-appearing 2- to 8-cell embryos are transferred from females of each of the two strains to females of either the same or the other strain. For the two strains of females there are four donor-recipient combinations. We found a higher incidence of deciduomata (by a factor of about 1.5 - 2) when the donor strain was the T-stock (regardless of the strain of recipient) than when the donor strain was (C3H  $\times$  C57BL)F<sub>1</sub>. However, the incidences of dead implantations in all cases were generally higher than expected from natural pregnancies, perhaps due to the stress caused by the transplantation procedure.

Experiments to compare development under culture conditions of embryos from T-stock and (C3H  $\times$  C57BL)F<sub>1</sub> females were performed, using embryos collected beginning either 24 or 48 hours after insemination of the mother. Two-cell embryos collected beginning 24 hours after insemination of the mother were incubated for 72 hours. Observations were made at 48 hours and at the end of the culture period. Observation at 48 hours already revealed a difference between the two stocks. Virtually all (97%) of the embryos from (C3H  $\times$  C57BL)F<sub>1</sub> mothers were at the 8-cell stage, compared to only 34% of the T-stock embryos. About 39% of the latter had remained at the two-cell stage, and the rest had progressed to the four-cell stage. By 72 hours of incubation, 84% of the embryos from (C3H  $\times$  C57BL)F<sub>1</sub> mothers had developed to the blastocyst stage, compared to only 9% of the T-stock embryos, again showing a marked difference between the two groups of embryos.

In the other in vitro experiment, early development was studied among embryos collected beginning 48 hours postinsemination and incubated in culture medium for 48 hours. Virtually all (95%) of the embryos from (C3H  $\times$  C57BL)F<sub>1</sub> females were at the 8-cell stage at the time of collection. By contrast, about one-third of the T-stock embryos were still at the 4-cell stage at this time. Similarly, after 24 hours of incubation relatively more embryos from (C3H  $\times$  C57BL)F<sub>1</sub> mothers had developed to the blastocyst stage. However, after 48 hours of incubation the difference

between the two stocks in the percentage of embryos that had developed to the blastocyst stage had become less pronounced.

The present in vivo and in vitro results both indicate that strain differences in the incidence of spontaneous decidiomata may be largely, if not wholly, accounted for by genetic differences between embryos themselves in their ability to survive the preimplantation and early implantation environment. Differences in the uterine environment appear to play a lesser role. A governing factor in early prenatal survival could be the inherent rate of development during the preimplantation period. If development is relatively slow, as it appears to be in T-stock embryos, the conceptus may be out of synchrony with the short period of uterine receptivity during which successful implantation can take place.

The differences observed between (C3H × C57BL)F<sub>1</sub> × T and T × T embryos in in vitro development from the 2-cell stage, and in their development in the fallopian tube, are much larger than the differences between stocks in the incidence of spontaneous decidiomata. Thus, if the rate of development during the preimplantation period is, indeed, a governing factor that determines differences in the incidence of spontaneous decidiomata, it follows that these differences are not attributable to newly occurring dominant-lethal mutations.

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#### ESTABLISHMENT OF AN EMBRYO-FREEZING PROGRAM FOR THE PRESERVATION OF GENETIC STOCKS

Liane B. Russeil                    Martha M. Larsen

During the past year, the embryo freezing laboratory has been fully operative, equipment and original technique problems having been solved during the previous year. However, we have only one person working less than full-time on the embryo-freezing project. A computer program for the pertinent data has recently been completed, and information has now been entered for 18 mutant stocks, comprising about 15,000 frozen 8-cell embryos. For each stock, freezing-success trials are performed in which a sample of frozen embryos is thawed, implanted into pseudopregnant host females, and raised to birth. A stock is not discarded unless the trial is successful.

When work with lethal genes was recently initiated, it became apparent that, in such stocks, progress in collecting the required number of embryos

for freezing will be slower, since the heterozygous lethal males often perform unsuccessful copulations, so that many mated females produce no embryos at all. In the case of autosomal lethals, or of sex-linked viabiles, we freeze at least 800 embryos per stock; in the case of sex-linked lethals, at least 1200; and in the case of autosomal viable genes, at least 400.

We have carried out two comparisons. (1) The success rate (% liveborns) obtained when thawed embryos are cultured to blastocysts which are then transferred to host females (a technique used at other laboratories) was compared with the success rate from direct transfer of thawed embryos. The latter was consistently larger, and direct transfer is now being routinely used. (2) The percentage of thawed and cultured embryos that form viable blastocysts (not transferred) was compared to the percentage of thawed and directly transferred embryos that survive to birth. It was hoped that, if a relatively constant ratio could be found for all stocks, the former procedure by itself could be used as a predictor of success. However, the ratios varied rather widely, and we have decided that frequency of liveborns after direct transfer must be used as our criterion of successful freezing.

## Toxicology Section

### SECTION OVERVIEW - H. R. WITSCHI

The main thrust of the work of the Toxicology Section is to understand mechanisms of disease caused by chemical and physical agents. Such a goal can essentially be approached in two ways: by studying the fate of and biological effects caused by a variety of well-selected chemicals or by trying to delineate and unravel disease processes. By and large, members of the Toxicology Section have chosen the second approach: to study the disease rather than individual compounds although chemicals are often used as probes.

Constraints in available resources, particularly personnel, made it necessary to limit our efforts. At present, we focus on two organ systems: the skin and the respiratory tract. The two organs play a most important role in defending the body against exogenous chemicals: skin and respiratory tract are the main barriers put up by the living body against most exogenous agents. Acute or chronic diseases of both skin and respiratory tract caused by exogenous agents play an important role in our present society.

Skin disease produced by chemicals is examined at several levels of biological organization. Painting skin of experimental animals with selected agents, mainly products associated with modern coal conversion technologies, informs us about the acute or chronic potential of the agent tested; the acute potential can be assessed by observing the test animal for either local effects such as skin irritation or skin sensitization, whereas the formation of benign or malignant skin tumors is indicative of the effects of chronic, low-level exposure. However, any such effects observed will eventually have to be understood in more mechanistic terms. An important element in our approach to understanding chemically-induced skin disease is experiments in which we explore how chemicals penetrate the intact skin, how they are transformed by the different cell layers of the skin and how they interact with critical targets within cells of the skin. These studies, carried out with techniques that make use of many advanced methods available in cell biology, biochemistry and morphology will provide a solid experimental basis for our understanding of chemically-induced skin lesions.

A similar approach is being pursued by several groups that study pathogenesis and mechanisms of acute and chronic lung disease. Again emphasis is placed on understanding a biological response to injury rather than merely studying compounds that are known or suspected to produce lung disease. An effort ongoing now for several years deals with induction and

progression of cancer in the tracheal epithelium. Methods have now been developed which allow us to combine the advantage of experiments with animals with advanced techniques of cell and tissue culture. This will allow us to probe deeper into mechanisms of respiratory carcinogenesis while, at the same time, offering a possible and scientifically sound alternative to cumbersome and expensive animal inhalation studies. In addition, progress has been made in studying mechanisms of other forms of chronic lung disease such as fibrosis and emphysema. A key element appears to be the cellular reactions in the lung and tracheobronchial tree to acute injury, as it begins to become evident that the nature of such acute responses may determine to no small extent the development of chronic lesions.

While most research groups within the Toxicology Section focus thus on skin or respiratory tract, one group concerns itself with fundamental aspects of the immune system and how they may be compromised by radiation. This work is necessary to better characterize the response of the many components of the immune system to acute injury. Eventually this will help to understand some of the lesions that may develop in either skin or lung if chemicals do not act directly on these tissues, but more likely produce damage by interfering with dermal or pulmonary elements of the immune system.

Finally it should be pointed out that the Toxicology Section is not a closed entity, but that many groups within the Section collaborate closely with other groups within the Biology Division and with other research divisions at ORNL. A formal toxicity testing program relies heavily on the help and expertise provided by members of the Analytical Chemistry Division for analysis of test agents, monitoring of aerosol generated in inhalation chambers and preparation of radiolabeled compounds, to be used in studies on metabolism and disposition of agents. An interdisciplinary group of workers from the Health and Safety Research Division, the Chemistry Division, the Comparative Genetics Section, the Molecular Genetics Section and the Toxicology Section share a common interest in the toxicity and mechanism of action of metals. Workers from the Cancer Section have been called upon to provide help in questions dealing with the metabolism and biotransformation of foreign compounds or to prepare monoclonal antibodies against proteins that might be released in certain forms of acute or chronic lung injury. Finally it should be mentioned that several projects, completed or still under way, are carried out in collaboration with workers from universities, national research institutes or private industry. While such projects may range from providing expertise not available to others to getting help not otherwise available to us, they nevertheless all have one common theme: to further advance our understanding of disease processes brought about by chemicals.

## SYSTEMIC TOXICOLOGY

|                           |                                  |
|---------------------------|----------------------------------|
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Work done by the Systemic Toxicology Group centered around three themes: (a) elucidation of mechanisms of acute and chronic lung injury, (b) a toxicology testing program in which acute and chronic effects of products originating from and associated with synfuel technologies were examined, and (c) completion of a 3-year effort concerning the validation of the mouse lung tumor assay as a potential short-term *in vivo* screening assay for suspected carcinogens. In addition to these efforts we initiated a series of new experiments on (d) the effects of the antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as modifiers of tumor development in mouse lungs.

(a) Work on potentiation of diffuse alveolar damage by oxygen continued. The focus shifted towards a detailed analysis of the accompanying changes in pulmonary cell proliferation. Mice were treated with a single intraperitoneal injection of BHT in corn oil or corn oil alone and immediately exposed to 70% oxygen or air for 6 days. The animals were injected with (<sup>3</sup>H) thymidine 90 minutes before being killed or implanted with thymidine-filled osmotic minipumps. Autoradiography on lung tissue was done 2 to 14 days after injection of BHT and the tissues were analyzed for cell proliferation. We found that exposure to 70% oxygen immediately after the administration of BHT initially delayed epithelial cell proliferation in lung. Once the animals were removed from oxygen, however, there occurred a compensatory burst of cell division. The proliferating cell population after removing animals from oxygen was primarily interstitial and not epithelial. This explains the ensuing massive fibrotic changes in the lungs of animals so treated (9).

A detailed knowledge of cell kinetics following acute lung injury is thus essential for predicting how lung damage progresses and eventually becomes modified by other agents. This led to a new series of experiments in which we further analyzed the sequence of cell proliferation in lungs damaged by agents other than BHT. As observed following injection of BHT or intravenous injection of oleic acid, we found that with inhaled CdCl<sub>2</sub> there is an initial burst of epithelial cell proliferation which can be partially blocked by exposure to 80% oxygen. Not unexpectedly, fibrosis develops. Epithelial cell proliferation is much less pronounced following inhalation of 3-methylfuran than after BHT or CdCl<sub>2</sub>; the most active cell population is in the small airways. A different pattern of cell proliferation was observed following administration of the antineoplastic agent cyclophosphamide. In contrast to what had been seen with all other agents, this drug virtually blocked division of pulmonary epithelial cells, and virtually all dividing cells in lung for up to 2 weeks following

100 mg/kg of cyclophosphamide were interstitial cells and capillary endothelial cells. Work along these lines continues and we hope to establish a solid base of information about the different patterns of response to pulmonary injury (3,6,8).

An effort was made to correlate acute lung injury with some measures of pulmonary function. Agents used were BHT, oleic acid, 3-methylfuran or cyclophosphamide. Lung damage was assessed in unanesthetized mice by measuring changes in respiratory rate with a total body plethysmograph. It was found that respiratory rate measured immediately prior to killing animals correlated well with the degree of pulmonary fibrosis defined as total amount of collagen per lung. However, prior to this timepoint, i.e., when fibrosis was developing, there was a peak and a trough in respiratory rate response that could not be directly correlated with the development of fibrosis. In an effort to determine the influence of pulmonary edema and lung cell proliferation on respiratory rate, oleic acid was used. Oleic acid produces lung injury followed by a high level of cellular proliferation with only minimal development of fibrosis. Good correlations between respiratory rate and either pulmonary edema or pulmonary cell proliferation were found 3 days after oleic injection. Liver and kidney toxicants and starvation produced either no change or a depression in respiratory rate, making an increase in respiratory rate a rather specific and sensitive index of acute pulmonary injury (2).

Experimental work on the acute and chronic toxicity of 3-methylfuran (3-MF) was completed. In rat, hamster and mouse, 3-MF produced extensive necrosis of bronchiolar epithelium, followed by extensive cell proliferation. Cells in the alveolar zone appeared to be much less damaged than were cells in the small airways. Many changes were transitory, however, and following a recovery period an essentially normal lung structure was established. One exception appeared to be the epithelium lining the nasal turbinates of rats. Here a single exposure to methylfuran produces extensive necrosis of the epithelial lining, breaks in the basal membrane and eventually development of fibrous tissue coating islands of cartilage which completely filled parts of the lateral nasal turbinates (7,8,10).

Analysis of one life-time study in which animals were exposed to a toxic dose of 3-MF once a week for ten consecutive weeks and then kept for 2 years provided no evidence for a carcinogenic action of 3-MF in mice (1).

(b) Toxicity Testing Program. An integral part of the Biology Division's mission is to provide a data base that will allow us to evaluate the potential health hazards of substances generated by energy technologies. Basic to this evaluation is the determination of acute toxic effects of substances in laboratory animals. For these determinations we routinely use five types of tests to estimate the toxicities of synthetic fuels and of substances related to their production: acute oral and intraperitoneal toxicity in mice ( $LD_{50}$ ); acute dermal toxicity in rats; skin and eye irritation in rabbits; and delayed-type allergic hypersensitivity in guinea pigs. The procedures used for these tests are essentially those employed

in many other toxicology facilities and in general conform to methods set forth in the OECD Guidelines for Testing Chemicals (Paris, 1981).

The substances submitted to us for toxicity testing were obtained from a coal liquefaction facility in which two kinds of coal feed stock were used to generate two groups of analogous samples. Each group consisted of two fuel oils and three samples collected at different in-line process ports. Oral and intraperitoneal LD<sub>50</sub> determinations of these substances showed that the fuel oils were moderately toxic while the three port samples were slightly toxic or nontoxic. After dermal application, none of the substances produced skin lesions or systemic effects. One of the fuel oils resulted in moderate-to-severe eye irritation that was reversible. However, none of the other substances produced more than a mild transient skin or eye irritation and none elicited delayed-type hypersensitivity.

When the current results with products from two types of coal were compared with those previously obtained from a third kind of coal (ORNL-5685), it appeared that with a minor exception acute toxicity of derived analogs is not related to the source of coal used as a feed stock.

The LD<sub>50</sub> was determined for two coal liquid blends that had been hydrotreated. The data suggest that reduction in nitrogen content in the liquids may relate directly to oral toxicity.

Seventeen substances associated with the synthetic fossil fuel program are currently under chronic test to determine their tumorigenic potential in mice. The procedures used are essentially those developed in the Biology Division over the last several years. It is anticipated that the results will provide data on tumor incidence and latency, substance potency relative to benzo(a)pyrene, and systemic effects as revealed by pathologic evaluation.

(c) Mouse Lung Tumor Assay. Assessment of the carcinogenicity of a substance is often made on the basis of data from prolonged or lifetime exposure studies, usually with rodents. Because of the time involved, these studies are expensive, and therefore, efforts have been made to devise other appropriate whole-animal assays that would be less costly and provide answers in a shorter period of time. Analysis of the data base strongly suggested that the mouse lung tumor assay developed by Shimkin and co-workers might serve as a reliable routine short-term assay for carcinogens. Therefore, under a contract with NCI-NTP, the Toxicology Group conducted a series of experiments (1980-1982) designed to validate the mouse lung tumor assay in terms of its use as a routine procedure to identify carcinogens. Validation of the assay was carried out with 30 coded compounds which had been tested previously in a standard 2-year NCI-type carcinogenesis bioassay. Two other groups of substances were also tested - five complex mixtures from modern synfuel processes and eight nitrated toluenes.

For the assay, male strain A/Jax mice 7 to 9 weeks old were injected intraperitoneally with each substance 3 times a week for 8 consecutive

weeks. After the last injection, the animals were left undisturbed for 4 months and then were killed. The carcinogenicity of each substance was evaluated from the number of mice per group with lung tumors (tumor incidence) and from the number of visible tumors on the lung surface (tumor multiplicity). Incidence and multiplicity for each treatment group were compared with appropriate vehicle control groups, and statistical significance at the 5% level was determined by the use of Chi-square for tumor incidence and Student's t-test for tumor multiplicity. A detailed report of the project has been published (11).

Among the 30 coded compounds there was one known human carcinogen and 17 for which there was very strong evidence for carcinogenicity in two animal species, strong evidence in one species and sufficient evidence in a second, or strong evidence in one species. Of these 18 compounds that we tested in the lung tumor assay, only 5 could be classed as carcinogens. If we add to this observation the fact that two potent animal carcinogens, benzo(a)pyrene and 4-nitroquinoline-1-oxide, were negative in our hands, it can be concluded that the mouse lung tumor assay resulted in a very high percentage of false negatives.

Some of the most definitive data were obtained with the complex mixtures from synfuel processes. Shale oil and two of its derivatives and two substances from an experimental coal gasifier were unequivocally positive in the lung tumor assay. None of the eight nitrated toluenes tested were positive.

