DOE Contractor's Meeting on Chemical Toxicity

June 23 -26, 1987

Monterey Beach Hotel
Monterey, Ca.

Organized and Sponsored by:
Office of Health & Environmental Research,
U.S. Department of Energy, and
Biomedical Sciences Division,
Lawrence Livermore National Laboratory

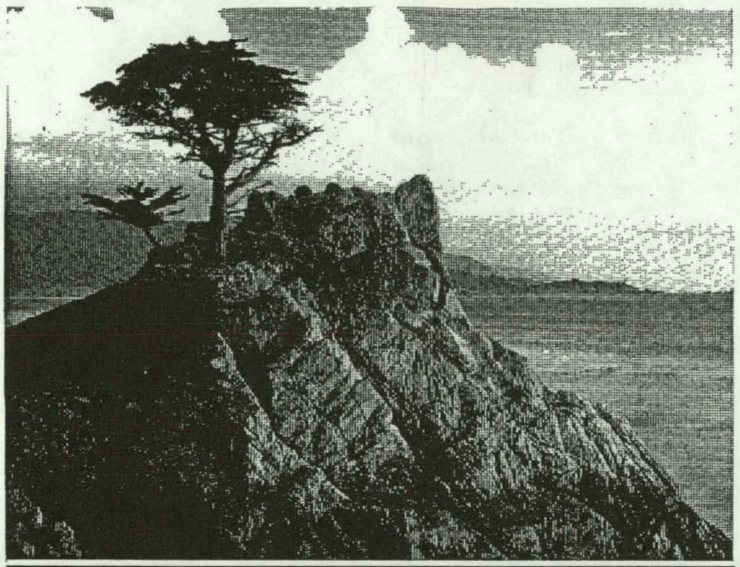
DISCLAIMER

Price: Printed Copy A11
Microfiche A01

DOE/ER-0343
UC-48

DOE/ER--0343

DE88 000256



DOE Contractor's Meeting on Chemical Toxicity

June 23 -26, 1987

Monterey Beach Hotel
Monterey, Ca.

Organized and Sponsored by:
Office of Health & Environmental Research,
U.S. Department of Energy, and
Biomedical Sciences Division,
Lawrence Livermore National Laboratory

THIS PAGE

WAS INTENTIONALLY

LEFT BLANK

FORWARD

The Department of Energy's Office of Health and Environmental Research held its Third Annual Contractor's meeting on the health and environmental effects of energy-related chemicals on June 24-26, 1987. Fifty one investigators, representing six universities and seven national laboratories, attended the meeting. The 49 presentations spanned two and one half days, focused on current research findings, and explored opportunities for future research.

The meeting covered a wide spectrum of disciplines and research supported by OHER. It provided a forum at which scientists from diverse, but related, areas summarized current OHER research activities. This successful meeting provided an exceptional opportunity for OHER supported scientist to exchange information and make new contacts for potential collaboration in research. Most of the research projects are directed towards understanding carcinogenic processes and the effects of chemicals on those processes. Some projects are directed towards chemical analyses and non-carcinogenic endpoints of toxicity. Details are contained in the abstracts of the presentations and the associated session reviews.

Robert W. Wood, Ph.D.
Acting Associate Director for
Health and Environmental Research
Office of Energy Research

This meeting was organized by Dr. Bart Gledhill and hosted by Lawrence Livermore National Laboratory. It was supported by DOE/OHER Contract No. LLSFENG4B.

Project Managed By:

Dr. James Beall and Dr. Chris Reilly

DOE/OHER CONTRACTORS' WORKSHOP ON CHEMICAL TOXICITY MONTEREY BEACH HOTEL, MONTEREY, CALIFORNIA

23 - 26 JUNE, 1987

TABLE OF CONTENTS

SESSION IV METABOLISM AND GENOTOXICITY

SESSION V INHALATION TOXICOLOGY

EXECUTIVE SUMMARY

The Office of Health and Environmental Research (OHER) of the Department of Energy (DOE) has a programmatic need to determine the potential health and environmental effects associated with energy production and use. This need is addressed through a limited number of fundamental research projects directed at understanding effects of chemicals on biological systems. To ensure appropriate communication among investigators and scientific disciplines that these research studies represent, OHER has sponsored a series of workshops. These workshops provide for exchange of ideas and information, stimulate improved collaboration, and form a basis for future research planning.

This document provides a compilation of activities at the Third Annual DOE/OHER Workshop; which was hosted by Lawrence Livermore National Laboratory and held in Monterey, California, on June 24-26, 1987. The first two workshops focused on activities specifically related to DOE's Complex Chemical Mixtures Program. With the termination of that program, this year's workshop was broadened to include all OHER activities identified as within the chemical effects area. It included 51 participants representing seven DOE national laboratories and six universities. The workshop consisted of eight sessions entitled: Isolation and Detection of Toxic Chemicals; Adduct Formation and Repair; Chemical Toxicity (Posters); Metabolism and Genotoxicity; Inhalation Toxicology; Gene Regulation; Metals Toxicity; and Biological Mechanisms. Dr. Alexander Wood of Roche Institute gave the keynote address. It described the metabolism of polyaromatic hydrocarbons and provided a common ground for the remaining presentations.

This document contains abstracts of the information presented by session. A summary of the observations described in each session precedes the abstracts. There were a number of research issues of particular significance. For example, the need in molecular dosimetry and molecular mechanism studies for methods applicable to trace quantities (e.g., parts per billion concentrations) has lead to new applications of chemical analyses (selected ion monitoring and fourier transform mass spectrometry) and biological based analytical methods (e.g. monoclonal antibodies). As an indication of the importance of chemical interactions with DNA, an entire session was devoted to DNA adducts. Papers in that session dealt with adduct distribution, mechanisms of adduct formation, physiocochemical and structural properties of adducts, and biological consequences of adduct formation. DOE probably supports the only theoretical research aimed at defining and simulating visually at an atomic level the structures of DNA adducts. This work utilizes DOE's super computing capabilities that allow development of molecular structures using minimized potential energy calculations.

The ability to manipulate metabolism of genotoxic and carcinogenic agents holds the potential for lowering the potential risk of humans to their effects. Thus, there were a number of important studies of the detoxification and metabolic mechanisms. The studies encompassed topics

ranging from molecular regulation to environmental monitoring and included such topics as imprinting (developmental induction resulting in alter gene expression in adult life), regulation of inducible genes needed for metabolism, and comparative metabolism in different organisms.

The inhalation studies that were reported at the meeting illustrate the breath of DOE's fundamental interest in the mechanisms by which inhaled chemicals produce adverse effects on human health. Work presented ranged from mechanisms of deposition and clearance to the mechanisms by which the dose delivered to tissues cause biological effects. These studies by their nature generally involve whole animals and the workshop allowed close interactions between the inhalation toxicologist and the molecular biologist helping to provide the necessary bridges between cellular and molecular studies and findings in animals.

The role metals, carcinogens, and promoters play in the modulation of the regulation of gene expression was a topic of considerable interest. Presentations in this area emphasized the importance of obtaining fundamental knowledge in the areas of regulation of transcription in normal systems as a prerequisite to understanding how metals and chemicals alter these controls and produce pathological changes. Efforts to identify specific sites of interactions have lead to fundamental insights into mechanisms of gene regulation that have broad importance.

Studies were reported that deal with animal models for evaluating reproductive consequences of chemical exposures. The importance of mutational effects in parental gametes and of interference with implantation and maturation were discussed. Finally, information on the carcinogenic process is coming from in vitro studies of both cell transformation and normal differentiation (cancer cells often appear as normal cells blocked in their normal differentiation).

For sake of completeness, abstracts that were submitted for the program, but were not presented, are included in the document.

---

1. Argonne National Laboratory, Battelle Pacific Northwest Laboratory, Lawrence Livermore National Laboratory, Lawrence Berkeley Laboratory, the Lovelace Inhalation Toxicology Research Institute, and Oak Ridge National Laboratory.

2. The University of California, Los Angeles; University of Chicago; Johns Hopkins University; Michigan State University; New York University; and University of Rochester.

Dr. Alexander Wood's Keynote Lecture

Dr. Wood focused on the prevalence and metabolism of polyaromatic hydrocarbons (PAH). He described the Roche/NIH overall approach to identifying the ultimate carcinogen metabolite(s) of PAH. This has required stereospecific synthesis of many of the intermediates, examination of the cytochrome P-450 and epoxide hydrolase metabolic pathways, examination of bacterial and mammalian mutagenicity, and analysis of whole-animal carcinogenicity. Their work has lead to the "bay region theory" of diol-epoxide metabolite formation for a number of PAHs. In addition, they have been able to demonstrate the specific response for Ames/*Salmonella*, V79, and tumorigenicity for the different stereoisomers of the B[a]P diol-epoxides. Current work is focusing on oncogene activation by a series of the PAH metabolites.

METABOLIC AND CHEMICAL DETERMINANTS OF POLYCYCLIC HYDROCARBON INDUCED CANCER

Alexander Wood[1], Wayne Levin[1], Allan Conney[1], Roland Lehr[2], Dhiren Thakker[3], H. Yagi[3] and Donald Jerina[3]

[1]Roche Research Center, Nutley, NJ 07110, [2]Univ. OK, Norman, OK 73019 and [3]NIADDK, NIH, Bethesda, MD 20892

Polycyclic aromatic hydrocarbons are one of the most prevalent classes of environmental carcinogens to which man is exposed. The hydrocarbons are produced during the incomplete oxidation of organic mattter and are found in automobile exhaust, cigarette smoke, burning garbage, and numerous other combustion processes. It is estimated that 1,300 tons of one pentacyclic hydrocarbon, benzo[a]pyrene (B[a]P), are released into the environment each year in the U.S.A. alone. The magnitude of this exposure is suggested by the fact that application of as little as 40 μg of B[a]P once every other week to the backs of mice can produce a 100% incidence of squamous cell carcinomas within a year.

Polycyclic aromatic hydrocarbons are relatively inert chemicals, and it has been known for some time that they must exert their carcinogenic activity through metabolically formed reactive intermediates, commonly termed ultimate carcinogens, which covalently interact with cellular macromolecules such as DNA, RNA, and protein.

For almost 15 years our laboratories have explored the nature of the ultimate carcinogens of polycyclic hydrocarbons and the chemical and metabolic determinants of their adverse biological effects. The overall approach to the problem has involved (i) the unequivocal synthesis of the known and potential oxidative metabolites of a number of polycyclic hydrocarbons (ii) examination

of the metabolism of the hydrocarbon and hydrocarbon derivatives by the cytochrome P450-dependent monooxygenase system and epoxide hydrolase, (iii) evaluation of the mutagenicity of the compounds in bacterial and mammalian cells, and finally (iv) determination of the carcinogenic activity of the hydrocarbons and their derivatives in vivo. Application of this approach to over a dozen polycyclic hydrocarbons has led to the formulation and verification of the bay-region theory which states that the formation of a bay-region diol epoxide metabolite of a polycyclic hydrocarbon and its subsequent covalent interaction with a cellular target is a critical initiating event in polycyclic hydrocarbon induced cancer. A bay-region of a polycyclic hydrocarbon is defined as the sterically hindered area that results from the juxtaposition of an angular benzo-ring with a nonadjacent benzene ring; for example, the area between carbon atoms 10 and 11 of benzo[a]pyrene.

Our studies have further revealed a number of metabolic and chemical determinants that form the basis for three orders of magnitude differences in the tumorigenic potencies of polycyclic hydrocarbons. These determinants include the extent to which the hydrocarbon is enzymatically converted by the cytochrome P450-dependent monooxygenase system and epoxide hydrolase to a dihydrodiol with a bay-region double bond, the extent to which this dihydrodiol is metabolized to its bay-region diol epoxide, the intrinsic chemical reactivity of the diol epoxide, the relative and absolute stereochemistry of the hydroxyl and epoxide moieties and the conformation of the hydroxyl groups.

Identification of a common ultimately carcinogenic metabolite of polyaromatic compounds has also stimulated studies on the nature of the critical cellular target(s) of these electrophiles, and on the identification of normal body constituents which may block diol epoxide interaction with these targets.

SESSION 1

Isolation and Detection of Toxic Chemicals

Isolation and Detection of Toxic Chemicals

Summary of Session 1

M. R. Guerin,

Oak Ridge National Laboratory

The focus of recent research on molecular dosimetry and molecular mechanisms of toxicity has increased the need for methods applicable to trace (e.g., ppb) quantities of chemicals in physiological samples. Methods capable of identifying and/or selectively detecting chemicals at the isomeric level in trace quantities are especially important. Research to relate chemical structure with toxicological properties, to develop highly selective measurement methods, and to identify biologically significant constituents continues to require the synthesis of specialty organic and biochemicals.

Progress on the identification of mutagens in prepared food (Felton *et al.*, LLNL) reaffirms the utility of biological testing in combination with chemical separations. The exceptionally high bacterial mutagenicity of aminomethylimidazoazaarenes, the principal mutagens studied, allows detection of the compounds at ppb concentrations in the original sample. Multiple-step chemical separation combined with mutagenicity testing of the separated fractions provides sufficient material for spectroscopic analyses and unambiguous assignment of isomeric structure. Final structural assignment generally requires that the suspect chemical be synthesized to allow spectral matching.

Mass spectrometry (MS) continues to be the most commonly used method for identifying toxic organic chemicals (Bean *et al.*, PNL; Guerin *et al.*, ORNL; Felton *et al*). Quantitative determination of polycyclic aromatic hydrocarbon adducts to DNA (Bean *et al.*) has shown that MS is inherently capable of detecting femtomole quantities of the adducts when the Selected Ion Monitoring (SIM) mode is used. The method is currently limited to approximately 600 pmole for actual samples because of difficulties with sample preparation at the femtomole range. The overall procedure involves isolation of DNA, liberation of the hydrocarbon adduct by acid hydrolysis, conversion of the resulting tetrols to methyl ethers, and GCMS analyses of the ethers using SIM. Benzo[a]pyrene, Chrysene, and benz[a]anthracene have been successfully analyzed using this approach. An important ancilliary finding is that epimerization of the tetrols occurs upon acid hydrolyses.

Fourier Transform Mass Spectrometry (FTMS) is also being studied for its applicability to identifying trace constituents of mixtures (Guerin *et al*). FTMS is particularly useful because of its high resolution, high mass range, and MS/MS capabilities. Laser-desorption negative-ionization conditions are found to yield molecular ions for nucleosides, nucleotides, and trinucleotides. Diagnostic fragmentation allows identification of the bases by exact mass measurement. The ability to trap preselected ions for subsequent reaction followed by mass spectrometry of the products promises to provide structural information as well. The approach has been shown applicable to environmental mixtures by resolving nominally isobaric ions and by collision-induced dissociation of nicotine instrumentally isolated from smoke tar. The study of gas-

phase ion processes has also resulted in a new detector for gas chromatography. The detector, basically on electron capture detector operated at variable reduced pressures, allows tunable selectivity based on the electron affinities of the analytes. Initial results suggest that the detector may also provide a means to experimentally measure the electron affinity of a chemical.

Major progress is being made on the development of immunoassays for environmental analyses and research is underway to apply the technology to measurements of DNA adducts (Vanderlaan *et al.*, Stanker *et al.*; LLNL). Immunoassay methods offer the promise to combine extreme sensitivity (ppb) with stereospecific molecular selectivity. Monoclonal antibody reagents have been prepared to allow the selective quantitative determination of dioxin. The reagent is specific to tetrachloro-and pentachloro-dioxins and especially responsive to the important 2,3,7,8-tetrachloro isomer (TCDD). A competition enzyme-linked immunosorbent assay (c-ELISA) has been developed which detects picogram quantities of 2,3,7,8-TCDD. This monoclonal antibody approach promises to be equally applicable to the development of assays for TCDD in physiological samples as well as assays for other toxic organic chemicals. Related research has already produced antibodies to aminomethylimidazoazaarene mutagens in foods. The technology promises also to be suitable for rapid, low-cost, field measurements.

Understanding chemical structure continues to be an important component of research attempts to predict biological activity and to explain mechanisms of chemical

toxicity. Nitrofluorenes and nitrofluorenones have been characterized in terms of toxicity and sister chromatid exchange rate using CHO cells and in terms of Ames mutagenicity (Brooks *et al.*, ITRI). Addition of a carbonyl group to nitro- and dinitrofluorenes greatly increases Ames activity in strains TA98 and TA100 without S9 activation. Addition of a second nitro group to a nitrofluorene or nitrofluorenone increases mutagenicity, while the addition of a third and fourth nitro group decreases mutagenicity. The compounds most commonly found associated with diesel exhaust particulates are among the most mutagenic of the class. Sister chromatid exchange studies showed a different sensitivity to structural changes than did the Ames mutagenicity study. No increase was seen in SCE frequency with the addition of a carbonyl group but an increase was seen in SCE with additional nitro-groups.

ISOMERIC IDENTIFICATION AND SELECTIVE DETECTION OF BIOLOGICALLY SIGNIFICANT ORGANICS*

Michael R. Guerin, Michelle V. Buchanan, Marcus B. Wise, and Robert L. Hettich

Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120

Research is under way to develop technologies allowing the unambiguous identification and/or selective detection of trace organics in toxicologically-relevant materials. Issues of particular interest are the quantitative determination of environmental carcinogens and the identification of biomolecule-adducts. Related research supported by the National Cancer Institute focuses on the characterization of environmental tobacco smoke.

Progress to date (e.g., 1-4) is based on earlier OHER research aimed at identifying biologically significant constituents of fossil fuels. It was found that ammonia chemical ionization mass spectrometry allowed the unambiguous assignment of polycyclic aromatic amines as primary, secondary, or tertiary. Similarly, methanol chemical ionization mass spectrometry allowed distinguishing alcohols from ethers and thiols from sulfides. In agreement with others, negative chemical ionization mass spectrometry was found promising for the high sensitivity and selective detection of polycyclic aromatic hydrocarbons.

A study of these observations suggested that gas phase ion chemistry coupled with mass spectrometric analysis offered the possibility of unambiguous identification at sufficiently high sensitivity. It also

---

*Research sponsored jointly by the Office of Health and Environmental Research, U.S. Department of Energy, and the National Cancer Institute, Interagency Agreement No. 0485-0485-A1 under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

suggested that Fourier Transform Mass Spectrometry (FTMS) was the most promising technology for this purpose. Resulting research has led to the development of a tunably selective detector for gas chromatography and has confirmed the utility of FTMS for both basic and applied research related to chemical toxicology.

The chromatographic detector[1] resulted in part from fundamental studies of negative ionization chemical ionization (NICI) mass spectrometry (MS). Approximately 40 polycyclic aromatic hydrocarbons (PAHs) were analyzed by NICI MS and it was found that only those PAHs with electron affinities greater than approximately 0.5 eV yielded molecular anions. The common electron capture detector (ECD) used in gas chromatography is known to also respond to chemicals depending on their electron affinities. This suggested a commonality between NICI and ECD processes.

The resulting detector consists of a standard ECD contained in a variable pressure vacuum chamber. At atmospheric pressure, the detector responds as does the standard ECD. At low pressure ($\leq$ 10 Torr), the detector functions similarly to an argon ionization detector and provides a universal electron emission response. At intermediate pressures (100-300 Torr), electron capture and electron emission processes compete to provide a response dependent on the electron affinity of the analyte. The net electron capture or electron emission response (a "positive" or "negative" chromatographic peak under normal ECD conditions) is a function of the analytes electron affinity and the detector cell pressure. The cell pressure may be adjusted so that analytes differing by less than 0.05 eV in electron affinities can be discriminated by virtue of yielding capture or emission responses. Important examples include the abilities to selectively discriminate between benzo(a)pyrene (- 0.63 eV) and benzo(e)pyrene

(- 0.35 eV), polychlorinated biphenyls from polycyclic aromatic hydrocarbons, and phenanthrenes from anthracenes.

A Nicolet FTMS-2000 has been acquired for fundamental studies of gas phase ion chemistry and for applied analytical research in chemical toxicology and molecular dosimetry. FTMS provides extraordinarily high mass resolution, MS/MS capabilities, and the opportunity to trap ions for chemical and physical reaction prior to daughter ion analysis.

We find FTMS to be especially suitable for the analysis of complex mixtures[2] and of biological macromolecules[3]. Analyses of smoke "tars" have demonstrated that quantitative determinations are practical and that nominally isobaric ions may be resolved to provide a high degree of isomeric discrimination. Studies of nucleotides and oligonuclides have demonstrated the possibility of DNA-adduct detection and identification. Trimers have been successfully introduced into the analyzer to provide molecular ions for further analysis and the promise exists for introducing higher molecular weight oligonucleotides. Nucleosides and nucleotides are readily analyzed using FTMS. Nanogram quantities of adenosine monophosphate are sufficient to provide appreciable ions for further analysis when laser desorption is used to produce the parent ions. The technique provides the opportunity to identify adducts and to provide sequence information for small oligonucleotides.

REFERENCES

1. Wise, M. B., Buchanan, M. V., Parks, S. K., and Haas, J. W. Rev. Sci. Instrum. 57, 3075; 1986.

2. Wise, M. B. Characterization of Constituents in Tobacco Smoke Using Fourier Transform Mass Spectrometry, Extended Abstracts, 35th ASMS Conference, May 1987.

3. Hettich, R. L. and Buchanan, M. V. Identification and Characterization of Nucleotides by Laser Ionization Fourier Transform Mass Spectrometry, Extended Abstracts, 35th ASMS Conference, May 1987.

4. Buchanan, M. V. and Wise, M. B. Negative Ion Processes for the Unambiguous Identification of Polycyclic Aromatic Compounds, Fuel, in press.

IDENTIFICATION AND PURIFICATION OF MUTAGENS FROM COMPLEX MIXTURES AT THE ONE PART PER BILLION LEVEL

J.S. Felton, M.G. Knize, N.H. Shen, and S.K. Healy

Lawrence Livermore National Laboratory, Livermore, CA 94550

The Ames/Salmonella mutagenicity assay is used in our laboratory to guide the chemical purification of genotoxic chemicals in a complex mixture. Our approach to this problem was to purify these biologically active compounds present in a matrix of natural constituents at concentrations below 1 part per billion (1ppb) using XAD adsorption, liquid/liquid extractions, and high performance liquid chromatography in concert with a bacterial mutagenesis assay. Mass spectrometry and nuclear magnetic resonance spectrometry were tied to chemical synthesis for the final identification of the mutagens.

At the present time, 8 extremely mutagenic heterocyclic amines have been identified in this specific complex mixture derived from cooked meat using this approach. Their abundance ranges from 15 to less than 0.1 PPB equivalent of starting material. These PPB purifications would not be possible without both the speed and the sensitivity of the Salmonella assay and the ability to recover sample for biological testing on purified fractions.

It is essential to identify the components responsible for the mutagenicity of a mixture of chemicals in order to evaluate the risk. This is especially important for the case we have chosen, cooked food, where human exposure information such as the dose and identity of the chemical(s) ingested is needed for risk analysis calculations. Once the amount and the toxicological response of the major components is known, the risk to the exposed population, can be estimated. This estimate could be based on the mass of each constituent consumed over a life-time or an occupational exposure period and the extrapolated potency of the mouse and rat carcinogenicity and mutagenicity data.

In this particular case, acidified water is used to extract the mutagenic amines from the matrix of biological material. The extract is centrifuged and the pellet is washed with acid and centrifuged again. The supernatants are combined, the pH adjusted to 7.0 and the liquid passed over an XAD-2 Amberlite column. The column is eluted with acetone followed by methanol and these eluates pooled, evaporated, diluted with water and acidified. The resulting concentrate is then extracted with methylene chloride. The remaining aqueous phase neutralized and passed over a second XAD-2 column, eluted with acetone and evaporated. This XAD-2 eluent was combined with the methylene chloride fraction, evaporated and prepared for Ames/Salmonella analysis and subsequent HPLC separations.

In the analysis of oil shale retort water (Strniste et al., 1983) and diesel engine soot (Yu and Hites, 1981), mutagenic liquid chromatography fractions were analyzed by gas chromatography/mass spectroscopy, giving numerous peaks, with the compounds responsible for the mutagenic activity characterized by only chemical class. This general information is not enough to understand which compounds are contributing to the risk from these mutagenic mixtures.

The gas chromatography analysis method assumes that the mutagens in the complex mixture will exhibit good chromatographic behavior. Many genotoxic compounds, including the pyrolysis mutagens characterized by Japanese workers (Hatch et al., 1984; Sugimura et al., 1982) do not separate well using gas chromatography. Most give poor peak shapes and yields, making gas chromatography for these types of unknown molecules a poor choice. Ideally, what one would like is a single pure mutagenic compound quantified and characterized by bacterial testing and available for MS, NMR, UV and IR specrometric analysis.

Our approach with the cooked-food problem was to use the Salmonella assay to monitor the chromatographic separation and purification of the fried-beef mutagens, saving the mutagenic fractions and further purifying them until pure mutagens are isolated. The purified compound is either identified by using analytical techniques to compare with known compounds,

or, in the case of the cooked-food mutagens only probable structures could be determined and a series of compounds synthesized to determine which one matched the unknown purified from the extract. This was primarily a problem of not enough recoverable mass for NMR determination with a starting concentration below the PPB level.

The HPLC separation(s) is the critical step in the purification. The fraction size was determined by the expected peak width. The optimum separation has two or more fractions per peak of mutagenic activity. Since the biological testing of many chromatographic separations is labor intensive, we opted to get the most information from the minimum number of samples. Experience with mutagenic aromatic amine standards routinely showed peak widths of 2-3 min; so fractions were collected at 2 min intervals for preparative separations and at 1 min intervals for the final analytical collections. The amount to be tested was determined by the stage of sample purification. The goal is to detect all significant active peaks while consuming the minimum amount of sample. A portion of each sample equivalent to 5,000 revertants, was used for samples expected to have multiple mutagenic components, and an aliquot representing 1,000 to 2,000 revertants was deemed adequate when only one peak was expected. These revertant numbers are dependent on the spontaneous revertant rate of the bacterial strain used for the screen; a higher background (>40) would require more revertants.

Aqueous samples for the Ames test are micro-pipetted directly from the HPLC fractions, diluted to 100 µl with dimethylsulfoxide (DMSO), and tested. Our experiments showed that water-miscible organic solvents such as methanol and acetonitrile have no effect on the Ames test up to 100 µl per plate. Water immiscible fractions from the normal-phase chromatography are treated somewhat differently; they are sampled, evaporated to dryness under a stream of nitrogen and then dissolved in DMSO for testing. Mutagenic fractions once identified, were pooled into peaks, evaporated to dryness under a stream of nitrogen at 50 deg. C and then dissolved into the appropiate mobile phase for the next HPLC purification step.

Recent work (Knize et al., 1984) has shown that for samples of a few µg or less, a semi-micro column (2.1 x 250 mm) works well to purify samples for mass spectrometric analysis by minimizing mobile-phase contaminants. The use of a spectrophotometric HPLC detector can aid in the assessment of sample purity by plotting absorbance ratios and detailed sample UV absorbance information.