In view of the results with the 30 coded compounds, we conclude that the lung tumor assay is not sufficiently reliable to be used as a routine test for carcinogenicity.

(d) A series of experiments was completed in which a systematic dose-effect and time-effect analysis was done on the enhancement of lung tumors by dietary BHT. The lowest concentration of BHT fed for 8 weeks and capable of promoting lung tumors was found to be 0.1%, and the shortest time of 0.75% BHT in the diet required to have such an effect was 2 weeks. The cell kinetics accompanying lung tumor development were examined with autoradiography. Urethan by itself is able to produce inhibition of cell proliferation in the first 2 weeks followed later by somewhat enhanced mitotic activity. BHT in the diet produces a substantial burst of cell proliferation within the first 2 to 4 weeks; later the animals become resistant to this effect of BHT.

While the relationship between tumor promotion and cell proliferation continues to be explored, some potentially important new observations on the modulation of murine lung tumors by dietary antioxidants were made. BHT fed before a carcinogenic dose of urethan has no effect and fed after urethan will enhance lung tumor development. Prefeeding of BHA, on the other hand, diminishes the response to urethan, whereas postfeeding has no effect. However, BHA given after benzo(a)pyrene diminishes the number of tumors which develop, whereas BHA under the same conditions substantially

increases the number of tumors found. Additional studies have shown that BHA has no promoting activity.

In conclusion, the common goal of the systemic toxicology group is to study in depth the reaction of one organ, the lung, to various forms of acute and chronic insults and how the biological response of the respiratory apparatus to injury can be modified by extraneous agents (12, 13, 15, 16).

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#### INDUCTION AND PROGRESSION OF NEOPLASIA IN TRACHEAL EPITHELIUM

A. C. Marchok                    D. H. Martin  
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The respiratory tract of man is continually accosted by hazardous agents in the environment that are potentially carcinogenic, cocarcinogenic or which may promote the development of lung cancer. Many of these hazardous agents are introduced into the environment as particulates, gases, aerosols, etc. during the refining and utilization of most energy sources. There is a great need to develop mammalian model systems that will test for these potentially hazardous agents under experimental conditions that are well controlled in terms of target site exposed, as well as dose and duration of exposure to the agent. It is also important to define and use endpoints that are relevant to the human situation. Therefore, in this program *in vivo*, *in vivo-in vitro* and *in vitro* models have been developed and are utilized to define and quantify cellular and biochemical changes that identify particular stages in the evolution of neoplasia in the epithelium of the rat trachea, a tissue which is similar to the human bronchus.

In Vivo Exposure to the Test Agent. Several years ago, the rat tracheal implant model was developed in this laboratory to study the induction and pathogenesis of lung cancer. In this model a well-defined target site is exposed to test agents released from cylindrical pellets molded to fit the tracheal lumen. This model has the disadvantages that a pellet can be introduced into a tracheal implant only a limited number of times, and release of the agent from the pellet is always initially abrupt and, most likely, highly toxic. To circumvent these problems, an open-ended, flowthrough tracheal implant (FTTI) was developed that allows unlimited numbers of exposures to 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. During the past year studies of the effects of the well known tumor promoter of skin carcinogenesis, tetradecanoyl-phorbol-acetate (TPA) have been initiated using the FTI. This open-ended tracheal model permits us to use the same experimental regimen commonly done with skin. We have carried out acute dose response studies with TPA. TPA given at 2, 6.7 or 20  $\mu$ g twice a week stimulates increasing levels of basal cell hyperplasia as evidenced by [<sup>3</sup>H]-thymidine incorporation and a marked inflammatory response in the tracheas. A long-term study has been started in which tracheal implants, initiated with dimethylbenz[a]anthracene (DMBA), are being exposed 2x/week to 6.7  $\mu$ g TPA. The progression of neoplasia is being followed by periodic sampling and pathologic diagnosis of the cells exfoliating into the tracheal lumen. Pilot studies are also being carried out in collaboration with Dr. Gary Braslawsky to determine whether alterations in the exfoliated cells can also be determined by flow cytometric analysis of different parameters including DNA content. The promotion of other markers of neoplasia, as described in the next section, is also being followed. The early toxic effects of formaldehyde (HCHO), an ubiquitous environmental pollutant, have also been studied with this model. Exposures for 1 hr twice a week to HCHO gas mixed with humidified air at doses of 5, 8 or 12 ppm HCHO induce basal cell hyperplasia and keratinizing squamous metaplasia within a few weeks. Experiments are being initiated to study the long-term effects of HCHO given alone and in various combinations with benzo[a]pyrene (B[a]P), a known carcinogen in the environment, to establish whether HCHO is a carcinogen, cocarcinogen or promoter of cancer in the respiratory epithelium. The development of the FTI model opens up the possibility of testing a wide range of hazardous agents for their toxic and carcinogenic effects on respiratory epithelium in a quantitative fashion not possible before.

In Vivo-In Vitro Model. In vivo models are essentially limited to morphological endpoints. To overcome this limitation a combined in vivo-in vitro model of carcinogenesis was developed which makes it possible to study a gamut of cellular and biochemical changes in cell populations derived from the tracheal tissue at different times after carcinogen exposure in vivo. The approach is to expose tracheal implants to known doses of carcinogen for pre-selected periods of time. The tracheas are then cut into explants and placed in organ culture for 24 hr. A cytopathological diagnosis is then made on the cells that exfoliated into the medium, and the explants are placed on the bottom of tissue culture dishes to initiate outgrowth of epithelial cells and establish primary cultures. Carcinogen-altered cells are selected out by placing the 14-day primary cultures in a medium deprived of pyruvate, a component we found to be necessary for the long-term growth of normal tracheal epithelial cells but not altered cells. Further alterations in the selected cell populations are identified by testing for ability of the cells to survive subculture, acquisition of anchorage-independent growth in agarose and tumorigenicity when inoculated back into a suitable host. We have found dose-dependent effects after exposure to dimethylbenz[a]anthracene (DMBA) in terms of the number of explants that yield carcinogen-altered cell populations and decrease in time to anchorage-independent growth as well as to tumorigenicity. Since lesions in the explants are initially identified from the cytopathology of

exfoliated cells, cellular and biochemical properties of cell populations derived from specific lesions can be studied and correlated directly with conventional morphological markers of the progression of neoplasia. We have 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.

Experiments are in progress to study the effects of TPA on the frequency of induction of preneoplastic cell populations and the progression of neoplasia in these cell populations following initiation with DMBA. Similar studies are planned for FTI exposed to B[a]P and HCHO. Quantifying the number of preneoplastic cell populations following exposure of tracheal implants to a test agent(s) appears to be a highly sensitive and relatively rapid method for determining the carcinogenic potency of the agent in respiratory epithelium. This assay should help to bridge the gap between long-term whole animal studies and short-term mammalian cell culture and mutagenesis assays in risk assessment.

We have found that the ability of cells to survive the 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, and have determined the following: (1) Pyruvate does not act on normal cells by directly stimulating cell proliferation. When pyruvate is removed from the medium, the normal cells continue to incorporate [<sup>3</sup>H]-thymidine into DNA at the same rate. However, cells increasingly terminally differentiate toward keratinization and are lost. (2) Normal tracheal cells take up at least 5 times more [<sup>14</sup>C]pyruvate than carcinogen-altered cells in most of the cell fractions. (3) There are no differences in the utilization of [<sup>14</sup>C]glucose or [<sup>14</sup>C]glutamine between the two cell populations. (4) Carcinogen-altered cells utilize more [<sup>14</sup>C]-alanine than normal cells. These findings point out the marked changes in metabolism that occur early in carcinogen-altered cells. Experiments will be continued to further elucidate these metabolic differences and how they relate to the changes in growth control in these cells. (5) Increase in uptake of [<sup>14</sup>C]-2-deoxyglucose is not an early marker of neoplasia, nor is it a definitive marker of tumorigenicity in tracheal epithelial cells as claimed for some cell types. [<sup>14</sup>C]-2-deoxyglucose uptake is actually lower in preneoplastic cells as compared to normal cells. This uptake remains low in some tumor cells, but markedly increases in others. The latter are from rapidly growing, undifferentiated carcinomas. (6) A good in vitro marker for tumorigenicity is the ability of the tracheal cells to form multinuclei in vitro in the presence of cytochalasin B.

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#### INHALATION TOXICOLOGY

Simon Lock                    Fred J. Stenglein, Jr.

The inhalation toxicology group has been active in two main areas of research: (a) exposure of rats to diesel fuel aerosol, and (b) exposure of mice to ethylene oxide.

(a) The work with the diesel fuel aerosol was part of an ongoing program supported by the Smokes and Obscurants program within the Health Effects Research Division of the United States Army Medical Bioengineering Research and Development Laboratory. Briefly diesel fuel aerosol is used as a smoke screen by the U.S. Army. In the laboratory we have produced a very similar aerosol to that used by the military by flash vaporization of diesel fuel in a 100% nitrogen atmosphere and subsequent recondensation in the chamber air stream. The first two phases of this work were undertaken to look at the acute effects following single exposure and to study the relative importance of concentration, duration of exposure and frequency of exposure when animals were exposed a total of nine times. The matrix design used included a range of concentrations from 0 to 6 mg/L, exposure durations of 2 or 6 hours and frequencies of once/week for 9 weeks or three times/week for 3 weeks. Both the single and repeat exposure studies were

used to define what parameters to measure and what exposure concentrations would be suitable for the final phase of this work: a subchronic study.

In the sub-chronic study male and female rats were exposed twice/week for 4 hours/day to concentrations ranging from 0 to 1.5 mg/L. Body weight was followed on a weekly basis over the 13-week exposure period and in those animals not sacrificed at the end of exposure body weight was followed for a further 8-week recovery period. Food consumption was also measured on a weekly basis and followed throughout the exposure and recovery periods in a sub-group of animals. Animals that were not scheduled for sacrifice until after the two month recovery period were also used to test for changes in startle reflex as a result of multiple exposure. Each animal used for this assay was tested for its reaction to an auditory stimulus (110dB, 13KHz for 10msec.) prior to the start of the exposures in order to obtain a baseline response for that animal. One day before the 14th and 26th exposures and at 1 and 2 months after treatment had ceased both male and female animals were tested to determine whether there was a performance decrement as a result of frequent exposure and if so whether there was evidence of recovery. Male animals were also tested immediately after the first, fourteenth and 26th exposures to see whether the low diesel fuel aerosol concentrations had acute effects similar to those observed previously at higher aerosol concentrations (i.e., greater number of non-responses, longer reaction time and reduced force exerted on the strain gauge). At the end of the exposures or after two months recovery animals were examined in a battery of pulmonary function tests, or alveolar free cell populations were studied. Blood samples were sent to the Medical Department for serum chemistry analyses and those animals used in the pulmonary function tests were subsequently necropsied.

The overall conclusion derived from these experiments was that low concentrations of diesel fuel aerosol had minimal effects on any of the parameters measured. However, there were some concentration related trends including slower growth with increasing aerosol concentration and changes in certain lung volumes immediately postexposure. The effects on the different lung volumes included a slight depression in total lung capacity as concentration increased and similar changes in vital capacity and inspiratory capacity. The trends were more evident in females than in males and immediately after exposure rather than after 2 months recovery.

(b) One branch of EPA has been mandated to study the mutagenic potential of industrial chemicals. A compound of particular interest to them because of its extremely extensive usage as an intermediate in a diversity of manufacturing processes is ethylene oxide (EtO). The current threshold limit value (TLV) for ethylene oxide has been set by OSHA at 50 ppm, but because of health problems associated with exposure to EtO, there are movements to lower the TLV to 1 ppm with a 5 ppm ceiling. One interest of the EPA is whether EtO can cause mutations or other toxic effects on the hematopoietic system.

A collaborative investigation of this matter was carried out in the Biology Division by the inhalation toxicology group, Dr. Raymond Popp's

group who studied the hematopoietic toxicology, Dr. Gene Perkin's group who studied immunocompetence following exposure and Dr. Julian Preston's group who looked at aberrations in sister chromatid exchange. Following modifications to an exposure chamber and the design of a continuous remote monitoring system based on a Miran 1A (infrared spectrometer), a sub-chronic study was carried out. Male C57BL/6 mice were exposed to 255 ppm ( $\pm$  10 ppm), 6 hours/day, 5 days/week for up to 10 weeks. The exposure concentration of 255 ppm was selected by the EPA based on the assumption that if all the inhaled ethylene oxide was absorbed by the animal then 255 ppm over 10 weeks would be approximately equivalent to exposing a man for one year to the current TLV (58 ppm). While the concentration-time (Ct) product does not necessarily hold true it is a good starting point for this type of experimental protocol. A parallel study was also carried out in which animals were exposed 6 hours/day for 1 to 14 days straight.

There were a few (4 animals) early mortalities in exposure groups. However, since they all occurred in one cage it is believed that infection might have been the primary cause of death, exacerbated by the stress of exposure. While no significant mutagenic effects were observed there were some important changes in various cell populations. These changes included loss of granulocytic elements in bone marrow at early time points followed by replacement and hyperproliferation, and a significant depression in lymphocytes in the peripheral blood.

It would appear that the immunocompetence of the mice is severely depressed during the first few days of exposure; however, there does seem to be a correction in this depression as the animals receive more exposures. Further studies are planned to look at the alteration in the immunological function as a result of exposure to EtO. Research will be directed toward the early time points and the effect of concentration on immunocompetence.

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### IN VITRO STUDIES OF CUTANEOUS TOXICITY AND CHEMICAL-SKIN INTERACTIONS

J. Y. Kao                    M. J. Whitaker  
 J. W. Hall

The aims and objectives of this research program are (a) to develop mechanistic and functional approaches for use in studying problems in dermatotoxicology, (b) to develop a better understanding of the factors involved in the interaction of chemicals with the skin and subsequent toxic responses, e.g., toxicokinetics of percutaneous absorption, cutaneous metabolism and disposition of topically applied chemicals, and biochemical and histological changes in the skin resulting from interaction with xenobiotics, (c) to identify possible biochemical and functional parameters for toxicity quantitation, and (d) to develop a quantitative basis for extrapolation from animal to man. Our experimental approach is to make use of in vitro methods utilizing mammalian skin in organ culture.

In order to better establish a quantitative basis for the extrapolation of cutaneous toxicity observations from animals to man, we have developed in vitro systems to study the toxicity, translocation and coupled biotransformation of topically applied materials in mammalian skin.

Two systems for maintaining metabolically viable and structurally intact mouse skin as short term organ cultures (24-48 hrs) are being used. An important property of our culture systems is that chemicals of interest can be applied to the skin surface in a manner similar to exposure in vivo and the material of interest reaches the epidermal cells by diffusion through the various strata of the skin.

In the "static" system disks of freshly excised skin, 1" in diameter, are maintained in a controlled optimal environment, supported on filter paper in individual organ culture dishes containing suitable culture medium (2). In the "dynamic" system, on the other hand, the skin disks form the upper seal of 1 ml wells of a compact, water jacketed multisample skin penetration chamber (1). Fresh, oxygenated culture medium is continuously passed through the wells and the well effluents are collected via a fraction collector. Metabolic viability and structural integrity of the cultured skin is evaluated based on histological examination, in vitro incorporation of radiolabeled precursors into cellular macromolecules (2) and the ability of the culture tissue to metabolize <sup>14</sup>C-glucose (3).

Studies using the "dynamic" system with <sup>3</sup>H-benzo(a)pyrene as a model aromatic hydrocarbon have shown that, at the low doses investigated, the

rate of translocation of benzo(a)pyrene across the intact, full thickness mouse skin was related to its metabolic viability; and translocation of benzo(a)pyrene in mouse skin was dose dependent and linear up to 5  $\mu$ g per skin disk (200  $\text{mm}^2$ ). Following in vivo topical or systemic induction with TCDD, a two-threefold increase in the overall penetration of benzo(a)pyrene was observed (1). Sex and strain differences were also apparent.

Preliminary analysis demonstrated the presence of essentially only polar metabolites of benzo(a)pyrene in the effluent medium from the penetration chamber. Subsequent studies with  $^{14}\text{C}$ -benzo(a)pyrene using the "static" system demonstrated similar overall penetration of in vitro topically applied benzo(a)pyrene. The majority of the radioactivity in the culture media was not extractable into ethylacetate. Chromatography of the organic fractions showed that all classes of benzo(a)pyrene metabolites were present. Dihydrodiols and "polar metabolites" predominated and negligible amounts of unmetabolized benzo(a)pyrene were found. Enzymic hydrolysis of the media showed that TCDD induction shifts the metabolism towards more water soluble conjugates. Differences in the degree of in vitro covalent binding of benzo(a)pyrene, via the diol epoxide intermediates, to epidermal DNA from control and induced tissues were observed. These differences may also reflect the change in the pathways of metabolism of benzo(a)pyrene as a consequence of TCDD induction (3).

It is important to note that these in vitro toxicokinetic studies can be performed under conditions where the influence of local cutaneous toxicity of the topically applied chemical can be monitored. It has been demonstrated that mouse skin maintained in our culture system responds to toxic insults with biochemical changes. These changes, which included leakage of intracellular enzymes out of damaged cells and reduced incorporation of radiolabeled precursor into tissue macromolecules, are sensitive indicators of cellular injuries; as such they may offer a simple measure for assessing cutaneous toxicity of chemicals in vitro (2). Since local cutaneous toxicity may have profound effects on the functional capabilities of the skin, an assessment of toxicity may be an important factor in skin penetration studies. Recent studies in our laboratory have demonstrated that following in vivo exposure to topical tributyltin chloride, a known skin irritant (Barnes, J. N., Brit. J. Industr. Med. 15: 15-22, 1958; Lyle, W. H., Brit. J. Industr. Med. 15: 193-196, 1958), the in vitro penetration of benzo(a)pyrene in mouse skin was significantly impaired (Kao, unpublished observations).

These studies with mouse skin demonstrated that percutaneous translocation of surface applied benzo(a)pyrene is coupled to cutaneous metabolism. In addition, it is dependent upon the metabolic status and biochemical viability of the skin. However, current studies with rabbit and guinea pig skin have indicated that not only is the overall skin penetration of benzo(a)pyrene in these species much lower than in the mouse, but also there are no differences in the translocation of benzo(a)pyrene between viable and nonviable tissues. In contrast, studies with testosterone have shown that penetration of this steroid through metabolically viable skin from all three species was significantly lower than

through the corresponding nonviable tissue (Kao, unpublished observations). Skin permeability is a complex phenomenon influenced by a variety of biological and physiochemical factors. For a great many compounds the stratum corneum appears to be the barrier of primary importance. For lipophilic chemicals, such as aromatic hydrocarbons and steroids, it is less clear that the stratum corneum is a barrier at all. The lipid rich stratum corneum and skin appendages such as the pilosebaceous apparatus may act as a "sink" for topically applied lipotropic material (Holland, J. M., *et al.*, Am. Ind. Hyg. Assoc. J. 40: 496-503, 1979). Rather than a barrier, the stratum corneum may function more as a sponge, capable of absorbing a quantity of lipophilic material limited only by the solubility of the substances in sebaceous and intrinsic lipids.

Much of the evaluation of *in vitro* penetration has been conducted using frozen or nonviable skin that is incapable of respiration. However, the recovery of material diffusing through dead skin may not be a useful measure of percutaneous penetration. Our studies suggest that percutaneous absorption may involve both diffusional and metabolic processes. The relative importance of these processes in skin penetration of chemicals will depend on the physiochemical properties of the compound and the metabolic capabilities of the epidermal cells toward the compound in question. The use of nonviable skin may provide information on the diffusional aspect of penetration, while the net results of the diffusional and metabolic components may be obtained by the use of metabolically viable and structurally intact skin. It should be noted, however, that a special problem associated with *in vitro* skin penetration studies of water insoluble compounds involves the inability of the chemical to partition into the aqueous receptor fluid. The role of a metabolically active viable epidermis is to convert a water insoluble compound to a water soluble metabolite capable of partitioning into the receptor fluid. This problem is currently being addressed in our laboratory.

Taken together, our studies strongly implicated cutaneous metabolism to be an important determinant in the percutaneous absorption of certain lipophilic chemicals. They also indicated that meaningful *in vitro* measures of skin penetration should take into consideration both the viability and metabolic status of the skin penetration.

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## DOSIMETRY OF PAH IN SKIN CARCINOGENESIS

L. R. Shugart  
J. M. HollandB. H. Chastain  
J. Y. Kao

Development of a synthetic petroleum industry raises concerns about potential exposures of workers and the general population to complex organic mixtures. Because one of the most probable routes of exposure is through the skin, we are interested in devising methods of cutaneous exposure to these materials that can be used in quantifying the overall extent of protocarcinogenic tissue insult. From such information we expect to obtain estimates of human cutaneous exposure risk.

We have demonstrated that mouse skin in organ culture is an ideal bioassay system because it possesses the pharmacokinetic and pharmacodynamic properties that are present in the intact whole animal (1). Currently we are evaluating this technique for screening potential chemical carcinogens. In particular we are focusing on the classical PAH compound benzo[a]pyrene (BaP) (4).

Since the metabolism of any carcinogen by mouse skin can be influenced by the interferences and/or interactions of all compounds present, our approach has been to attempt to measure the covalent interaction of the chemical in question with cellular DNA. The rationale for this approach is based on the hypothesis that this represents the critical step in chemical carcinogenesis (Harvey, R., Am. Sci. 70: 386-393, 1982). For example, subsequent to topical application of BaP to mouse skin, metabolic activation occurs stereospecifically to the chemically reactive diol epoxides (DE), which can then bind covalently to cellular macromolecules. The primary adduct formed via BaPDE with DNA is anti-BaPDE/deoxyriboguanosine, and there is good correlation between the amount of BaPDE bound to DNA of mouse skin and the carcinogenic activity of BaP. We have demonstrated that tetrols liberated from BaPDE/DNA adducts by acid hydrolysis are easily quantified by HPLC and fluorescence detection (2,3), which allows femtomole amounts of BaPDE associated with the DNA isolated from a single mouse to be detected using conventional instrumentation (4).

Recently we were able to use the mouse skin in organ culture system to demonstrate its suitability for testing the effect of ellagic acid on adduct formation (5). Recent studies had shown that ellagic acid, a product of the degradation of certain plant tannins, inhibited the mutagenicity of anti-BaPDE in Salmonella typhimurium as well as the mutagenic and cytotoxic effects of the compound in Chinese hamster 79 cells, without any detectable harmful activity by itself (Wood, A. W., et al., Proc. Natl. Acad. Sci. USA, 79: 5513-5517, 1982). Since the inhibitory effect of ellagic acid had been postulated to be due to its direct and facile interaction with anti-BaPDE to form an inactivated adduct we sought to determine whether or not in our system subsequent covalent binding of anti-BaPDE to DNA also occurred. We found that ellagic acid did not interfere with the metabolism of BaP but did affect the binding of anti-BaPDE to cellular DNA (5).

Other factors must be considered in attempting to relate the amount of adducts formed with BaPDE carcinogenicity, and we are proceeding to answer the question of the significance of the persistence of adducts once formed, and the importance of functional toxicity resulting from the carcinogen, the vehicle or the metabolic products accumulating in the target cells.

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#### RADIATION IMMUNOLOGY

E. H. Perkins                    W. C. Klima

The purpose of this research is to define the role of the immune system and immunologic dysfunction in radiation carcinogenesis, i.e., (1) the role of acute immunodepression in the induction of leukemia and late arising tumors, (2) the significance of autogenous immunity, (3) the possible restriction of tumor induction by immunoregulation, (4) the enhancement of the immunogenicity of leukemic cells and solid tumors by in vitro cultivation and selective cloning, and (5) correlation of immunocompetence and target cell susceptibility. Current studies center on two models of leukemogenesis to evaluate both immunologic and nonimmunologic mechanisms in the shifts of leukemia incidence. In nonirradiated RFM mice four primary types of leukemia occur (reticulum cell sarcoma, myeloid leukemia, thymic lymphoma and nonthymic lymphoma) almost exclusively in the last third of life, reticulum cell sarcoma accounting for 90% of the total. After irradiation thymic lymphoma reaches >60% with an induction period of 5-6 months. Myeloid leukemia also increases but the time of occurrence is only slightly advanced. Two studies to shift and increase the incidence of myeloid leukemia are in progress. Both attempt to reduce early thymic lymphoma so more animals are at risk for the induction of myeloid leukemia. Young-adult animals were thymectomized and then irradiated, or mice were irradiated at six months of age (at an age when the induction of thymic

lymphoma is sharply decreased). Replicate groups of both male and female mice have been included because radiation-induced thymic lymphomas are significantly higher in RFM females than in males, whereas myeloid leukemia is more common in males. Immune parameters are monitored because immune competence is significantly reduced in thymectomized mice and also declines with age. An incidence (>50%) of myeloid leukemia is desirable for conducting immunological reconstitutive and immunoregulatory studies.

Concurrently we are investigating the effects of spleen and bone marrow reconstitution on myeloid leukemia in RFM mice. It is important to determine if immunohematopoietic reconstitution after irradiation will significantly alter the incidence of radiation induced myeloid leukemia. If immediate immunological restoration is of significance in interrupting the inductive process, spleen cells should prove more effective than bone marrow cells. With thymic lymphomas restoration of immunocompetence was more rapid among spleen-cell reconstituted animals, but spleen cells failed to protect against the irradiation-induced thymic lymphoma. In contrast, immunologic recovery of bone marrow-reconstituted animals was not enhanced but mortality from thymic lymphomas was both delayed and reduced as a result of marrow injection. The present investigation permits us to compare the effects of immunologic and immunologic and cellular restoration on radiation disruption of cellular homeostasis in myeloid leukemia with the cellular perturbation observed in thymic lymphomas and to evaluate the significance of immunological dysfunction in the induction of two leukemias where different susceptible target cells are implicated.

Cell transfer experiments demonstrate a high incidence of potential lymphoma (preleukemic) cells in the bone marrow of irradiated older (8 months) mice; however, these mice never develop overt leukemia. These observations have allowed us to further investigate the significance of the immune system in the age-related resistance to thymic lymphoma. Old mice were grafted with a neonatal thymus under the kidney capsule. Thirty days later, when the thymus graft had grown and differentiated into a fully functional organ, mice were exposed to irradiation and observed for the development of both thymic lymphoma and myeloid leukemia. Some animals die of massive lymphomas, as early as 90 days after irradiation. Hence, when a target organ with a suitable microenvironment is restored, susceptibility to thymic lymphomas occurs. Measured immune competence is significantly less in these older animals but to relate the apparent increased susceptibility to decreased immune competence is premature. In another experiment assessing the effects of humoral factors in the induction of thymic lymphoma, injection of serum obtained from old RFM mice (which are refractory to induction of thymic lymphoma) reduced the incidence and delayed the onset of thymic lymphoma among these normally highly susceptible young adult animals. This finding implicates the humoral immune system and autogenous immunity perhaps to the murine leukemia virus. The experiment has not progressed sufficiently to assess the effect on myeloid leukemia. In this context, considering myeloid leukemia, loss of a susceptible target population is probably not a causal factor, and autogenous immunity may not have a dramatic effect if age-dependent immune competence is of importance.

It has commonly been accepted that malignant cells may in some situations be killed by the body's defense systems. Specific immunization generally augments these defense mechanisms, provided sufficient immunogenicity is present in the tumor cell inoculum. Immunization was attempted by intravenous injection of  $2 \times 10^7$  X-irradiated spleen cells of an in vivo passaged myeloid leukemia line (harvested when the spleen was twice its normal size). Mice were injected thrice at 3-week intervals and challenged 4-6 weeks later. Mortality for the "immunized" and control mice did not differ. In other studies using a diethylnitrosamine-induced squamous cell forestomach carcinoma, we observed that a tumor line is heterogeneous in cellular makeup and consists of cells with different immunogenic properties. Furthermore, studies using different in vitro propagated clones isolated from the same tumor cell line showed that some highly immunogenic cells are masked or are too infrequent to be demonstrable in the primary tumor cell population but can be selectively cloned out. Cloning experiments will be carried out to provide more conclusive evidence for the presence or absence of demonstrable tumor immunogenicity for both myeloid leukemia and thymic lymphoma.

We have also examined what effect an endogenous retrovirus (RFM V-1) induced and isolated in vitro from embryonic RFM cells might have on the induction of myeloid leukemia. In non-irradiated RFM mice, the virus does not produce leukemia. Mice received 300R X-irradiation and half the animals were injected intraperitoneally with  $4.7 \times 10^5$  plaque forming units of the virus. Mortality curves to date from the virus infected and noninfected animals appear to be quite comparable. While most animals have died of thymic lymphoma, the relative incidence of thymic and myeloid leukemia (as judged from gross pathology) between the two groups may be different. Conclusions must be deferred until all animals are dead (the remaining 30% are expected to die primarily from myeloid leukemia) and until microscopic examination can confirm types and incidences of leukemia.

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## Cancer Section

### SECTION OVERVIEW - R. J. M. FRY

The genetic nature of cancer has long been known and the molecular intricacies of this association are becoming clearer from the results of the extensive and exciting work on oncogenes and the control of their transcription. Molecular changes do not alone make a cancer because cellular, tissue and systemic factors play a major role in whether or not and when an overt cancer occurs. Chemical carcinogens, radiation and viruses interact with cells in very different ways but at some stage of the cascade of events there must be a common pathway that leads to neoplasia. To understand such a complex process it is necessary to study many facets of the biology of cells, tissues and whole animals.

Although the number of members of this section is much smaller than in former years the spectrum of studies remains broad. The ability to maintain the breadth of the program stems in part from collaboration both within the division and with colleagues in other institutes.

The study of radiation-induced leukemia has required some very basic studies of the various elements that are involved in the susceptibility to leukemia and the process of its development. For example, the Molecular Genetics Carcinogenesis group has cloned the genome of the ecotropic retrovirus found on chromosome 5 in RFM/Un embryonic cells and its characteristics have been dissected by restriction endonuclease mapping. There is reason to believe that this viral genome is associated with the genesis of myeloid leukemia that is found to occur naturally and that can be induced in RFM mice. The nucleotide sequence of the long terminal repeats (LTR) of the viral gene has now been determined.

The search continues for unequivocal evidence of the role of movable gene elements in the transformation process that may follow the interaction of a carcinogenic agent and the genome of a cell. There are genes that have certain sequence characteristics found in retroviral genes but that occur in numbers that cannot be accounted for on the basis of expression of the viral genes. The multiplicity of these genes may be due to the transposability of these genes and certain characteristics of the control mechanisms that govern integration. These elements must be considered as the possible targets affected by various agents including carcinogens.

An important aspect of understanding the mechanisms of the action of viruses is concerned with Fv-1 gene restriction and how Fv-1 gene products result in the inhibition of replication of the retroviruses. The recent studies have revealed which part of the viral genome the host gene

controls. A new restriction mechanism that inhibits most but not all endogenous murine leukemia viruses has been identified. Various nucleotide sequencing experiments have shown that restriction or lack of it may depend on a change in a single amino acid.

Central to the mechanism of carcinogenesis are changes in gene expression. Related and of even greater importance is the understanding of the control of the role of gene expression in differentiation. The Molecular Carcinogenesis group has continued the study of the control of the enzyme tyrosine aminotransferase (TAT). This gene that transcribes for the enzyme begins to be expressed very actively about a day before birth in rats. It has been established that the changes prior to birth indicate accelerated transcription of the gene. Since this gene expression is not reflected in a functional form of the enzyme until after birth it is clear that the control of translation is also important. Investigations have revealed the nature of this control and the changes that take place when functional enzyme is produced.

An interesting aspect of this translational control has been seen in studies of the hormonal control of the TAT gene. Glucocorticoids previously thought not to be involved in the activation of TAT gene expression may in fact be of importance.

The control at the translational level has been altered by 5-azacytidine and using this compound the role of methylation in the control of TAT expression has been studied.

The well established studies of hormonal regulation of gene expression in the adult have continued. Recent results suggest that the effect of steroids may involve modification in the specific messenger RNA. Such a mode of control may be involved in the effects of other hormones.

Cloning of the TAT gene has proven to be a considerable challenge. A clone has been identified that may contain the complete gene and certainly contains part of it.

The Molecular Immunology group continues its study of changes in cells that result from malignant transformation or damage and that can be detected with a high degree of sensitivity and specificity. Identification, quantitation and manipulation of such changes can be used in a number of ways. For example, use can be made in the study of the changes that result in malignant transformation, the identification of transformed cells and the targeted delivery of drugs to tumor cells, and lastly to detect and quantify damage to tissues such as the lung.

A number of techniques, including monoclonal antibodies and solid state radioimmunoassay, have been used in the characterization of tumor antigens. The aim is to determine the way in which these antigens arise in the process of transformation and whether their identification can be used for quantifying transformation.

Rat tracheal cells are used to investigate markers of transformation of epithelial cells exposed to chemical agents. In this system changes in DNA content have been used to detect pre-malignant cells. This approach, added to the tracking of the changes in surface antigens, has been used in a mouse lung cell system and is providing information about both the nuclear and the surface changes that characterize the transformed cell phenotype.

Monoclonal antibodies can be bound to liposomes for targeting to tumor cells or other target cells. (Ehrlich would certainly have appreciated this tailored form of chemotherapy with the so-called immunoliposomes.) A fundamental point about whether the encapsulated drug will be effective has been established. It was found that some chemotherapeutic agents are inactivated by lysosomes, and this rendered these types of drugs ineffective whereas others are not inactivated and therefore may be suitable for therapy. Such information is essential in the design of targeted drug techniques.

For years the detection of tissue damage has been approached in a number of ways, including detection of serum enzymes or other proteins by sophisticated chromatographic techniques. A new approach to this old problem has begun in this division and involves the potential use of monoclonal antibodies for the sensitive detection of proteins released from damaged lung into the blood.

The study on the changes that occur in the process of malignant transformation that involve detection of the alterations in DNA content requires techniques for studying large cell populations with sufficient sensitivity to detect relatively small changes in a small number of cells. Flow cytometry (FCM) provides the means to carry out such an experimental approach. The study on the tracheal epithelial system is just one example of an expanding number of collaborative studies using FCM techniques that are now in progress with members of the staff of various sections. Besides these collaborative studies, the group has been developing methods of data storage and also methods of cell cycle analysis.