There are several techniques that can be used to identify the chemical structure of the mutagens. UV absorbance spectrometry can be useful for comparison to known mutagens and to determine sample purity. The technique is nondestructive and can be utilized with as little as 0.1 µg of compound. Mass spectrometry is essential for sample identification, and for compounds like aromatic amines it will give the molecular weight and some structural

details. Mass spectra can be compared to known compounds by searching available libraries containing more than 40,000 spectra. For unknown molecules, such as many we had to identify from cooked foods, a high-resolution mass spectrum will give information on the elemental composition. Only 50-100 nanograms of material are required for definitive analysis. Mass spectrometry has the shortcomings that it normally cannot distinguish isomers from each other, and the sample is unfortunately not recoverable. Nuclear magnetic resonance (NMR) spectrometry is also essential for the structural characterization of these new mutagens. It gives unique structural information and in many cases will resolve isomeric ambiguities. With a 200 MHz instrument a minimum of 10 µg is needed, but fortunately, the technique is non-destructive so samples are recoverable. Synthesis of the proposed molecule is perhaps the most important confirmatory technique. Proposed structures and any isomers that can not be ruled out by other analytical techniques must eventually be synthesized and compared to the unknowns for definitive structural identity.

In summary, the identification of mutagens from complex mixtures requires a multidisciplinary approach. The microbiologist, the analytical chemist and synthetic chemist must all work together on the identification problem. Once the mutagenic, carcinogenic, or toxic composition has been determined, the tasks of understanding the toxicological importance of both the potency and mass abundance of the biologically active substances in the mixture can begin.

References:

1. Hatch, F.T., J.S. Felton, D.H. Stuermer, and L.F. Bjeldanes. 1984. Identification of mutagens from the cooking of food. In: Chemical Mutagens: Principles and Methods for their detection (deSerres, F.J., ed.) Vol. 9, pp 111-164, Plenum Publ. Corp. New York.

2. Knize, M.G., B.J. Wuebbles, and J.S. Felton. 1984. The advantages of a semimicro HPLC system for the purification of small amounts of biologically active material. LC 2:222-224.

3. Strniste, G.F., J.M. Bingham, W.D. Spall, J.W. Nickols, R.T. Okinaka, and D.J.-C. Chen. 1983. In: Short-term bioassays in the analysis of complex environmental mixtures III (Waters, M.D., Sandhu, S.S., Lewtas, J., Claxton, L., Chernoff, N., and Nesnow, S. eds.) pp139-151, Plenum Press, New York.

4. Sugimura, T. 1982. Mutagens in cooked food. Basic Life Sci. 21:243-269.

5. Yu, M.-L. and R.A. Hites. 1981. Identification of organic compounds on diesel engine soot. Anal. Chem. 53:151-154.

Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48 and supported by an Interagency Agreement (NIEHS 2122Y01-ES-10063) between the National Institute of Environmental Health Sciences and the Department of Energy.

IMMUNOASSAYS FOR MONITORING EXPOSURE TO TOXIC CHEMICALS

M. Vanderlaan, L. Stanker, and B. Watkins
Lawrence Livermore National Laboratory, Livermore, CA 94550

Immunoassays for pesticides and toxic chemicals are finding increasing acceptance as rapid, sensitive means of quantifying small organic molecules in environmental studies[1]. Immunoassays of carcinogen-DNA adducts allow quantification of dose to target cells, and can provide dose estimates in epidemiologic studies. By providing cost-effective screening means, immunoassays allow experiments on energy-related pollutants not previously possible. These include improved detection of the migration of trace organics in ground and surface waters and the characterization of emerging energy technologies (e.g. co-combustion of municipal wastes) from the standpoint of their impact on the environment.

Our group is a major center for the development of monoclonal antibody reagents to toxic organics for use in these studies, and the application of immunoassays to studies on human and environmental samples[2,3]. Examples of assays that we have developed include the detection of dioxins in soils, the detection of pesticide residues in foods, the detection of cooking-induced mutagens in meats, and the detection of modified nucleic acids in cells. In each of these cases the antibodies provide sensitivity of about a part-per-billion, and minimal sample cleanup is required for the assay.

In this presentation we will illustrate the basic principles of monoclonal-antibody-based immunoassays for toxic chemicals using examples from our laboratory. A background to the current state-of-the-art will be provided. Results of the immunoassay of aromatic amines in cooked beef will be discussed as an illustration of monoclonal antibody development.

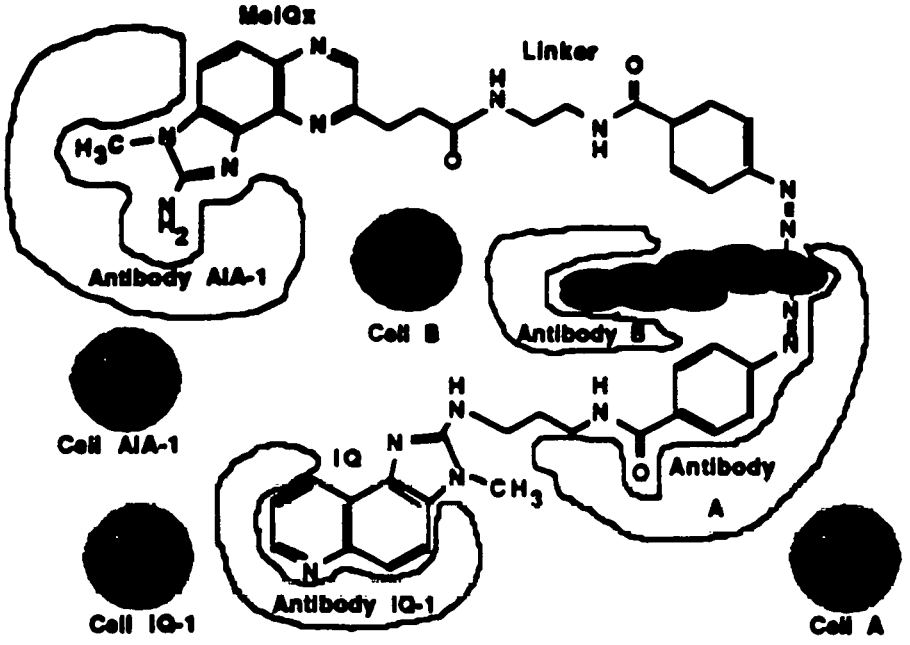
A family of 2-amino-3-methylimidazoazaarene (AIA) mutagens produced in meats by cooking have been described by a previous speaker in this session (Dr.

James Felton), and of concern as potential carcinogens in the human diet. Conventional analytical chemistry methods have allowed the identification of six members of the AIA class, but are much too labor intensive to allow screening large numbers of meat samples cooked under a variety of conditions. Small molecules will not produce an immune response if injected alone into an animal, but can be made immunogenic by conjugating them to carrier proteins. Two different linkages were used for conjugation in this study, in an effort to direct the specificity of the antibodies produced. The immunogens are illustrated in the figure, along with a schematic representation of the clonal nature of the immune response to hapten-protein immunogens. Individual clones of cells (e.g. IQ-1 and AIA-1) can be selected that secrete antibodies which bind to specific regions of the immunizing molecule. In all, a set of six monoclonal antibodies (named IQ-1, IQ-2, AIA-1, AIA-2, AIA-4, and AIA-7) were selected. Each antibody shows a unique binding selectivity pattern. For example, IQ-1 binds to IQ and 2-nitro-IQ, suggesting that it will react not only with the parent compound produced by cooking, but also N-hydroxyl metabolites of IQ. AIA-1 shows broad specificity, recognizing most AIAs and quinoxoline. AIA-2 is imidazoquinoxoline specific, recognizing only quinoxoline-containing AIAs but irrespective of their degree of methylation. None of the antibodies recognize PhIP. Taken as a set, these antibodies will allow screening of foods for AIA mutagens, and aid in the isolation and characterization of other AIAs and metabolites of AIAs.

1. Vanderlaan, M., Watkins, B., and Stanker, L. Environmental Sci and Technology (in press) Aug. 1987.
2. Vanderlaan, M., Van Emon, J., Watkins, B., and Stanker, L. In: Pesticide Science and Biotechnology, R. Greenhalgh and T.R. Roberts, Eds. Blackwell Scientific Publications, London. 1987. p 597-602.
3. Stanker, L.H., Watkins, B., Rogers, N., and Vanderlaan, M. Toxicology (In press) 1987.

Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48. Funding provided by NCI grant No. 1 RO1 CA-40811-01.

Submitted to OHER Toxicology Research Meeting, Monterey, CA June 24-26, 1987 UCRL-96550



This figure illustrates the clonal nature of the immune response to hapten-protein conjugates. The mutagens IQ and MeIQx were conjugated to a carrier protein for injection into mice. IQ was linked through the amino group in the two position, and MeIQx was linked through the methyl group in the eight position. Following immunization antibodies of all the types illustrated can be found in the serum. These include antibodies like A, that recognize portions of the protein and linker chemistry in addition to the hapten of interest, and antibodies like B that recognize the protein. Neither of these are suitable for the immunoassay of the haptens. Other antibodies, however, will bind selectivily to the haptens. These, in general, bind to the unrestricted, native end of the hapten, farthest from the site of conjugation. In the case of the 2-linked IQ, antibodies like IQ-1 will be formed which recognize the quinoline ring preferentially. In the case of the 8-linked MeIQx, antibodies like AIA-1 will be formed which are most influenced by the amino-imidazo portion of the molecule. Corresponding to each antibody, there is a B-lymphocyte which produces it. In the polyclonal immune serum, all antibodies will be present, along with many others not illustrated. The task of isolating the useful minority of anitibodies is formidable. With the advent of monoclonal antibodies, however, it is possible to select the clones secreting the antibodies of interest, a far easier task. Thus, clones of cells AIA-1 and IQ-1 can be isolated which produce as pure reagents their correspondingly named antibodies.

DEVELOPMENT OF AN IMMUNOASSAY FOR CHLORINATED DIOXINS

Larry Stanker, Bruce Watkins and Martin Vanderlaan

Biomedical Sciences Division, Lawrence Livermore National Laboratory, P.O. Box 5507, Livermore CA 94550

Polychlorinated dibenzo-p-dioxins (PCDDs) belong to a group of chemicals collectively referred to as halogenated aryl hydrocarbons. The toxicology of PCDDs is complicated by the existence of 75 isomers. Each isomer, when individually studied, has a unique, species specific, toxicological profile. 2,3,7,8-Tetrachlorodibenzodioxin (2,3,7,8-TCDD) is considered the most toxic isomer. In addition to acute toxicity, chronic toxicity, genotoxic and immunotoxic effects also have been reported for 2,3,7,8-TCDD, and vary with species. Finally, 2,3,7,8-TCDD is capable of inducing several enzymes including, microsomal monooxygenases, glucuronyl transferase,and glutathione S-transferase. As with toxicity, microsomal monooxygenases induction is species and age dependent.

Dioxin is not manufactured, but is found as a by-product in many commercial chlorinated organics, (e.g., polychlorinated biphenyls, chlorophenols, and pesticides). Another source of dioxin exposure is emissions from municipal waste incinerators, thermal power plants, and automobile exhaust. Studies in Sweden indicate that many incinerators generate 5-10 g/year of PCDDs and PCDFs counted as TCDD equivalents.

As a result of the variable toxicity of dioxin isomers, meaningful analysis requires identification and quantification of the various isomers and congeners thus necessitating the use of sophisticated analytical techniques such as gas chromatography and mass spectroscopy. These techniques are not only expensive, but are time consuming, require specialized equipment, and highly trained personnel. Alternative assays which are specific for the toxic isomers, less expensive, rapid and amenable to automation are thus desirable. Immunoassays meet these criteria. Immunoassays have proven effective in medicine for detecting and quantifying infectious agents and compounds of medical interest. They have only recently, however, been applied to the analysis of compounds of toxicological interest.

We report here the development of a set of monoclonal antibodies (mabs) to dioxin, referred to as DD-1, DD-3, DD-4, DD-5, and DD-6. Antibody binding is related to the level of chlorine substitution. The antibodies preferentially bind the tetrachloro- and pentachlorodibenzodioxins including 2,3,7,8-TCDD. Thus, they bind the most toxic isomers. Dibenzofurans also are recognized but for the most part the mabs do not recognize PCBs or the chlorinated pesticides that have been analyzed.

Using these antibodies we have developed a competition enzyme-linked immunosorbent assay (c-ELISA) which can easily detect a few hundred picograms of 2,3,7,8-TCDD. We are adapting this assay to detect dioxin in soils and in fly ash from municipal waste incinerators and cogeneration plants. We ultimately hope to be able to apply this assay to biological matrixes.

This work supported by the U.S. Environmental Protection Agency through interagency agreement No. DW89931433-01-0 and performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48.

DETERMINATION OF ADDUCTS OF POLYCYCLIC AROMATIC HYDROCARBONS TO DNA

R. M. Bean, E. K. Chess, B. L. Thomas, D. B. Mann, D. A. Dankovic, J. A. Franz and D. L. Springer

Pacific Northwest Laboratory, Richland, WA 99352

Adducts to deoxyribonucleic acid (DNA), formed from metabolites of polynuclear aromatic compounds, are relatively persistent and correlate with bioresponse (carcinogenicity). Therefore, qualitative and quantitative analysis of adducts in the DNA of individuals may provide valuable information as to recent exposure to carcinogenic hydrocarbons. Further, the ability to detect adducts in a large segment of a population may have significant epidemiological significance. A major problem with the analysis of adducts is that they are formed at extremely low concentrations. Everson et al.[1] reported detecting concentrations of an individual adduct in the placenta of human cigarette smokers as low as 1 adduct per 10^{10} DNA base units, corresponding to one adduct per cell. Although data on humans is sparse, we may expect, allowing for initial rapid decay after exposure, adduct levels of 0.1 to 0.01 nanograms per milligram of DNA. Methods currently being used for analysis of DNA adducts have been reviewed by Wogan and Gorelick[2]. In general, the currently available methods suffer either from a lack of sufficient sensitivity for environmental screening, or from a lack of qualitative specificity.

The current thrust of the analytical development at PNL is to isolate the DNA, liberate the adducted hydrocarbon residue from the DNA with acid hydrolysis, and prepare derivatives of the hydrolyzed species that will enhance its detection, quantitation, and characterization using gas chromatography/mass spectrometry (GC/MS). We have initiated the development of the necessary techniques using benzo[a]pyrene (B[a]P). Samples of DNA adducts of radiolabeled B[a]P have been prepared for study by reacting DNA isolated from calf thymus with benzo[a]pyrene-7,8-diol-9,10-epoxide (the ultimate carcinogenic form of B[a]P). Other DNA/B[a]P samples have been prepared by painting the skin of mice with radiolabeled B[a]P. The ability to prepare research quantities of adducts using the hepatocyte preparation method reported by Dankovic et al.[3] is a significant development to our DNA adduct analysis program.

The liberation of the adducted hydrocarbon residue as the tetrahydrotetrol (tetrol) from a B[a]P/DNA adduct has been shown by Rahn et al.[4] to be facile using 0.1 N hydrochloric acid at 80° C for 6 hr. We have found that the hydrolytic conditions also cause epimerization of the tetrol. About 12 % of the principle tetrol expected from the anti-B[a]P diol epoxide (I-1) is isomerized to the I-2 form during hydrolysis; and the II-1 isomer expected from the syn-B[a]P diol epoxide is extensively isomerized to the II-2 isomer. NMR examination of the acid isomerizates have confirmed that the epimerization occurs exclusively at the 10-position of the B[a]P tetrol, although the 7-position would also seem to be a likely candidate for isomerization, being also adjacent to the aromatic ring. The product distributions of the isomerized tetrols are similar to those observed by Yagi et al.[5] during the hydrolysis of B[a]P diol epoxide in

acetone/water at 37 °C. These experiments have shown that while the recoveries of tetrols under hydrolytic conditions are acceptable, some information about the conformation of the adduct when attached to the DNA is lost during the cleavage step.

Once the tetrols are removed from the DNA polymer and isolated, they are reacted with reagents that make them sufficiently volatile to permit their analysis by GC/MS methods. Permethyl and peracetyl derivatives have been prepared and have been tested with the tetrol metabolites of B[a]P, chrysene, and benz[a]anthracene (B[a]A) to determine which type of derivative will offer the best performance in terms of stability and sensitivity for the analysis at trace levels. Permethylated derivatives were prepared using the method of Hakamori[6] and were examined by GC/MS using relatively short (<15 m) fused silica capillary columns. The EI mass spectra provided the most diagnostic fragmentation information related to the structure of the ion. For example, the prominent molecular ion (m/z 376) of the permethylated B[a]P tetrol undergoes a retro-Diels Alder reaction, expelling a molecule of 1,2-dimethoxyethene containing C-8 and C-9 from the B[a]P ring structure, resulting in the fragment ion at m/z 288. This ion then loses a methyl radical, primarily originating from the methoxy group attached to C-10, to produce the base peak fragment ion at m/z 273. This fragmentation pathway has been confirmed by deuterium labeling experiments. Electron impact spectra obtained from the permethylated chrysene and benzanthracene tetrols could be predicted from the spectra of the methylated B[a]P metabolite. The ability to predict *a priori* the principal ions formed from metabolite tetrols of PAH has important application in the qualitative aspects of DNA adduct analysis. These results have been accepted for publication in *Biomedical & Environmental Mass Spectrometry*.

Peracetate derivatives of the tetrols of B[a]P, Chrysene, and B[a]A were prepared using standard derivatization procedures with acetic anhydride. Since the B[a]P trifluoroacetates (prepared from N-methyl-N-trimethylsilyltrifluoroacetamide) were very susceptible to hydrolysis, the corresponding derivatives of the other two compounds were not investigated. On-column injection has been shown to improve the analysis both qualitatively and quantitatively by reducing both injector discrimination and thermal decomposition. The peracetate derivatives were stable in solution for several days. The permethyl derivatives were found to be the most stable of the three types.

Initial experiments to determine the detection limits of the derivatized metabolites indicated that < 2 fmol of permethylated or peracetylated tetrol can be detected during selected ion monitoring (SIM) GC/MS analyses with a mass selective detector. The practical detection levels in actual samples are functions of recovery during analyte isolation, and degree of interference during the analysis. For analytical levels in the femtomole range, neither of these considerations is trivial. Using 600 pmol quantities of B[a]P tetrol, over 80 % is recovered after exposure to acid hydrolysis, and recovery of permethyl or peracetyl compound after derivatization is also over 80 %. At levels approaching the femtomole range, more elaborate precautions as to glassware pretreatment and reagent preparation are required to maintain good recoveries. Although SIM "filters out" interferences from many sources, it does not completely eliminate problems due to sample contamination. Although further research is required before reliable analyses can be made at the required sensitivity, our results indicate that direct SIM analysis of adducted hydrocarbon metabolites will be a valuable

contribution to the field of carcinogen dosimetry.

In addition to studies related to dosimetry of hydrocarbon/DNA adducts, the PNL adduct analytical team is also engaged in an exploratory research project to identify forms of hydrocarbon adducts that do not exhibit the behavior of adducts formed through normal diolepoxide addition to DNA bases. A number of researchers, for example Shen et al.[7], and Ashurst and Cohen[8], have reported that when radiolabeled B[a]P adducts are isolated by the conventional method (enzymatic hydrolysis of DNA to the adducted nucleosides and adsorption of the adducts on LH-20), 1/3 to 1/2 of the radiolabeled nucleoside material is not retained on the LH-20. Although some preliminary work has been done on this material, it has not been identified. We have examined this "non-classical" adduct material by treating it with 0.12 N HCl in an attempt to release it from the residual DNA structure, followed by solid reverse-phase adsorption of the hydrolyzed product. Elution of the reverse-phase resin with methanol resulted in isolation of 66 % of the original "non-classical" radioactivity. Reverse phase chromatography of this material revealed that in addition to tetrols, from 10 to 33 % had retention times consistent with B[a]P-3,6-quinone and B[a]P-6-12-quinone. Since "classical" B[a]P-adducted nucleosides, B[a]P tetrols, and B[a]P quinones are strongly retained on LH-20, it is apparent that the tetrols and quinones were released from the nonclassical adducts during the acid hydrolysis step. We believe that determination of the structure of the adducted material from which the quinones are derived is important; the quinones comprise as much as 10 % of the radioactivity in the isolated DNA.

REFERENCES

1. Everson, R. B., Randerath, E., Santilla, R. M., Cefalo, R. C., Avitts, T. A., and Randerath, K. *Science* **231**, 54-57; **1986.**

2. Wogan, G. N., and Gorelick,N. J. *Environ. Health Perspec.* **62,** 5-18; **1985.**

3. Dankovic, D.A., Springer, D. L., Thomas, B. L., Mann, D. B, and R. M. Bean. Preparation of DNA adducts for chromatin and chemical characterization studies using freshly isolated rat hepatocytes. Proceedings, 1987 DOE Contractor Conference on Chemical Research, Monterey, CA, June 24-26, **1987.**

4. Rahn, R. O., Chang,S. S., Holland, J. M., and Schugart, L. R. *Biochem. Biophys. Res. Commun.* **109**, 262-68; **1982.**

5. Yagi, H., Thakker, D. R., Hernandez, O., Koreeda, M., and Jerina, D. M. *J. Amer. Chem. Soc.* **99**, 1604-1611; **1977.**

6. Hakamori, S. *J. Biochem. (Tokyo)* **55,** 205-8; **1964.**

7. Shen, A. L., Fahl, W. E., and Jefcoate, C. R. *Arch. Biochem. Biophys.* **204**, 511-523; **1980.**

8. Ashurst, S. W., and Cohen, G. M. *Carcinogenesis* **3**, 267-273; **1982.**

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE OF NITROFLUORENONES AND NITROFLUORENES AND THEIR GENOTOXIC ACTIVITY.

[1]A.L. Brooks, [2]R.M. Kitchin, [1]W.E. Bechtold,

[1]Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM,
[2]University of Wyoming, Laramie, WY

Derivatives of nitroflourene are an important class of environmental mutagens which have served as model compounds and positive controls for many studies using the Ames mutagenicity assay. These compounds have also been used as models to determine the mechanisms of interactions of nitroaromatics with DNA and to establish structure-function relationships for mutation induction in bacteria.

In the current study, the genotoxicities of 2-nitrofluorene (2-NF), 2,7-dinitrofluorene (2,7-DNF), 3-nitroflorenone (3NFone), 2-nitrofluoreneone (2NFone), 2,7-dinitrofluorenone (2,7-DNFone), 2,4,7-trinitrofluorenone (2,4,7-TNFone), and 2,4,5,7-tetranitrofluorneone (2,4,5,7-TNFone) were related to chemical structure. By limiting this study to a single class of compounds it was possible to compare chemicals with similar molecular size, a constant plane for the aromatic rings, but with different numbers and types of electron withdrawing groups on the parent compound. Mutations were evaluated using the Ames Salmonella bacterial test. Toxicity, mutations at HGPRT gene loci and the induction of sister chromatid exchanges were measured using CHO cells.

Our data on the induction of mutations in the Ames test agreed with that previously reported indicating that the addition of carbonyl groups to mono or di nitrofluorenes to form the nitrofluorenones increased the mutagenic activity in Ames bacteria strains TA-98 or TA-100 without the addition of S-9. The addition of a nitro group to the 2-nitrofluorene or 2-nitrofluorenone to form 2,7-dinitro compounds produced a marked increase in the mutagenic activity in TA-98 without S-9 from 18 to 2900 revertants/nmole for

the dinitroflourene and from 59 to 3900 revertants/nmole for the dinitroflourenone. Further addition of nitro groups to form the tri and tetra nitroflourenone resulted in a decrease in mutagenic activities to 3500 and 1100 revertants/µg in TA-98 without S-9. The pattern observed in TA-100 without the addition of S-9 was again similar to that observed by McCoy et al 1981, an increase level of activity for 2,7-DNFone which decreased for 2,4,7-TNFone then increased again for the 2,4,5,7-TNFone. When a single nitro group was placed in the 3 position of mononitrofluorenone, mutagenic activity was less than observed when it was in the 2 position. Values have not been reported for many of these compounds with the addition of S-9, since they are direct acting mutagens. In our studies, the addition of S-9 decreased the mutagenic activities of all the compounds except 2-nitroflourene, 2-nitrofloueneone and 3-nitrofloueneone, where there was an increase in activity with the addition of S-9.

The mono- and dinitroflourenes or mononitrofluorenones were not toxic to CHO cells when tested at levels up to 10 µg/ml. The di, tri and tetranitrofluorenones were cytotoxic and delayed in the cell cycle, as measured by the frequency of second division cells, at concentrations of 5 µg/ml or above.

The addition of carbonyl groups with or without the addition of S-9 did not change the slopes of the SCE concentration-response relationships for 2-nitrofluorene and 2-nitrofloureneone, which were 0.22 and 0.21 with S-9 and 0.21 and 0.18 SCE/cell/µg/ml without S-9, respectively. The addition of a carbonyl group to the dinitro compounds did not alter the mutagenic activity with the addition of S-9 (0.37 vs 0.33 SCE/cell/µg/ml) but increased it from 0.32 to 0.89 SCE/cell/µg/ml without S-9. The dose-response relationship for SCE induction was complicated by the increased level of cytotoxicity observed without the addition of S-9 for both the 2,4,7

TNFone and 2,4,5,7 TNFone. With the addition of S-9, it was possible to test the compounds with a minimum amount of cytotoxicity. Under these conditions, the slope of the SCE concentration-response relation increased with the addition of nitro groups from 0.07, 0.21, 0.33, 1.33 and 4.6 SCE/-cell/μg/ml for the 3-NFone, 2-NFone, 2,7-DNFone, 2,4,7-TNFone and 2,4,5,7-TNFone. Again placement of the nitro group in the 3 position was less effective in the induction of SCE than when it was in the 2 position.

These studies indicate that SCE induction is less sensitive than the Ames test to minor alterations in the structure of nitroaromatics. This was noted especially for the addition of carbonyl groups. The addition of a second nitro group caused a marked change (about 30 fold increase) in mutagenic activity in the Ames test, while addition of a second nitro group caused only a 1.5-4 fold increase in the SCE frequency. In bacteria, additional nitro groups did not continue to increase the response, while the SCE frequency increased with increasing number of nitro groups. The complex interaction between cell killing and cell cycle delay with SCE induction needs to be further evaluated to define the structure-function relationships which exist for nitroaromatics in mammalian cells. These studies illustrated that the type of the biological system and its responsiveness to damage are important determinants of the relationships between chemical structure and predicted genotoxicity.

Acknowledgements: This work was sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76-EV01013

SESSION 2

Adduct Formation and Repair

Adduct Formation and Repair

Summary of Session 2

N.E. Geacintov

New York University

Many toxic chemicals are by-products of various energy-generating processes and are known to be mutagenetic and/or tumorigenic. The mechanism of action of such chemicals is widely believed to involve the binding of these toxic compounds (or their metabolically activated forms) to DNA, the genetic material of the cell. Considerable experimental evidence has been accumulated that some of these DNA adducts are persistent *in vivo*, and that their presence in bacterial and mammalian cells is correlated with the observed frequencies of mutations. The formation of DNA adducts in test animals exposed to carcinogenic chemicals is believed to represent the critical initial event in the complex, multi-stage tumorigenesis process; similar mechanisms of tumor initiation are suspected to be the cause of many human cancers of exogeneous chemical origin.