The Chemical Carcinogenesis group has continued and extended its studies of benzo(a)pyrene metabolism found in cells of different species, especially human cells. The metabolism of B(a)P by human cells is similar to that by many animal cells. However, of importance are the tissue-specific differences and the individual variations in metabolism that have been found. The information that is accumulating about the heterogeneity of humans is important in deciding whether or not there really are subgroups in populations that are quite different in their susceptibility to certain chemical carcinogens.

The determination of the precise differences in metabolic pathways in different cells within a tissue or between tissues will lead to selecting ways of amplifying or inhibiting metabolic events critical for transformation by a specific chemical agent. Development and culture of clones

of cells that vary markedly in their metabolic activities will help to provide test systems for further studies.

The studies of the Radiation Carcinogenesis group are concerned with both the aspects of the estimate of the risks and the mechanisms of induction of cancers by radiation. Currently there is a particular interest in neutron radiation carcinogenesis. There have been suggestions that the estimates of risk of cancer as a result of exposure to neutrons have been too low and should be changed. It was hoped that data from the survivors of the atomic bomb at Hiroshima would provide the information required for the risk estimates since the radiation from the bomb dropped on Hiroshima was thought to consist of a mixture of neutrons and gamma rays. It is now thought that there was only a very small contribution of neutrons in the doses of particular interest. As a result it is probable that any changes in protection standards will have to be based on the results of animal studies.

The important questions are how to estimate the risk at low doses and what model can be used for extrapolation from data obtained at high doses. A new analysis of the data for the life-shortening effect indicates that the initial slopes of the dose-response curves for both neutron and gamma radiation are linear. The importance of this finding is that there must be maximum values for the relative biological effects (RBE) of neutrons. The maximum values for RBEs will vary for cancer induction in different tissues and for specific tissues in different strains and species.

New information has also been obtained about the effects of fractionation and protraction of neutrons. In the case of gamma radiation there is less than additivity when fractionation regimens are used. Our current studies are aimed at explaining the greater than additivity found for some endpoints with some regimens of neutron irradiation.

The radiation carcinogenesis program has been strengthened by the transfer of Dr. M. Terzaghi from the Toxicology Section. The epithelial focus assay system developed by Terzaghi and Nettesheim is being applied to the in vivo - in vitro studies on the radiation-induction of mammary tumors as well as the epithelial cells of the trachea. The method extends the ability to detect phenotypic changes and the sequence of alterations that occur in carcinogenesis.

In the study of ultraviolet radiation (UVR) carcinogenesis, we have used a number of approaches. First, the development of experimental animal systems that are suitable for the investigation of different aspects of the mechanisms involved. For example, an opossum that is smaller than a rat has been introduced in order to determine the role of pyrimidine dimers in various UVR-induced effects, including cancer. The opossum has an efficient photoreactivating system. Unfortunately, Dr. R. D. Ley, who has carried out these studies, left for a post with the Lovelace Medical Foundation but will remain as a consultant and as a collaborator.

We have introduced the mutation at the hr locus into SENCAR mice and have found that this hairless SENCAR mouse is markedly susceptible to the induction of papillomas by UVR. However, the susceptibility to carcinoma induction is not greater than in one of the other hairless stocks, although the time of appearance is considerably earlier.

In an attempt to discover why different stocks of hairless mice show such differences in susceptibility to squamous cell carcinoma induction by UVR, we are studying the effects of UVR on the immune system. As yet no stock-dependent difference in the UVR sensitivity of the immune system has been found.

The factors that influence the expression of tumors in skin are of importance both from a practical point of view and for the understanding of mechanisms. Skin appears to be able to suppress the expression of initiated cells, but the ability to do so and the effect of promoters in increasing the expression are strain-, sex- and age-dependent. For example, sex hormones influence cell proliferation in skin. It has been found that a progestin is an effective inhibitor of promotion by the phorbol ester TPA. In contrast, testosterone may enhance promotion by TPA.

The members of the section are involved in directing the research of graduate and postdoctoral students, some of whom are supported by the two existing Carcinogenesis training programs. In the last year a new training program in Radiation Studies with special emphasis on radiation carcinogenesis has been funded by NIH, and we look forward to recruiting students in this field of research.

#### MOLECULAR GENETICS OF CARCINOGENESIS

|                           |                           |
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The long-range goal of our research is to understand the genetic mechanisms of carcinogenesis at the molecular level. Particular emphasis is placed on the search for critical gene elements involved in the multiple genetic processes which lead ultimately to neoplastic expression of the cell, on how these gene elements are controlled in the cell and on what environmental agents may affect their expression. Progress of the three specific research projects ongoing in the laboratory are summarized in the following.

Radiation-induced Leukemias of RFM/Un Mice. The RFM/Un mouse model is used because radiation causes an increased incidence of myeloid leukemias

in these mice similar to man (8). The results of our current work on the possible role played by endogenous retroviral genes in the radiation-induced leukemogenesis are summarized in the following:

(i) An N-tropic type C retrovirus, which can be induced in RFM/Un cultured embryo cells by iododeoxyuridine treatment and which presumably represents the expression of the single ecotropic type C retroviral gene locus on chromosome 5 in this mouse strain, was isolated. The genome of this endogenous viral gene was molecularly cloned by using unintegrated form I viral DNA preparations from acutely infected cells and characterized by restriction endonuclease mapping. Its ecotropic type-specific env gene sequence was subcloned. Its long terminal repeats (LTR) sequence is 527 base pairs long and presumably the smallest of murine endogenous type C retroviral LTRs. The nucleotide sequence of this LTR has been determined (4).

(ii) In our previous study, cells of an established myeloid leukemic cell line originally developed from leukemic tissues of an irradiated RFM/Un mouse (Upton line) were found to contain additional copies of the ecotropic murine leukemia virus gene integrated in DNA sites distinct from the original single copy, located on chromosome 5. To determine whether this observation represents an early event in radiation-induced leukemogenesis in this mouse strain, we made similar analyses with primary tumor tissues of irradiated RFM/Un mice. Of about 40 individual tumors examined, about half of them showed additional ecotropic proviral integration. Histological analyses of the tumors revealed that reticulum cell sarcomas and myeloid leukemias consistently contained additional ecotropic MuLV proviral copies, whereas thymic lymphoma and a few other lymphomas showed no such phenomenon.

(iii) A lambda phage genomic library of HindIII DNA fragments prepared from normal and leukemic cell DNAs was constructed for subsequent screening and isolation of clones containing ecotropic MuLV proviral sequences or a complete AL-10 type provirus. More than two dozen individual clones have been isolated for further characterization.

Mechanism of Fv-1 Gene Restriction (13). In the past year we have concentrated our efforts on localizing the genetic determinant in N- and B-tropic murine leukemia virus genomes, which render the retroviral replication susceptible to inhibition by Fv-1 gene products. Having isolated the genomes of individual N- and B-tropic viruses we have been employing for Fv-1 studies, we made the following progress:

(i) In vitro recombinants were constructed by exchanging the homologous restriction endonuclease fragments between cloned N-tropic and B-tropic viral genomes; this provided a means for recognizing which particular gene segment is responsible for the N-tropic and B-tropic host range property. Our results revealed that genetic determinants of N-tropism and B-tropism were located in a BamHI-HindIII DNA fragment, which codes for two gag gene proteins (most of the p30 proteins except its

amino-terminal peptides, and the p10 protein and 36 amino acid residues at the amino terminus of the pol protein [3]).

(ii) Nucleotide sequence analysis (6) revealed that there is more than 95% sequence homology in the BamHI-HindIII DNA fragment between N-tropic and B-tropic viruses. Amino acid sequences predicted from the nucleotide sequences of N-tropic and B-tropic viruses revealed four differences: the 109th and 110th amino acid positions of p30 were glutamine-arginine in N viruses versus threonine-glutamic acid in B viruses; the 159th amino acid position of p30 was glutamic acid in N viruses versus glycine in B viruses; the 36th amino acid position of viral polymerase protein was threonine in N viruses versus isoleucine in B viruses. The sequence data and the predicted amino acid changes could explain the different protein properties of the N virus p30 and the B virus p30 and also served to predict the possible protein conformational changes which may be responsible for recognition of this presumed virion target by the Fv-1 gene product.

(iii) We have found in RFM/Un mice a novel restriction mechanism, called Emvr-1 locus, which inhibits most, if not all, endogenous ecotropic murine leukemia viruses such as RFV (endogenous ecotropic virus of RFM/Un), WNL802N and WNL802B viruses (10). However, Gross passage A N-tropic murine leukemia virus is not susceptible to restriction by this mechanism. To elucidate the location of the genetic determinant for the susceptibility to this restriction mechanism, we performed fragment exchange experiments, as described above in (i), and found that the genetic determinant of the susceptibility to Emvr-1 restriction was present in the BamHI-HindIII genomic fragment corresponding to p30-p10- $\Delta$  pol region of the viral genome. Results of nucleotide sequence analysis indicated that single amino acid changes might be responsible for the resistance of Gross virus to this restriction.

Carcinogen-cell Genome Interaction. Our current research efforts are focused on isolation and characterization of germ-line associated heritable retrovirus-like genes which are not known to come out of the cell as a virus, and yet contain structural features characteristic of a movable gene element. We have used BALB/c mouse DNA as a source for isolating several such individual genes as recombinant DNA clones. Detailed analyses were performed with a particular recombinant DNA clone, called AL-10 (7). Restriction endonuclease mapping revealed that the 10.5 kbp EcoRI insert consists of a 3.6 kbp left flanking cellular DNA region and a 6.9 kbp MuLV related region that has a typical proviral LTR-gag-pol-env structure up to the EcoRI site in the env gene region. Comparison of the AL-10 map with ecotropic and xenotropic virus isolates revealed many common restriction sites in the LTR and pol gene regions, but much fewer in the leader gag region. A stretch of 1,700 nucleotides containing the cell provirus functional region was sequenced and revealed transcriptional consensus signals and other structural features characteristic of MuLV LTRs, as well as two distinctive features. First, a sequence of about 170 bp with direct and inverted terminal repeats not seen in infectious MuLV LTRs was identified in the U3 region between the "enhancer" region and the "CAT" box. This novel segment or its homologous sequences appear to be present in most of

the endogenous MuLV-related LTRs and in other chromosomal locations of the mouse. Second, the tRNA primer binding site is not complementary to proline tRNA, the primer for all known MuLVs, but is a 17/18 match with the 18 nucleotide sequence at the 3' end of rat glutamine tRNA. The integration site of AL-10 provirus was in a unique DNA region but contained an "Alu"-like short interdispersed repeat in the 5' adjacent cellular region. The AL-10 proviral integration found in BALB/c was also apparent in RFM, AKR and SENCAR mouse cells but not in cells of NFS/N, C3H, HRS/J, SC-1, and a California Lake Casitas wild mouse.

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#### REGULATION OF GENE EXPRESSION

|                                 |                             |
|---------------------------------|-----------------------------|
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The objective of this research is to define in molecular terms the mechanisms controlling expression of specific genes in mammalian cells. We study how gene expression is activated in the process of differentiation, how tissue-specific expression is effected, how expression is modulated by hormones and other specific effectors, and how genetic control mechanisms are altered in the dysfunction of gene expression in cells transformed to malignancy. Much of our work is focused on expression of the rat liver enzyme tyrosine aminotransferase (TAT), a convenient model since its expression is modulated in several important ways and the system is amenable to detailed biochemical analysis. Significant progress has been made in a number of experimental approaches.

Differentiation. Our analysis of the activation of TAT gene expression during the perinatal period of development in rat liver now provides a clear picture of the sequence of events, as follows: (1) the TAT gene is expressed to a very limited extent in late term fetal livers (18, 19, 20 days gestation), hybridizable transcripts, functional mRNA, and enzyme all being present at levels 10- to 15-fold below adult; (2) hybridizable transcripts begin to increase in the 21st day, are several-fold above fetal levels in the 22nd day (usual day of delivery), and are at or above adult levels in newborns. These changes are virtually identical in both nuclear and cytoplasmic RNAs, in accord with the conclusion that the increase reflects accelerated transcription of the TAT gene. However, these transcripts are not functional in the liver cell (as no increase in enzyme is observed at this time), and they are similarly non-functional when assayed by translation in reticulocyte lysates; (3) the transcripts are converted to functional form beginning in the first few hours after birth. By 12 hours both mRNA<sub>TAT</sub> (assayed by translation) and enzyme are well above adult levels, to which they decline as the steady-state is established 24 to 36 hours after birth.

These results provide a clear indication of translational control superimposed upon transcriptional control and temporarily operating to limit full expression of the TAT gene products. Post-mitochondrial supernatants of homogenates of livers from newborns were fractionated on sucrose gradients and analyzed for TAT sequences by cytoplasmic dot hybridizations. This analysis reveals that the non-functional sequences are not polysome-bound, but rather appear to be present as mRNP particles that sediment between the ribosomal subunits and the intact 80 S ribosome. In the hours after birth we see a shift of these sequences into the polysomes, in accord with increased enzyme production and translatability as assessed in the lysate system. It is significant that these changes in specific TAT production are mirrored by what appears to be a corresponding change in the total capacity for translation. In newborns the polysome population is markedly reduced, the bulk of the ribosomes being present in monomeric form. The relationship between this indication of a generalized defect in initiation and our observations on TAT expression remains to be established.

Earlier analyses have shown that TAT expression in fetal liver is refractory to induction by glucocorticoids, in terms of either enzyme production or translatable mRNA. However, we now find that gene transcripts are indeed synthesized in response to hydrocortisone in 20 or 21 day fetal livers; dot hybridizations reveal an increase in transcripts that is quite comparable to that seen in adults. That these transcripts are not functional either in vivo or in vitro again points to a translational control mechanism operating to limit full expression and perhaps involving a modification of mRNA<sup>TAT</sup> structure. These results also reintroduce the question of a glucocorticoid involvement in developmental activation of TAT gene expression, which we had thought could be excluded from consideration.

Changes in DNA methylation have been implicated in differentiation-associated activation of gene expression in a number of experimental systems. We have found that direct treatment of 20-day fetuses in utero with the methylation inhibitor, 5-azacytidine, has profound effects on hepatic differentiation. TAT expression is fully activated 18 hours after treatment. Hybridizable transcripts are at or above adult levels, indicating a demethylation-induced acceleration of TAT gene transcription. These transcripts are fully active in translation assays and in the hepatocyte, as enzyme levels are increased some 70-fold above age-matched fetuses. These results indicate that the drug somehow obviates the translational block normally operating in fetal liver. Analogs of cytidine that are also toxic but do not affect DNA methylation have no effect on TAT expression. The drug is not very effective if given to fetuses earlier than 20 days of gestation, implying a temporally programmed change in "competence" that is independent of methylation, and in accord with results of others that demethylation may be necessary but not sufficient for transcriptional activation.

It was observed that the gross appearance of fetal livers was changed after 5-azacytidine treatment, which led to evaluation of several

parameters other than TAT expression. Histological studies show a change in liver structure that is truly dramatic. Hematopoietic elements are not obviously affected, but hepatocytes are greatly enlarged, basophilic, and arranged in the sinusoidal pattern that is not apparent in untreated controls. Glycogen is markedly reduced and stored lipid droplets have increased, as seen both by histochemical staining and EM studies; the latter also show an enhancement of both smooth and rough ER following treatment. Assays for other developmentally activated "liver-specific" enzymes show a marked increase in phosphoenolpyruvate carboxykinase but little or no change in glucose-6-phosphatase or tryptophan dioxygenase, but these analyses must be supplemented by measurements of gene transcripts to be fully understood. Taken together, the data indicate that the demethylating agent advances by several days the programmed maturation of the hepatocyte.

Tissue-Specific Gene Expression. This aspect of our studies in differentiation was initiated this year. TAT is said to be "liver-specific" but previous studies of its expression in other tissues are not definitive. We have scanned a variety of rat organs for TAT gene expression, assaying for two parameters. Presence of the TAT protein is assessed by "Western" blotting of electrophoretic gels and probing with antibody to TAT followed by  $^{125}\text{I}$ -protein A to detect the immobilized antibody; this immunological assay overcomes difficulties in interpretation of activity measurements. TAT gene transcripts are measured by dot hybridizations and, when appropriate, by "Northern" blotting to determine the size of the transcripts. Three organs have been selected for further analysis. Liver, in which expression is maximal, is considered a positive control, and testis, wherein expression of either parameter is at background levels, is representative of tissues in which the gene is essentially silent. Kidney has been found to display a most interesting intermediate level of expression. Enzyme is not detectable. Total RNA preparations, or poly(A)RNAs extracted from total RNA, contain a level of hybridizable sequences nearly equivalent to those in liver. However, when nuclei were excluded by preparation of cytoplasmic RNAs from kidney, the sequence level was far below (perhaps 100-fold) the liver. These results imply that in kidney the gene is being transcribed but, probably as a consequence of defective nuclear processing, very few or no functional mRNA molecules enter the cytoplasm and are translated.

Hormonal Regulation. Our analyses of induction of TAT synthesis in adult liver by hydrocortisone have revealed some interesting complexities that are as yet poorly understood but hold the promise of explaining some of the unexpected phenomena we observe in studies of differentiation. First, the accumulation of cytoplasmic transcripts detectable by hybridization is much more rapid and extensive than are increases in either translatable mRNA or enzyme. Secondly, as the induction process proceeds, there appears to be accumulation of a second form of mRNA<sup>TAT</sup> as seen on "Northern" blots; this species is somewhat smaller (3000 vs 3400 nucleotides) and appears to correlate with increased translational activity of the mRNA and enzyme production. These results, currently being confirmed and extended, suggest a requirement for a cytoplasmic modification of

mRNA<sup>TAT</sup> resulting in both its shortening and activation of its capacity to be translated; perhaps such a mechanism is involved in the developmental shift of mRNA<sup>TAT</sup> from mRNP particles to functional polysomes. An alternative, that steroid induction involves transcription from a different promoter and thereby a different (smaller) mRNA, cannot be excluded.

Studies of induction by cyclic AMP and by insulin are nearing completion. In both we see a very rapid accumulation of hybridizable transcripts, again preceding accumulation of translational capacity or of enzyme. The enhancement of TAT synthesis induced by cAMP is completely accounted for by increased accumulation of mRNA<sup>TAT</sup> in both cytoplasmic and nuclear RNA preparations, making it most likely that this induction mechanism is also primarily transcriptional. Surprisingly, cAMP treatment renders the already labile mRNA<sup>TAT</sup> (intracellular  $t_{1/2}$ , ca 90 min) even more labile *in vivo* ( $t_{1/2}$  ca 20 min). This effect is not seen with the other hormonal inducers, and as yet we can offer no explanation. Analyses of these inductions in terms of the newly-discovered multiple species of mRNA<sup>TAT</sup> have not yet been done.

Genomic Cloning. To define fully expression of the TAT gene, changes in expression during development, as induced by hormones or its expression in different tissues, it will be necessary to isolate the gene and to characterize its structure. Restriction analysis of rat DNA has established that TAT is a single copy gene. A library of fragments of rat DNA cloned in phage lambda was kindly made available by Dr. James Bonner, California Institute of Technology. After extensive screening of nearly  $2 \times 10^6$  plaques, one was identified (by hybridization to  $^{32}P$ -labeled cDNA<sup>TAT</sup>) as carrying at least a portion of the TAT gene. This clone is currently being purified preparatory to characterization of the fragment of rat DNA by restriction mapping. It is conceivable, but we think unlikely, that this fragment contains the entire gene; it will probably be necessary to use the fragment now identified to rescreen the library for contiguous fragments.

To characterize the gene structure it will be necessary to have available a full length cDNA<sup>TAT</sup>. The cloned cDNA now available is representative of only the 3' third of mRNA<sup>TAT</sup> and will not recognize the 5' portion of the gene, the region wherein key regulatory elements could be expected. We have now worked out procedures for producing a cDNA<sup>TAT</sup> representing the region of the messenger not recognized by our current probe. This was done by primer extension of a small fragment, and the product is full length or nearly so. This cDNA is now being prepared for cloning to produce the reagent in quantity. When available, it will also be very significant in analyzing intermediates in processing of TAT gene transcripts, a research objective that has not been successful with our current cDNA probe.

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#### TUMOR CELL SURFACE PROTEINS

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

A tumor surface protein of 180,000 M<sub>r</sub> (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 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. MoAb to several epitopes on TSP-180 are being used to identify normal cell components related to the TSP-180 molecule through quantitative absorption and immunohistochemical techniques.

Drug Targeting and Tumor Imaging. Antibody reagents reacting specifically with tumor antigens should "home" to target cells in vivo. Using radiolabeled antibody, tumor cells and their metastases can be located by radioimaging. Pure antibody reagents needed for these studies are now available in the form of MoAb. A detailed study of interaction of MoAb with cell surface antigens has been done in three different tumor systems.

These results show that quantitatively the interaction follows a simple mass action expression for bimolecular reactions. Thus for effective tumor imaging, the factors that are important are (i) the purity of the labeled antibody, (ii) the affinity constant of the antibody, and (iii) the antigen concentration of the tumor cells.

If antibodies can localize at tumor cell sites, they should be useful for delivery of cytotoxic chemicals to the tumor cells. Intermediate carriers, such as liposomes, should allow large numbers of drugs to be delivered by a limited number of antibody molecules. Monoclonal antibodies have been derivitized with fatty acids and attached to liposomes.

With this method we have covalently attached a monoclonal antibody (11-4.1) against the murine major histocompatibility antigen, H-2K<sup>k</sup>, on the surface of liposomes. The interaction of these antibody-coated liposomes (immunoliposomes) with target cells, RDM-4 lymphoma (H-2K<sup>k</sup>), was investigated. About 90% of the immunoliposomes taken up by target cells at 4°C could be removed by a mild protease treatment of the cells; whereas only 30% of the uptake at 37°C was labile to the same treatment. Furthermore, the uptake of immunoliposomes at 37°C was inhibited by cytochalasin B or by a combination of 2-deoxyglucose and NaN<sub>3</sub>. These results suggest that immunoliposome binding to the target cell surface is the primary uptake event at 4°C and that the surface bound liposomes are rapidly internalized by the cells at 37°C, probably via an endocytic pathway. Studies with fluorescence microscopy of target cells treated with immunoliposomes containing carboxyfluorescein also supported this conclusion. If endocytosis is the mechanism by which immunoliposomes gain entry into target cells, the efficacy of a cytotoxic drug encapsulated would depend on the resistance of the drug to lysosomal inactivation and its ability to escape from the lysosomal system. Consistent with this notion, we observed that methotrexate (MTX) encapsulated in liposomes bearing 11-4.1 antibody specifically inhibited deoxy [<sup>6-3</sup>H] uridine incorporation into DNA in target RDM-4 cells but not in P3-X63-Ag 8 myeloma cells (H-2K<sup>d</sup>) at the same doses. The observed cytotoxic effect of encapsulated MTX could be reversed by the treatment of cells with a lysosomotropic amine, chloroquine, which has been shown to increase the intralysosomal pH of mammalian cells. On the other hand, cytosine-β-D-arabinofuranoside (ara C) encapsulated in immunoliposomes showed no target specific killing, probably because the drug is readily inactivated in the lysosomal system. This basic information is important for future design of a more efficient drug delivery system.

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

In another system, syngeneic monoclonal antibodies (MoAb) to Muloney sarcoma cells (MSC) were produced by fusion of spleen cells from MSC

regressor mice to myeloma SP2/0. MoAb 244-19A, an immunoglobulin G<sub>2b</sub> bound to MSC cells and did not bind to two other sarcomas (K-BALB and Ha2), a carcinoma (Line 1), a fibroblast (A31) or a fibroblast infected with C-type virus (A31-Moloney leukemia virus). 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. Optimal dose, schedule, and mechanism studies concerning this therapy are in progress.

Solid State Radioimmunoassay for Carcinogenesis. Tumor-specific proteins represent cell phenotypes characteristic of cell transformation. Quantitation and sensitive detection of these proteins should provide a method of early detection of transformation. Automated solid state radioimmunoassay techniques have been developed that can be used to screen thousands of cell clones for transformed phenotypes. Assays using monoclonal antibody coupled to solid supports should allow assay of more than 10<sup>6</sup> cells at a time. Details of solid state assays with MoAb have been worked out. Affinity constants of antibodies for antigen in fluid phase or coupled to a solid support do not vary significantly; however, antigen presentation on solid supports depends on the configuration of the support as well as the mode of attachment of the antibody. Monoclonal antibody to fragment D (FgD) of human fibrinogen has been used to develop a solid state assay for FgD which should be diagnostic of plasminogen activator activity. Although the affinity constant of the specific MoAb is relatively low (10<sup>7</sup> 1/mole), the assay has been made sensitive at the ng/ml range by coupling the specific MoAb with non-specific antibodies of high affinity (1.1 x 10<sup>10</sup> 1/mole). This assay should be useful in screening clones of cells for plasminogen activator activity and for analysis of serum FgD levels in cancer patients.

Monoclonal Antibody for Lung Toxicology. Analysis of toxic injury to lungs can be obtained chiefly through invasive procedures, 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 Developing 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. Identification of such markers would not only aid in understanding preneoplastic disease, but also offer new approaches for detection and quantitation of such alterations in cells exposed to putative oncogenic agents well before the appearance of the neoplastic phenotype. Our approach has been to define early phenotypic changes in short-term cultures of rat tracheal epithelial cells exposed to chemical carcinogens. Altered phenotypes have been identified on the basis of an increase in 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 and correlate with the initial breakdown of those mechanisms which regulate normal growth and differentiation. Our goals are to be able to predict the probability of neoplastic cell types appearing in exposed cell populations well before the appearance of the malignant cell phenotype.

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## CYTOMETRICS

R. 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. During the third year of its existence, the group has continued to pursue two major goals: (1) to engage in collaborative work with different groups in the Division applying FCM, and (2) to perform independent research developing new cytometric techniques. Efforts towards achieving the latter goal have been concentrated mainly on problems in FCM data analysis and interpretation, and on new sample preparation techniques for FCM.

We have addressed three problem areas in FCM data analysis: (1) analysis of one- and two-parameter histograms, (2) comparison of histograms, (3) efficient processing of large volumes of multi-parameter data.

Histograms frequently consist of several components that show varying degrees of overlap. The main task of histogram analysis is to identify these components. Our major progress has been in the area of analysis of DNA-histograms. Several algorithms in the literature were implemented on the computer system in the FCM laboratory to analyze DNA distribution of homogeneous cell populations. They are being used for research by several investigators (W. Au, G. R. Braslawsky, D. M. Popp, R. A. Popp, J. K. Selkirk). The need to analyze DNA-histograms obtained from heterogeneous cell populations (in collaboration with G. R. Braslawsky) led to the formulation of a new method for the identification of histogram components. The method is based on maximum likelihood estimation, and is also applicable to two-parameter histograms. Preliminary investigations have shown that the two-dimensional version of this method will most probably be used for the measurement of spot intensities in the automatic electro-phoretic gel analysis system that will be available soon in the Chemical Carcinogenesis Group.

The detection of significant differences between histograms or groups of histograms is another problem in FCM data analysis that arises in many different applications. We have adapted Fisher's randomization test to compare two groups of one-parameter histograms. The method is not restricted to a particular form of histogram, and is therefore applicable to any type of one-parameter data pattern measured with our instrument.

Research is under way to develop a corresponding method for the comparison of two-parameter data.

Multi-parameter (>2) measurements are being made in more than half of the experiments using FCM. Data are collected in LIST mode, i.e. data are stored in form of a list containing the parameter values for each individual cell measured. The amount of memory required for these measurements is therefore proportional to the product of the number of cells and the number of parameters per cell. In a typical experiment 50,000 to 100,000 cells are measured per sample. Data from the different investigators' experiments are currently stored on 18 magnetic disks (5 Mbyte/disk) and 8 magnetic tapes, and these numbers are increasing. We are therefore investigating methods for compact data storage, data dimensionality reduction, and optimal display of multi-parameter data. Progress has been made in compact storage, and the application of projections for dimensionality reduction of FCM data.

A major improvement has been achieved in the speed of analysis of large volumes of data by establishing a "soft" link via magnetic tape between the minicomputer in the FCM laboratory and the main computer in the Division.

We were fortunate to obtain from Dr. Howard Gratzner, Institute for Cell Analysis, Miami, Florida, a sufficient amount of bromodeoxyuridine (BrdU) specific monoclonal antibody that enabled us to implement a new assay for cell cycle analysis (recently developed at Lawrence Livermore National Laboratory), and to explore further FCM assays involving BrdU. BrdU is a compound that is incorporated into DNA during synthesis or repair as a substitute for thymidine. Therefore it quenches the fluorescence of the adenine/thymidine-specific Hoechst 33258 stain. This effect can be measured, thereby quantifying the amount of incorporated BrdU into DNA, and hence, repair or synthesis. There are problems with this assay, however, due to low quantum yield and the need for high power UV excitation. With the BrdU specific monoclonal antibody that is labeled with fluorescein isothiocyanate (FITC), it is possible to measure the amount of incorporated BrdU by excitation with visible light (488 nm). So far the antibody has been used for cell cycle analysis measuring the amount of incorporated BrdU during S-phase and total DNA (stained with propidium iodide). The addition of the second (FITC-labeled antibody) fluorescence parameter allows a determination of the fraction of cells in S-phase much more accurately than by measuring DNA content alone. Preliminary experiments (in collaboration with J. D. Regan) suggest that DNA repair in human fibroblasts after irradiation with different doses of UV light can be quantified by flow cytometry with the BrdU-specific antibody.

Together with Wayne Greene, DiaClin Laboratory, Nashville, Tennessee, we organized the first regional FCM users meeting which was held on September 23, 1983 in Oak Ridge. The goal of this meeting was to provide a regional forum in which all levels of laboratory personnel can gather to discuss current instrumentation, techniques, and applications relating to

flow cytometric analyses in both research and clinical situations. It is planned to hold these meetings semi-annually in the future.

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#### METABOLIC ACTIVATION AND CARCINOGEN METABOLISM

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Research into the metabolism of chemical carcinogens has clearly shown a striking similarity for all eukaryotes with regard to the enzymatic pathways that are utilized to metabolize xenobiotics, and the final array of metabolic products. Our studies over the past year have been designed to more carefully define the metabolic differences in diverse tissues, both human and rodent, for the activation and detoxification of polycyclic carcinogens as major environmental contaminants of energy production from fossil fuels. Our goal is to probe and possibly amplify differences in metabolic pathways in order to determine what portions of the metabolic activation profile are critical to forming, maintaining, and directing the ultimate carcinogenic species toward its final fate. Benzo(a)pyrene is being used as the prototype molecule to formulate a mechanism which describes how individual systems transform the parent chemical into a highly reactive electrophile. Benzo(a)pyrene forms a particularly active diol-epoxide as one of its many metabolites. This reactive intermediate is thought to be the principal carcinogenic species for this polycyclic molecule, and is based on enhanced carcinogenic and mutagenic activity when compared to the parent molecule. The diol-epoxide is also the major form of benzo(a)pyrene that binds to nucleic acid. However, this structural information has been insufficient to formulate an explanation of how this alkylation process triggers malignant transformation. The fact that both resistant and susceptible tissues form the same carcinogenic diol-epoxide intermediate points to quantitative variation in the amount of the carcinogenic intermediate formed as a critical parameter, which helps determine whether a tissue or cell will become malignant transformed. During the past year, we examined benzo(a)pyrene metabolism and DNA binding in both human and rodent cells. Since human exposure to carcinogenic polycyclic hydrocarbons continues to be a major environmental hazard, it is important to know if specific tissues in the body are at greater risk than

other tissues or organ systems. We studied metabolism in explants of four human tissues: bladder, skin, bronchus and esophagus from eight donors sampled 4 hours after death. Explants were incubated with tritiated benzo(a)pyrene and the metabolites extracted and analyzed by high pressure liquid chromatography. Fibroblasts were grown from explants from two patients and also incubated with benzo(a)pyrene in order to compare differences between differentiated and undifferentiated cells. Averaging the percent yield of each metabolite for each individual tissue between the eight donors showed remarkably comparable ratios for all the major metabolites. In general, the human tissue profiles were quite similar to the majority of the non-human profiles reported in the literature, including the formation of the 9,10-dihydrodiol as the major cellular metabolite. Undifferentiated fibroblasts cultured from esophagus, skin, bladder and bronchus from two patients yielded the same metabolite profile as seen in the epithelial cultures. The amount of carcinogen bound to the DNA in these four tissues showed a wide variability in specific activity of binding between individual donors. There also seemed to be differences in variance between the four tissue studies. Variability between individual tissues showed bladder to have the widest range of specific activity (10-183; skin, 5-82; bronchus, 10-60; esophagus, 2-42). Interestingly, the level of activity for the four tissues was relatively the same in a given individual (e.g. four high values or four low values), which may reflect a genetic variance at the Ah locus for monooxygenase induction. These results clearly show that the heterogeneous human population possesses a wide range of metabolic competencies for inducing the drug metabolizing enzymes, and concomitantly the formation of carcinogenic intermediates. Assuming qualitative patterns of carcinogen activation to be the same, we must increasingly probe individual differences between resistant and susceptible cells for metabolic activation. This will help to determine if there are sub-groups in the population more susceptible to environmental exposure to carcinogens. Since liver cells are the prime site for metabolism of xenobiotic chemicals and are not favored sites for tumor formation by environmental carcinogens, we probed the high metabolic competency of normal and malignant liver cells. Furthermore, we utilized the liver cell metabolic products in a short term mutagenesis assay. These studies utilized different cell lines derived from BD-IV and BD-VI rats in collaboration with the International Agency for Research on Cancer in Lyon, France. We utilized a non-transformed liver line (IAR-20) and compared it against four tumor lines derived from that parent cell (IAR-6-1, IAR-19, IAR-27, IAR-28). Cultures were treated over a 5-day period, and the cells and medium analyzed for the level of metabolism and the distribution between organic soluble intermediates and water-soluble excretion products. The various cell cultures were tested for survival over a 5-day period to see if the transformed lines processed the carcinogen to more cytotoxic products in a parent (IAR-20) cell line. Survival rates over the 5-day incubation period and overall benzo(a)pyrene metabolism produced an inverse relationship. More metabolism resulted in less survival due to the greater production of toxic intermediates. However there were some inconsistencies over the 5-day period. Analysis at the 5-day time period showed some of the cell populations were producing the same level of metabolism with only half the cell population as the parent cell line. Time course studies of

the formation and disappearance of each metabolite in the array of benzo(a)pyrene diols, phenols, and quinones showed continual buildup of the benzo(a)pyrene diols which appeared to be less active substrates for water-soluble conjugation to glutathione or glucuronide. Phenols and quinones peaked at 24 hours, and either maintained a steady state of formation and disappearance or in some cases, exhibited a gradual decline over the 5-day period. Our previous studies have shown the phenols of polycyclic carcinogens to be good substrates for conjugation to water-soluble products.