Polycyclic aromatic hydrocarbons (PAH), PAH derivatives, and aromatic amines are well-known classes of chemical carcinogens and mutagens. These molecules are metabolized *in vivo* to potent electrophiles that bind covalently to DNA. The metabolic pathways of many aromatic compounds have been established, and the products of the chemical reactions with DNA can be identified by established analytical

techniques. Recombinant-DNA and molecular-biology techniques are increasingly utilized to investigate the nature of the mutations induced by different DNA adducts. However, little is known, on a molecular level, of how these DNA adducts are processed by the cellular machinery. In particular, it has been observed that different types of adducts, sometimes derived from structural or geometric isomers of the same reactive PAH metabolites, are characterized by strikingly different biological activities. The causes underlying these fascinating differences in the properties of chemically and structurally related DNA adducts are not known.

The papers presented in this session deal with several different but related subjects regarding adduct distributions, the mechanisms of formation of adducts, their physicochemical and structural properties, and some biological consequences of adduct formation (DNA damage).

The papers by Sega (ORNL) and Jones *et al.* (LLNL) deal with specific model systems devised to study the effects of relatively small DNA-alkylating agents.

Sega's work focuses on measuring the amounts of chemical mutagens (e.g., ethylene oxide) that reach the germ cells in the mouse, the amount of DNA strand breakage, and the induction of unscheduled DNA synthesis. At levels of ethylene oxide exposure below the limits presently allowed for humans, a strong increase in DNA adduct formation and strand breakage was noted in the late spermatid stages. Furthermore, the level of unscheduled DNA synthesis was found to depend on the dose rate. The advantage of these approaches is that DNA damage can be assessed

at exposure levels far below those needed to produce detectable mutation events. The techniques developed should be useful for human genetic risk estimation at the low exposure levels usually encountered by humans.

Jones *et al.* developed a mouse lymphocyte model system designed to evaluate the genetic effects of *in vivo* exposure to toxic chemicals. In this system, sister chromatid exchange is used as a biological dosimeter for exposure to toxic agents (ethylnitrosourea was used as a model alkylating agent). The relative exposure levels of cells to this mutagen and thus the formation of adducts, repair capacities, and dilution of adducts by cell division, are all tissue specific. A time dependence of the frequency of mutants following exposure was noted. The sister chromatid exchange effect was used to demonstrate that some adducts persist for weeks after exposure to ethylnitrosourea, thus accounting for the appearance of mutants on these time scales. The methods of mutant analysis described by Jones may be useful in modeling the consequences of *in vivo* exposures of human populations to toxic chemicals.

Bond and co-workers (Lovelace) described detailed results on the regional distribution of metabolizing enzymes and DNA adducts in the respiratory tract of dogs. The substances studied included benzo[a]pyrene, nitropyrene, and dilute diesel exhaust (rats were used in this latter case). There are significant differences in distributions of metabolizing enzymes and DNA adducts along the respiratory tract; these results may explain why certain portions of the respiratory tract are more susceptible to tumor formation than others.

Studies on adduct formation and damage in mouse lung DNA after intratracheal instillation of benzo[a]pyrene and 1-nitropyrene (and other compounds) were reported by Mitchell *et al.* (ITRI). The binding of 1-nitropyrene to DNA was about 30 times greater than that of benzo[a]pyrene, and gave rise to a dose-dependent increase in single-strand breaks. It was shown that a significant portion of the DNA may have been repaired early, while other adducts were removed at a slower rate. Future studies involving the characteristics of these different adducts and their susceptibility to repair and excision by enzymes should be important in assessing the biological effects of these nitroaromatic compounds.

One of the important aspects of assessing the effects of exposure of living systems to carcinogens and mutagens is the quantitative determination of the DNA adducts generated. However, the number of adducts formed in cells is extremely low (one adduct per 10^6 bases or less is typical), and a chemical characterization of such adducts is generally difficult. One approach is to synthesize DNA adduct standards in quantities sufficient for chemical characterization. These standards then serve to identify adducts obtained *in vivo*. Dankovic and co-workers (PNL) described an approach that can be used to synthesize relatively large (microgram) quantities of DNA adduct standards. This method involves an "amplification" of DNA adduct production by incubating a PAH compound (benzo[a]pyrene) with freshly isolated rat hepatocytes and calf thymus DNA mixtures.

A strong correlation between the tumorigenic activities of 16 different PAH diol epoxide derivatives (the ultimate carcinogenic forms of these polycyclic aromatic compounds) and the conformation of the DNA adducts formed was described by Geacintov (NYU). For example, the highly active diol-epoxide stereoisomer of benzo[a]pyrene forms external adducts, while the other inactive stereoisomers form intercalative, carcinogen-base stacked conformations. These results may ultimately be of importance for an understanding, on a molecular level, of the conformational features and local structures of the DNA adducts that distinguish mutagenic or carcinogenic lesions from those which are not. Furthermore, the results of these characterizations and classifications suggest that it might be possible, using physicochemical techniques, to distinguish between biologically harmful PAH-DNA adducts and DNA lesions that do not give rise to mutations or tumorigenesis.

The theoretical work of Broyde (NYU) and Hingerty (ORNL) is presently the only available approach for visualizing the structures of such adducts on an atomic level. Their minimized potential energy calculations produce molecular views and indicate that the lowest energy form of the adduct derived from the chemical binding of the most tumorigenic stereoisomer of benzo[a]pyrene diol epoxide has an external conformation, in agreement with the experimental findings of Geacintov and co-workers.

Recombinant DNA techniques were used by both Weiss and Kokontis (Univ. of Chicago) and Felton *et al.* (LLNL) to study the DNA alterations induced by adducts derived from benzo[a]pyrene diol epoxide and other aromatic mutagens. Weiss and

Kokontis found two major types of modifications in DNA sequence arrangements: large deletions and illegitimate (nonhomologous) recombinants that resulted in complete removal of the inserted oligomers. It was speculated that these phenomena are common denominators of different types of DNA damage produced by agents that induce malignancies and tumors. Future work is designed to test this hypothesis, and possible applications to eukaryotic systems are contemplated.

Felton *et al.* found that anti-benzo[a]pyrene diol epoxide caused large deletions; this was in marked contrast to the effects caused by several aromatic amines from cooked food, where only the single-type (dinucleotide) lesion was observed. The mechanisms for the induction of these types of deletions, or the illegitimate recombinants observed by Weiss and Kokontis, are not known.

In summary, this session dealt with a variety of approaches to the study and characterization of carcinogen-DNA and mutagen-DNA adducts, and the types of DNA damage induced by toxic chemicals. These approaches varied from basic chemical (Geacintov) and theoretical (Broyde) studies of adducts, to the molecular biology techniques of Weiss and Kokontis and Felton and co-workers. However, it is evident that we still do not know which physicochemical and/or biochemical properties of adducts determine mutagenicity, nor the mechanisms by which the observed mutation and deletions are induced by some of these adducts.

MECHANISMS OF INTERACTIONS AND BINDING OF CARCINOGENIC POLYCYCLIC AROMATIC METABOLITES TO DNA AND RELATIONSHIPS TO BIOLOGICAL ACTIVITIES.

Nicholas E. Geacintov

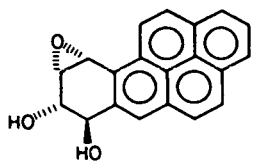
Chemistry Department and Radiation and Solid State Laboratory, New York University, New York, NY 10003.

Polycyclic aromatic hydrocarbons (PAH) undergo metabolic activation in living cells to epoxides and diol epoxides, which are the ultimate tumorigenic and mutagenic forms of these compounds. The covalent binding of these metabolites to DNA is widely believed to represent the critical initial event in mutagenesis and in the multi-step tumorigenesis process. Some, but not all of these DNA lesions are believed to result in mutations and/or overexpression of certain genes. The activation of oncogenes by certain chemicals has been established [1]. For example, benzo(a)pyrene diol epoxide (BPDE), the ultimate mutagenic and tumorigenic metabolite of the ubiquitous environmental pollutant benzo(a)pyrene, can transform some protooncogenes into oncogenes by a process which involves a point mutation [2].

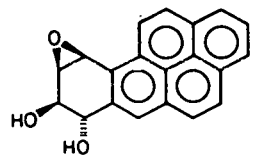
The conformations of the carcinogen-DNA adducts are believed to be of critical importance in determining (1) whether DNA repair can occur, (2) whether such repair is error-free or error-prone, or (3) whether the adducts can cause the replication machinery to err, thus giving rise to mutations. A basic understanding of the conformational features and local structures of DNA around the carcinogen binding sites is essential for an understanding of these phenomena on a molecular level.

The stereoisomeric bay region diol epoxide derivatives of PAH compounds are ideal systems for adduct-structure biological activity studies, because the different stereoisomers of the same compound are characterized by astonishingly different biological activities [3]. Each aromatic diol epoxide consists of a family of two diastereomers (syn and anti), each of which can be further resolved into the two enantiomers (+) and (-). The stereochemical

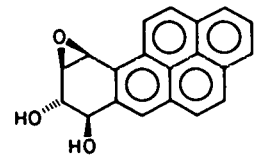
properties of the four enantiomers of BPDE are illustrated below.



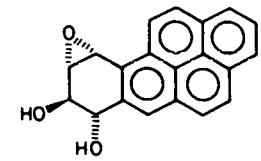
(+) anti-BaPDE



(−) anti-BaPDE



(+) syn-BaPDE



(−) syn-BaPDE

Out of the four optical isomers of BPDE, the (+)anti enantiomer is by far the most tumorigenic one. Similar characteristics are displayed by other aromatic diol epoxides, thus associating the tumorigenic activities of these compounds with (R,S,S,R) stereochemistry. In mammalian cell lines, the mutagenic efficiencies of the different stereoisomers of BPDE follow the same pattern as the tumorigenic activities: (+)anti >> (−)anti = (+)syn; however, in bacterial test systems, the order of mutagenic efficiencies is exactly reversed [4]! While the N2 exocyclic amino group of deoxyguanine is the primary target for covalent binding of BPDE in both prokaryotic and eukaryotic DNA, these results suggest that the DNA lesions are processed quite differently in these two systems [4].

Utilizing a variety of physico-chemical techniques, we have been investigating the conformations of adducts derived from the binding of the different stereoisomers of various polycyclic aromatic diol epoxide derivatives with DNA. In chemically well defined aqueous model systems, two types of adduct conformations can be distinguished [5]:

Site I: In this conformation, the aromatic portions of the covalently bound diol epoxide residues are stacked or partially stacked with adjacent DNA bases (quasi-intercalative complex).

Site II: the polycyclic aromatic residues appear to be tilted at large angles ($>65^{o}$) with respect to the planes of the DNA bases. The flexibility of the DNA at the binding site is increased significantly. The adducts appear to be exposed to the solvent environment (external adduct conformations).

We have found striking correlations between mutagenic (in mammalian cells) and tumorigenic activities of at least 16 different aromatic diol epoxides and/or stereoisomers, and the conformations of the DNA adducts formed. All of the highly tumorigenic and mutagenic (R,S,S,R, or (+)anti) enantiomers form predominantly site II adducts, while the less active or inactive stereoisomers form predominantly site I, quasi-intercalative adducts. More recent work shows that the site I/site II distribution of BPDE adducts in synthetic polynucleotides also depends on the nature of the base-sequences [6].

Computer modelling studies suggest that BPDE can form both base-stacked and external adducts [7]. The intercalative, base-stacked conformers (site I) appear to give rise to a more severe disruption of the local DNA structure, than the external adducts (site II). It has been suggested that the site II adducts thus escape repair, and interfere with the normal processes occurring at the replication fork. Site I adducts, because they perturb the local structure of the DNA helix more severely, are recognized by repair enzymes and are excised [7]. The validity of these hypotheses, the site I/site II correlation with biological activities, and the base-sequence specificities of these effects, are presently being investigated in our laboratory.

Acknowledgements: This research is supported by The Department of Energy and The National Institutes of Health (National Cancer Institute).

REFERENCES

[1] Weinberg, R.A. (1985) Science 230, 770-776.
[2] Marshall, C.J., Vousden, K.H. and Phillips, D.H. (1984) Nature 310, 586-589.
[3] Conney, A.H. (1982) Cancer Res. 43, 4875-4917.
[4] Stevens, C.W., Bouck, N., Burgess, J.A. and Fahl, W.E. (1986) Mutat. Res. 152, 5-14.
[5] Geacintov, N.E. (1985) in: Polycyclic Hydrocarbons and Carcinogenesis (R.G. Harvey, ed.) ACS Symposium No. 283, The American Chemical Society, Washington, D.C.
[6] Roche, C.J. and Geacintov, N.E. (1987) to be published.
[7] Hingerty, B.E. and Broyde, S. (1983) Biopolymers 24, 2279-2300.

PREPARATION OF DNA ADDUCTS FOR CHROMATIN AND CHEMICAL CHARACTERIZATION STUDIES, USING FRESHLY ISOLATED RAT HEPATOCYTES

David A. Dankovic, David L. Springer, Berta L. Thomas, David B. Mann, and Roger M. Bean

Department of Biology and Chemistry, Pacific Northwest Laboratory, Richland, WA 99352

Carcinogenic polycyclic aromatic hydrocarbons (PAH) are known to be metabolically activated to reactive intermediates that bind covalently to DNA and other macromolecules. Even though tumor initiation by PAH carcinogens has been shown to correlate with covalent binding of reactive intermediates to DNA, the critical target macromolecules have not been unequivocally identified. Because of the inadequacy of the current understanding of the tumor initiation process, we have recently submitted a proposal to study PAH carcinogenesis at the chromatin level; the emphasis of this work will be on the location, identification, and persistence of carcinogen binding to DNA and to the protein components of chromatin. In addition, we have an ongoing project to study the chemistry of DNA adducts. The needs of these two projects include (1): methods to isolate and characterize the PAH adducts formed with DNA subregions, histones, and other non-histone proteins; and (2): methods to prepare large quantities of adducts for structural characterization studies. To fill these needs we have been examining the use of freshly isolated rat hepatocytes for the preparation of DNA adducts, using benzo[a]pyrene (BAP) as our model carcinogen.

The preparation of DNA adduct standards in quantities sufficient for chemical characterization has generally been a difficult matter. Although chemically-reactive carcinogen metabolites (such as diol-epoxides) are available for BAP and a few other PAH, thus facilitating <u>in vitro</u> chemical synthesis of adduct standards, the appropriate reactive metabolites are <u>not</u> availa-

ble for the vast majority of carcinogenic PAH. Numerous investigators have therefore used biosynthetic methods of adduct preparation, often either using rat liver homogenates (S9) or microsomes, plus calf-thymus DNA.

Unfortunately, the HPLC profile of BAP adducts obtained using microsomes plus CT-DNA has been shown to differ substantially from that observed in mouse skin in vivo.[1] In contrast, the major adducts formed with endogenous DNA by isolated rat hepatocytes in vitro are identical to those observed in mouse skin in vivo.[2] In addition, it has also been reported that the formation of BAP-DNA adducts by isolated rat hepatocytes can be "amplified" by the addition of exogenous CT-DNA.[3] Although the adducts obtained in the latter study were not characterized by HPLC, these data suggested that isolated rat hepatocytes in vitro could be utilized effectively for the preparation of relatively "large" (ie, μg) quantities of DNA adduct standards. In the present paper we describe the results of experiments investigating the amount of DNA adduct formation and the HPLC profile of the BAP-DNA adducts obtained using freshly isolated rat hepatocytes incubated with CT-DNA.

Viable rat hepatocytes were prepared by collagenase perfusion, and incubated with one mg/ml CT-DNA as described by Shen et al[3]. DNA was isolated by a series of phenol extractions, and RNA and protein digested enzymatically and removed by solvent extraction. The DNA was enzymatically digested into deoxyribonucleosides, and the BAP-DNA adducts were analyzed by HPLC, using the method of Smolarek and Baird.[4]

In Experiment #1, hepatocytes were prepared from a 3-methylcholanthrene induced male rat, and the time course and dose-response curve of BAP adduct formation with exogenous CT-DNA were determined. Maximal DNA binding was obtained in two hour incubations with 180 μM BAP, yielding 151 pmol of adduct per mg DNA.

In experiment #2, hepatocytes were prepared from a β-naphthoflavone induced rat and used in a full-scale DNA adduct preparation experiment. Twelve flasks of 10 ml of hepatocyte suspension (containing one mg/ml CT-DNA) were incubated for two hours with 246 μM BAP, and the BAP-DNA adducts isolated. Significantly lower levels of DNA binding were observed than in experiment #1, resulting in an average yield of 52 pmol per mg DNA. Despite the lowered binding, 3.5 nmol of BAP-DNA adducts (approx. 1.6 μg) were obtained.

HPLC profiles of the BAP-DNA adducts obtained in experiments #1 and #2 were determined, and compared to those isolated from the skins of mice treated with BAP in vivo. The HPLC profiles of the adducts prepared using isolated rat hepatocytes plus exogenous calf-thymus DNA were essentially identical to the profiles of mouse skin adducts; in all cases, the predominant peak was the benzo[a]pyrene-anti-diol-epoxide adduct of deoxyguanosine.

Preliminary experiments have also been carried out to establish conditions for the preparation of 7,12-dimethylbenzanthracene (DMBA) adducts, using isolated rat hepatocytes plus CT-DNA. Although optimal conditions have not yet been established, the covalent binding of 42 - 72 pmol DMBA per mg DNA has been observed, and the HPLC profile of the DNA adducts is similar to that observed in vivo, in mouse skin. The preliminary results using BAP and DMBA are therefore satisfactory, and it is anticipated that this method will prove useful for other PAH as well.

REFERENCES

1. Ashurst, S. W. and Cohen, G. M. Carcinogenesis 3, 267-273; 1982.

2. Ashurst, S. W. and Cohen, G. M. Chem. Biol. interact. 29, 117-127; 1980.

3. Shen, A. L., Fahl, W. E. and Jefcoate, C. R. Arch. Biochem. Biophys. 204, 511-523, 1980.

4. Smolarek, T. A. and Baird, W. M. Carcinogenesis 5, 1065-1069; 1984.

IDENTIFICATION OF DNA DAMAGE IN MOUSE LUNG AFTER INTRATRACHEAL INSTILLATION OF NITROAROMATIC AND POLYCYCLIC AROMATIC HYDROCARBONS

C. E. Mitchell, O. S. Martin, and J. A. Bond

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Nitroaromatic and polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants produced from the incomplete combustion of fossil fuels. 1-Nitropyrene (1-NP), a representative nitroaromatic, produces mutations in bacterial cells, sister chromatid exchanges and mutations in mammalian cells and cancer in rats and mice. The N-(deoxyguanosin-8-yl)1-aminopyrene (C8-dG-AP) adduct has been identified in Salmonella typhimurium cells after metabolism of 1-NP, and in rat kidney, liver, and mammary gland after intraperitoneal administration of 1-NP. This adduct is thought to be responsible for the induced mutation found in Salmonella typhimurium after 1-NP treatment. The purposes of these studies were to determine the nature of the covalent binding and DNA damage of polycyclic aromatic and nitroaromatic compounds to mouse lung DNA after intratracheal instillation, and the isolation and characterization of DNA adducts in mouse lung, liver and kidney after 1-NP administration.

DNA alkaline elution, total covalent binding, and DNA adduct formation (using the ^{32}P-postlabeling assay and HPLC) were used to determine the extent of DNA damage after instillation of chemicals. Pretreatment of male B6C3F$_1$ mice (12-16 weeks old) with benzo(a)pyrene (BaP) (24 hours prior to instillation of labeled compounds) was effective in increasing the binding of chemicals to DNA. Four hours after instillation of [^{14}C]-1-NP, [-^{14}C]dinitropyrene, [^{3}H]BaP, or [^{3}H]-2-aminoanthracene, the amounts of labeled compounds bound to DNA were 160-, 7.6-, 5.4-, and 5.2-fold above uninduced mice. The amount of 1-NP bound to DNA was 30 times greater than

BaP. The average amount of label bound one week later ranged from 43-62% of the activity bound at four hours. Pretreatment with diesel exhaust extract increased only BaP binding, and 1-NP pretreatment did not affect the bound activity.

1-NP caused a dose-dependent increase in single strand breaks (SSB) at 2 hr after treatment as measured by the alkaline elution assay. The DNA elution rate (a measure of SSB) at the highest dose (0.3 mmol 1-NP/Kg) was 22, 12, 3.5, and 1.2 times the controls at 2 hr, 1, 3, and 7 days, respectively. DNA repair of SSB appeared complete by 7 days after treatment; however, covalently-bound radioactivity was present at 7 days and at 28 days after treatment. Although dose related increases in SSB and in bound radioactivity were observed after 1-NP administration, the times at which the highest levels of SSB and bound radioactivity occurred suggest that the repair of SSB in DNA and the loss of DNA adducts were not related. The t1/2s for DNA turnover in cells prelabeled with [^{3}H]thymidine in newborn mice labeled when adults were ~22 and 9 days, respectively, at the time of the 1-NP treatment.

To isolate and characterize the DNA adducts in mouse tissue after 1-NP administration, enzymatically digested DNA was separated by HPLC analysis. Multiple DNA adducts were present in the lung, liver, and kidney at 1 day after administration. One of the major DNA adducts in the lung (20% of the total eluted radioactivity), coeluted with the C8-dG-AP adduct. This adduct (10% of the total eluted radioactivity) and others were still present in the lung at 28 days after administration of 1-NP. One of the adducts in liver and kidney digests also coeluted with C8-dG-AP. Treatment of the adducts with 1 M NaOH resulted in earlier eluting peaks containing radioactivity, indicative of an imidazole ring-opening adduct. A portion of the original

peak of radioactivity that coeluted with C8-dG-AP and other adducts, however, was not affected by NaOH. Thus, the chromatographic properties and chemical behavior of the adducts formed in vivo is consistent with the properties of guanine-8-arylamines and suggests that one of the adducts in lung is C8-dG-AP, which is formed by nitroreduction of 1-NP. Other data, obtained using ^{32}P-postlabeling techniques, indicate that three major and three minor adducts are formed (one of which is C8-dG-AP) after incubation of 1-NP with xanthine oxidase and calf thymus DNA. The presence of other adducts in lung DNA indicates that they may be formed via ring oxidation and/or reduction.

These studies show that different classes of polycyclic aromatic compound metabolites bind to lung DNA and that after induction of lung enzymes, 1-NP is bound much more extensively than other compounds investigated. These studies also indicate that DNA adducts of 1-NP metabolites may be formed in lung (a primary site for inhaled particles), liver and kidney following inhalation of airborne particles containing 1-NP and that both the slower proliferating and rapidly proliferating cells are targets for 1-NP binding. In addition, a significant portion of the DNA adducts may be repaired early whereas other adducts are removed at a slower rate which is similar to that of normal DNA turnover. It will be important in future studies to determine the level, type and effect of DNA adducts in tissues and cells and the role of DNA repair enzymes in the excision of DNA lesions induced by 1-NP and other nitroaromatic compounds.

Acknowledgements: Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

CHEMICAL BINDING, DAMAGE AND REPAIR IN MAMMALIAN GERM CELLS

Gary A. Sega

Biology Division, Oak Ridge National Laboratory*, P.O. Box Y
Oak Ridge, TN 37831

Many chemicals have been found to be mutagenic in mammalian germ cells. However, both the types of mutational events and their frequencies are dependent on the chemical being used and the germ-cell stage being considered. At least part of this variation in germ-cell stage sensitivity can be attributed to differences in the amount of chemical reaching genetically significant targets and to variations in the repair capabilities of different stages.

Our work has focused on measuring the amounts of selected chemical mutagens that reach the germ cells in the mouse, determining if the molecular targets in the various stages may be different, how much DNA breakage is occurring in the different stages, and whether or not repair of DNA damage occurs in the germ cells.

In the course of our research we have found that: (1) Protamines as well as DNA are important molecular targets for chemical attack that may lead to chromosome damage. (The sperm protamines of both mouse and man are simple proteins that are intimately associated with DNA and found only in late-spermatid and mature-sperm stages.) (2) DNA repair can occur in a number of germ-cell stages in the male, but not in the most mature stages. (Our observation of a mutagen-induced DNA repair response in mouse germ cells has led to its development as a fast, sensitive and inexpensive screen for potential germ-cell mutagens.) (3) A pattern of DNA breakage can be measured in developing spermiogenic stages exposed to certain mutagens which parallels the pattern of sperm head alkylation and the pattern of induced dominant lethals and heritable translocations.

For human genetic risk estimation, knowledge of the molecular events occurring within the exposed germ cells is of great importance. Furthermore, the fact that these molecular events can be measured at exposures far below those needed to produce a detectable mutagenic effect in the mouse allows the extrapolation of any genetic data to be made down to the low exposure levels usually encountered by humans. (*Operated by Martin Marietta Energy Systems, Inc., under contract DE-AC05-840R21400 with the U.S. DOE.)

ILLEGITIMATE RECOMBINANTS AND DNA DELETIONS INDUCED BY BENZO[a]PYRENE DIOL EPOXIDE IN ESCHERICIA COLI

Samuel B. Weiss and John Kokontis

The Franklin McLean Memorial Research Institute, The University of Chicago, Chicago, IL 60637

The alkylation of cellular macromolecules by activated polycyclic aromatic hydrocarbons (PAH) is believed responsible for the mutagenic and carcinogenic responses induced. While different cellular molecules may react with activated PAH, DNA is probably the most critical target in living systems. Our studies have employed the use of infectious viral nucleic acids as a probe to study modifications in DNA sequence arrangement induced by alkylation with (+)-BPDE [(+)-anti-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene].

In an earlier report from this laboratory,[1] short DNA oligomers, Bam HI linkers 10 bp in length, were alkylated with (±)-BPDE, inserted into M13 viral DNA, and then used to generate progeny virus in transfected E. coli JM103 cells. The DNA of these progeny virus could then be sequenced at the site of oligomer insertion to determine if nucleotide sequences had been altered. Our studies indicated that the alkylated oligomer inserts induced deletions ranging from 1 to 24 base pairs in length which included both target and non-target nucleotides. We speculated that the high localized concentration of BPDE adducts in our Bam HI linker inserts might be responsible for the large deletions observed. Therefore, it was decided to prepare synthetic duplex DNA oligomers containing a single alkylated site in each duplex DNA strand. Several ds oligomers were synthesized such that the nucleotide base alkylated and the distance between the alkylated residues would vary for each oligomer construct. The (+)-BPDE constructs prepared are indicated below.