We also monitored this metabolism for the amount of carcinogen bound to nuclear macromolecules. While alkylation of DNA/RNA nuclear protein is dependent upon a complex series of enzymatic and chemical steps that control the amount of available activated intermediate at any given time, it is important to know if the attack on nuclear material can be directly approximated to the amount of metabolism by the cellular monooxygenases. Interestingly, the protein/DNA ratios were fairly constant, but there were major differences in the amounts of DNA bound. As an example, IAR-20 and IAR-6-1 showed an equivalent DNA and protein binding ratio (1.55 vs. 1.57) in which the IAR-6-1 was able to accomplish this with half the metabolism of the IAR-20. IAR-2-19 showed a relative increase in binding with an increase in overall metabolism, although not proportionately since the binding increase was about 30%, while metabolism increased only 13%. In addition, we saw further variation with the spontaneously transformed line IAR-27, which showed the lowest DNA and protein binding along with a significant change in the protein/DNA binding ratio. That was coupled to a metabolism equivalent to IAR-6-1. Primary hamster embryonic fibroblasts which were utilized for comparison showed greater than twice the binding of the most active liver epithelial line, but was accompanied by an overall metabolism roughly equivalent to the parent liver line, IAR-20.

The complex metabolism seen in these liver cell lines shows that interrupting a single step of the complex biochemical scheme that is responsible for controlling metabolism of chemical carcinogens may inhibit the activation pathway in favor of accumulation of one or more hydroxylated intermediates. This was observed when these liver cell lines were used as activators in the V79 mutation cell assay where, as predicted, the more active parent cell, IAR-20, induced a three-fold higher mutation rate than in the poorly metabolizing spontaneous transformant, IAR-27. Our present approach to the concept of selective susceptibility and resistance to chemical carcinogens has enabled us to formulate a new approach to study subpopulations of cells within target tissues or organs in order to isolate and amplify those metabolic parameters that are critical for directing a cell to become malignantly transformed. We are now utilizing the Fluorescence Activated Cell Sorter in the Biology Division to sort populations of cells based on their ability to clear polycyclic hydrocarbons from the cytoplasm after short term incubations. This is indicative of varying metabolic activity in the cytoplasmic monooxygenases. In this manner, we will separate clones of cells in vastly different biochemical competencies from a high background of "normal cell" metabolism. By selecting out these biochemically aberrant cells, we hope to determine which portions of the

metabolic scheme have been altered. We can then propagate these cell lines to compare their transforming ability and their complete metabolic profiles.

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#### RADIATION CARCINOGENESIS

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|---------------|-------------------|
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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 approaches. A common theme among all of these studies is the examination of mechanisms and the establishment of general principles which may allow a better understanding of the risks to humans from radiation exposure. Data from all of these studies are also being used to examine more direct methods of extrapolation of animal data to human risks.

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

Neutron Carcinogenesis. The largest effort is directed toward the study of carcinogenesis after exposure to neutrons. The objective of these studies is to define dose response relationships, RBE-dose relationships and dose rate relationships for the induction of several mouse tumors. Over the last several years the effects of acute, protracted or fractionated neutron exposures on life shortening and tumor induction have been examined and compared in a series of studies. In these studies particular emphasis has been placed on the study of life shortening, and the induction of lung and mammary adenocarcinomas and ovarian tumors in BALB/C mice.

For life shortening the dose response rose rapidly and then began to plateau at doses in the 25-50 rad dose range. Fractionation with an extended time between fractions (30 days) or protraction diminished this plateauing in the 25-50 rad range. This resulted in enhanced life shortening after protraction or fractionation in this dose range. No differences were observed between acute and fractionated protracted exposures at doses in the 0-25 rad range. As a result fractionation or protraction resulted in a more nearly linear response over the 0-50 rad dose range. These

results support a linear dose response for life shortening after neutron exposures at doses below 20 rad.

Additional evidence for a linear response for neutrons comes from our recent analysis of data from the studies in our laboratory and from studies at Argonne National Laboratory. This analysis established that the initial slopes of both neutron and gamma dose-response curves for life shortening are linear. Such linearity enforces limiting values of RBEs. The maximum estimated RBEs for life shortening which are sex- and mouse strain-dependent range from 13-22.

The induction of lung adenocarcinomas, mammary adenocarcinomas and ovarian tumors in female BALB/c mice was also examined after whole-body neutron irradiation delivered at a high dose rate, in a single exposure, or delivered in two equal fractions separated by intervals of 24 hours or 30 days, and these effects were compared with those after neutron irradiation at low dose rates. The dose-response for ovarian tumorigenesis after the split-dose fractionation regimen was similar to that observed after single high dose-rate neutron exposure. However, lowering the dose rate reduced the incidence over the 0-50 rad dose range. For lung and mammary tumors the results were more complex. One observation of particular interest was that protraction of neutron exposures enhanced the mammary tumorigenic effect of neutrons after doses as low as 2.5 rad. This enhancement at low doses was not observed for lung tumor induction. These data suggest that fractionation and dose-rate effects are different for different tumor types presumably because of the different mechanisms of tumorigenesis that may be involved.

In addition to these studies, the induction of myelogenous leukemia after neutron exposures is also being examined. 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 Drs. Preston and Au (Biology Division). In another collaborative study Dr. Yang of this Division is examining the possible role of transposition of genetic elements in the development of myelogenous leukemia. Mechanistic studies are also being pursued using an in vivo/in vitro system to study mammary carcinogenesis. This system allows the study of cell killing, transformation and progression of mammary epithelial cells after neutron exposure.

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 a combination of the mammary cell dissociation (CD) system originally developed by DeOme and his coworkers (Cancer Res. 38: 2103, 1978) and the epithelial focus assay system developed by Terzaghi and Nettesheim (Cancer Res. 39: 4003, 1979). The CD system takes advantage of

the fact that mammary fat pads of 3-week-old mice, from which the mammary gland rudiments are removed and which therefore contain no mammary cells of their own, serve as an ideal site for the growth and differentiation of mammary cells from a donor. Further, the injection of enzymatically dissociated cells into gland-free fat pads yields classifiable outgrowths characteristic of the state of the cells. By injecting cells from irradiated mice into these fat pads within 24 hours after irradiation, we have shown that this technique can be used to detect and quantify early alterations in mammary cells induced by radiation. By allowing cells to remain *in situ* for various time periods prior to injection, the progression of these altered cell populations is also being studied.

The epithelial focus assay was originally developed for purposes of identification and isolation of "carcinogen-altered" epithelial cells from rat tracheas, esophagi, and lungs exposed *in vivo* to carcinogen. These carcinogen altered cells appear soon after the target tissues have been exposed to carcinogen and can be found throughout the tumor latency period. They are considered to be preneoplastic, since compared with unexposed cells, they have an increased likelihood to become neoplastic. These early preneoplastic cells can be identified because of the acquisition of a markedly increased *in vitro* growth capacity. Under culture conditions, which are "non-permissive" for most normal tracheal epithelial cells, they not only survive but proliferate rapidly, forming expanding epithelial foci (EF) at a time when the cultures of normal tracheal cells have ceased to proliferate and senescence has set in. Some of the epithelial colonies escape permanently from senescence (or terminal differentiation); they become "immortal" and can be propagated *in vitro* indefinitely. After repeated subculturing, some of the cell line cultures become neoplastically transformed, producing invasive carcinomas upon inoculation into compatible hosts. The progenitor-progeny relationship between the cells of the epithelial focus and the malignant cells of the late immortalized cell cultures is, if not proven, strongly suggested by previous studies.

The epithelial focus assay (EF-assay) has been modified for the analysis of the cellular events of neoplastic development in mouse mammary gland. With this assay one can distinguish and quantify three different "growth-phenotypes" of carcinogen altered clonogenic cells: (i) the clonogenic unit giving rise to an epithelial focus ( $EF_o$ ); (ii) the clonogenic unit giving rise to an epithelial focus that permanently escapes senescence and is subculturable ( $EF_s$ ); and (iii) the clonogenic unit that gives rise to subculturable foci ( $EF_{s,n}$ ) that are tumorigenic when injected into the mammary fat pad. In general, we have shown that the first two of these phenotypes of carcinogen altered cells are detectable in carcinogen exposed mammary epithelium immediately after carcinogen exposure and that the number of subculturable focus-forming units increases with time even in the absence of any further carcinogenic stimulus. Further, the data suggest differences between the effects of radiation and chemical carcinogens on this sequence of events. This approach is now being applied to studies of neutron carcinogenesis and time-dose relationships as well as a study designed to compare chemical and radiation mammary carcinogenesis.

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 essential. Over the last year we have initiated studies designed to examine time-dose relationships for radiation carcinogenesis. The objectives of this project are to: (i) examine time-dose relationships for the induction of lung adenocarcinomas and mammary adenocarcinomas in BALB/c mice after gamma ray irradiation, including the influence of dose rate and fractionation; (ii) determine whether dose rate effects are a result of repair or recovery from initial carcinogenic events or due to changes in mechanisms related to tumor expression; and (iii) examine the persistence of latent carcinogenic effects. For the second and third objectives, experiments are examining 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.

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

a. Mechanisms of Carcinogenesis. It has become widely accepted that the induction of pyrimidine dimers is the initiating event in skin cancer caused by exposure to sunlight. However, unequivocal experimental evidence of the causal relationship between the specific type of DNA damage and subsequent skin cancer has not been produced. There are at least two approaches that appear appropriate for investigating the role of pyrimidine dimers. First, the use of an experimental model in which the cells have the capability of photoreactivation. This was the basis of the experiments carried out by Hart, R. W., et al. (Proc. Natl. Acad. Sci. USA 74: 5574, 1977) on fish. Second, to determine whether there is a concordance between the action spectrum for UVR-induced skin cancer and the induction of pyrimidine dimers. Both approaches, although relatively simple in concept, are extremely difficult to carry out experimentally. In the last report we described the investigations of the action spectrum for the induction of pyrimidine dimers in mouse skin that are necessary for the design of the carcinogenesis study and these results have been published. This year we report progress in identifying a suitable animal model system for investigating the role of pyrimidine dimers in UVR-induced skin cancer. The animal selected is Monodelphis domestica, a small opossum. We have demonstrated that the cells of this animal are capable of photoreactivating the UVR-induced DNA damage identified by assay of endonuclease sensitive sites. Evidence of the photoreactive capability in the skin has been obtained by determining the comparative doses required to induce erythema-edema in opossum skin with and without exposure to photoreactivating wavelengths. It is clear that photoreactivation occurs in the skin of the animals. The tumor studies have begun at Lovelace Institute and are being carried out by R. D. Ley.

The induction of overt skin cancer involves a great deal more than just the initial events. Skin appears to be very efficient in suppressing the expression of initiated cells, and we have shown that expression is strain-dependent.

We have used mice with a mutation at the hr locus on different backgrounds to investigate the nature of these genetically determined differences in expression of initiation. We have now compared the susceptibility of hairless SENCAR mice (developed from SKH:hr-1 and SENCAR mice) with that of SKH:hr-1 mice to both UVR and DMBA induction of skin tumors and with HRS/J/Anl mice to induction by UVR. Following 36 exposures (3/week for 12 weeks) to 1000 J/m<sup>2</sup> (280-400 nm) papillomatous lesions occurred in 70% of the SENCAR:SKH mice (7 papillomas/mouse) by 30 weeks, and in 60% of SKH:hr-1 mice (0.7 papilloma/mouse) by 80 weeks, whereas none occurred in HRS/J//Anl mice. In the case of squamous cell carcinomas the cumulative incidence was not different between SENCAR-SKH and SKH:hr-1 but the time of appearance was earlier in the former stock. The latent period (time to 50% of mice with carcinomas) was about 20 weeks shorter in the SENCAR-SKH. The incidence of carcinomas was 2% at 70 weeks in the HRS/J/Anl mice. These results suggest that different genetic factors influence the susceptibility to papillomas and carcinomas and also the rate of expression of the tumors.

In the case of SKH:hr-1 and HRS/J/Anl, we have eliminated some of the potential causes for a difference in the expression of initiation events and now we are concentrating, in collaboration with E. Perkins, on the question whether the immune system plays a role in determining susceptibility. Although it is clear that both UVR, and psoralen plus UVA (PUVA), do affect Langerhans cells, and such indicators of cellular immunity as delayed-type hypersensitivity (DTH), there is no direct evidence that such changes play a role in epidermal carcinogenesis.

We have carried out a preliminary study on the action spectrum for effects on DTH. It is thought that Langerhans cells are the target for the UVR effects on the immune system. It is of some importance to establish whether the target is the DNA in the Langerhans cells. Such studies can only be carried out meaningfully if equivalent doses of the different wavelengths are used. We used the assay of dimer induction at the different wavelengths in order to adjust the effective fluences to be equivalent in our study. For example, the surface fluence with 289 nm was  $667 \text{ J/m}^2$  but  $1000 \text{ J/m}^2$  with 302 nm. Both these fluences induced about  $4 \times 10^5$  pyrimidine dimers in the basal layer. We found that the longer wavelengths and the broad spectrum 280-400 nm radiations were more effective than the wavelengths absorbed in DNA. The results suggest a non-DNA target.

b. Cocarcinogenesis. In the study of cocarcinogenesis (X ray and PUVA) we have found that protracted low dose PUVA treatment enhances the carcinogenic effect of X-irradiation, the carcinogenic effect of the combination of treatments being clearly dependent on the total dose of X rays. This study is also examining effects of TPA and UVR as cocarcinogens relative to PUVA. Since most of the actions of these three agents involve different mechanisms, it provides a way to probe the factors involved in cocarcinogenesis. It may be of importance that all three agents appear to depress delayed-type hypersensitivity.

The importance of UVR in sunlight as a cause of skin cancer is now undisputed. However, the role of UVR as a cocarcinogen is not clearly defined. For example, does exposure to UVR increase the probability of skin cancer in persons exposed to chemicals either topically or systemically? The question of the importance of interactions between UVR and the components of the process streams in the production of synthetic fuels and the final products is a practical one. We are investigating whether exposure to UVR after exposure to single chemical agents, such as B(a)P or complex mixtures such as shale oil at dose levels that individually would either produce no tumors or a very low incidence of skin cancer, results in a marked enhancement in tumor expression.

The preliminary results with B(a)P and UVR indicate a synergistic effect when exposures to UVR follow topical treatment with B(a)P.

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#### EFFECTS OF ANDROGENS AND ANTIANDROGENS ON THE INDUCTION OF MOUSE SKIN PAPILLOMAS BY PHORBOL ESTER TUMOR PROMOTERS

K. A. Davidson

The skin is a target organ for androgenic steroids. These steroids stimulate cell proliferation in the epidermis and sebaceous glands. The skin also has the capacity to convert preandrogens to active androgens and metabolize active androgens to inactive forms that can be excreted. Cyproterone acetate (CPA) is a progestin that has antiandrogenic activity. This agent has been tested clinically in the treatment of abnormal hair growth in hirsute women, acne, prostate cancer and other conditions associated with abnormal androgen production or the response of target tissues to androgens. CPA and other antiandrogens block the stimulatory effects of androgens on cell proliferation in the skin. The antiandrogenic

activity of CPA is mediated by its ability to competitively inhibit the binding of androgens to intracellular receptors.

This project was initiated in order to (1) determine if CPA would inhibit the production of papillomas induced by the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), (2) study the mechanism by which CPA inhibits the production of papillomas, and (3) study the influence of host factors (androgens) as modulators in the induction of papillomas.

The initiation/promotion protocol was used to induce skin papillomas in the highly sensitive SENCAR mouse. 7,12-dimethylbenz(a)anthracene (10 N Mol) was applied to the dorsal skin (initiation) followed in one week by twice weekly applications of 1  $\mu$ g of TPA for 20 weeks (promotion). CPA was applied simultaneously with 1  $\mu$ g of TPA for the duration of the experiment. A second protocol for inducing papillomas, 2-stage promotion, was also used. TPA (2  $\mu$ g) was applied to the dorsal skin of initiated mice twice weekly for two weeks (1st stage) followed by twice weekly applications of 2  $\mu$ g of mezerein (a nonpromoting hyperplasiogenic agent) for 18 weeks (2nd stage). CPA was applied during the first stage with TPA or during the second stage with mezerein. All agents were applied in 0.2 ml acetone.

The results of these experiments revealed that 500  $\mu$ g of CPA inhibited the incidence of papillomas/mouse by 95% and 91% after 29 and 37 weeks of promotion, respectively. In a second experiment the incidence of papillomas/mouse was inhibited by 84% (female mice). In male mice 500  $\mu$ g and 1 mg of CPA inhibited tumor promotion by 77% and 88%, respectively. The androgen, testosterone propionate (TP) (500  $\mu$ g), applied simultaneously with TPA and 500  $\mu$ g of CPA partially blocked the inhibitory effect of CPA (female mice) suggesting that the antiandrogenic activity of CPA is involved in its antipromotion activity. In the 2-stage promotion protocol, CPA inhibited the incidence of papillomas/mouse when it was applied during the 2nd stage with mezerein (71% inhibition) but not when it was applied during the 1st stage with TPA.

Phorbol ester tumor promoters induce inflammation (infiltration of leucocytes and edema) in the skin and DNA synthesis, cell proliferation that results in hyperplasia, and increased ornithine decarboxylase activity in the epidermis. These parameters show very good correlation with promoting activity of phorbol esters. Inflammation, epidermal DNA synthesis and hyperplasia were inhibited by a single application of 500  $\mu$ g of CPA given simultaneously with 2  $\mu$ g of TPA.

These results suggest that CPA may be an effective agent for chemo-prevention of chemically induced papillomas and possibly other proliferative lesions in the skin. It is also suggested that androgens have either a direct or indirect influence on the induction of skin papillomas. Two experiments were carried out to determine the effect of exogenous androgens on the induction of papillomas by TPA. TP (500  $\mu$ g) applied simultaneously with 1  $\mu$ g of TPA increased the incidence of papillomas/mouse by 2.3-fold and 1.5-fold at 20 weeks and 37 weeks of promotion, respectively. However,

in a followup experiment, 250  $\mu$ g, 500  $\mu$ g and 1 mg of TP did not significantly alter the incidence of papillomas/mouse. The discrepancy in the magnitude of the results from the two experiments makes it impossible to form definite conclusions at this time.

## Educational Activities

### University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences

W. E. BARNETT - DIRECTOR

The University of Tennessee-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 42 students working toward the Ph.D. degree and 3 in the Master's program. As of August 1983, 88 students have been awarded the Ph.D. degree.

The fall quarter of 1983 marks the beginning of the School's eighteenth 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 Union Carbide Corporation and a postdoctoral training grant in Carcinogenesis from the National Cancer Institute. As of July 31, 1983, there were nine 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. Ten to twenty students who possess the educational qualifications and the potential for a successful scientific career spend 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) doing research under the guidance of Biology Division staff members.

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

## Appendices

### Advisory Committee - FY 1984

|                      |   |
|----------------------|---|
| Dr. Robert Barker    | Vice President for Research, 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       |
| Dr. Gerald N. Wogan  | Head, Department of Nutrition and Food Sciences, Massachusetts Institute of Technology, Cambridge, Maryland |

### 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 August 1, 1982 through September 30, 1983.

| Speaker                       | Affiliation  | Subject   |
|-------------------------------|--|---|
| Anderson, Norman              | Argonne National Laboratory<br>Argonne, Illinois   | Human protein index.  |
| Bachhawat, B. K. <sup>a</sup> | Indian Institute of<br>Chemical Biology<br>Calcutta, India   | Use of liposomes in drug delivery and<br>immunology                   |
| Bantle, Jack                  | Oklahoma State University<br>Stillwater, Oklahoma  | The use of Xenopus oocytes as a gene toxicity<br>and expression assay |
| Brodie, Arnold <sup>a</sup>   | National Institute of<br>Environmental Health<br>Sciences<br>Research Triangle Park,<br>North Carolina | Initial pathogenesis of asbestos-induced<br>lung disease              |
| Chirgwin, John                | Washington Medical School<br>St. Louis, Missouri   | Molecular genetics of insulin and renin in<br>man and mouse           |
| Collins, Andrew               | University of Cambridge<br>Cambridge, England  | Inhibition of DNA repair — why bother?                                |
| Crespi, C. L.                 | Massachusetts Institute of<br>Technology<br>Cambridge, Massachusetts                                   | Human lymphoblasts that metabolize mutagens                           |

|                               |   |   |
|-------------------------------|---|---|
| Doyle, Darrell                | Roswell Park Memorial<br>Institute<br>Buffalo, New York                     | Metabolism of plasma membrane proteins in<br>hepatocytes  |
| DuFrain, R. J. <sup>b</sup>   | Oak Ridge Associated<br>Universities<br>Oak Ridge, Tennessee                | Cytogenetic analysis of somatic and female<br>germ cell damage in neonatal rabbits:<br>A preliminary report |
| Farkas, W. R. <sup>a</sup>    | University of Tennessee<br>Memorial Research Center<br>Knoxville, Tennessee | Role of diets in the synthesis of Q family<br>transfer RNAs   |
| Fuscoe, James C. <sup>b</sup> | Baylor College of Medicine<br>Houston, Texas                                | Deletion and amplification events at the<br><u>hprt</u> locus in Chinese hamster cells                      |
| Gratzner, Howard <sup>c</sup> | University of Miami<br>School of Medicine<br>Miami, Florida                 | DNA replication and cell kinetics by<br>immunofluorescence techniques                                       |
| Hosokawa, K. <sup>a</sup>     | Kawasaki Medical School<br>Kurashiki, Japan                                 | Structure and function of adenovirus<br>chromatin   |
| Lehrach, H.                   | European Molecular Biology<br>Laboratory<br>Heidelberg, Germany             | Cloning of gene regions containing<br>developmental mutations: The T-t complex<br>in the mouse              |
| Levine, Arnold <sup>a</sup>   | State University of<br>New York at Stony Brook<br>Stony Brook, New York     | Regulation of cellular gene expression in<br>transformed cells  |
| Lewis, Susan E. <sup>b</sup>  | Research Triangle Institute<br>Research Triangle Park,<br>North Carolina    | Electrophoretically detected specific locus<br>mutations in mice  |

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|-------------------------------|--|--|
| Lieber, H. L.                 | Massachusetts Institute of Technology<br>Cambridge, Massachusetts                      | Mutagenicity of 1-2-5 iododeoxyuridine and tritiated thymidine in human lymphoblasts                             |
| Lindenschmidt, R. C.          | Indiana University<br>Indianapolis, Indiana  | Paraquat-induced pulmonary edema in the perfused lung  |
| Maio, Joseph J.               | Albert Einstein College of Medicine<br>New York, New York                              | Kpn families, interspersed DNA's and human gene organization   |
| Morgan, K.                    | Chemical Industry Institute of Toxicology<br>Research Triangle Park,<br>North Carolina | The nose: Structure, function and toxicity, with special reference to formaldehyde and the mucociliary apparatus |
| Moseley, E. B. <sup>a</sup>   | University of Edinburgh<br>Scotland  | DNA repair in <i>micrococcus</i>   |
| Natarajan, A. T.              | University of Leiden<br>The Netherlands  | Relationship between sister chromatid exchanges, chromosome aberrations and point mutations                      |
| Nicholas, Robert <sup>d</sup> | University of California<br>at San Diego<br>San Diego, California                      | Purification of membrane-spanning peptides from Na/K-ATPase  |
| Pincheira, Guido              | University of Chile<br>Santiago, Chile   | The current status of basic research in Chile  |
| Plaa, G. L.                   | Faculty of Medicine<br>University of Montreal<br>Montreal, Quebec, Canada              | Chemical interaction in the potentiation of toxic liver damage   |

|                           |  |   |
|---------------------------|--|---|
| Plewa, M. J.              | Institute for Environmental Studies<br>University of Illinois<br>Urbana, Illinois                | The activation of promutagens by plant cells in culture   |
| Revis, N. W. <sup>a</sup> | Oak Ridge Research Institute<br>Oak Ridge, Tennessee   | The relationship of Pb, Cd and Cl to the induction of hypertension and atherosclerosis  |
| Schleich, Thomas          | University of California<br>Santa Cruz, California   | Biochemistry as studied by proton NMR spectroscopy: A survey of recent results with specific application to damage in whole tissue including the intact eye |
| Skow, L. C. <sup>b</sup>  | National Institute of Environmental Health Sciences<br>Research Triangle Park,<br>North Carolina | A mouse model of human beta thalassemia   |
| Thilly, W. G.             | Massachusetts Institute of Technology<br>Cambridge, Massachusetts                                | Mutational spectra in human lymphoblasts  |
| Tyrrell, R. M.            | Swiss Institute of Experimental Cancer Research<br>Lausanne, Switzerland                         | Repair of ultraviolet radiation damage in cultured human cells  |

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<sup>a</sup>Cancer Research Seminar

<sup>b</sup>Genetics Seminar

<sup>c</sup>Genetics and Developmental Biology Seminar Series

<sup>d</sup>Biochemistry Journal Club

### Research Conferences

A Workshop on Neutron Radiation Carcinogenesis, sponsored by the Office of Health and Environmental Research of the U.S. Department of Energy and the Low-Level Radiation Effects Branch of the National Cancer Institute, and hosted by the Biology Division, was held at The Oak Ridge Associated Universities conference center on September 19-20, 1983. The Workshop was attended by research workers from Europe and the United States, as well as representatives of many government agencies. This Workshop was an activity of the newly formed External Radiation Carcinogenesis Interlaboratory Group.

A round table discussion on "In vitro Radiation Transformation" was organized and hosted by the Biology Division and chaired by Dr. M. Terzaghi. The discussion took place on September 21, 1983, and was attended by radio-biologists from across the country.

The first Regional Flow Cytometry User's Meeting took place on September 23, 1983 under the sponsorship of the Biology Division. The chairman of the organizing committee was Dr. Reinhold Manz of the Biology Division. The meeting was held at the conference center of the Oak Ridge Associated Universities.

### 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
- D. M. Skinner - Chairperson-Elect, Section G, Biological Sciences, American Association for the Advancement of Science, 1983

## 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 - USA National Committee for the International Union of Physiological Sciences (Vice Chairman), 1977-1982  
Publications Committee, Society of General Physiologists, 1982-1986  
Publications Committee, Federation of American Societies for Experimental Biology, 1982-1985  
Membrane Biophysics Subgroup (Chairman), Biophysical Society, 1984
- 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
- F. C. Hartman - Nominating Committee, American Society of Biological Chemists, 1982  
Nominating Committee, American Chemical Society, Division of Biological Chemistry, 1982

- 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 - Awards and Honors Committee, Environmental Mutagen Society, 1983-present
- L. B. Russell - Publications Policy Committee, Environmental Mutagen Society, 1981-1982  
Program Committee (Chairman), Environmental Mutagen Society, 1983-1984
- D. M. Skinner - Committee on Equal Opportunities for Women, American Society of Biological Chemists, 1980-1983  
Public Policy Committee, American Society for Cell Biology, 1981-present

### 3. Advisory Committees

- 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)  
Member of the Corporation, Mount Desert Island Biological Laboratory  
Advisory Board to Current Topics in Membranes and Transport, 1983
- 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  
Program Advisory Committee (Chairman), Lawrence Berkeley Laboratory, 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 - Pesticide Information Review and Evaluation Committee, National Research Council, 1981-present  
 Subcommittee to Evaluate Effects of Short-Term Exposures to Drinking Water Contaminants, Committee on Toxicology, National Research Council, 1982-present  
 Committee on Toxicology, National Research Council, 1983-1986  
 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  
 Working Group on Shale Oils, Soots, Creosote, Coal Tars and Pitches, and Asphalt, International Agency for Research on Cancer, Lyon, 1983

A. W. Hsie - Member of Scientific Directorate, Coordinating Council for Cancer Research, Villejuif, France, 1978-present

S. J. Kennel - Study Section on Health Effects Research, Environmental Protection Agency, 1982-present

F. T. Kenney - Advisory Committee for Personnel in Research, American Cancer Society, 1978-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  
 Molecular Biology Study Section (ad hoc member), National Institutes of Health, 1983

Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-  
 Associate, Helicon Foundation, LaJolla, California, 1983-  
 Faculty Senate, University of Tennessee, 1983-1986  
 Gordon Conference on Chromatin, 1984, 1986 (Co-chairman)

R. J. Preston - Cytogenetic Adviser to Ethylene Oxide Council and to Health Industry Manufacturers Association, 1981-present  
 Health Effects Adviser to Clinch River Breeder Reactor Project, 1981-1983  
 Genetics Working Group, American National Standards Institute, 1983-

L. B. Russell - Committee I, International Commission for Protection Against Environmental Mutagens and Carcinogens, 1977-present  
 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

G. A. Sega - Site Visit Review Committee, National Institutes of Health, Environmental Health Services Center Grant, 1983

J. K. Selkirk - Breast Cancer Task Force, National Institutes of Health, 1979-1982  
 Committee on Pyrene and Analogs, National Academy of Sciences, 1981-1982  
 Biochemistry Study Section, American Cancer Society, 1983-1988

D. M. Skinner - Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-present  
 Peer Review Committee, Visiting Professorships for Women, National Science Foundation, 1983-

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-

M. Terzaghi - Clinical 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  
 Safe Drinking Water Committee, National Research Council, 1981-1982  
 Review Committee (Chairman), Inhalation Toxicology Division, Health Effects Research Laboratory, Environmental Protection Agency, 1982-present  
 Ozone Program Project Committee, Primate Center, University of California-Davis, 1983

#### 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*, 1981-present  
*Cell and General Physiology*, *American Physiological Society Handbook Series*, 1982-1984

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 - *Mutation Research*, 1976-1982  
*Fundamental and Applied Toxicology*, 1983-  
*Teratogenesis, Carcinogenesis, and Mutagenesis*, 1983-

P. Mazur - *Cryobiology*, 1967-present  
*Cryo-Letters*, 1979-1982  
*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*, 1982

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-

L. B. Russell - *Mutation Research*, 1976-present  
*Environmental Mutagenesis*, 1978-1983

J. S. 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

### 5. Awards

**S. Mitra** - **NSF U.S./India Scientists Exchange Fellowship, 1982**

**A. L. Olins** - **Naito Foundation of Japan Award, 1982**

**L. B. Russell** - **Corporate Research Fellow, Union Carbide Corporation, 1983**

**J. B. Storer** - **Distinguished Service Award, The University of Chicago, 1983**  
- **Corporate Research Fellow, Union Carbide Corporation, 1983**

**H. R. Witschi** - **Diplomate, Board of Toxicological Science, 1982**

ABSTRACTS FOR TECHNICAL MEETINGS HELD  
AUGUST 1, 1982 - SEPTEMBER 30, 1983

Aardema, M. J., W. W. Au, and R. J. Preston. A comparison of the sensitivity to X-ray induced chromosome aberrations in myeloid leukemia cells and normal bone marrow cells. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Adler, Howard I., and Weldon D. Crow. An unusual method for producing anaerobic conditions. 31st Annual Meeting, Radiation Research Society, San Antonio, Texas, February 27-March 3, 1983.

Allen, R. L., and D. E. Ollins. Examination of chromatin replication in the ciliated protozoa (hypotricha) with silver staining and thiol-specific coumarin maleimide. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Amsler, K., and J. S. Cook. Development of  $\text{Na}^+$ -dependent phlorizin binding sites in a clone of LLC-PK<sub>1</sub> cells. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Au, William W., Henry E. Luippold, J. Patrick O'Neill, and R. Julian Preston. Neocarzinostatin induces chromosome aberrations and mutation but not sister chromatid exchanges in CHO cells. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Bhattacharyya, A., B. C. Pal, and S. Mitra. Synthesis of radio-labelled  $\text{O}^4$ -ethylthymidine 5'-triphosphate: A novel approach to study the repair of  $\text{O}^4$ -alkylthymidine. American Chemical Society, Washington, D.C., August 29-September 2, 1983.

Bonnewell, V., R. F. Fowler, and D. M. Skinner. Sequence augmentation by amplification of an (A+T)-rich 142 bp segment in a (G+C)-rich satellite DNA. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Boone, L. R., C.-Y. Ou, F. E. Myer, D.-M. Yang, R. W. Tennant, and W. K. Yang. Analysis of the Fv-1 gene target in N-tropic and B-tropic MuLV by restriction fragment exchange and nucleotide sequence analysis. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 25-29, 1983.

Boone, L. R., W. K. Yang, and R. W. Tennant. Fine Structure Analysis of Retroviral LTRs: Implications for Induced Gene Transpositions in Mammalian Cells. New Building Dedication, NIEHS, Research Triangle Park, North Carolina, November 15, 1982.

Bunick, G. J., E. C. Uberbacher, and D. E. Olins. Neutron scattering and X-ray diffraction of nucleosomes and nucleosome - HMG complexes. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Carrier, William L., William H. Lee, and James D. Regan. Studies on ultraviolet light induction and excision repair of pyrimidine dimers in human skin fibroblasts. International Congress of Radiation Research, Amsterdam, The Netherlands, July 3-8, 1983.

Carrier, W. L., and J. D. Regan. Studies on UV-light induction and excision of pyrimidine dimers in the DNA of mammalian cells. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Castellani, Amleto, William L. Carrier, and James D. Regan. The effect of hydroxyurea (HU) on excision repair of ultraviolet light induced pyrimidine dimers in proliferating and quiescent human fibroblasts. International Congress of Radiation Research, Amsterdam, The Netherlands, July 3-8, 1983.