1. $(T_{12}AT_2)$

```
                    BP
                    |
5'P-CTTTTTTTTTTTTATTAATAAAAAAAAAAAAG 3'
3' GAAAAAAAAAAAAATAATTATTTTTTTTTTTTC-P 5'
                    |
                    BP
```

2. $(T_{12}GT_2)$

```
                    BP
                    |
5' P-CTTTTTTTTTTTTGTTAACAAAAAAAAAAAAG 3'
3' GAAAAAAAAAAAAACAATTGTTTTTTTTTTTTC-P 5'
                    |
                    BP
```

3. (T_7GT_7)

```
                  BP
                  |
5' P-CTTTTTTTGTTTTTTTAAAAAAACAAAAAAAG 3'
3' GAAAAAAACAAAAAAATTTTTTTGTTTTTTTC-P 5'
                  |
                  BP
```

The above (+)-BPDE alkylated constructs, 32 bp in length, were ligated into M13mp19 RF viral DNA which were used to transfect E. coli, plated on agar containing YT medium, IPTG, and XGal, and incubated overnight at 37°C. Progeny virus were selected on the basis of colorless and light blue plaques. Selected virus were amplified and their DNA isolated for sequence analysis.

The results of the DNA sequence analysis for the progeny virus recovered from cells transfected with the above constructs are summarized below.

				M13mp19 DNA Sequence Modifications		
Treatment of insert oligomer	Type of oligomer	Cells transfected	Total clones	None	Deletions	Illegitimate recombinants
None	$\begin{bmatrix} T_{12}AT_2 \\ T_{12}GT_2 \end{bmatrix}$	JM105	294	294	0	0
BPDE	$T_{12}AT_2$	JM105	20	1	6	13
BPDE	$T_{12}GT_2$	JM105	20	7	8	5
BPDE	$T_{12}GT_2$	JM109 (recA⁻)	3	0	2	1
BPDE	T_7GT_7	JM105	17	9	7	1

Our findings indicate that when nonalkylated constructs were employed no modifications in DNA sequence arrangement were detected. When the construct contained BP-adducts, two major types of modifications in DNA sequence arrangement were found, large deletions and illegitimate (nonhomologous) recombinants which resulted in complete removal of the inserted oligomer. Both types of modifications appear not to be under control of the RecA gene since recA$^-$ cells (JM109) transfected with the (+)-BPDE alkylated insert, $T_{12}GT_2$, induced the same kinds of DNA sequence rearrangements. As the distance between the alkylated residues in the duplex strands was increased, the number of recombinants detected was reduced. In addition to deletions and nonhomologous recombinants, the E. coli repair system is capable of restoring the original nucleotide sequence of the alkylated construct.

The appearance of large deletions at the viral DNA site of BP-oligomer insertion is similar to our previous observations. However, the detection of BP-DNA repair by illegitimate recombination, with recombinants ranging from 104 to 698 base pairs in size, is unique. All of the DNA recombinant segments examined so far are derived from Alu I sites (AGCT) in M13 DNA. The mechanism by which DNA BP-adducts induces the exchange of DNA strands which requires double strand breaks, as is also the case with DNA deletions, is not presently clear.

REFERENCES

1. Wei, S-J. C., Desai, S.M., Harvey, R.G., and Weiss, S.B. Proc. Natl. Acad. Sci. USA, 81, 5936-5940; 1984.

REGIONAL DISTRIBUTION OF XENOBIOTIC METABOLIZING ENZYMES AND DNA ADDUCTS IN THE RESPIRATORY TRACT--RELATIONSHIP TO RESPIRATORY EPITHELIAL POPULATIONS

J. A. Bond, J. R. Harkema, C. E. Mitchell, L. Martin, V. I. Russell, J. L. Mauderly, and R. K. Wolff

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Several factors could be responsible for the apparent differential susceptibility of respiratory tract tissues to carcinogens. One important factor that probably plays a crucial role is metabolism. The objectives of this research were three-fold: 1) to characterize the metabolic capacities of various regions of the respiratory tract, 2) to determine if regional variances in metabolic capability are associated with morphologic differences of surface epithelium among airways, and 3) to investigate the build-up and persistence of DNA adducts in select regions of the respiratory tract during and following the end of exposure to carcinogens.

Specific regions from one-half of the nasal, tracheal, bronchial, and pulmonary airways of dogs were excised and analyzed for the presence of xenobiotic metabolizing enzymes. Complementary halves of airways were fixed and processed for light microscopy. Substrates for different isozymes of cytochrome P-450, including benzo[a]pyrene (BaP), nitropyrene (NP), ethoxycoumarin, and ethoxyresorufin as well as selected Phase II enzymes were measured. The data for BaP and NP were qualitatively similar in that there was high metabolic activity in the nasal tissue and in the intrapulmonary airway generations 3 - 18 compared to the major conducting airways (e.g. larynx, trachea, and bronchi). Most of the ethoxycoumarin O-deethylase activity was in the nasal region, with much less activity observed in the major airways or the pulmonary airways. The specific activity of ethoxycoumarin O-deethylase in the ethmoid turbinates was, in

general, 10 times that observed for the other portions of the nasal cavity sampled. Only the ethmoid turbinates showed evidence of ethoxyresorufin metabolism. Both epoxide hydrolase and glutathione transferase activity were greatest in the various tissues of the nasal cavity and in the pulmonary airways. UDP-Glucuronyl transferase was relatively evenly distributed throughout the respiratory tract. All regions of sampled respiratory tract tissue had unique epithelial populations ranging from squamous in proximal nasal cavity, to olfactory in distal nasal cavity, and ciliated epithelium in distal pulmonary airways.

Experiments were also conducted in which rats were exposed nose-only to dilute diesel exhaust (10 mg particles/m^3) for up to 12 weeks. DNA adducts were measured in various regions of the respiratory tract after the 12-week exposure, using the highly sensitive ^{32}P-postlabeling assay. The data indicate that there were both quantitative and qualitative differences in the distribution of DNA adducts along the rat respiratory tract. A minimum of 6 separate adducts were detected. The level of modification ranged from about 1 to 5 adducts in 10^7 normal bases (mainstem bronchi) to nearly 12,000 adducts in 10^7 normal bases (parenchyma). The nasal tissue, including both the maxilloturbinates and ethmoturbinates contained 1 major adduct, whereas the rest of the respiratory tract contained several adducts. For example, one adduct, which was present at levels of 50 to 100 adducts in 10^7 normal bases in the trachea and nasal tissue, was absent in the major conducting airways (mainstem bronchi and axial airway) but was present in very high concentrations in the parenchyma (about 12,000 adducts in 10^7 normal bases). We have not yet identified the observed adducts, although they are probably adducts of polycyclic aromatic hydrocarbons (PAH) and substituted PAH.

The data presented here indicate that there are significant differences in the metabolic capability and DNA adduct distribution along the respiratory tract. Quantitative differences that were noted along the respiratory tract are probably due to both differences in the capability of a given region to metabolize the organic chemicals associated with diesel exhaust and differences in the deposition patterns of the diesel exhaust. Differences in the metabolic capability, and therefore DNA adduct distribution, along the respiratory tracts of different species could explain, in part, why certain portions of the respiratory tract are more susceptible to tumor formation than are other portions.

Acknowledgements: Research sponsored by the U.S. Department of Energy's Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

EVALUATION OF THE GENETIC EFFECTS OF IN VIVO EXPOSURE TO TOXIC AGENTS: THE MOUSE LYMPHOCYTE MODEL

Irene M. Jones, James D. Tucker, and Anthony V. Carrano

Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94550

Evaluating the biological significance of exposure to toxic chemicals is a demanding task. One must establish that exposure produces alterations in a predictable way, and that the alterations are related to the welfare of exposed individuals. To accomplish these objectives one must quantify the exposure to both the individuals and the specific cellular target system, quantify a biological effect of the exposure, and establish the predictive significance of this effect. For predictive purposes, it is important to understand at the DNA level the nature of genetic damage induced in vivo by exposure to toxic substances. Genetic damage in somatic cells might increase the risk of cancer, whereas genetic damage in germ cells may contribute to diminished reproductive fitness, and potentially to increased mutation frequency in the gene pool.

We have developed a mouse lymphocyte model to study mechanisms of mutation induction and the physiological and other factors that affect quantitation of somatic mutation in vivo. *The mouse lymphocyte model system offers many advantages for studying mutations.* The use of live mice for exposures insures, in ways in which in vitro studies cannot, that many critical parameters are normal, eg. metabolism of the agent, the distribution of the agent and metabolites, and the cell cycle state, karyotype and DNA repair capacities of the target cells. With mice, controlled exposures are possible, whereas with human populations this is seldom true. The use of lymphocytes provides: cells that are easily accessible in mice and humans; cells that can be cultured in vitro for analysis of genetic changes; the ability to define factors that affect the frequency, nature and persistence of particular genetic alterations in cells; the ability to study tissue specific differences by comparing the tissues of lymphocyte ontogeny.

In our present mouse model we use sister chromatid exchange (SCE) to monitor the formation and persistence of DNA adducts (1,2), and measure the frequency and spectrum of mutations of the hprt gene as an indicator of genetic consequences of exposure to toxic agents (1,3,4,5). Together these approaches give us the ability to quantify the dose received, and the magnitude and molecular nature of specific induced genetic changes, in the target cells.

We use sister chromatid exchange as a biological dosimeter for chemical exposures. SCE results from a DNA recombination event that occurs in dividing cells both naturally (6) and, in a

dose reponsive manner, in response to adducts in DNA (7). We selectively study SCE of thymus derived ("T") lymphocytes by using T lymphocyte-specific mitogen in the cell cultures. By using ethylnitrosourea (ENU) as a test compound, we have determined that tissue, dose, and time since exposure affect the frequency of SCE (1,2). In general, the frequency of SCE was greatest immediately after exposure to ENU, and decayed linearly to control values within 25 weeks. The frequency of SCE in spleen lymphocytes was greater than in peripheral blood lymphocytes, but the patterns were similar. These results demonstrate that accessibility of mutagen, repair capacities, and dilution of adducts by cell division, are tissue specific, and that a proportion of ENU-induced adducts persist in vivo in lymphocytes for many weeks. Though SCE analysis does not reveal the identity of adducts, it monitors the exposure of particular cells to an agent, and the persistence of lesions. Persistence, in turn, indicates the level of repair, of cell division and turnover, and increases our ability to interpret the results with other endpoints.

We study both the molecular nature (5) and frequency of mutations of the hprt gene (1,3,4) in order to understand the genetic consequences of exposure to mutagen. Hprt is the target gene of choice because mutants of it can easily be selected by resistance to thioguanine, and knowledge and techniques are available for determination of the specific genetic changes present in mutants. In addition, use of hprt facilitates interspecies, and in vitro/in vivo comparisons.

Our studies of the molecular nature of mutations of the mouse hprt gene have revealed that a wide variety of genetic lesions can be recovered as mutant lymphocytes (5). This is a critical attribute of our model system for it indicates that almost all types of genetic alterations that occur in somatic cells can be recovered as mutants by selecting for thioguanine resistant lymphocytes. We have used both conventional and field inversion electrophoresis techniques in Southern analysis of spontaneously occurring, cloned, HPRT-deficient spleen lymphocytes to reveal deletions (15/23) that range up to 100 kilobases in length, as well as rearrangements of the gene (3/23), and mutants whose alterations are too small to be resolved by Southern analysis (5/23).

Our assay for the frequency of thioguanine-resistant mouse lymphocytes (1) has enabled us to analyze factors that affect the mutant frequency. Using ENU as a model alkylating agent, we have deduced that the efficiency of mutation induction was greater in the mitotically active thymus, the producer of T lymphocytes, than among mature spleen lymphocytes. This tissue difference, coupled with the steady replenishment of spleen lymphocytes by the thymus, led to a time-dependence of the frequency of mutants following exposure. Persistent adducts, indicated by SCE analyses, also may have contributed to formation of new mutants for a period of weeks after exposure. ENU-induced elevations of mutant frequency persisted for up to 1 year,

suggesting that stem cells were mutated as well as maturing and mature lymphocytes. Other important factors that require further study are dose rate and toxicity. We have demonstrated that dose rate affects mutant yield; a fractionated dose gave more than an additive effect, and the highest acute dose tested (72mg/kg) gave reduced mutant yield.

In our mouse lymphocyte model system we are addressing issues central to in vivo mammalian mutagenesis by using state-of-the-art methods of molecular analysis, and conventional methods of mutant analysis and cytogenetics. The system is responsive to a wide variety of genetic alterations. The ability to analyze not only the frequency of induced genetic changes, but also the molecular nature of mutations, will lead to valuable knowledge of the spectrum of mutations induced in vivo, and hence of the biological significance of elevated mutant frequencies. The choice of hprt as the target gene will allow this system to contribute to a wide range of comparisons, of metabolism of toxic agents in vitro and in vivo, for example, and to model the consequences of in vivo exposures of human populations.

This work conducted under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under contract W-7405-ENG-48.

References

1. Jones, I.M., K. Burkhart-Schultz, and A.V. Carrano (1985) Mutat. Res. 147: 97-105.
2. Tucker, J.T., C.L. Strout, M.L. Christensen, and A.V. Carrano (1986) Environ. Mutagen. 8: 345-355.
3. Jones, I.M., K. Burkhart-Schultz, and A.V. Carrano (1985) Mutat. Res. 143: 245-249.
4. Jones, I.M., K. Burkhart-Schultz, C.L. Strout, and T.L. Crippen (1987) Environ. Mutagen. 9: 317-329.
5. Jones, I.M., K. Burkhart-Schultz, and T.L. Crippen (1987) Som. Cell Mol. Genet. (in press).
6. Tucker, J.D., M.L. Christensen, C.L. Strout, and A.V. Carrano (1986) Cytogenet. Cell Genet. 43: 38-42.
7. Latt, S.A. and others (1981) Mutat. Res. 87: 17-62.

POSTER SESSION 3

Summary of Poster Session (3)

M. H. Bhattacharyya

Argonne National Laboratory

and

T. J. Mast

Pacific Northwest Laboratory

The poster session brought together presentations on diverse aspects of chemical toxicity. The majority were aimed at understanding the molecular events responsible for the toxic effects of the chemicals under study.

A number of studies were conducted to identify the critical molecules responsible for the carcinogenicity and mutagenicity of polynuclear aromatic hydrocarbons (PAH). Recent work from Springer, *et al.* (PNL) reported a lack of correlation between the tumorigenicity of PAH-containing mixtures and the amount of benzo[a]pyrene-DNA binding detected after exposure to those mixtures. They propose further examination of the formation of DNA adducts and alterations in chromatin structure observed following exposure to complex chemical insults. Pelroy and Stewart (PNL) further demonstrated the complexity of the toxic response to chemical mixtures by reporting the existence of synergism between mixtures of hydrocarbon PAH's and several nitrogen-containing PAH's causing either frameshift or base-substitution mutations.

Human mammary epithelial cells (HMEC) were shown by Bartley *et al.* (LBL) to readily metabolize benzo[a]pyrene (BaP) to the 7,8-dihydrodiol-9,10-epoxy-BaP, and to form the PAH-DNA adduct with deoxyguanosine. Furthermore, repeated treatment of these cells with BaP resulted in the isolation of two immortal cell lines that maintained the characteristics of the original mammary epithelial cells. Continued experimentation with these cell lines demonstrated the induction of oxidative damage to DNA by BaP as determined by detection of thymine glycols with monoclonal antibodies. (The thymine glycols are indicative of oxidative damage to DNA.) When compared to equitoxic doses of ionizing radiation, BaP was shown to induce a 35-fold greater concentration of thymine glycol in HMEC cultures.

The genetic influence on an organism's response to a toxicant was clearly shown by Popp and Niemann (ORNL) when they demonstrated the differential sensitivity of C57BL/6 and SEC/R1 mice to inhalation exposures of ethylene oxide (EtO), the C57BL/6 strain being the more resistant. Crossmating studies indicated that the sensitivity to EtO may be controlled by a single pair of recessive genes. It was further shown that germinal as well as somatic cells of the SEC/R1 mice were more susceptible to EtO toxicity. Attempts to identify the specific gene and the gene product(s) that are responsible for controlling the toxic response to EtO are underway.

In studies of molecular events associated with exposure to carcinogens, Felton, *et al.* (LLNL) developed a method to identify the DNA changes in the HisD gene of *Salmonella* caused by a number of mutagens. All of these changes were deter-

mined to be deletions of 2 to 26 nucleotides in length. Results also demonstrated that exposure to all of the aromatic amines resulted in 2-base deletions, while BaP caused the larger base deletions. The technique developed by Felton, *et al.* can be applied to understanding the molecular changes in DNA responsible for the reverse mutations in *Salmonella* that are studied by investigators in numerous laboratories around the world. Broyde (NYU) reported results of calculations on the Cray-2 computer at Livermore that yielded views of carcinogen-DNA adducts with resolution at the atomic level. One such calculation showed the first molecular view of how 2-acetylaminofluorene (AAF) could induce the 2-base deletion mutations that have been identified in AAF-modified *E. coli* DNA, and another calculation provided an explanation of why aniline lacks carcinogenic activity while the structurally related 4-aminobiphenyl is carcinogenic. Such calculations are important since no experimental data at the level of atomic resolution are yet available for any carcinogen-DNA adduct because of the experimental difficulties in synthesizing and crystallizing these substances. In another presentation, Haugen and Myers (ANL) reported analytical methods for isolating and quantitating adducts between carcinogens, metabolites, and hemoglobin. Most carcinogens react with the two equivalent sulfhydryl groups in hemoglobin. The method being developed involves treatment of hemoglobin with an ionic reagent that reacts with the free but not the substituted sulfhydryl groups and subsequent resolution of the adduct-containing hemoglobin from the "charge-shifted" native protein by ion-exchange liquid chromatography. Because carcinogen-hemo-

globin adducts are stable, their quantitation may ultimately provide a measure of human exposure to carcinogens by analysis of blood samples.

Two presentations directed attention to the importance of the nasal cavity in the handling of inhaled toxicants. Yeh *et al.* (ITRI) demonstrated that 10 to 50% of ultrafine particles were deposited in the nasal region of a human nasal cast, with deposition efficiency being greatest for the smallest particles (<0.01 μm diameter). Because most inhalation deposition models assume zero deposition in the nasal-oral regions for small particles, these data demonstrate the necessity for reevaluating existing inhalation models. The presentation of Dahl *et al.* (ITRI) provided information on the importance of nasal enzymes in the metabolism of inhaled chemicals. In a third study from ITRI on response to inhaled toxicants, Henderson, *et al.* reported on the role of glutathione depletion in the differential response of rats and mice to inhaled diesel soot particles. In earlier work they had shown that rats appear to develop more extensive pulmonary fibrosis than do mice, despite a higher relative lung burden of diesel soot in the mouse.

Two laboratories reported on changes occurring during fetal and neonatal development. Investigators at PNL demonstrated that the PAH fraction of complex mixtures is teratogenic in both rats and mice, and consistently produces severe pulmonary hypoplasia in the offspring. T.J. Mast *et al.*, (PNL) investigating the temporal development of this abnormality at the ultrastructural level, demonstrated that the treated lungs underwent accelerated biochemical and morphological maturation of the

alveolar region without concomitant proliferation of the alveolar epithelium. This maturational defect will serve as a model to study mechanisms of initiation of developmental differentiation and maturation.

Bhattacharyya *et al.* (ANL) observed mouse dams after exposure to environmental levels of dietary cadmium during pregnancy and lactation. They hypothesized that increases in metallothionein (MT) concentrations in tissues of the lactating dam might explain the increases in the absorption and subsequent deposition of cadmium. Results with two different MT assays showed clear increases in MT concentrations in the liver, kidney, and duodenum of the mouse dam during lactation, with MT concentrations falling to levels comparable to those of nonpregnant controls within 5 days after weaning of the pups. Additional investigations may elucidate the role of the metal-binding protein, metallothionein, during lactation and identify the as yet unknown agent responsible for its induction.

Although most of the posters dealt with the biological aspects of chemical toxicity, Angel *et al.* (LLNL) reported on a significant advancement in analytical technology. The necessity of performing on-site measurements of low concentrations of toxic contaminants prompted the development of an optical chemical sensor, or "optrode," to function as a portable spectrometer. This portable instrument is a sensitive and specific detector capable of analyzing chlorinated organic chemicals in ground water at levels of less than 100 ppb. Efforts are also underway to develop even more specific sensors by using immobilized enzymes. Enzyme-based optrodes will monitor concen-

trations by measuring the rate of formation of specific products enzymatically catalyzed. Future plans also include the development of specific sensors based on antigen–antibody interactions.

In summary, the poster session provided an opportunity for presentations in areas ranging from molecular changes in response to carcinogen or teratogen exposure to sensitive new techniques for the monitoring of toxicants in the environment. The format allowed for in–depth discussions not afforded by the platform presentations and formed the basis for the development of a number of collaborations among investigators at the workshop.

OPTICAL CHEMICAL SENSORS FOR ENVIRONMENTAL MONITORING

Stanley M. Angel, Paul F. Daley, Thomas J. Kulp

Environmental Sciences Division, Lawrence Livermore National Laboratory, P.O. Box 808, L-524, Livermore, CA 94550

At Lawrence Livermore National Laboratory we are investigating methods for measuring low concentrations of toxic contaminants in-situ using optical fibers and fieldable spectrometers. This requires the development of optical chemical sensors - Optrodes - that have high sensitivity and selectivity. It also requires developing portable spectrometer systems that are specially suited to make measurements of low signals over optical fibers.

The optrode readout system is a key component of this technology. There are as yet no commercially available systems that can make measurements over single optical fibers with sufficient sensitivity to be useful with most optrodes. Laser-based systems have generally been used in the laboratory and are capable of making measurements over up to a kilometer of optical fiber. Although the laboratory instruments are very versatile they are not portable. Recently, portable fiber-optic instruments have been developed using incandescent sources and solid state detectors. These are housed in a single aluminum briefcase and will fit under an airplane seat. One such instrument is very sensitive and can be used to make optrode measurements over many tens of meters of optical fiber. This instrument is used in our laboratory for making routine measurements and has also been used in the field.

Organochlorides are major contaminants in groundwater. Because of this, chloroform and trichloroethylene (TCE) have been our primary targets in developing optical sensors. We have developed an optrode that is sensitive to trace quantities of these two contaminants[1]. One version is shown schematically in Figure 1. The optrode is constructed by inserting a stripped

200 um optical fiber into a 500 um diameter glass capillary and sealing one end with epoxy. With a microsyringe, 10 M KOH solution is placed around the exposed fiber, followed by a slug of pyridine. An interface forms between the KOH solution and pyridine since the two are immiscible. A mylar membrane is then used to seal the capillary, leaving an air gap between it and the pyridine. Organochloride vapors are detected as they pass through the membrane, cross the air gap, and dissolve in the basic pyridine layer where a fluorescent red product is formed. The same optical fiber is used to deliver the excitation light and to collect the red fluorescence.

The organochloride optrode is sensitive to very low levels of chloroform and TCE. A typical response curve is shown in figure 2 and a detection limit of less than 100 ppb has been demonstrated for chloroform[1]. This optrode has been field tested on several occasions at a chloroform-contamination site in Henderson Nevada. In these tests the optrode is placed above the water layer where chloroform vapors accumulate above the contaminated aquifer. Quantitative measurements of sub ppm levels of chloroform using this technique usually take 1 to 2 hours. The measured levels are in good agreement with independent measurements obtained by gas chromatography.

We are studying ways to improve the organochloride sensor. For example, by changing the chemistry slightly, and using an organic base, the reagents become single phase and the sensor responds much faster. Also, it is possible to enhance the sensitivity of the probe toward specific organochlorides, such as TCE, by changing the active reagents in the sensor. In addition, we are looking at ways to make the sensor more robust by incorporating the reagents in a polymer.

Enzyme optrodes have recently been developed at LLNL[2]. These probes exploit the inherent ability of the biomolecule to selectively recognize a

particular chemical species in a complex mixture of other chemicals. In the case of enzyme sensors the signal produced is a result of selective enzyme-catalyzed chemical reaction of the analyte resulting in a product that is transduced by the probe. The reaction, and thus the analyte concentration, is monitored either through the rate of formation of products or the steady state concentration of products. Because the enzyme is catalytic, the probe is inherently reversible.

Data demonstrating an enzyme optrode used to detect penicillin will be presented. The enzyme, penicillinase, selectively catalyzes the cleavage of the penicillin beta-lactam ring to form penicilloic acid, which dissociates into penicilloate and a proton and thus produces a pH change in the probe microenvironment. The physical transducer with which the enzymatic reaction is monitored is a dye whose fluorescence intensity is pH dependent. Both the enzyme and the pH sensitive dye are immobilized in a thin polymer membrane on the tip of a 200 um core optical fiber. The 95% response time for this sensor, as can be seen in figure 3, is 40-60 s. This is much faster than the response times (2-3 minutes) reported for penicillin electrodes[3].

Optical fiber sensors using antibodies have recently been demonstrated by several groups[4,5,6]. This type of sensor was first studied at LLNL by Tomas Hirschfeld and is currently being pursued in a collaborative effort between our group in Environmental Sciences and a group in the Biomedical Sciences Division at LLNL. This sensor is shown schematically in figure 4. The antibody is bound to the surface of a short length of unclad optical fiber which is placed inside a small diameter glass capillary. Fluorescently tagged antigen is then attached to the antibody binding sites to produce a steady state fluorescence signal in the optical fiber. The concentration of unknown (and untagged) antigen is determined by introducing a solution of it into the capillary tube.

After diffusing to the surface of the optical fiber the untagged antigen will compete for antibody binding sites with the tagged antigen resulting in the displacement of the latter and a decrease in the fluorescence signal. Detection of femtomolar concentrations of selected antigens have been reported by Hirschfeld et al.[7] using this type of sensor.

References:

1. Milanovich, F. P., Garvis, D. G., Angel, S. M., Klainer, S. K., and Eccles, L. Anal.Inst., 15(2), 137; 1986.
2. Kulp, T. J., Camins, I., Angel, S. M., Munkholm, C., and Walt, D. R. In press.
3. Tor, R., and Freeman, A. Anal. Chem. 58, 1042; 1986.
4. Van Wegenen, R. A., Andrade, J. D. Fed. Proc., Fed. Am. Soc. Exp. Biol. 41, Abstract 7094; 1982.
5. Hirschfeld, T. U. S. Patent 4,447,546, 8 May 1984.
6. Sutherland, R. M., Dahne, C., Place, J. F., Ringrave, A. S. Clin. Chem. 30(9), 1533; 1984.
7. Hirschfeld, T., Block, M., Anderson, W., Glass, T., and Eckerson, D. Abstract 675, paper presented at the Pittsburgh Conference and Exposition, Atlantic City, New Jersey, March 1986.

MOLECULAR EVENTS DURING TUMOR DEVELOPMENT

David L. Springer, David B. Mann, and D. Dennis Mahlum

Biology and Chemistry Department, Battelle Pacific Northwest Laboratories. Richland, WA 99352

Even though there is considerable information on the events leading to tumor initiation by organic compounds the critical target molecules involved have not been unequivocally identified. It is known that certain carcinogens are metabolically activated to reactive intermediates which covalently bind to macromolecules and that there is a correlation between binding to DNA and tumor initiation. Because of this information, it is widely held that tumor initiation begins with binding of the carcinogen to DNA. Despite this information there is another body of data which suggests that events other than DNA binding may contribute to the initiation process (Jenson et al., 1982). These data include: (1) on a qualitative and quantitative basis the correlation between mutagenesis and carcinogenic potency is incomplete, (2) transformation events are more frequent (10^2 - 10^3) than mutagenic events after chemical insults (Parodi and Brambilla, 1977), (3) certain tumors undergo spontaneous regression to normal tissue.

Recent work from our laboratory, showing a non-linear relationship between carcinogenic activity and covalent binding of benzo[a]pyrene(BaP), is consistent with an epigenetic component in chemically induced tumor initiation. In this this work, we initiated mice dermally with BaP alone or with BaP in the presence of polycyclic aromatic hydrocarbon (PAH)-containing mixtures. Two weeks after initiation the animals were given twice weekly applications of 12-O-tetradecanoylphorbol acetate (TPA) for 6 months. The initiating activity of BaP was decreased by 10-60% when it was coadministered with the mixtures. Because co-administration of the mixtures decreased the initiating activity of BaP, we examined the binding of labeled BaP to DNA to determine if binding was decreased by the presence of the mixture. We found that the binding of BaP to DNA was inhibited to even a greater degree than was the initiating activity. Even a mixture that only minimally depressed initiating activity decreased binding by 50% while others that inhibited initiating activity by 40-60% decreased binding to approximately 20% (Table 1). These data indicate that there is a non-quantitative relationship

between DNA binding and tumor initiation, suggesting that initiation involves processes other than a simple interaction between the carcinogen and DNA.

The effects of the mixtures of PAH on BaP binding to DNA and on tumor initiation by BaP could occur by altered metabolism, increased persistence of DNA adducts or altered location of damage on the genome. Our present data indicate that alterations in metabolism are primarily quantitative rather than qualitative and therefore probably not responsible for the lack of correlation between BaP-DNA binding and tumorigenicities. We are therefore proposing to concentrate our efforts on examining the role of alterations in DNA adduction, adduct persistence, and changes in chromatin structure in the initiation process. We plan to simplify our experimental system by using simple combinations of PAHs to examine the relationship between tumor initiation and binding of carcinogens to macromolecules. We will assess the role of total DNA adduct formation, alterations of DNA adduct profiles, adduct persistence, and binding of carcinogen to proteins in mediating any observed interactions. We will also determine the location of adducts in the genome by comparing levels in bulk chromatin with those in actively expressed regions, and determine adduct levels in linker and nucleosomal regions (Obi, 1986). We have instituted the methods for isolation of the chromatin fractions of interest in our laboratory and we should soon have preliminary results to suggest which of the above options are the most likely.

Table 1. Data showing the non-linear relationship between covalent binding and skin tumor initiating activity for benzo(a)-pyrene co-administered with polycyclic aromatic hydrocarbon-containing mixtures.

Initiator[a]	Tumor/Mouse[b]	DNA Binding[c]
Solvent	0.17 ± 0	0.09
BaP	7.07 + 0.67	5.72 ± 1.3
300-700°F + BaP	6.63 ± 0.50	2.92 ± 1.0
700-750°F + BaP	4.14 ± 0.49	1.24 ± 0.2
750-800°F + BaP	2.93 ± 0.33	1.26 ± 0.2
300-700°F	0.37 ± 0.13	---
700-750°F	0.57 ± 0.14	---
750-800°F	0.60 ± 0.18	---

[a] 25 ug BaP administered in 50 ul of acetone; 5 mg polycyclic aromatic hydrocarbon-containing mixture administered in 50 ul acetone
[b] 30 mice/group
[c] pmole/mg DNA

REFERENCES

Jenson, J. C., J. FitzGibbon, L.A. Brennan, and G. W. Litman, 1982. Binding of benzo(a)pyrene to histones and altered affinity of modified histone 1 for deoxyribonucleic acid. Biochem. 21 (4): 601-607.

Obi, F. O., A. J. Ryan and M. A. Billett, 1986. Preferential binding of the carcinogen benzo(a)pyrene to DNA in active chromatin and the nuclear matrix. Carcinogenesis 7: 907-913.

Parodi, S. and G. Brambilla, 1977. Mutat. Res. 47: 53-74.

A RECOMBINANT DNA METHOD FOR UNDERSTANDING THE MECHANISMS BEHIND *SALMONELLA* REVERSE MUTATIONS

J.S. Felton, R. Wu, N.H. Shen, S.K. Healy, and J.C. Fuscoe

Lawrence Livermore National Laboratory, Livermore, CA 94550

We investigated the specific sequence changes in the DNA of *Salmonella* revertants induced by a set of mutagens and metabolites. Most of these compounds were chosen because of their specificity to the frameshift strains TA1538 and TA98. DNA lesions in these strains reside in the *hisD* gene and can lead to reversion of histidine dependence by either deletions, insertions, or suppressor mutations. The understanding of the types of DNA base changes induced by both a mutagen and its metabolites can lead to a better understanding of not only the mutational process and repair mechanisms, but also the potency and mode of action of specific metabolites and their corresponding DNA adduct(s).

The compounds used in our study were B[a]P, B[a]P diol-epoxide, B[a]P 4,5-epoxide, Aflatoxin B_1, and the food mutagens, IQ, MeIQ, and PhIP. The DNA of the *Salmonella* revertants induced by these compounds was extracted and cleaved with restriction enzymes (*Sau*3A and *Eco*R1) so that the portion of the *hisD* gene of interest was incorporated in a 650 bp fragment. The 600-700 bp fragments produced following enzyme cleavage are purified from the

smaller and larger fragments by agarose gel electrophoresis. These size fractionated fragments were ligated to the bacteriophage vector *M13mp8* and this mixture was used to transform *E. coli*. The recombinants were screened with a nick-translated *hisD* gene probe to identify the revertant gene sequence. Single-stranded DNA from the recombinants was purified using the Sanger/dideoxynucleotide chain termination method.

All IQ (13), MeIQ (3), PhIP (5), and Aflatoxin B_1 (3) induced revertants isolated had a 2-base (CG dinucleotide) deletion. This deletion is situated in a run of 4 CG-dinucleotide pairs (a hot-spot) ending 10 bases upstream from the original mutation in TA1538 and TA98. This original auxotrophic mutation is a single-C deletion designated *hisD3052* that confirms the histidine dependence on these strains.

In contrast, 9 of 24 revertants induced by B[a]P had extensive deletions varying from 8 to 26 nucleotides in length and located at various sites along a 45 base sequence beginning at nucleotide 2085 of the operon. No pattern could be determined for either the 3' or 5' splice sites associated with the deletions. The other 15 deletions had a CG deletion at the same location as that for the other mutagens. In the case of one large deletion of 14 bases, not only are 5 amino acids not translated due to the deletion, but 8 more are mistranslated because of the change in reading-frame that occurs up to the correction at the *his*D3052 single-base deletion. The ability of the enzyme histidinol dehydrogenase (the product of the *hisD*

gene) to still function with 13 amino acids either deleted or miscoded suggests, either the enzymatic active site is quite distant or independent from this region or domain, or possibly the deletion and subsequent miscoding occur in a looped-out region of the peptide. The amino acid sequence of this portion of the peptide suggests the secondary structure is primarily alpha helical. Measurement of the histidinol dehyrogenase activity of individual revertants showed no clear correlation with the size or the location of the deletion. All revertants had decreased activity from wildtype and varied from 11 to 89% of control. As might be expected, smaller colonies tended to have a higher percentage of large deletions suggesting the synthesis of histidine may have been impaired slightly more in the revertants with the large deletions. These smaller colonies when subcloned still remained small, thus supporting the conclusion a genetic event is responsible for the slow growth. Finally, it is interesting that all revertants sequenced to date are deletions. One might expect a certain number of 2-base insertions to appear; as they could just as easily correct the reading-frame as the 1-base deletions. Additional mutagens are being studied with the possibility they might induce insertions.

Analysis of the metabolites of B[a]P to understand which of the many mutagenic metabolites may be responsible for the large deletions showed clearly that the "anti" 7,8-diol-9,10-epoxide was the major inducer of large lesions (more than 60% were large deletions compared to 38% for B[a]P alone). The 4,5-epoxide showed no large deletions, only CG dinucleotide deletions in the hotspot

region. This effect with the diol-epoxide is in marked contrast to that for the aromatic amines from cooked-food where only the single type (dinucleotide) lesion was seen in more than 20 revertants colonies.

The mechanisms for induction of both the dinucleotide deletion and the larger deletions are not clear, but the aromatic amines have been shown to form C-8 adducts which could lead to a CG deletion. Studies are in progress to determine the role of repair on the types of deletions induced by PAHs and aromatic amines.

Research funded under a special award from the LLNL Insitutional Research and Development Program.

STUDIES OF THE CONTRIBUTION OF RESPIRATORY TRACT METABOLISM TO THE TOXICITY OF INHALED CHEMICALS.

A. R. Dahl, J. Petridou-Fischer, P. J. Sabourin, S. Whaley, and J. A. Bond

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

The major hypotheses being tested is that the respiratory tract, including the nasal region, contributes substantially to the metabolism of inhaled organic compounds. If a substantial portion of an inhaled compound is metabolized in the respiratory tract, then the metabolites are either more or less toxic than the parent compound. Important toxicological effects could follow. For example, the tissues at risk might shift from the liver to the respiratory tract or to tissue exposed to metabolites formed in the respiratory tract. Research to test this hypothesis has focused on respiratory tract enzymology and the role that specific respiratory tract enzymes play in the metabolic fate of inhaled chemicals. Because many compounds are well absorbed in the nasal cavity when inhaled, and the nasal tissues are the first tissues that are exposed to inhaled vapors (and a certain fraction of inhaled particles), the nasal tissues are of particular interest with regard to their capacity to metabolize inhaled materials. The capacity to scrub out inhaled vapors in the nasal cavity may be enhanced by metabolic capacity within the nasal cavity. This, in turn, adds to the protective role of the nose towards the sensitive pulmonary tissues. In consideration of these factors, we have devoted effort to identifying specific nasal enzymes. The nasal enzymes identified and characterized within this project include the cytochromes P-450, primary xenobiotic metabolizing enzymes for PAH's, nitrosamines, some halogenated compounds, alkenes, alkanes, and a large variety of other organic compounds. In addition, flavin containing monooxygenase (FMO), has been identified in the nasal cavity and characterized. These enzymes are responsible for oxidation of compounds

containing heteroatoms, especially nitrogen and sulfur. Carboxylesterases, and rhodanese have also been found in the nasal cavity. These enzymes are respectively responsible for the hydrolysis of carboxylic acid esters, a common class of inhaled materials, and for the metabolism of cyanide to thiocyanate. Other nasal enzymes identified include: catechol methyltransferases, phenol methyltransferases, epoxide hydrolase and glutathione and glucuronyl tranferases. Each of these enzyme systems may have important toxicological effects on inhaled materials.

To move the role of nasal enzymes from in vitro enzymology one step closer to the situation as it occurs in an inhalation exposure, we have tested the ability of nasal enzymes to metabolize substrates in vivo. In these experiments, radiolabeled substances were placed on the nasal mucosas of monkeys, dogs, or Syrian hamsters. Mucus was then collected and analyzed for metabolites. It was found that benzo(a)pyrene and dihydrosafrole are both metabolized on the nasal surface following instillation by this method. These experiments show that, in fact, the nasal enzymes are responsible for transformation of inhaled materials deposited on them.

Currently, experiments are planned to test the role of nasal enzymes in the fate of inhaled materials. The test substances are dimethylnitrosamine (DMN) and dimethylsulfate (DMS). DMN is a methylating agent requiring prior metabolism by cytochrome P-450, and DMS is a direct alkylating agent. Radiolabeled vapors of these compounds will be used for exposure of rats at various concentrations. The deposition and fate within the respiratory tract of these two compounds will be compared to give an indication of how respiratory tract metabolism may alter the binding of methyl fragments to macromolecules when inhaled.

Acknowledgements: Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013).

DIFFERENTIAL EFFECTS OF ETHYLENE OXIDE IN TWO STRAINS OF MICE

R. A. Popp and S. L. Niemann

Biology Division, Oak Ridge National Laboratory and University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, TN 37831

C57BL/6 males survive and remain fertile during 50 exposures to 255 ppm of EO for 6 hours a day and 5 days a week, but SEC/R1 males similarly exposed become sterile after 5 exposures and die during less than 20 exposures. The genetic and biochemical basis for the differential effects of EO in these strains of mice is being studied.

The cause of death of EO-exposed mice is not known but their urine becomes bloody and histological sections of kidney reveal proximal tubule necrosis. There is proteinaceous fluid in the Bowman's capsule and collecting tubules. Calcium oxalate crystals have also been observed in the kidney tubules of SEC mice, which suggests that EO may be metabolized through ethylene glycol, glycol aldehyde, glycolic acid, glyoxylic acid and oxalic acid pathway. We have also shown that SEC mice are more sensitive than C57BL mice to ethylene glycol and that 5% ethylene glycol in the drinking water causes SEC mice to die with symptoms similar to those induced by EO. Toxicity of other EO metabolites is being tested in these mice. Tissues from C57BL and SEC mice are being examined for enzymatic differences that might produce a toxic intermediate in the kidney of SEC but not of C57BL mice.

Standard mating schemes are being used to determine the genetic basis for the differential sensitivity of C57BL and SEC mice to EO. F1 males survive 20 exposures to EO, which suggests that sensitivity to EO is a

recessive trait. About half (88 of 148) of a backcross population of C57BL X SEC)F1 X SEC mice survive 20 exposures to 255 ppm of EO, which indicates that sensitivity of SEC mice to EO is controlled by a single pair of recessive genes. Once the genetic and biochemical basis for EO sensitivity in mice is known, it should be possible to determine whether homologous factors cause some humans to be more sensitive than others to EO or its metabolites.

The germinal tissue of SEC males is also uniquely affected by EO. SEC males exposed to 255 ppm of EO become sterile and histological sections reveal extensive tubular degeneration in the testis. When SEC males are exposed to 150 ppm of EO, there is exfoliation of cells, the numbers of primary spermatocytes and mature sperm are reduced, spermatogenesis is abnormal, and many pycnotic and nucleated cells appear in the tubule lumen. A significantly higher loss of preimplantation embryos was observed when non-exposed females were mated to exposed SEC males compared with exposed C57BL males. Similar numbers of eggs are fertilized by sperm from both strains of exposed males but a larger number of unequal blastomere cleavages were observed when eggs were fertilized by EO-exposed SEC sperm, particularly when these sperm were exposed at the late primary or early secondary spermatocyte stage of spermatogenesis. Exposure of mice to EO did not affect sperm motility but half of the normal number of sperm was recovered from the caput and vas deferens of SEC males after 10 exposures to 150 ppm of EO.

In summary, we have observed that somatic and germinal cells of two strains of mice are affected very differently by exposure to EO. Genetic studies reveal that these differences are controlled by a single pair of recessive genes. We are attempting to identify the gene, to map it to a specific chromosome, to learn how the gene product metabolizes EO to cause

lethality in SEC but not in C57BL mice, and to determine whether homologous genetic and biochemical factors exist in humans to detoxify EO and its metabolites.

(Research sponsored by the Office of Health and Environmental Research, U. S. Department of Energy under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.)

DEPOSITION OF ULTRAFINE PARTICLES IN A HUMAN NASAL CAST

H. C. Yeh[1], Y. S. Cheng[1], Y. Yamada[1], and D. L. Swift[2]

[1]Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185
[2]Johns Hopkins University, Baltimore, MD 21233

The regional respiratory deposition of inhaled aerosols has been recognized as a critical factor in the evaluation of health efects of exposure to airborne material. Previous studies of upper airway deposition have emphasized the role of inertial deposition of particles > 0.5 μm in aerodynamic diameter (AD). However, the deposition of particles <0.5 μm AD has not been well studied. Most deposition models assume zero deposition in the nasal-oral regions for particles less than 0.2 μm. However, in a recent study of local deposition (1), evidence of upper airway deposition of submicron aerosols by diffusional transport was reported. Pattle studied nasal deposition with polydisperse aerosols and gases (2), and reported that the deposition efficiency increased with decreasing particle size and that nearly 100% deposition for iodine and ammonia gas were observed. To better understand the deposition mechanisms of ultrafine particles, we used a human nasal cast (1) and monodisperse aerosols to determine deposition of ultrafine particles with diameters between 0.004 and 0.2 μm. The inspiratory flow rates were between 4 and 60 1/min.

A clear polyester resin cast of the upper airways of a normal human adult was made from a post-mortem negative cast (1). The cast was prepared at Johns Hopkins University. This model includes nasal hairs, the nasal airways, nasopharynx and larynx. The pressure drop through the cast was measured by an inclined manometer. Monidisperse NaCl aerosols were generated using a condensation-mobility classified method (3). The aerosolized material was placed in a quartz boat inside a tube furnace operated between 200 to 600°C, depending on output aerosol size required. The material was

vaporized into a regulated N_2 carrier gas stream and condensed in a glass condensation chamber. The monodisperse aerosols of known size were obtained by using an electrostatic classifier (TSI Model 3071). The monodisperse aerosol was neutralized in a Kr-85 discharger before being delivered to the nasal cast. Additional flow was added to make up the required flow. The deposition efficiency was obtained by measuring the aerosol concentrations at the inlet and outlet of the cast, using a continuous flow condensation nucleus counter (TSI Model 3020).

Similar pressure drops were obtained for both inhalation and exhalation modes. These results are comparable to those obtained in vivo (2,4), suggesting that the cast will be a useful model for deposition studies. The deposition efficiency in the nasal passage for the inspiratory mode is shown in Figures 1 and 2 as functions of particle size and flow rate. The deposition efficiency increased with decreasing particle diameter and flow rate, confirming that diffusional transport is the dominant mechanism of deposition of submicron particles. Our results show substantial deposition in the nasal airways for ultrafine particles and this information should be included in future regional deposition models.

Acknowledgements: Research sponsored by the U.S. Department of Energy Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

REFERENCES

1. Itoh, I., Smaldone, G. C., Swift, D. L., and Wagner, Jr., H. N., J. Aerosol Sci. 16: 529-534, 1985.

2. Pattle, R. E., in Inhaled Particles and Vapors I. (C. N. Davies, ed.), Pergamon Press, Oxford, pp. 302-309, 1961.

3. Cheng, Y. S., Keating, J. S., and Kanapilly, G. M., J. Aerosol Sci. 11: 549-556, 1980.

4. Hounam, R. F., Black, A., and Walsh, M., J. Aerosol Sci. 2: 47-61, 1971.

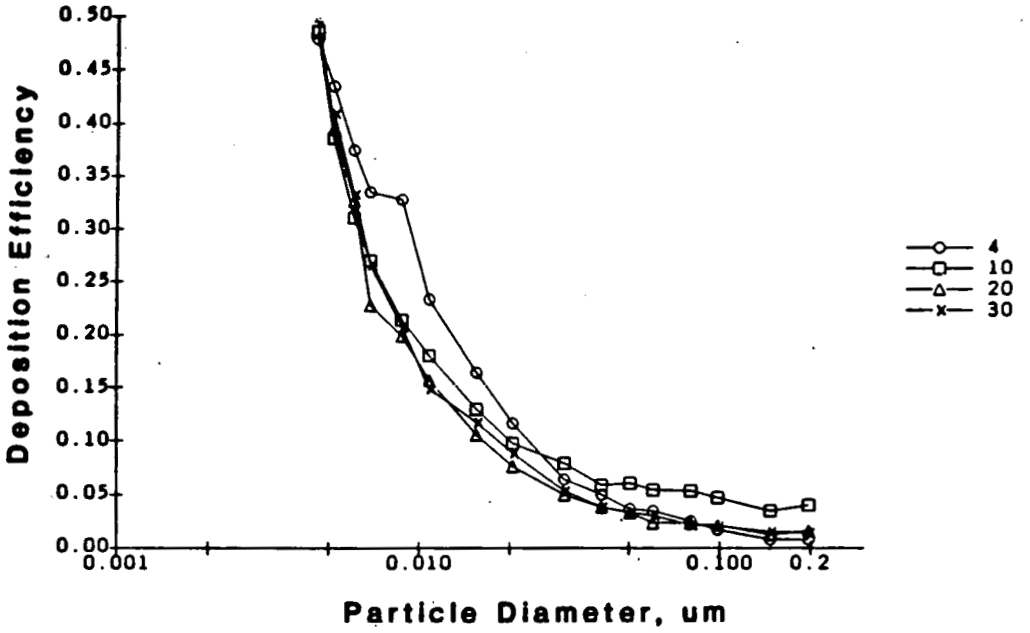


Figure 1. Deposition efficiency of particles in the cast for inspiratory flows of 4, 10, 20, and 30 L/min.

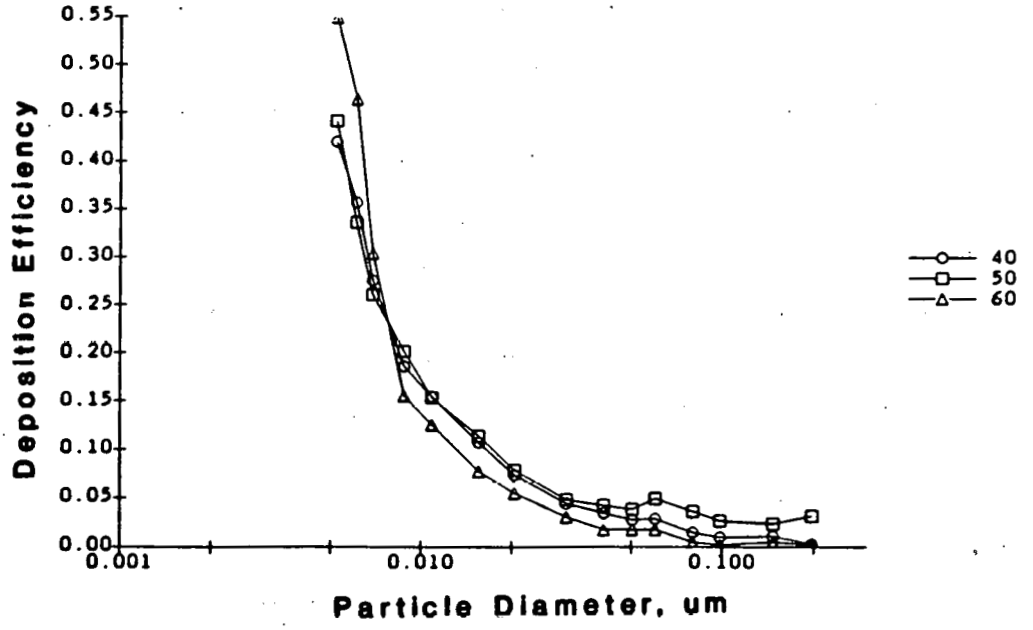


Figure 2. Deposition efficiency of particles in the cast for inspiratory flows of 40, 50, and 60 L/min.

METALLOTHIONEIN: A LACTATION-INDUCED CADMIUM-BINDING PROTEIN

Maryka H. Bhattacharyya°, Daniel Solaiman+, Justine S. Garvey*, and Wesley Y. Miyazaki°

°Biological and Medical Research Division, Argonne National Laboratory, Argonne, IL 60439-4833

+Chemistry Department, Dusquesne University, Pittsburgh, PA 15282

*Biology Department, Syracuse University, Syracuse, NY 13244

Toxic metals accumulate in the body at accelerated rates during early childhood, when the gastrointestinal absorption of metals is significantly greater than in later life, and also during pregnancy and lactation, when demands for minerals by the mother are increased.

Previously we demonstrated striking increases during midlactation in ^{109}Cd absorption (2- to 3-fold) and retention by the duodenum (12-fold), kidney (5-fold), and mammary tissue (12-fold) of mouse dams receiving environmental levels of cadmium/^{109}Cd via drinking water, with little change in ^{109}Cd retention in liver and jejunum compared to nonpregnant controls[1-3]. Here we report results of a study conducted to test the hypothesis that these increases in cadmium deposition during midlactation are caused by an induction of the metal-binding protein, metallothionein (MT). (The postulated induction of metallothionein would be a response to normal biochemical changes occurring during midlactation and not a response to cadmium administration, because, in the above studies of cadmium deposition during midlactation, the amounts of cadmium as ^{109}Cd received orally were negligible and would not have induced MT.)

A cadmium/hemoglobin (Cd/Hb) assay[4] for MT (which measures heat-stable cadmium binding capacity in tissues) was used to determine MT concentrations in the heat-treated supernatant fractions of kidney, liver, duodenum, and jejunum from five groups of B6CF1/ANL female mice (Table 1). Portions of the same supernatants were also analyzed for MT by radioimmunoassay (RIA) in the laboratory of Dr. Justine Garvey. As shown in Table 1, results obtained by RIA showed greater MT concentrations in liver and kidney from mice on lactation days 13 and 20 (L13 and L20 mice) and lower MT concentrations in all other tissues and groups than did the Cd/Hb assay. However, both assays demonstrated clear lactation-induced increases in MT concentrations in liver, kidney, and duodenum, with MT concentrations falling rapidly to control levels after weaning.

Table 1. Changes in Metallothionein Concentrations During Lactation in Mice[a]

Organ	Method	MT Concentration (ug/g tissues)				
		0-Time	NP	L13	L20	W5
Liver	Cd/Hb	11.5 ± 1.0	15.2 ± 1.4	64.2 ± 3.3	38.7 ± 2.6	11.2 ± 0.7
	RIA	2.7 ± 0.5	6.8 ± 1.2	161. ± 54.	32.5 ± 4.1	2.7 ± 0.9
Kidney	Cd/Hb	6.1 ± 0.6	11.3 ± 0.6	22.3 ± 1.2	21.7 ± 1.1	11.0 ± 0.6
	RIA	2.4 ± 0.1	5.9 ± 1.3	125. ± 25.	53.3 ± 8.9	3.7 ± 0.4
Duodenum	Cd/Hg	11.6 ± 1.5	19.1 ± 1.1	41.1 ± 3.8	37.7 ± 2.1	19.4 ± 1.9
	RIA	3.0 ± 0.4	2.1 ± 0.2	9.2 ± 2.6	5.2 ± 1.0	2.4 ± 0.2
Jejunum	Cd/Hb	6.5 ± 1.0	9.9 ± 0.5	13.9 ± 1.3	11.1 ± 0.8	10.7 ± 0.8
	RIA	<1.8	<1.7	5.4 ± 1.0	5.3 ± 0.7	<1.9

[a]Heat-treated supernatants were prepared from tissue homogenates from nonpregnant control mice on the day the experimental mice were mated, and 37 days later (0-Time and NP groups, respectively). Supernatants were also prepared from mouse dams on days 13 and 20 of lactation and day 5 after weaning (L13, L20, and W5 groups, respectively). Pups were weaned on lactation day 20. Values shown are means ± SE (n = 10).