DeMarini, D. M., T. K. Rao, J. L. Epler, and A. W. Hsie. Mutagenicity of crude and neutral fractions of liquified coal oils in the *Salmonella* and CHO/HGPRT assays. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Dodson, L. A., R. S. Foote, S. Mitra, and W. E. Masker. Mutagenesis of bacteriophage T7. UCLA Symposia, Keystone, Colorado, April 10-15, 1983.

Dodson, Lori A., and Warren E. Masker. In vivo and in vitro mutagenesis of bacteriophage T7. American Society for Microbiology, Nashville, Tennessee, November 5-6, 1982.

Donnelly, Mark I., Fred C. Hartman, and V. R. Ramakrishnan. Activation of ribulosebisphosphate carboxylase/oxygenase from Rhodospirillum rubrum occurs without large conformational changes. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Donnelly, Mark I., V. R. Ramakrishnan, and Fred C. Hartman. Chemical and physical characterization of the  $\text{CO}_2/\text{Mg}^{2+}$ -induced activation of ribulosebisphosphate carboxylase/oxygenase from Rhodospirillum rubrum. Sixth International Congress on Photosynthesis, Vrije Universiteit Brussel, Brussels, Belgium, August 1-6, 1983.

Donnelly, M. I., C. D. Stringer, and F. C. Hartman. Characterization of the activator site of Rhodospirillum rubrum ribulosebisphosphate carboxylase/oxygenase. American Chemical Society, Washington, D.C., August 28-September 2, 1983.

Dumont, J. N., J. L. Epler, and T. W. Schultz. Mutagenic, embryotoxic and teratogenic effects of complex mixtures of synfuel materials. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Dumont, J. N., T. W. Schultz, and R. G. Epler. The response of the FETAX model to mammalian teratogens. Teratology Society, Atlantic City, New Jersey, June 26-29, 1983.

Epler, J. L. Cancer in the workplace: chemical industries - petroleum. International Conference on "Cancer in the Workplace," Vancouver, British Columbia, Canada, May 16-18, 1983.

Epler, J. L. Identification of hazardous components within complex energy effluents and products. Third International Congress on Toxicology, San Diego, California, August 28-September 3, 1983.

Ethier, S. P., and R. L. Ullrich. Factors influencing expression of mammary ductal dysplasia in BALB/c mice. American Association for Cancer Research, San Diego, California, May 25-28, 1983.

Fishman, J. B., and J. S. Cook. Heterogeneous recycling of surface sialoglycoproteins in HeLa cells. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Foote, Robert S., Abraham W. Hsie, Bimal C. Pal, and Sankar Mitra.  $\text{O}^6$ -Methylguanine-DNA methyltransferase in mammalian cells. UCLA Symposia, Keystone, Colorado, April 10-15, 1983.

Fowler, R. F., V. Bonnewell, and D. M. Skinner. Unusual sequence features in the repeat unit of a (G+C)-rich crab satellite DNA. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Francis, Andrew A., and James D. Regan. Photosensitivity of human DNA following UV irradiation. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Fry, R. J. M., and D. S. Nachtwey. Health effects of the radiation environment in space. Radiation Research Society, San Antonio, Texas, February 27-March 3, 1983.

Fry, R. J. M., and J. B. Storer. Late effects of radiation: Host factors. International Congress of Radiation Research, Amsterdam, The Netherlands, July 3-8, 1983.

Fujimura, Robert K., and Barbara C. Roop. Interactions of the product of gene D5 of bacteriophage T5 with DNA and DNA polymerase. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Fujimura, Robert K., and Barbara C. Roop. Mechanism of inhibition of DNA synthesis in vitro by a double-stranded DNA binding protein induced by bacteriophage T5. UCLA Symposium, Keystone, Colorado, April 3-9, 1983.

Glover, P. L., and E. H. Perkins. Antigen-induced proliferation of thioglycolate-elicited mouse peritoneal macrophages in vitro under normal cultural conditions. 19th National Reticuloendothelial Society, St. Louis, Missouri, October 16-20, 1982.

Hearl, W. G., D. Dorsett, and K. B. Jacobson. Synthesis of the common precursor of sepiapterin and drosopterins in *Drosophila*. 7th International Symposia on Pteridines and Folic Acid Derivatives, Chemical, Biological and Clinical Aspects, St. Andrews, Scotland, September 21-24, 1982.

Heartlein, M. W., and R. J. Preston. An explanation of interspecific differences in sensitivity to X-ray-induced chromosome aberrations. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Heckman, C. A., S. E. Ludemann, A. E. Campbell, and Y. B. Shah. Cell shape changes in oncogenic transformation. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Hellwig, R. J., S. N. Sinha, and S. K. Niyogi. Specific transcription of ordered nucleoprotein complexes containing the adenovirus 2 major late promoter by RNA polymerase II in a HeLa cell-free system. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Herndon, Cinda S., and Fred C. Hartman. Affinity labeling of an active-site histidyl residue in ribulosebisphosphate (RuBP) carboxylase/oxygenase. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Hitotsumachi, S., D. A. Carpenter, and W. L. Russell. Dose fractionation as a means of increasing the mutagenic effectiveness of ethylnitrosourea in mouse spermatogonia. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Hotchkiss, J. A., and S. J. Kennel. Monoclonal antibodies to normal mouse lung. Second Annual Congress for Hybridoma Research, Philadelphia, Pennsylvania, February 6-10, 1983.

Hsie, A. W. Quantitative analysis of mammalian cell mutagenesis. 13th Annual Meeting, European Environmental Mutagen Society, Montpellier, France, September 5-9, 1983.

Isham, Kenneth R., Robin Rothrock, Kai-Lin Lee, and Francis T. Kenney. Transcriptional and translational control in development of tyrosine aminotransferase. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Jacobson, K. B., E. H. Lee, J. M. Flanagan, D. R. Hare, and Brian R. Reed.  $Zn^{2+}$  causes unique alterations of tRNA conformation. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Jacobson, K. B., E. H. Lee, J. M. Flanagan, B. R. Reid, and D. R. Hare. Alteration of tRNA conformation by zinc ions. tRNA Workshop, Hakone, Japan, March 22-25, 1983.

Jacobson, K. B., J. J. Yim, and C. R. Wobbe. Enzymatic localization of the site of action of a suppressor mutant that restores the biosynthesis of drosopoterins in the purple mutant of *Drosophila melanogaster*. 7th International Symposium on Pteridines and Folic Acid Derivatives, Chemical, Biological and Clinical Aspects, St. Andrews, Scotland, September 21-24, 1982.

Jamasbi, Roudabeh J., and Eugene H. Perkins. Production of vaccine to DEN-induced carcinomas by in vitro attenuation. American Association for Cancer Research, San Diego, California, May 25-28, 1983.

Kao, J., J. W. Hall, and J. M. Holland. A method for in vitro cutaneous toxicity evaluation. Joint meeting of the American Society for Pharmacology and Experimental Therapeutics, and the Society of Toxicology, Louisville, Kentucky, August 15-19, 1982.

Kao, J., L. R. Shugart, and J. M. Holland. An in vitro approach to studying cutaneous metabolism and disposition of topically applied xenobiotics. 3rd International Congress on Toxicology, San Diego, California, August 28-September 3, 1983.

Kennel, S. J., and P. K. Lankford. Two site double monoclonal antibody assay for fragment D of human fibrinogen. Second Annual Congress for Hybridoma Research, Philadelphia, Pennsylvania, February 6-10, 1983.

Lalley, P. A., and J. M. Chirgwin. Mapping of mouse insulin genes. VIIth International Workshop on Human Gene Mapping, Los Angeles, California, August 21-27, 1983.

Lalley, P. A., and J. A. Diaz. Comparative gene mapping in the mouse involving genes assigned to human chromosomes 7 and 20. VIIth International Workshop on Human Gene Mapping, Los Angeles, California, August 21-27, 1983.

Lalley, P. A., J. A. Diaz, A. A. Francis, W. C. Dunn, W. L. Carrier, and J. D. Regan. The expression and chromosomal assignments of genes required for repair of UV-induced DNA damage. VIIth International Workshop on Human Gene Mapping, Los Angeles, California, August 21-27, 1983.

Lalley, Peter A., J. A. Diaz, A. A. Francis, W. C. Dunn, W. L. Carrier, and James D. Regan. DNA repair: genetic dissection and chromosomal location of the genes required for ultraviolet radiation induced DNA damage in man using human X mouse somatic cell hybrids. International Congress of Radiation Research, Amsterdam, The Netherlands, July 3-8, 1983.

Lalley, P. A., J. A. Diaz, A. A. Francis, W. C. Dunn, and J. D. Regan. Genetic analysis of repair of UV-induced DNA damage in man using somatic cell hybrids. Environmental Mutagen Society. San Antonio, Texas, March 3-6, 1983.

Lalley, P. A., J. A. Diaz, A. A. Francis, W. C. Dunn, and J. D. Regan. The expression and chromosomal assignments of genes required for repair of UV-induced DNA damage in man. Radiation Research Society, San Antonio, Texas, February 27-March 3, 1983.

Larimer, F. W., A. A. Hardigree, M. Fillenwarth, R. S. Foote, and S. Mitra. Characterization of N-methyl-N-nitrosourea mutagenesis in Saccharomyces cerevisiae: allele-specificity and repair dependence. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Laughlin, T. J., R. L. Allen, A. P. Butler, A. L. Herrmann, C. L. Cadilla, and D. E. Olins. The chromatin organization of the active and inactive genes of the binucleated eukaryote Oxytricha. Symposium in Molecular Biology, University Park, Pennsylvania, July 27-29, 1983.

Laughlin, T. J., J. M. Henry, A. P. Butler, and D. E. Olins. The structure of gene-sized chromatin fragments from the macronucleus of Oxytricha nova. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Lewis, Susan E., F. M. Johnson, L. C. Skow, Lois B. Barnett, and R. A. Popp. A mutation in the Hbb<sup>8</sup> gene induced in a female by ethylnitrosourea. Twelfth Biochemical Genetics Workshop, Bar Harbor, Maine, August 29-September 2, 1983.

Lewis, S. E., R. A. Popp, F. M. Johnson, and L. C. Skow. A spontaneously arisen  $\beta$ -thalassemia mutant in mice. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Ley, R. D., and R. J. M. Fry. Ultraviolet radiation-induced changes in the optical properties of hairless, albino mouse epidermis. International Congress of Radiation Research, Amsterdam, The Netherlands, July 3-8, 1983.

Machanoff, Richard. Enzyme degradation as a factor in phenotypic expression. UCLA Symposia, Keystone, Colorado, March 26-April 1, 1983.

Mann, R. C., G. R. Braslawsky, and R. E. Hand, Jr. Statistical analysis of DNA histograms measured in flow systems. Combined International Conference on Analytical Cytology IX, Schloss Elmau, Mittenwald Bavaria, Federal Republic of Germany, October 17-23, 1982.

Marchok, A. C., S. F. Huang, and D. H. Martin. Growth dynamics and progression of neoplasia in presumptive neoplastic cell populations (PNCP) from rat tracheas exposed to dimethylbenz[a]anthracene (DMBA). American Association for Cancer Research, San Diego, California, May 25-28, 1983.

Mazur, Peter. Fundamentals of the freezing of organs and cells: similarities and differences. Symposium on the Cryopreservation of Organs, Society for Cryobiology, Cambridge, England, August 30-September 2, 1983.

Mazur, Peter. Responses of living cells to the events occurring during freezing and thawing. American Association for the Advancement of Science Symposium on "The Preservation of Germplasm," Detroit, Michigan, May 29, 1983.

McKinley, Marcus D., and Bimal C. Pal. Environmental pollutant 5-chlorouracil is incorporated in mouse liver RNA. American Chemical Society, Washington, D.C., August 29-September 2, 1983.

Mitra, Sankar, Robert S. Foote, Michael J. Fillenwarth, Bimal C. Pal, Abraham W. Hsie, and Charles T. Hadden. Adaptive response of bacteria and mammalian cells to N-methyl-N'-nitro-N-nitroso-guanidine. Radiation Research Society, San Antonio, Texas, February 27-March 3, 1983.

Mitra, Sankar, Robert S. Foote, and Charles T. Hadden. Adaptive response in Bacillus subtilis. UCLA Symposia, Keystone, Colorado, April 10-15, 1983.

Moore, K. L., and P. A. Lalley. Comparative mapping in man and baboon (Papio papio) involving genes assigned to human chromosomes 4, 6, 14, 15 and 20. VIIth International Workshop on Human Gene Mapping, Los Angeles, California, August 21-27, 1983.

Mykles, Donald L., and Dorothy M. Skinner. Crab calcium-dependent proteinase that degrades actin, myosin, and other myofibrillar proteins. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Mykles, D. L., and D. M. Skinner. Crustacean  $Ca^{2+}$ -dependent proteinase that degrades actin, myosin, and other myofibrillar proteins. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

Mykles, D. L., and D. M. Skinner. Role of  $\text{Ca}^{2+}$ -dependent proteinase in proecdysial claw muscle atrophy. American Society of Zoologists, Louisville, Kentucky, December 27-30, 1982.

Nix, C. E., and L. C. Waters. Influence of genotype on mutation induction in Drosophila melanogaster. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Olins, D. E., A. L. Olins, H. A. Levy, R. C. Durfee, S. M. Margle, E. P. Tinnel, and S. D. Dover. Electron microscope tomography: transcription in 3-D. American Society for Cell Biology, Baltimore, Maryland, November 30-December 4, 1982.

O'Neill, J. P., M. W. Heartlein, and R. J. Preston. SCE and gene mutation are induced by the replication of BrdU and Cldu-substituted DNA. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Ou, C. Y., L. R. Boone, Gloria Boone, and W. K. Yang. A novel mouse-specific interdispersed short repetitive sequence found in the LTK of mouse MuLV-related proviral sequences of mouse genome. Cold Spring Harbor Symposium, Cold Spring Harbor, New York, May 25-29, 1983.

Ou, C. Y., L. R. Boone, and W. K. Yang. Sequence characterization of the genetic determinants of N- and B-tropism in murine leukemia viruses. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Perkins, E. H., and P. L. Glover. Sequential thymus grafting of aged mice: characterization of growth, regression and enrichment of immune competence. Gerontological Society of America, Boston, Massachusetts, November 19-23, 1982.

Perkins, E. H., K. K. Peterson, P. L. Glover, and R. E. Hand, Jr. Macrophage dependency of the proliferative response of human T cell subpopulations stimulated with PHA and Con A: Quantitative differences. Federation of American Societies for Experimental Biology, Chicago, Illinois, April 10-15, 1983.

Popp, D. M., and R. A. Popp. The use of an in vivo mutation assay system to quantitate somatic mutation rate in murine hemopoietic stem cells. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Popp, R. A., E. G. Bailiff, L. C. Skow, F. M. Johnson, and S. E. Lewis. Analysis of a mouse  $\alpha$ -globin gene mutation induced by ethylnitrosourea. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Popp, R. A., L. C. Skow, F. M. Johnson, S. E. Lewis, W. F. Anderson, and S. Z. Goldberg. Mouse model of beta thalassemia. Gordon Research Conference, New London, New Hampshire, August 15, 1983.

Preston, R. J. In vitro and in vivo cytogenetic effects. Seminars on Ethylene Oxide Worker Safety, Chicago, Illinois, October 4-5, 1982.

Recio, L., B. Chastenay, R. J. Albertini, and A. W. Hsie. Detection of 6-thioguanine resistant spleenocytes from Chinese hamsters. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Rothrock, Robin, Kenneth R. Isham, Kai-Lin Lee, and Francis T. Kenney. Activation of hepatic differentiation by 5-azacytidine. American Society of Biological Chemists, San Francisco, California, June 5-9, 1983.

Russell, W. L., and P. R. Hunsicker. Extreme sensitivity of one particular germ-cell stage in male mice to induction of specific-locus mutations by methylnitrosourea. Environmental Mutagen Society, San Antonio, Texas, March 3-6, 1983.

Schneider, U., and P. Mazur. Changes in cell volume of mammalian zygotes under hyperosmotic or hypoosmotic conditions. Society for Cryobiology, Cambridge, England, August 30-September 2, 1983.

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| <b>Funding Source</b>                                  | <b>Funding in<br/>thousands</b> | <b>% of total<br/>budget</b> | <b>Person-years</b> |
|--|---------------------------------|------------------------------|---------------------|
| Department of Energy                                   | 13,265                          | 76.3                         | 153.0               |
| National Cancer Institute                              | 1,437                           | 8.3                          | 18.4                |
| Environmental Protection Agency                        | 667                             | 3.8                          | 12.0                |
| National Institute of Environmental<br>Health Sciences | 1,168                           | 6.7                          | 13.9                |
| Department of Defense                                  | 506                             | 2.9                          | 7.4                 |
| National Institute of General<br>Medical Sciences      | 65                              | 0.4                          | 0.9                 |
| Department of Agriculture                              | 46                              | 0.3                          | 0.1                 |
| Miscellaneous  | 228                             | 1.3                          | 4.3                 |
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