Sephadex G-75 chromatography of the ^{109}Cd-containing supernatants from the Cd/Hb assays revealed that the majority of the ^{109}Cd radioactivity from all tissues of all groups appeared in a peak with a Ve/Vo of 2.1 -2.2, the same elution position as determined for Cd-induced mouse liver MT (Fig. 1C). The profiles from the L13 and L20 mouse livers (but not the NP livers) contained additional smaller peaks: void volume, 5-10%; Ve/Vo = 1.6, 20-30% of the recovered ^{109}Cd (Fig. 1). Surprisingly, the 1.6 peak from liver showed a

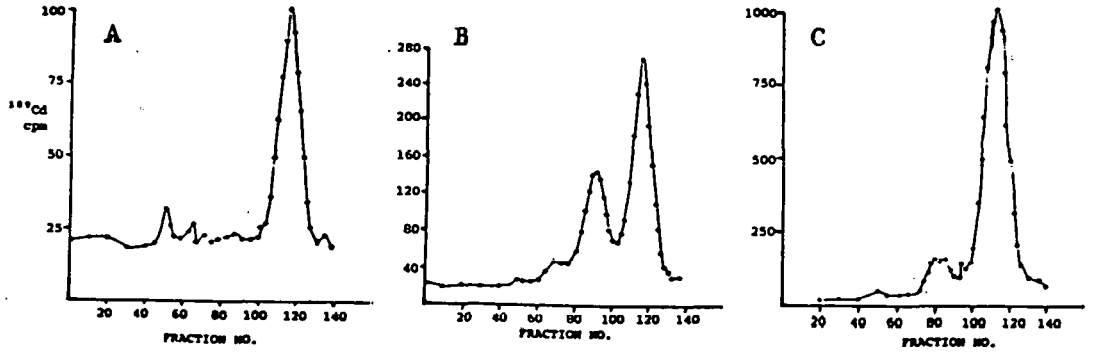


Fig. 1. Sephadex G-75 chromatograms of the ^{109}Cd-containing liver supernatants from Cd/Hb assays for MT. Each sample was applied to a 1.5 x 100 cm Sephadex G-75 column and eluted with 10 mM Tris, 5 mM 2-mercaptoethanol, pH 8.6 at 4°C with a 10 mL/h flow rate and 1.5 mL fractions. A = NP liver; B = L20 liver; C = Cd-induced MT.

much higher specific binding to the MT antibody than did the 2.2 peak; the ratio of MT concentration estimated by RIA to that estimated by the Cd/Hb assay was ~ 1:1 for the 2.2 peak of liver and was ~ 10:1 for the 1.6 peak of liver. Possibly both the 1.6 and 2.2 peaks from liver are MT, with the 2.2 peak a smaller, more condensed form of the molecule.

In summary, we have demonstrated that metallothionein (or an MT-like cadmium-binding protein) increases in concentration in liver, kidney, and duodenum of mouse dams during lactation and decreases to levels found in nonpregnant controls by 5 days after weaning. We have evidence that the MT that appears in the liver of midlactating mice exists in two forms, a native form that chromatographs with a Ve/Vo = 1.6 and reacts strongly with MT antibody, and a second form that appears with heating or storage in the freezer (data not shown), reacts less strongly with MT antibody, and chromatographs with a lower apparent molecular weight (Ve/Vo = 2.2). Additional investigations are needed to identify the role of this MT-like cadmium-binding molecule(s) that appears during a specific time course in the tissues of the lactating mouse dam.

Acknowledgments: This work was supported by the U. S. Department of Energy and the National Institutes of Health.

REFERENCES

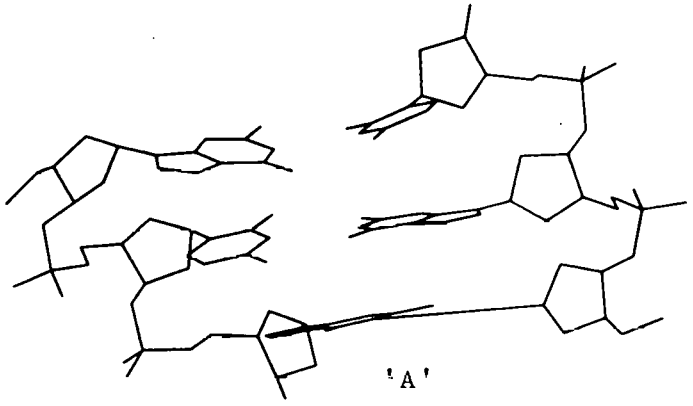
1. Bhattacharyya, M.H., Whelton, B.D., and Peterson, D.P., Toxicol. Appl. Pharmacol. 61, 335-342; 1981.
2. Bhattacharyya, M.H., Whelton, B.D., and Peterson, D.P., Toxicol. Appl. Pharmacol. 66, 368-375; 1982.
3. Bhattacharyya, M.H., Sellers, D.A., and Peterson, D.P., Environ. Res. 40, 145-154; 1986.
4. Eaton, D.L., and Toal, B.F., Toxicol. Appl. Pharmacol. 66, 134-142; 1982.

PREDICTION OF DNA STRUCTURE FROM SEQUENCE

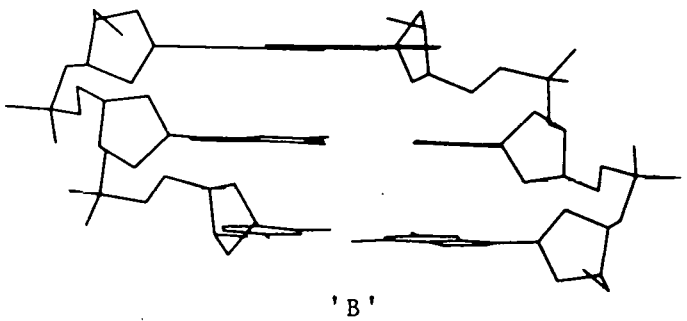
S. Broyde[1], B. E. Hingerty[2], S. Figueroa[3] and T. Hayden[3]

Biology Department[1], New York University, New York, New York 10003, Health and Safety Research Division[2], Oak Ridge National Laboratory, Oak Ridge, TN 37831, and Mathematics Department[3], University of Kentucky, Lexington, KY 40506

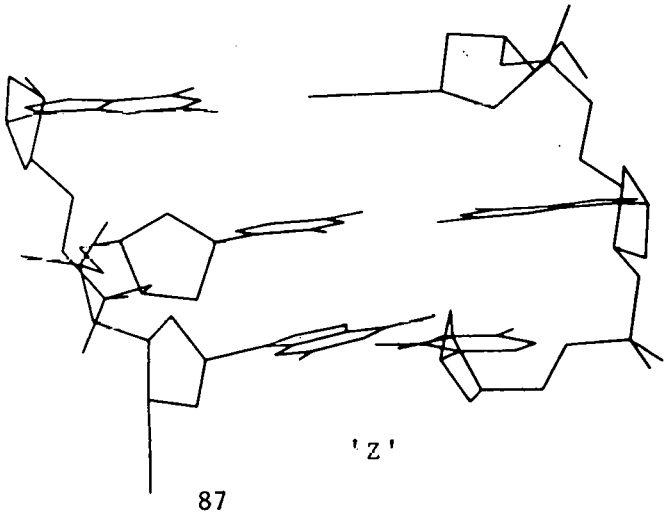
A build-up technique has been developed which predicts DNA structures from sequence using minimized semi-empirical potential energy calculations. In the first stage of the calculations a global search of the conformation space of all sixteen deoxydinucleoside monophosphates was made. This entailed 2,000 trials for each of these dimeric DNA conformational building blocks. In the next stage the minimum energy dimer conformations below 5 kcal./mole are combined to form trimers of any chosen sequence. Larger single stranded polymers can be generated by further build-up. Duplexes are produced by combining the single strands. By this method canonical A, B and Z form duplex helices have been computed a priori for the double stranded trimer d(CpGpC)·d(GpCpG), as well as novel duplexes.



'A'



'B'



'Z'

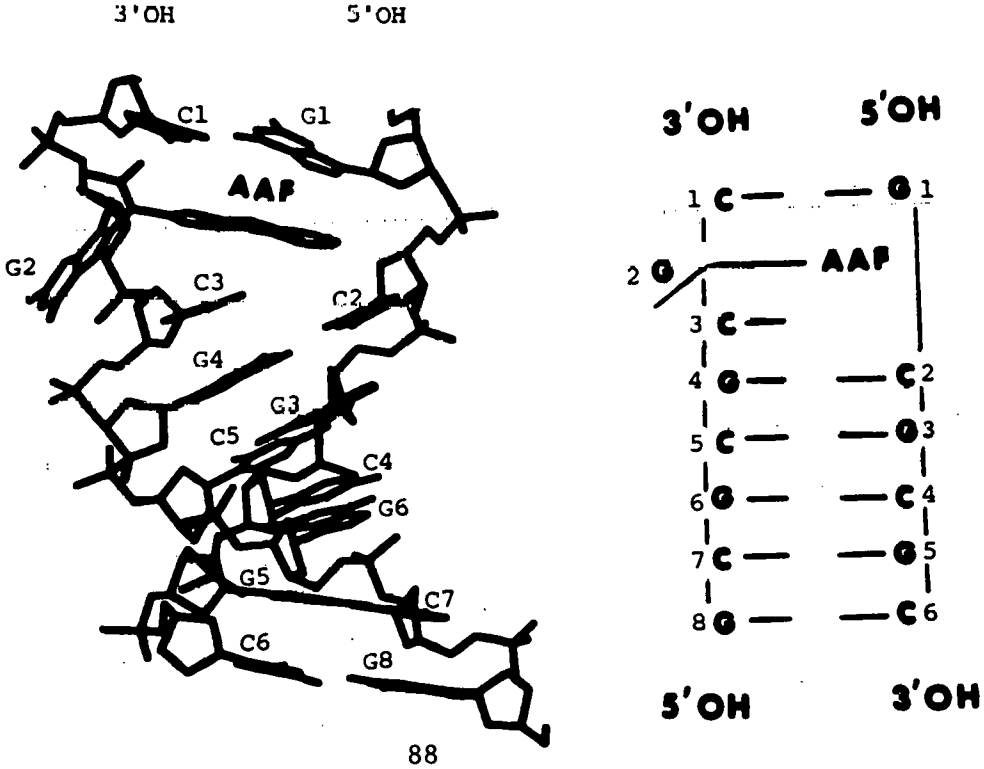
BASE DISPLACEMENT BY AAF IN A DOUBLE STRANDED B DNA DODECAMER: MOLECULAR VIEWS FROM MINIMIZED POTENTIAL ENERGY CALCULATIONS

S. Broyde[1] and B. E. Hingerty[2]

Biology Department[1], New York University, New York, New York 10003 and Health and Safety Research Division, Oak Ridge National Laboratory[2], Oak Ridge, TN 37831

We have computed energy minimized structures of base displacement in an AAF modified B DNA dodecamer. A rational search strategy, beginning with a global search of the conformation space of the modified deoxydinucleoside monophosphate, together with model building by computer graphics, have been employed. A number of different minimum energy conformations have been located which reveal base displaced structures. These show fluorene interstrand stacking, fluorene inter-and intra-strand stacking, and non-stacked fluorene situated in the denatured bulge. One structure of especial interest offers a molecular view that suggests how AAF can induce the -2 deletion mutation observed in AAF modified E. coli (1). Other interesting structures are compatible with the large mutations associated with AAF modification in Chinese Hamster Ovary Cells (2).

(1) Koffel-Schwartz, et al. J. Mol. Biol. 177, 33 (1984)
(2) Carothers, et al. PNAS 83, 6519 (1986)

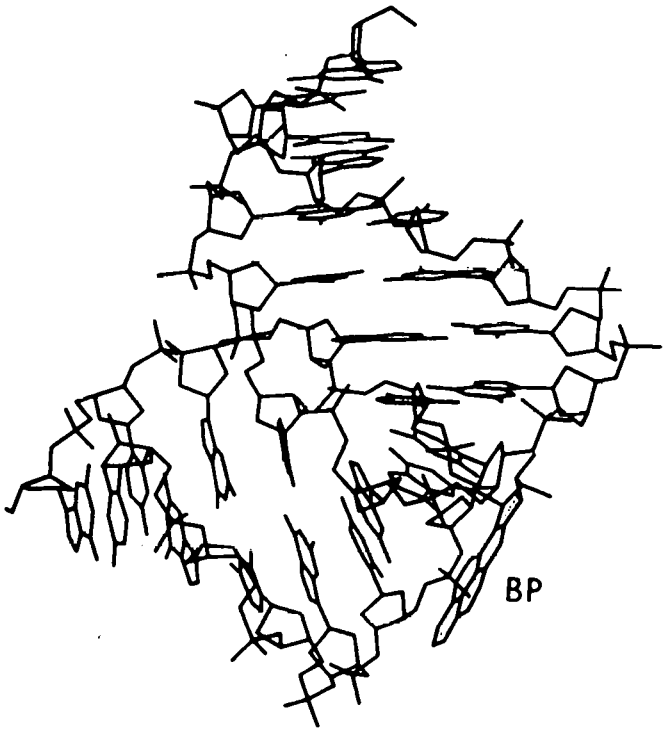


STRUCTURE OF THE TUMORIGENIC GUANINE N-2 ADDUCT WITH (+) ANTI BPDE FROM MINIMIZED POTENTIAL ENERGY CALCULATIONS

B. E. Hingerty[1] and S. Broyde[2]

Health and Safety Research Division, Oak Ridge National Laboratory[1], Oak Ridge, TN 37831 and Biology Department[2], New York University, New York, New York 10003

We have employed a rational search strategy to compute structures of the tumorigenic adduct of (+)-7β,-8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene [(+) anti BPDE] to a DNA duplex dodecamer. In previous work we had made a global search of the conformation space of the (+) anti BPDE adduct to the deoxydinucleoside monophosphate dCpdG. Both carcinogen-base stacked states and base-base stacked states of low energy were found. We have now incorporated these important forms into a duplex dodecamer in the B DNA conformation and have minimized the energy of the larger polymers. In these carcinogen modified duplexes, forms with carcinogen at the helix exterior are energetically preferred to those that place the carcinogen in an intercalated situation.



SYNTHESIS AND CONFORMATION OF A DINUCLEOSIDE MONOPHOSPHATE MODIFIED BY ANILINE

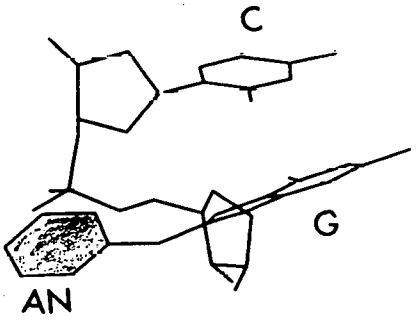
M. D. Jacobson[1], R. Shapiro[1], G. R. Underwood[1], S. Broyde[2], L. Verna[1], and B. E. Hingerty[3]

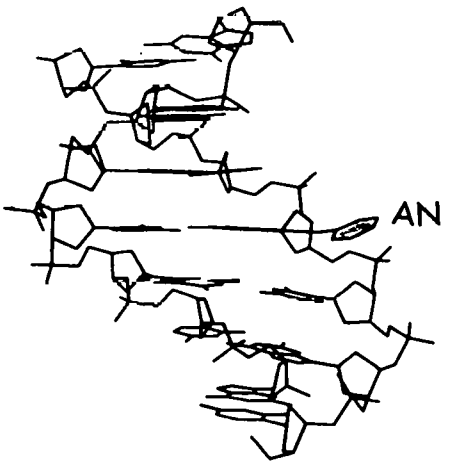
Departments of Chemistry[1] and Biology[2], New York University, New York, New York 10003 and Health and Safety Research Division, Oak Ridge National Laboratory[3], Oak Ridge, TN 37831

The modified dinucleoside monophosphate, N-[deoxycytidilyl-(3'-5')-guanosin-8-yl)-aniline (dCprG-An) has been prepared by the phosphotriester synthesis approach, using suitably blocked derivatives of dCp and N-(guanosin-8-yl)-aniline (rG-An). The latter compound was synthesized by a route that featured nucleophilic displacement by aniline upon an 8-bromoguanosine derivative. A number of attempts to prepare N-(deoxyguanosin-8-yl)-aniline (dG-An) by electrophilic substitution, using activated aniline derivatives, failed. Nucleophilic substitution reactions of aniline with 8-bromodeoxyguanosine derivatives afforded only the base, N-(guanin-8-yl)-aniline.

The conformation of dCprG-An has been studied by circular dichroism, proton magnetic resonance and minimized potential energy calculations. A flexible molecule with a mixture of conformers is indicated. Base-base stacked states predominate, in contrast to the case of a dimer containing 4-aminobiphenyl bound to the 8-position of guanine, where carcinogen-base stacked states are dominant.

The mutagenic and carcinogenic activities of aniline are much less than those of many polycyclic aromatic amines. The diminished stacking ability of the aniline ring, as well as the weak electrophilic reactivity of activated aniline derivatives, may be a cause of this weak biological activity.



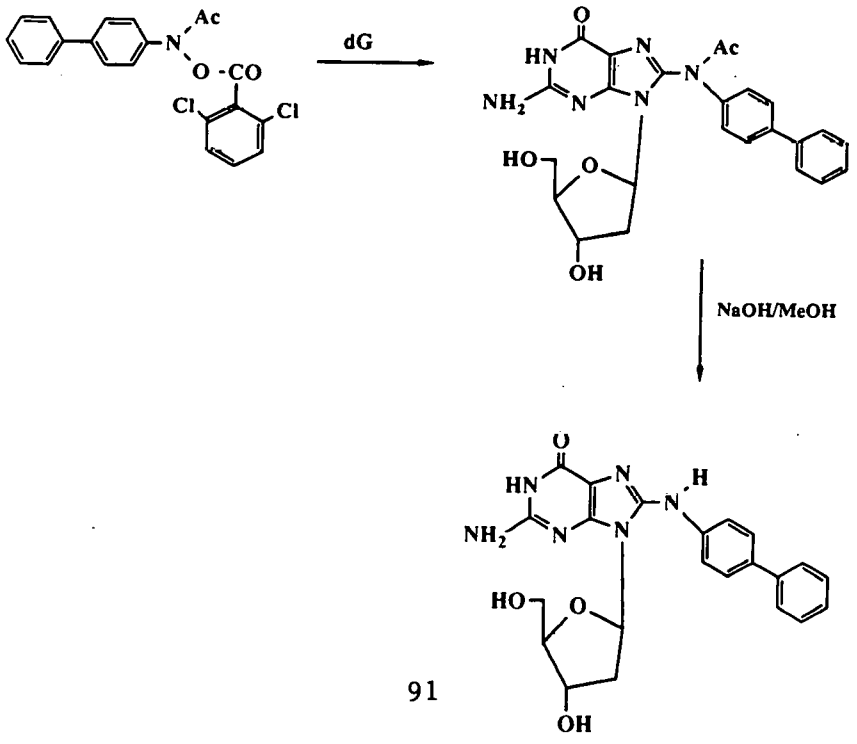


A NEW ROUTE TO ADDUCTS OF NUCLEOTIDES WITH CARCINOGENIC AROMATIC AMINES

G. Underwood, M. Price and R. Shapiro

Chemistry Department, New York University, New York, New York 10003

Investigations of carcinogen-DNA adducts in the aromatic amine series have been severely hampered by the lack of appropriate synthetic routes to these adducts. Adducts of acetylaminobiphenyl to guanine C-8 that were previously synthetically inaccessible have been prepared in high yield by a new synthetic route. N-hydroxy-4-acetylaminobiphenyl has been reacted with 2,6 dichlorobenzoyl chloride to produce the corresponding hydroxamic acid ester. Unlike the acetate ester, which undergoes acyl oxygen scission, this analog reacts in aqueous solution with nitrenium ion formation. When reacted with deoxyguanosine, good yields of an adduct are produced in which the nitrogen of the biphenylamido moiety forms a covalent bond to the C-8 position of guanine. In this manner, the analogous adducts of d(CpG), d(GpC) and d(ApG) have also been synthesized. Treatment of these adducts with NaOH in methanol results in removal of the acetyl group, thus providing access to both the acetyl and desacetyl derivatives from one precursor.



PULMONARY HYPOPLASIA IN RATS PRENATALLY EXPOSED TO COAL-DERIVED COMPLEX MIXTURES: HISTOLOGICAL AND SUBCELLULAR DEVELOPMENT

Terryl J. Mast, Ramona L. Rommereim, and James S. Young. Battelle-Pacific Northwest Laboratory, Biology and Chemistry Department, Richland, WA 99352.

Coal-derived liquids have previously been shown to be teratogenic when pregnant rats or mice were exposed to these complex mixtures by either inhalation, oral, or dermal routes [1]. Subsequent studies on the chemical class fractions showed that this teratogenic activity was due almost entirely to the polynuclear aromatic hydrocarbon fraction [2]. The major abnormalities induced were pulmonary hypoplasia, cleft palate, edema, cutaneous syndactyly, or hemorrhages in the sagittal suture area.

The pulmonary hypoplasia, defined in fetuses whose lung to body weight ratios are at least two standard deviations below the mean ratio for the controls, is of particular interest in that it may serve as a model to elucidate molecular mechanisms regulating pulmonary differentiation and maturation. Electron micrographs of 20-dg hypoplastic fetal rat lungs have shown them to be abnormal with respect to alveolar and interstitial tissues; however, the temporal sequence of the abnormal development was not known. In order to provide a framework for further research into the molecular mechanisms regulating pulmonary maturation this comparative study of temporal development was undertaken.

Timed-pregnant (2-hour matings) Sprague-Dawley rats were dermally exposed to coal-derived complex mixture (CM) or to a vehicle control. The CM, 500 mg/kg in acetone, or vehicle (acetone) was applied to the shaved backs of dams in a constant dosing volume of 1.0 ml/kg, on 11-15 days of gestation (dg). Groups of dams (n=5) were serially sacrificed on 16-22 dg.

For comparative purposes another group of dams was given the synthetic glucocorticoid, triamcinalone, as a subcutaneous injection, days 11-14 dg, 0.25 mg/kg/day. This group was sacrificed on 20 dg.

Fetal lungs were inflated *in situ* with McDowell-Trumps glutaraldehyde-cacodylate) buffer, excised and refrigerated in the buffer. In preparation for scanning-electron microscopy (SEM) the fixed lungs were dehydrated then cryofractured in a brass cup cooled with liquid nitrogen. Fractured pieces were critical-point dried, mounted with the fractured side up, and sputter-coated with gold/palladium. Transmission electron microscopy (TEM) samples were post-fixed in osmium tetroxide, dehydrated and embedded in resin. Thin sections, ca. 700 Å, were mounted on copper grids and stained with uranyl acetate and lead citrate.

Examination of electron micrographs taken of the serially sacrificed lungs revealed that differences in alveolar epithelial cell development between the treated and control lungs was evident as early as 17 dg. Columnar alveolar epithelial cells (AEC) had already begun to flatten in the CM-treated lungs, were slightly vacuolated and showed signs of disorganization. By 19 dg the AEC of the treated lungs were in an advanced stage of differentiation into Type I and Type II alveolar cells. This stage is not reached by the control until 21 dg. By 22 dg, term in the rat, the CM-treated lungs showed abnormal AEC and very thick septal walls while the control lungs had thin septal walls with AEC differentiated into Type I and Type II cells. Electron micrographs of 20-dg TAC-treated lungs showed very disorganized alveolar and interstitial tissue with a precocious surfactant production. A strong similarity was noted between 22-dg CM-treated lungs and the 20-dg TAC-treated lungs.

In summary, when compared to controls, hypoplastic lungs had less organization in the interstitial tissue, an increased septal thickness. However, bronchial and bronchiolar regions of the hypoplastic lungs appeared normal. These data indicate that functional impairment may result from abnormal development of the alveolar region. The similarity between the overall effects of the glucocorticoid treatment and those noted as a result of treatment with complex mixtures may imply a common mechanism. Results of this study will be used as a basis for future work intended to elucidate the molecular mechanisms regulating lung maturation and differentiation.

Work supported by U.S. Department of Energy under Contract DE-AC06-76RLO 1830.

REFERENCES

1. Hackett, P.L. 1985. Teratology of complex mixtures, pp. 61-62. In: Pacific Northwest Laboratory Annual Report for 1985 to the DOE Office of Energy Research, Part 1, Biomedical Sciences, PNL-5750. NTIS, Springfield, VA.
2. Mast, T.J. 1987. Teratology of complex mixtures, pp. 65-69. In: Pacific Northwest Laboratory Annual Report for 1985 to the DOE Office of Energy Research, Part 1, Biomedical Sciences, PNL-6100. NTIS, Springfield, VA.

METABOLISM OF BENZO(A)PYRENE IN HUMAN MAMMARY EPITHELIAL CELLS AND ITS CONSEQUENCES

J. C. Bartley, S. A. Leadon, and M. R. Stampfer, Division of Biology and Medicine, Lawrence Berkeley Laboratory, Berkeley, CA 94720

We have demonstrated that human mammary epithelial cells (HMEC) derived from normal glands readily metabolize benzo(a)pyrene (BaP) to the ultimate carcinogen when placed in culture. As a consequence of this metabolism, adducts between 7,8-dihydrodiol-9,10-epoxy-benzo(a)pyrene are formed mainly with deoxyguanosine of DNA. Repeated treatments of these cells in culture with BaP has resulted in the production of a large populations of cells with extended life span. In addition, two immortalized cell lines have been isolated in separate experiments. These cell lines have been characterized with respect to growth requirements, morphology, karyology, and metabolic activity typical of mammary epithelial cells. The two lines are quite different from one another, but neither one is truly malignant.

More recent studies have been initiated on the role of environmental carcinogens in the induction of oxidative damage in HMEC, particularly to DNA. A monoclonal antibody that recognizes thymine glycols in DNA has been developed by Dr. Leadon for this purpose. The electrophiles formed during metabolism of BaP can, of course, give rise to free radical intermediates and reactive reduced oxygen species. Our studies demonstrate that the induction of oxidative damage to DNA by BaP exposure is apparently equal to or greater than that due to direct formation of bulky adducts. The fact that superoxide dismutase (SOD) provides protection against the induction of oxidative damage to DNA, without affecting the overall metabolism of BaP, indicates that generation of super oxide is likely involved. When compared to equitoxic doses of ionizing irradiation, in which free radicals are responsible for the majority of the DNA damage, the extent of thymine glycol formation was approximately 35-fold greater in HMEC cultures exposed to BaP than to gamma irradiation. Current investigations on the possible mechanism(s) for production of reduced oxygen species from BaP metabolism will be discussed.

MECHANISMS OF LATE-OCCURRING DEGENERATIVE LUNG DISEASE

R. F. Henderson, J. M. Benson, J. A. Pickrell, J. D. Sun, J. R. Harkema, and F. F. Hahn

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Inflammatory processes in the lung may lead to normal repair or to late-occurring degenerative diseases such as fibrosis or emphysema. Two animal models are being used to investigate the mechanisms controlling the sequelae of inflammatory processes.

In the first model, rodents were exposed for up to 30 months to diluted diesel exhaust. Rats developed more extensive pulmonary fibrosis than mice, despite a higher relative lung burden of diesel soot in mice. One hypothesis to explain the species differences was that rat lung tissue, particularly pulmonary phagocytic cells, responded to the diesel soot with a greater release of inflammatory mediators than did the mouse lung tissue. This hypothesis was tested by analysis of arachidonic acid metabolites in bronchoalveolar lavage fluid (BALF) of rats and mice exposed for 12 days to diluted diesel exhaust. Rat BALF had five-fold increases in $PGF_{2\alpha}$ and LTB_4 in response to the exposure, compared to only two-fold increases in these parameters in mice. Additional research is required to link these initial events with the late occurring disease.

A second hypothesis was that the rats were more susceptible to development of soot-induced fibrosis because their lungs were depleted of glutathione (GSH) by the diesel exposure while the mouse lungs were not. It was proposed that the release of reactive oxygen radicals by pulmonary phagocytic cells in the rat had depleted the rat lung of GSH and allowed a sustained lung injury that led to fibrosis. To test this hypothesis, the resistant species, mice, were exposed to a known fibrogenic agent, ozone,

and then were made deficient in GSH by administration of buthionine sulfoximine (BSO) in the drinking water. Ozone was used as the fibrogenic agent instead of diesel soot to speed up the fibrogenic process, which had required a year to develop in response to diesel soot. If the proposed mechanism of GSH protecting against oxidant injury in the lung was correct, the depletion of lung GSH should enhance the ozone-induced fibrogenic processes. The BSO treatment greatly exacerbated the fibrosis induced by ozone exposure of mice.

In a second animal model, rats and mice exposed to several metallic compounds were found to develop either fibrosis (mice) or emphysema (rats). This species difference provides an opportunity to study the factors controlling the development of each type of disease. A greater inflammatory response was observed in the rats than the mice exposed to the compounds in vivo. In vitro studies indicated that the metallic compounds were more toxic to rat than to mouse pulmonary macrophages. We are currently testing the hypothesis that for emphysema to occur, the inflammation must cause breakdown of both components of the extracellular matrix (ECM), the collagen and the fibronectin (FN), while in fibrosis, only the collagen turns over. Because the FN determines the size, shape and extent of the ECM, its loss should lead to the tissue destruction seen in emphysema. If rat lung cells release greater amounts of inflammatory mediators in response to the metallic comounds (as was found with diesel soot), and if these mediators include proteinases, the rat response to the metallic compounds may overwhelm the capacity of the antiproteinases present, leading to emphysema. We will test this hypothesis in rats and mice exposed to the metallic compounds by observing changes in FN, growth factors, proteinases/antiproteinases and other markers of inflammation in BALF with time after exposure. If our

hypothesis is correct, we should observe higher ratios of FN to hydroxyproline-containing peptides and of proteinases to antiproteinases in BALF from rats than from mice.

Acknowledgements: Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

ABSTRACT

SYNERGISM BETWEEN CHEMICAL MUTAGENS AND
HIGHLY AROMATIC CHEMICAL MIXTURES

R. A. Pelroy and D. L. Stewart
Biology and Chemistry Department
Pacific Northwest Laboratory
Richland, Washington 99352

Using Salmonella typhimurium, mutational synergism was demonstrated between components in highly aromatic chemical mixtures and several classes of mutagens including aminopolycyclic aromatic hydrocarbons (aminoPAH), nitro aromatic hydrocarbons (nitroPAH), and the DNA intercalating agent, ICR191. The complex aromatic mixtures were highly enriched pyrenes, flouroanthenes, benzopyrenes in addition to other larger condensed ring structures. Mutational synergism was shown for several of the aromatic fractions (nitrogen and oxygen containing), but was not observed for nonaromatic chemical fractions enriched in aliphatic compounds. Mutational synergism was shown for both frameshift and base substitution mutagens. In contrast to results for the aminoPAH, nitroPAH and ICR191, benzo(a)pyrene, a neutral PAH showed less activity or no change in activity when assayed for mutagenicity in the presence of complex aromatic mixtures. In quantitative terms, the aminoPAH and nitroPAH showed the greatest, and ICR191, the least susceptibility to mutational synergism.

Liquid incubation experiments where survival and mutagenesis were both measured showed that apparent rates of increased mutation actually represented increases in mutational frequencies due to interactions between mixtures and mutagens. In no case were the nonadditive effects accompanied by extensive killing of target cells by the complex mixtures. However, at the levels used in these experiments, the complex mixtures showed little mutagenicity for any of the target cells.

AminoPAH were studied in some detail for mutational synergism. Compounds examined included: aminochrysenes, aminoflouranthenes, aminophenanthrenes and aminopyrenes. The strongest nonadditive effects were observed for 6-aminochrysene (6-AC) and 3-aminoflouroanthene. For these aminoPAH both maximum activity was increased and the threshold concentration capable of inducing mutagenesis was lower in the presence of aromatic mixtures. The effects for both aminoPAH were observed over a wide concentration range of P450 enzyme preparations. Ordered addition experiments showed that both aminoPAH and complex mixtures had to be present together for maximum levels of mutational synergism suggesting that the major effect was external to the target cells and primarily due to differential rates on P450 degradation vs activation of the aminoPAH.

In the presence of metabolic activation by P450 enzymes, 1-nitropyrene (1-NP) and 6-nitrochrysene (6-NC) plus aromatic mixtures both showed more mutagenic activity than the nitroPAH alone or the aromatic mixtures alone. 6-NC was subject to higher levels of synergism than 1-NP, parallelling results obtained with the aminoPAH. However, in the absence of metabolic activation, the nitroPAH were more active as mutagens than either mixtures of the nitroPAH plus aromatic mixtures or the aromatic mixtures alone. This is consistent with the aromatic mixtures preventing or inhibiting metabolic deactivation of these nitroPAH.

The aminoPAH and nitroPAH both require activation of exocyclic nitrogens by oxidation and reduction, respectively. ICR191 acts directly on the DNA of the target without intervening metabolic activation reactions. One probable site of action of the aromatic mixtures is to inhibit P450 reactions that would otherwise attack the PAH portions of the aminoPAH and nitroPAH. However, ring oxidation per se does not necessarily result in deactivation of 1-nitropyrene (El-Bayoumy and Hecht, 1986) and in some cases, gives rise to K-region derivations (dihycrodiols, lactones and quinones) of comparable mutagenicity to the parental 1-nitropyrene. Thus, synergism due to the complex aromatic mixtures may involve more than competition for the P450 enzymes that attack the PAH nucleous of the aminoPAH and nitroPAH.

Work supported by the U.S. Department of Energy under Contract DE-AC06-76RLO 1930.

CHARACTERIZATION OF HEMOGLOBIN-BENZO[a]PYRENE ADDUCTS

David A. Haugen and Steven R. Myers

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439-4833

The human population is exposed to low levels of many genotoxic chemicals from a variety of sources, including products of energy generation, synthetic chemicals, and natural products; however, causal links between non-occupational exposure to chemicals and human carcinogenesis have yet to be established. Thus, there is a growing interest in epidemiologic studies to evaluate the effects of environmental chemicals on human health. These studies require information about both the chemicals in external human environments and the biologically effective doses as determined by "chemical dosimetry" for individual people.[1]

We have recently begun to address the second requirement by studying hemoglobin-carcinogen adducts. Because hemoglobin (Hb) is long-lived (120 days) and hemoglobin-carcinogen adducts are stable in vivo, levels of hemoglobin-carcinogen adducts can represent the integrated effects of both external variables (levels of exposure, frequency and duration of exposure) and internal variables, especially patterns of biotransformation as affected by genetic factors and by exposure to exogenous chemicals.[1,2]

We began to develop general approaches for isolation of hemoglobin-carcinogen adducts by examining the chromatographic characteristics of hemoglobin-benzo[a]pyrene (Hb-BP) adducts. Hb-BP adducts have been detected in rodents,[3] but the amino acids involved in formation of these adducts and those with other polycyclic aromatic hydrocarbons have not been identified.

Cultures of Syrian hamster embryo (SHE) cells were supplemented with human Hb (0.2 mM heme) and [^{3}H]BP (1 μM). After a 24-h incubation, the medium

was removed and subjected to cation-exchange liquid chromatography (CM-Sepharose) to resolve hemoglobins from serum proteins in the medium. The BP-treated Hb was subjected to analysis in each of three column chromatographic systems (enumerated below) established for isolation and characterization of human hemoglobin and its genetic and post-translationally modified variants.

(1) The BP-treated Hb was subjected to cation-exchange chromatography on Bio-Rex 70. The naturally occurring major and minor Hb tetramers ($\alpha_2\beta_2$) were resolved as expected. The results demonstrated the presence of at least three Hb-BP adducts more acidic than Hb A (the major form in normal adults).

(2) The globins obtained by removing heme from the BP-treated hemoglobin were subjected to cation-exchange chromatography (CM-Sepharose) in the presence of 8 M urea to resolve β- and α-globins (in that order). The results indicated the presence of multiple (at least two) globin-BP adducts that either co-eluted with β-globin or were more acidic than β-globin.

(3) BP-treated Hb was also extracted with ethyl acetate and then subjected to reversed-phase HPLC (VYDAC C-4 column eluted with 0.1% trifluoroacetic acid in acetonitrile/water), in a system that resolves β- and α-globins (in that order). The results indicated the presence of multiple (four or more) globin-BP adducts; their increased retention times (relative to β- and α-globins) were consistent with the presence of a BP moiety.

Thus, multiple hemoglobin-BP or globin-BP adducts were detected in each of three chromatographic systems already established for analysis of hemoglobin. These techniques may be generally applicable for isolation and characterization of hemoglobin-carcinogen adducts.

Our observation that Hb-BP adducts are more acidic than Hb A is consistent with the reaction of BP-diol epoxides[3] with N-terminal amino groups (which decreases their pK values) but may also be attributed to perturbation

of intramolecular charge-charge interactions (as for iodoacetamide) accompanying reaction with the cysteine SH group at position 93 of the β-hemoglobin chain (β93 Cys) known to react with other electrophilic agents, including carcinogenic nitrosoarenes.[2]

Under conditions similar to those we used, Preuss-Schwartz et al. demonstrated the formation of multiple DNA-BP (guanosine-BP) adducts in SHE cells exposed to BP,[4] which is consistent with our finding of the extracellular formation of multiple Hb-BP adducts. Multiple adducts may also arise, in part, by reaction of a BP metabolite(s) at more than one hemoglobin site.

Our results demonstrate that hemoglobin-carcinogen adducts can be resolved from native hemoglobin by established conventional and high-performance liquid chromatographic procedures, suggesting the basis for development of general approaches for isolating and characterizing hemoglobin-carcinogen adducts. Our results also suggest the basis for a model system in which adducts between carcinogens and human hemoglobin are formed in cultures of mammalian cells or tissues.

Acknowledgements: This work was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under Contarct No. W-31-109-Eng-38.

REFERENCES

1. Wogan, G. N. and Gorelick, N. J. Environ. Health Perspec. 62, 5-18; 1985.
2. Bryant, M. S., Skipper, P. L., Tannenbaum, S. R. and Maclure, M. Cancer Res. 47, 602-608; 1987.
3. Shugart, L. Toxicol. 34, 211-220; 1985.
4. Preuss-Schwartz, D., Sebti, S. M., Gilham, P. T. and Baird, W. M. Cancer Res. 44, 4104-4110; 1984.

SESSION 4

Metabolism and Genotoxicity

Metabolism and Genotoxicity

Summary of Session 4

J. S. Felton

Lawrence Livermore National Laboratory

The significant role of metabolism in studies of genotoxicity and carcinogenicity is clear. Together with DNA repair, no other factors can modulate the effects of a dose of a chemical to such an extent. The knowledge of how to manipulate metabolism to lower risk can only come from understanding the mechanisms behind metabolic differences. This knowledge, in turn, can only come from studying the genetic regulation, protein regulation and pharmacodynamics related to specific exposures to genotoxic/carcinogenic insults.

The session on Metabolism and Genotoxicity demonstrated some critical scientific approaches to understanding different aspects of metabolism. The possibility that inducers given during development can imprint (effect gene expression during adult life) has major consequences in how drugs, endogenous substrates, and xenobiotic chemicals are metabolized. In a second study, to understand which genes are responsible for induction and metabolism and how they are regulated, mutant cell lines were established that can dissect out specific mechanistic differences through complementation analysis. With this system cloning of specific genes is quite feasible and very productive. Understanding the protein chemistry of a major metabolizing component such as cytochrome P-450 is also important. This is addressed by studying the

regulation of an apo-cytochrome P-450 from bacteria. Cloning into *E.coli* leads to understanding the expression of the gene, specifically the stability of the m-RNA. A different approach to understanding metabolism can come from comparing different metabolic pathways and genotoxic responses in different organisms. The advantages from this type of study are 1) comparison of different enzymes, different kinetics, and ultimately different toxic effects can lead to understanding events in man; 2) environmentally relevant organisms can give us insight into how xenobiotic chemicals are handled in aquatic and terrestrial environments; 3) many of these organisms can act as environmental sensors for toxic effects; and 4) understanding the metabolic pathways involved can make interpretation of the responses more meaningful. Finally, studies designed to understand the ubiquitous toxic effects from compounds like the dicarbonyls are also important. Measurement of their genotoxic response and determination of critical biochemical pathways are the potential advantages of looking at many different biological endpoints. The five studies reported in this session clearly represent different aspects of metabolism, encompassing molecular regulation to environmental monitoring; all are attempting to answer important questions about metabolism, and ultimately, chemical toxicity.

Springer *et al.* (PNL) proposed a study of altered imprinting during fetal/neonatal development in an effort to better define the role of cytochrome P-450 dependent monooxygenases in tumor development. The effect on carcinogen-metabolizing enzymes in the adult by xenobiotic exposures during early development will be assessed by measuring P-450 gene expression (employing specific m-RNA probes) and activi-

ties of several cytochrome P-450 dependent monooxygenases. Ultimately, the contribution of fetal/neonatal imprinting on a specific set of factors influencing susceptibility to tumors in the adult will be better understood if the studies are successful.

The presentation by Hankinson *et al.* (UCLA) described the isolation of four classes of recessive mutants (representing four distinct genes), all dominant mutants in a murine hepatoma cell line. These mutants are all involved in the induction of aryl hydrocarbon hydroxylase by polyaromatic hydrocarbons (PAH). One complementing class, the A mutants, represent the structural gene for a specific cytochrome P-450 responsible for AHH activity. The B,C, and D mutants are involved in the structure or function of the PAH or TCDD receptor. Transfected human DNA was shown to revert the four classes of recessive mutants. A portion of the C gene (a gene responsible for the receptor translocation process) has been isolated. The locus of action of the dominant factor has been mapped to the AHH promoter region, using promoter deletion-CAT gene constructs.

Although barbiturates are potent inducers of specific P-450 monooxygenases in mammalian cells, their mode of action is unknown. Fulco and Wen (UCLA) purified from *Bacillus megaterium* a soluble fatty acid P-450 monooxygenase that is barbiturate inducible. The P-450 enzyme is catalytically self-sufficient, in contrast to other bacterial or liver monooxygenases. However, it can be cleaved by trypsin into functional reductase- and substrate-binding domains. The gene for the enzyme was cloned by

immunochemical techniques. In a heterologous *E. coli* host, the cloned gene is constitutively expressed; while in *B. megaterium,* the cloned gene is subject to barbiturate regulation. The presence of a portion of the regulatory region on a plasmid prevents induction of the endogenous gene, suggesting a requirement for the presence of an additional trans-acting regulatory factor.

The metabolism and genotoxic effects of aromatic amines was examined by Knezovich *et al.* (LLNL) in aquatic organisms with the premise that mammalian findings are not analogous to lower organisms because of differences in the basic biochemical and physiological processes. Both mussels and tadpoles were exposed by immersion in aqueous solutions containing labeled aromatic amines. Metabolites of *o* and *p*-toluidine, and 2-AAF were identified by HPLC and GC/MS methods. In mussels exposed to 2-AAF, low levels of protein adducts, but no DNA adducts or SCEs were seen. Presumably, the metabolism in the mussel shunts the activation (detoxification) away from the genotoxic intermediates represented by hydroxylation on the primary amine. The tadpoles excreted a number of 2-AAF metabolites, but did manage to form DNA and protein adducts in the liver. This organism also showed clear induction of micronuclei in the peripheral red blood cells by 2-AAF. The tadpole may turn out to be a very sensitive sentinel organism for aromatic amine contamination in aquatic ecosystems.

Taylor *et al.* (LLNL) reminded us that the study of dicarbonyls has wide ranging

significance because of the foods we eat (both from roasting of coffee beans and heating of foodstuffs), the irradiation of foods, and the photooxidation of aromatic hydrocarbons in fossil fuels. These compounds form, without activation, direct acting base-substitution mutations in *Salmonella* and are reported to form fused-ring adducts with DNA-guanine bases. The 1,2-dicarbonyls are moderate genotoxins in mammalian cells. They induce GAT+ revertants at the structural gene locus for folypolyglutamate synthetase (FPGS). The mechanism seems to be by a G-C to A-T base-substitution. The fact that these compounds preferentially react with guanine bases makes them potent in this assay. The 1,2-dicarbonyl causes a dose-dependent increase in DNA-protein crosslinking and single-strand breaks. Bisulfite co-treatment negates the latter lesions. Isolation and identification of dicarbonyl induced exocyclic-DNA adducts is in progress. This study is a good example of the development of a special genotoxic assay which can give mechanistic data as well as mutagenic potency.

MOLECULAR MARKERS DURING DEVELOPMENT

David L. Springer

Biology and Chemistry Department, Battelle, Pacific Northwest Laboratories, PO Box 999, Richland, WA 99352

Mammalian development consists of a series of complex interrelated programs of gene expression and regulation. Normal factors or stimuli (such as hormones) present in the developing organism during certain sensitive periods determine the capability of the organism for gene expression/regulation during later life. This process is known as imprinting. Prenatal or neonatal exposure to chemicals or radiation may result in altered imprinting causing shifts in key biochemical pathways. These shifts, while not lethal, may be manifested as physiological imbalances and can have important consequences for the organism. This has been forcibly brought to our attention during the past few years by the discovery that female offspring of women given the synthetic steroid diethylstilbestrol (DES) developed cervical adenocarcinoma. While several mechanisms have been proposed for this phenomenon, including adduction of DNA by DES, more recent views favor an epigenetic model in which the DES disturbs normal imprinting causing hormonal or other imbalances which lead to tumor development.

Evidence for the involvement of the epigenetic mechanism in tumor development is beginning to accumulate. For example, it has been shown that in utero exposure to benzo(a)pyrene (and other xenobiotics) results in increased susceptibility of the adult offspring to a challenge with the carcinogen, 3-methylcholanthrene (Soyka, 1980). Moreover, it was also found that the animals prenatally treated with phenobarbital had higher concentrations of aflatoxin-DNA adducts than the controls, a finding consistent with the increased tumor response. Moreover, levels of cytochrome P-450-dependent monooxygenases, key enzymes in the activation of PAH and aflatoxin, were elevated in the treated animals (Faris and Campbell, 1983; Bagley and Hayes, 1985).

The above observations suggest that endogenous levels of P-450-dependent enzymes may be altered by xenobiotic administration during development and that these changes may play an important role in susceptibility to cancer. Altering P-450 levels may have other important consequences as well since

certain isozymes catalyze reactions in the biosynthesis of steroid and glucocorticoid hormones; in fact, several rate limiting or key regulatory steps in these pathways are occupied by specific P-450-dependent isozymes. Thus, altered imprinting could change the isozyme patterns as well as the total levels P-450-dependent monooxygenases. However, this key issue has not been experimentally addressed. We therefore propose to study the relationships among imprinting, P-450-dependent enzyme levels, and steroid concentrations.

Recently, molecular probes have been developed to isolate and characterize genes responsible for cytochrome P-450 dependent monooxygenase isozymes. Four families of genes have been identified and are usually classified according to their responsiveness to phenobarbital, 3-methylcholanthrene (and TCDD and PAH), pregnenolone and pituitary hormones (Whitlock, 1986). The phenobarbital and 3-MC gene families have been identified, sequenced and mapped, and molecular probes have been pre- pared to measure the cellular concentrations of the specific DNA responsible for coding for two phenobarbital inducible isozymes, P-450b and P-450e. The genes that respond to pituitary hormones have been characterized to a lesser extent, because their gene products are present at much lower concentrations; therefore the difficulty of obtaining purified isozymes was greatly increased. Despite these difficulties partial cDNA for two of the five genes that respond to pituitary hormones have been prepared and this information is available from the literature.

Despite the obvious importance of the P-450-dependent systems, little has been done to apply these approaches to developmental biology or to test the validity of the epigenetic/imprinting model of tumor development as described above. Studies on the influence of altered imprinting have been limited to measurement of enzyme activities. Similarly, measurement of the amount of radiolabelled carcinogen which copurifies with DNA as an indicator of carcinogen binding and susceptibility to tumor development provides valuable data; however, correlations may be improved when specific DNA adducts are examined.

We therefore plan to study altered imprinting during fetal/neonatal development in an effort to better define the role of cytochrome P-450 dependent monooxygenase enzymes in tumor development. We will determine whether 1) exposure to xenobiotics, including energy derived PAH, alter

normal imprinting patterns as well as the specific mRNA concentrations coding for these isozymes and 2) altered imprinting changes the susceptibility of animals toward tumor development as indicated by specific DNA adducts known to be associated with the appearance of tumors. Our approach will be to examine the expression of cytochrome P-450 dependent monooxygenase genes using molecular probes for specific mRNA species. Since the polynucleotide sequences for the phenobarbital inducible (P-450b and P-450e) probes are available (Giachelli and Omiencinski, 1986), these can be readily and inexpensively synthesized at PNL using capabilities currently on hand. Sequence information is available for both 3-MC inducible forms, however probes for these and other forms of P-450 will need to be prepared and characterized. In addition, data for the activities of several cytochrome P-450 dependent monooxygenases enzymes will be determined. Several of these enzyme assays are currently in place in our laboratory as well as methods to study enzymatically released DNA adducts following exposure to BaP and other PAH carcinogens. The combination of these methods should allow us to determine the extent to which altered fetal/neonatal imprinting contributes toward lifelong susceptibility to tumor development.

REFERENCES

Bagley, D. M. and J. R. Hayes, 1985. Xenobiotic imprinting of the hepatic monooxygenase system. Biochem. Pharm. 34: 1007-1014.

Faris, R. A. and T. C. Campbell, 1983. Long-term effects of neonatal phenobarbital exposure on aflatoxin B1 disposition in adult rats, Cancer Research, 43: 2576-2583.

Giachelli, C. M. and C. J. Omiencinski, 1986. Regulation of cytochrome P-450b and P-450e mRND expression in the developing rat. J. of Bio. Chem., 261 (3): 1359-1363.

Soyka, L. F., 1980. Hepatic drug metabolizing enzyme activity and tumorigenesis in mice following perinatal exposure to benzo(a)pyrene. Pediatric Pharm. 1: 85-96.

Whitlock, Jr., J. P., 1986. The regulation of cytochrome P-450 gene expression. Ann. Rev. Pharmacol. Toxicol. 26: 333-369.

MODULATION OF GENE EXPRESSION BY POLYCYCLIC AROMATIC HYDROCARBONS AND POLYHALOGENATED AROMATIC HYDROCARBONS

O. Hankinson, N.M. Carramanzana, F.-F. Chu, J. Nanthur, F.C. Sander and A.J. Watson.

Laboratory of Biomedical and Environmental Sciences, University of California, Los Angeles.

Polycyclic aromatic hydrocarbons (PAH's), generated by burning fossil fuels, and certain polyhalogenated aromatic hydrocarbons (HAH's), such as polychlorinated dioxins, biphenyls and dibenzofurans, are major environmental pollutants. Carcinogenesis by PAH's largely depends upon their metabolism by the aryl hydrocarbon hydroxylase (AHH) activity of cytochrome P_1-450. PAH's and HAH's induce cytochrome P_1-450. Induction is mediated by the soluble Ah receptor (reviewed in Ref. 1). Although it has been suggested that the unoccupied Ah receptor is located in the nucleus, we and others have provided evidence that it is cytosolic (2). After binding PAH or HAH ligands, the receptor translocates to the nucleus where it stimulates cytochrome P_1-450 transcription by binding to specific sequences 5' to the cap site of this gene (3). Pathogenesis by HAH's also hinges on action of the Ah receptor, but in this case P_1-450 activity is not involved and the mechanism of toxicity is not known.

Cytochrome P_1-450 and associated AHH activity are highly inducible by PAH's and HAH's in the mouse hepatoma cell line, Hepa-1. We previously isolated mutants of Hepa-1 that are no longer inducible for P_1-450 and lack AHH activity. The majority of the mutants are recessive, while a few are dominant. The recessive mutants have been assigned to four complementation groups (i.e. genes).(4) Gene A is the P_1-450 structural gene.(5) Mutants in gene B and D have much reduced levels of the Ah receptor, while mutants in gene C are

defective in nuclear translocation of the Ah receptor-ligand complex.(6) We have now isolated human DNA-derived AHH-positive transfectants from representative mutants of each complementation group using our previously described (7) "reverse selection procedure" for isolating P_1-450 inducible cells. A fragment of the C gene has been cloned from a secondary transfectant of the C mutant by screening a genomic library of the secondary with a human repetitive Alu sequence. Secondary transfectants of a B mutant, and primaries of a D mutant have also been isolated. We are therefore progressing towards cloning the B and D genes.

The dominant mutants synthesize a trans-acting repressor of P_1-450 mRNA transcription. Using the cloned rat P_1-450 gene in transient transfection experiments, we have demonstrated that a site (or sites) of action of the dominant repressor lies downstream of 1184 bp 5' to the cap site. We also treated wild-type Hepa-1 cells with a calcium phosphate precipitate of DNA from a dominant mutant and selected AHH-negative clones in benzo(a)pyrene. The lack of AHH inducibility in these clones was shown to be dominant when they were hybridized to Hepa-1 cells, thus providing convincing evidence that they are indeed bona fide transfectants for the dominant gene. We have now set out to isolate the dominant gene by means of transfection and a marker rescue strategy.

We will use the cloned genes to study the structure and regulation of the endogenous genes, to isolate the corresponding cDNA's and proteins, and to investigate possible interactions of the different proteins with each other and with the cloned P_1-450 gene. A major effort will also be directed towards ascertaining whether genetic differences in expression of any of these genes

are associated with different susceptibilities to PAH-induced cancer in humans. Our studies should therefore provide considerable insight into the mechanism of pathogenesis by PAH's and HAH's and methodologies for reducing their deleterious effects.

Acknowledgments: This research was supported by Contract DE-AC03-76-SF00012 from the DOE and PHS grant CA 28868 awarded by the National Cancer Institute.

REFERENCES

1. Nebert, D.W. and Gonzalez, F.J. Annual Rev. Biochem. **56**, in press (1987)
2. Gudas, J.M., Karenlampi, S.O., and Hankinson, O. J. Cell Physiol. **128**, 441-448 (1986)
3. Jones, P.B.C., Galeazzi, D.R., Fisher, J.M., and Whitlock, J.P.Jr. Science **227**, 1499-1502 (1985)
4. Hankinson, O. Somat. Cell Genet. **9**, 497-514 (1983)
5. Montisano, D.F. and Hankinson, O. Mol. Cell Biol. **5**, 698-704 (1985)
6. Legraverend, C., Hannah, R., Eisen, H., Owens, O., Nebert, D., and Hankinson, O. J. Biol. Chem. **257**, 6502-6507 (1982)
7. Van Gurp, J.R. and Hankinson, O. Cancer Res. **43**, 6031-6038 (1983)

MODULATION OF GENE EXPRESSION BY BARBITURATES: THE CLONING, FUNCTIONAL EXPRESSION AND REGULATION OF THE GENE ENCODING A CATALYTICALLY SELF-SUFFICIENT CYTOCHROME P-450 FATTY ACID MONOOXYGENASE INDUCED BY BARBITURATES IN *BACILLUS MEGATERIUM*

Long Ping Wen and Armand J. Fulco

Department of Biological Chemistry, School of Medicine, and the Laboratory of Biomedical and Environmental Sciences, University of California, Los Angeles, California 90024-1786

In recent publications (1-4) we described the characterization of a soluble 119,000-dalton P-450 cytochrome ($P\text{-}450_{BM\text{-}3}$) that was induced by barbiturates in *Bacillus megaterium*. This single polypeptide contained 1 mol each of FAD and FMN/mol of heme and, in the presence of NADPH and O_2, catalyzed the oxygenation of long-chain fatty acids without the aid of any other protein. Limited trypsin proteolysis in the presence of substrate cleaved $P\text{-}450_{BM\text{-}3}$ into two polypeptides (domains) of about 66,000 and 55,000 daltons. The 66 kDa domain contained both FAD and FMN but no heme, reduced cytochrome c in the presence of NADPH and was derived from the C-terminal portion of $P\text{-}450_{BM\text{-}3}$. The 55 kDa domain contained heme and showed a P-450 absorption peak in the presence of CO and dithionite. This domain also bound fatty acid substrate with a concomitant low spin to high spin shift and had an N-terminal amino acid sequence identical to that of intact $P\text{-}450_{BM\text{-}3}$, an indication that the 55 kDa domain constitutes the N-terminal portion of the intact (i.e. 119 kDa) protein. Removal of the first 9 amino acid residues of the 55 kDa domain abolished its ability to bind substrate.

The gene encoding cytochrome $P\text{-}450_{BM\text{-}3}$ was cloned by an immunochemical screening technique. The *Escherichia coli* clone harboring the recombinant plasmid produced a 119,000 dalton protein that appeared to be electrophoretically and immunochemically identical to the *B. megaterium* enzyme and contained the same N-terminal amino acid sequence. The recombinant DNA product also exhibited the characteristic cytochrome P-450 spectrum and was fully functional as a fatty acid

monooxygenase. In E. coli, the synthesis of P-450$_{BM-3}$ was directed by its own promoter included in the DNA insert, proceeded constitutively at a very high rate but was not stimulated by pentobarbital. However, when the cloned P-450$_{BM-3}$ gene, either intact or in a truncated form, was introduced back into B. megaterium via an E. coli/B. subtilis shuttle vector, its expression was constitutively repressed but was induced by pentobarbital. This finding demonstrated that the regulatory region of the P-450$_{BM-3}$ gene that responds to barbiturates was included in the cloned DNA. The evidence also indicated that pentobarbital cannot directly act on the gene to cause induction but presumably interacted with another component such as a respressor molecule that is present in B. megaterium but is absent in the E. coli clone.

We have now initiated a more detailed study of the mechanism of barbiturate-mediated induction of cytochrome P-450$_{BM-3}$ by inserting large putative regulatory sequences from the cloned gene upstream from a promoterless chloramphenicol acetyl-transferase (CAT) gene contained in a shuttle vector. We have found that a DNA fragment encoding the N-terminal 28 amino acids of the protein and encompassing 1.5 kb 5' to the N-terminal, when placed in the correct orientation upstream from the CAT gene, allows CAT induction by barbiturates. When the construct is introduced into E. coli, CAT activity is expressed at a high level constitutively but is not increased by barbiturate addition to the growth medium. However, when the construct is introduced into B. megaterium, there is a low but significant constitutive expression of CAT activity (relative to a control) but a high induction of CAT activity by barbiturates. The observed levels of induction by various barbiturates (2-thiobarbituric acid, phenobarbital, pentobarbital, secobarbital and methohexital) correlated very well with the previously determined potency of these barbiturates as inducers of cytochrome P-450$_{BM-3}$.

Acknowledgements: This work was supported by National Institutes of Health Research Grant GM 23913 and by Contract DE-AC03-76-SF00012 between the Department of Energy (Director of the Office of Health and Enviornmental Research) and the University of California.

REFERENCES

1. Narhi, L. O., and Fulco, A. J., J. Biol. Chem. 261, 7160-7169; 1986.

2. Fulco, A. J., and Ruettinger, R. T., Life Sci. 40, 1769-1775; 1987.

3. Wen, L.-P., and Fulco, A. J., J. Biol. Chem. 262, 6676-6682;1987.

4. Narhi, L. O., and Fulco, A. J., J. Biol. Chem. 262, 6683-6690; 1987.

THE METABOLISM AND GENOTOXICITY OF AROMATIC AMINES IN AQUATIC ORGANISMS

John P. Knezovich, Paula W. Krauter, Michael P. Lawton, and Florence L. Harrison.
Environmental Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94550

Aquatic organisms may be exposed to a variety of organic contaminants that originate from energy-related activities. Concentrations of these contaminants that are high enough to cause mortality are rare, however, and effects caused by continuous exposure to low concentrations are a more likely environmental problem. The tolerance of an organism to such sublethal exposures will be determined largely by the presence or absence of biochemical and physiological mechanisms that result in the metabolism and elimination of contaminants. The metabolism of aromatic amines is of primary concern because these compounds must be metabolically activated before they can interact with DNA and elicit genotoxic damage. This process has been well defined in mammals and has formed a sound basis for understanding the hazard posed by exposure to these compounds. Unfortunately, little is known of analogous processes and effects in aquatic organisms, and extrapolation of results for mammals cannot be made until basic biochemical and physiological processes are defined.

We used marine mussels (Mytilus edulis) and bullfrog tadpoles (Rana catesbeiana) to investigate the comparative metabolism and genotoxicity of aromatic amines in vivo. These organisms were selected because they possess distinctly different metabolic capabilities: mussels lack an active mixed-function-oxidase enzyme system that is present in most other organisms, including amphibians. Using ^{14}C-labeled chemical probes (o- and p-toluidine, 2-aminofluorene (2-AF), and 2-acetylaminofluorene (2-AAF)), we dosed mussels and tadpoles with individual compounds by direct immersion in aqueous solutions. The identities of metabolites were then determined by the analysis of depurated products and tissue residues according to previously defined HPLC and GC/MS methods[1].

Marine mussels converted p-toluidine, aniline, and 2-AF to their corresponding N-acetyl derivatives as the only products of metabolism[2]. These results contrast with those determined for o-toluidine, which was converted to N-formyl, N-methyl, and mutagenically active N-oxidized metabolites[1]. The N-conjugating pathways used by the mussel for the metabolism of aromatic amines are also those that are required for normal biochemical roles. The toxicological significance of these pathways was investigated by isolating covalently bound metabolites in mussels exposed to 2-AAF. Low levels of protein adducts (111 pmole/mg), but no DNA adducts, were detected. The formation of low levels of 2-AF DNA adducts have recently were demonstrated *in vitro*, however, with a ^{32}P-postlabeling assay[3]. Additionally, 2-AAF failed to induce sister chromatid exchanges (SCEs) in a larval assay that previously was used to detect increased SCEs that result from exposure to direct-acting chemical mutagens[4]. These results indicate that the N-conjugating pathways used by mussels result primarily in the detoxification of aromatic amines by limiting the amount of primary amine available for activation.

Bullfrog tadpoles rapidly accumulated 2-AAF from the water column and excreted 2-AF, N-hydroxy-2-AF, and 2-nitrosofluorene when they were transferred to clean water. Analyses of liver tissues revealed the presence of ring-hydroxylated metabolites and relatively high concentrations of nonextractable residues. Covalently bound protein adducts (959 pmole/mg) and DNA adducts (34 pmole/mg) were isolated from the liver and indicated that this haemopoetic center was susceptible to damage from 2-AAF. The extent of micronuclei (MN) formation in peripheral red blood cells[5] was used subsequently as an index of cytogenetic damage caused by 2-AAF exposure. A significant increase from the spontaneous (background) number of MN occurred in blood cells of tadpoles exposed to doses as low as 10^{-6}M 2-AAF (Figure 1.).

The results from these studies indicate that tadpoles, in contrast to mussels, can metabolically activate aromatic amines to concentrations that are sufficient to cause cytogenetic damage. Information on such basic processes is necessary for the identification of potentially susceptible organisms within a contaminant-impacted ecosystem.

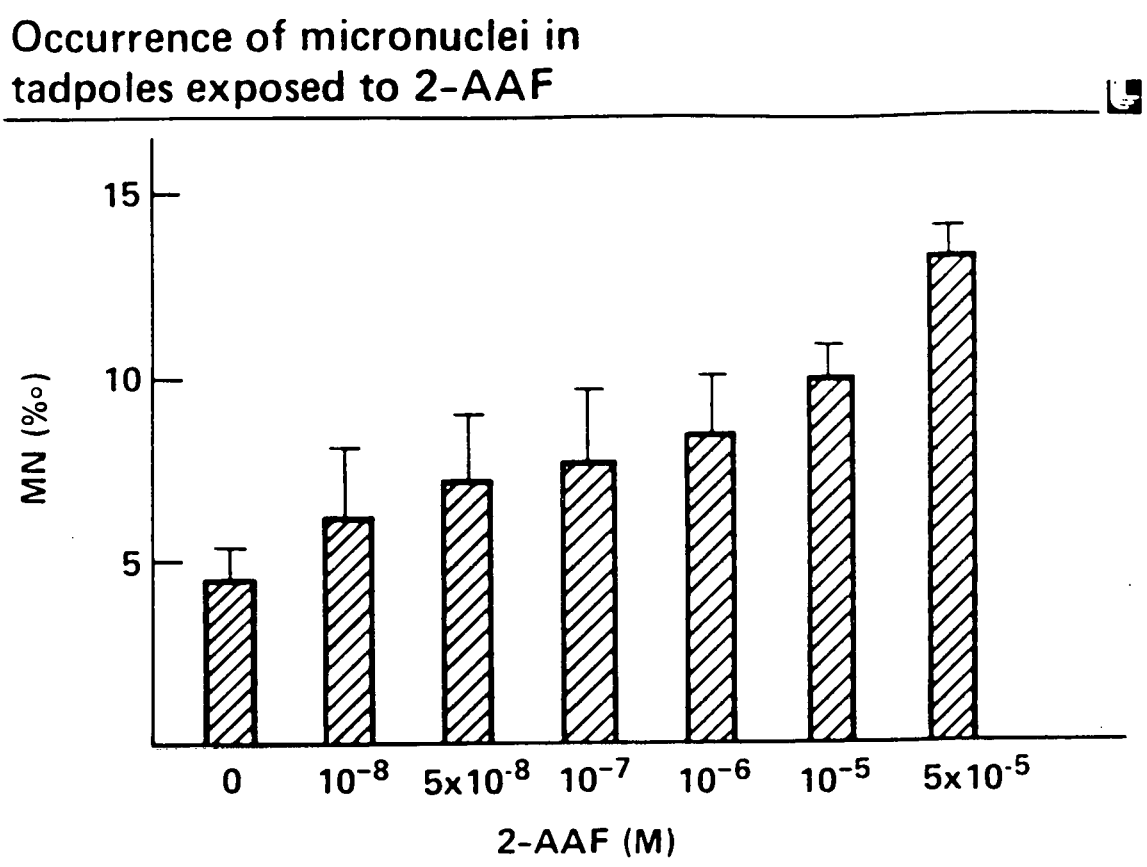


Figure 1. Incidence of micronuclei in peripheral erythrocytes of bullfrog tadpoles exposed to 2-acetylaminofluorene.

Acknowledgements: Work performed under the auspices of the Ecological Research Division of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under Contract W-7405-Eng-48.

REFERENCES

1. Knezovich, J. P., and D. G. Crosby. Env. Toxicol. Chem. 4, 435-446; 1985.
2. Knezovich, J. P., Lawton, M. P., and Harrison, F. L. Mar. Env. Res. (in press).
3. Kurelec, B., Chacko, M., and Gupta, R. C. Mar. Env. Res. (in press).
4. Harrison, F. L., and Jones, I. M. Mutation Res. 105, 235-242; 1982.
5. Krauter, P. W., Anderson, S. L., and Harrison, F. L. Environ. Mutagen. (in press).

MEASUREMENT OF MULTIPLE ENDPOINTS OF GENOTOXICITY IN A CHINESE HAMSTER OVARY TRIPLE AUXOTROPH: RESPONSE TO 1,2-DICARBONYL COMPOUNDS

Robert T. Taylor, M. Leslie Hanna, James D. Tucker, and Stephen J. Rinkus.

Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA 94550

Auxotrophic reversion has been a neglected research area in *in vitro* mammalian mutagenesis. Yet, it offers a sensitive and unique way to compare genetic damage potency and specificity, especially if additional types of genotoxicity can be assayed in the same cell line. A triple auxotrophic folate mutant that was originally described in 1974[1] can be used to assay not only nutritionally corrective reverse mutations,[2] but also several other endpoints of genetic alteration. The Chinese hamster ovary auxotroph (CHO AUXB1) requires glycine + adenosine + thymidine (GAT) for growth and survival[1] due to a defect in the structural gene for folylpolyglutamate synthetase (FPGS).[3] Its FPGS catalyzes the reaction below,

$$H_4PteGlu_n + \text{L-glutamate} + ATP \xrightarrow{Mg^{+2}} H_4PteGlu_{n+1} + ADP + Pi$$

which is essential for the intracellular retention of functional reduced folates.[4] We report here the utilization of CHO AUXB1 to characterize the mammalian cellular genotoxic properties of 1,2-dicarbonyl compounds.

It is well established that 1,2-dicarbonyls are produced *via* the Maillard reaction sequence during the heating of foodstuffs (e.g. coffee bean roasting); however, they are also formed from carbohydrates during the irradiation of foods.[5] Moreover, recent studies show that three 1,2-dicarbonyls (glyoxal, methylglyoxal, and diacetyl) are generated in high yields from the NO_x-air photooxidative-ring-cleavages of the aromatic hydrocarbons in fossil fuels.[6] 1,2-Dicarbonyls are direct acting base-substitution mutagens in the Ames/*Salmonella* assay[7] and are reported to form fused ring adducts with DNA-guanine bases.[8]

Upon 20-22 h exposures to varying concentrations of the 1,2-dicarbonyls, conditions were devised to measure quantitatively five endpoints of genotoxicity in CHO AUXB1. Briefly, (1) GAT^+ revertants at the FPGS locus were selected by plating into -GAT medium, (2) sister chromatid exchanges (SCEs) and (3) cells with endoreduplicated four-stranded diplochromosomes (ERs) were scored by incorporating a mixture of BrdU (30 μM) + T (10 μM) into the culture medium, (4) Na^+/K^+ ATPase locus mutants were selected in +GAT medium containing 3.0 mM ouabain, and (5) Pro^+ revertants resulting from re-expression of the gene for pyrroline-5-carboxylate synthase[9] were selected in -Pro/+GAT medium. To assess directly DNA damage (DNA-protein cross-links and DNA single-strand breaks), the cells were labeled with [2-^{14}C]T, the treatment time was shortened to 90 min, and the technique of DNA alkaline/filter elution was employed.[10] Among seven 1,2-dicarbonyls that have been studied, dose-dependent increases above the spontaneous GAT^+ revertant frequency (average = 5.0×10^{-7}) were induced with kethoxal (>194-fold), glyoxal (50-fold), methylglyoxal (91-fold), diacetyl (17-fold), phenylglyoxal (16-fold), and cyclohexanedione (21-fold), but not benzil. On a cell survival basis, kethoxal and glyoxal are more efficient mutagens than 4-NQO, while the other four are comparable in potency to *cis*-$Pt(NH_3)_2Cl_2$. These seven 1,2-dicarbonyls are either negative or weakly positive in parental CHO-S cells at the HGPRT forward mutation locus, using 200 μM 8-AG or 10 μM TG as selection drugs. In addition, at least two 1,2-dicarbonyls (glyoxal and methylglyoxal) are only slightly positive at the CHO AUXB1 ATPase locus, which yields the expected strong responses to EMS and

4-NQO. Bisulfite (1.0 mM), a carbonyl attacking nucleophile, protects CHO AUXB1 cells against the cytotoxicity and FPGS locus mutagenicity of 1,2-dicarbonyls. Titration curves show that about one equivalent of bisulfite is required to suppress maximally GAT^+ revertant induction by methylglyoxal and kethoxal. Increasing concentrations of added culture medium T, but not A, progressively enhance up to 5-6 fold the FPGS mutagenic activities of the three 1,2-dicarbonyls that we examined (glyoxal, methylglyoxal, and kethoxal). A 2'-deoxycytidine concentration of only 2 μM counteracts these increased mutagenicities that are promoted by excess T levels as high as 1.0 mM.

With respect to SCEs and ERs, detailed dose-response curves were generated for methylglyoxal, glyoxal, and kethoxal. Approximately 2-3 fold increases above the baseline SCE frequency (10.6 ± 0.8/cell) are elicited by 0.3-1.6 mM concentrations of these compounds. Against a spontaneous baseline frequency of ~0.1% ERs, each of these three agents also induces 2.4-12% ERs in a dose-dependent manner. An ER is an unusual and abnormal process of cell replication which involves two rounds of DNA replication without an intervening cytokinesis.

Metabolizable cytidines with nitrogen substituted at the C-5 position in the pyrimidine ring can not be methylated by AdoMet when they are incorporated into cellular DNA. Consequently, the high percentages of stable CHO-K1 Pro^+ revertants that are induced by 5-azacytidine (5-AZC) have been attributed to DNA hypomethylation, rather than a true gene mutational event.[9] It was therefore of interest to compare the effects in CHO AUXB1 of 5-AZC on Pro^+ reversion, versus GAT^+ reversion at the FPGS locus. Over the concentration range of 0-0.6 μM, 5-AZC increases the GAT^+ frequency ~30 fold to 12.3×10^{-6} (maximum), while over the range of 0-5.0 μM it elevates the Pro^+ frequency ~700 fold to 14.4×10^{-2} (maximum). 2'-Deoxyazacytidine behaves similarly, except that the GAT^+ frequency reaches 300×10^{-6}. Thus, although the CHO AUXB1/FPGS locus readily detects these cytidine derivatives as mutagens, it behaves like a typical mutated gene, rather than a silent hypermethylated gene.[9] It is noteworthy that these same agents are very weak acting at the CHO-S/HGPRT locus.

Thus far, the only 1,2-dicarbonyl that we have examined in detail for direct DNA damage is methylglyoxal. It causes a dose-dependent increase in both DNA-protein crosslinking, as well as single-strand breaks. A methylglyoxal concentration of 1.0 mM induces single-strand breaks in CHO AUXB1 with a potency that is equivalent to about 190 Rads of ^{137}Cs irradiation. Bisulfite (10 mM) co-treatment completely negates both types of methylglyoxal DNA-lesions. Other 1,2-dicarbonyls are currently under study by the alkaline elution method.

In summary, our results demonstrate that, as a class of compounds, 1,2-dicarbonyls are moderately potent genotoxins to cultured mammalian cells. They (1) suggest that 1,2-dicarbonyls induce reversion at the FPGS locus by a G-C to A-T base-substitution mechanism, (2) support our view that this mutation assay is sensitive preferentially to agents that are known to react with guanine bases in DNA, and (3) illustrate the versatility of the CHO AUXB1 cell line as a supplemental genotoxin assay system. Future work will be directed at the isolation and identification of exocyclic-ring DNA-base adducts from 1,2-dicarbonyl treated CHO AUXB1 cells.

Acknowledgements: This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48.

REFERENCES

1. Mc Burney, M.W. and Whitmore, G.F. Cell 2, 173-182; 1974.
2. Taylor, R.T., Wu, R., and Hanna, M.L. Mutation Res. 151, 293-308; 1985.
3. Taylor, R.T. and Hanna, M.L. Arch. Biochem. Biophys., 197, 36-43; 1979.
4. Taylor, R.T. and Hanna, M.L. Arch. Biochem. Biophys., 181, 331-344; 1977.
5. Mauron, J. in: Progress in Food Nutrition Service (C. Eriksson, Ed.), Vol. 5, pp. 5-35, Permagon Press, Oxford, 1982.
6. Tauzon, E.C., Mac Leod, H., Atkinson, R., and Carter, W.P.L. Environ. Sci. Technol., 20, 383-387; 1986.
7. Bjeldanes, L. and Chew, H. Mutation Res., 67, 367-371; 1979.
8. Shapiro, R. and Hachmann, J. Biochemistry 5, 2799-2808; 1966.
9. Harris, M. Somatic Cell and Molec. Genetics, 10, 615-624; 1984.
10. Kohn, K.W., Ewig, R.A.G., Erikson, L.C., and Zwelling, L.A. in: DNA Repair, A Laboratory Manual of Research Procedures (E.C. Friedberg and R.C. Hanawalt, Eds.), Ch. 29, pp. 379-401, Marcel Dekker, New York, 1981.

SESSION 5

Inhalation Toxicology

Inhalation Toxicology

Summary of Session 5

C. H. Hobbs

Lovelace Inhalation Toxicology Research Institute

Inhalation is one of the primary routes of exposure of people to energy-related chemicals. Following inhalation, chemicals deposited in the respiratory tract remain at the site of deposition for variable lengths of time, may be metabolized by the respiratory tract, and have the opportunity to interact with macromolecules of the respiratory tract. Thus, the health effects from inhaled material often occurs within tissues of the respiratory tract. Conversely, inhaled materials also may be translocated from their sites of deposition within the respiratory tract by blood and lymph to other organs and body tissues where health effects may occur.

The work presented in this session and in the other sessions that related directly or indirectly to inhalation toxicology illustrates the relatively large breadth of work being sponsored by DOE relating to the mechanisms by which inhaled chemicals may produce health effects in man. The primary emphasis of the work is to provide an understanding of how inhaled materials interact with the critical biological units to result in health effects.

The work presented ranged from mechanisms of deposition and clearance of inhaled materials, which determine the dose of the materials to the critical biological

units (i.e., DNA for cancer), to the mechanisms by which the dose delivered to tissues cause biological effects such as cancer. An important aspect of this workshop was the opportunity for those working in inhalation toxicology, where the studies are often conducted in whole animals, to interact with scientists conducting studies on disease mechanisms using cells as the research tool. An important aspect of future work is to provide ties between these areas of research to reach the goal of understanding and predicting health effects from exposure of people to energy related chemicals.

In order to predict the health effects to man from inhaled chemicals, it is essential that basic studies be undertaken to provide an understanding of mechanisms by which inhaled chemicals are deposited in the respiratory tract and how they are handled by the respiratory tract once deposited. It is also important to develop an understanding of how the inhaled chemicals interact with the cells to produce health effects including chronic respiratory disease and cancers of the nose, lung, and other organs. In this regard bridges must be built between studies such as those using *in vitro* systems, studies of adduct formation and repair, studies of gene regulation, and studies in which animals (as a model for man) are exposed via inhalation to chemicals. This symposium provided an excellent forum for the investigators conducting these different types of studies to interact and exchange research ideas.

The paper by Popp *et al,* (ORNL) presented the results of studies in which mice were exposed to ethylene oxide via inhalation. In this case, the health effects of interest were perturbations in the peripheral blood and bone marrow of adult mice and the

hemopoietic progenitor cells of fetal mice. The exposures caused an increase in granulocytes and resulted in depressed numbers of lymphocytes in the peripheral blood of adult mice. Inhalation of ethylene oxide by pregnant female mice caused reduced fetal weight, reduced cellularity of fetal livers, and a reduction of fetal hemopoietic stem cells.

Witschi (ORNL) reported on studies of lung cell kinetics and the relationship between cell injury and repair in lungs damaged by inhaled toxic materials and blood-borne agents. These studies showed possible correlations between defective epithelial repair and the development of pulmonary fibrosis. The studies also demonstrated that agents that cause lung cell proliferation do not always enhance (promote) lung tumor development in the mouse lung tumor model. This is in contrast to models for two-stage carcinogenesis models in mouse skin. Additional research is needed to clarify differences in these two tumor models.

The third paper in the session, by Bice *et al.* (ITRI), reported on local immune responses in the lung and the immune cells at risk following inhalation of chemicals. These studies demonstrated that following instillation of antigen into portions of the lungs, antibody-forming cells accumulate predominantly in the immunized portion of the lungs and also demonstrated that memory cells are present that result in intense antibody responses when that area of the lung is rechallenged with antigen. These studies show an important mechanism of defense from inhaled materials; they provide the basic information needed to determine if inhaled chemicals will decrease local

antibody production in the lung and if immune memory will be altered by inhaled chemicals.

The paper by Harkema *et al.* (ITRI) reported on the mechanisms by which toxic materials induce mucous hypersecretion by respiratory epithelium. They demonstrated that toxicant–induced neutrophil attraction and migration through the epithelium results in a stimulus for increased mucous secretion from respiratory epithelium. Presumably this increased mucous secretion is one protective mechanism by which the respiratory tract attempts to dilute the harmful effects of an inhaled toxicant.

Snipes *et al.* (ITRI) described the mechanisms of clearance of inhaled particles from the respiratory tract. Following deposition of particles in the deep lung, below the level of clearance by ciliary action such as occurs in the conducting airways, particles that are relatively insoluble are retained for long periods of time. One route of clearance of these particles is to lymph nodes associated with the lung. These studies demonstrated that clearance of particles from lung alveoli to lymph nodes is by phagocytic cells. The studies also demonstrated that some particles are also cleared via the mucociliary escalator within these phagocytic cells. Additional studies are needed to better understand the differences in the rates of clearance of relatively insoluble particles demonstrated to exist between different species, including man.

In the final paper in the session, Mauderly *et al.* (ITRI) reported on levels of lung DNA adducts detected in rats exposed over their lifespan to either diesel exhaust

or oil shale dusts and possible relationships of these adducts to the incidence of lung tumors observed. Although the two types of particles (diesel exhaust and oil shale) have markedly different chemical compositions and their extracts have markedly different *in vitro* mutagenicity, 24-month exposures of rats to either material resulted in similarly elevated lung DNA adducts. However, the pattern of DNA adducts, as determined by ^{32}P post-labeling techniques, differed between the two materials. Lung tumors were observed in the animals exposed to diesel exhaust. These studies illustrate the complexities of the relationships between DNA adduct formation and the formation of tumors. Current results suggest that total DNA adduct levels in an organ are not, by themselves, a good predictor of carcinogenicity.

In summary, papers were presented in this and other sessions of this symposium that demonstrate the diversity of biological effects that may result from the inhalation of energy-related chemicals. The studies being conducted are concerned with the mechanisms by which inhaled materials cause the health effects. These studies also provide a bridge between *in vitro* and *in vivo* studies on the mechanisms of chemical toxicity.

HEMOTOXICITY OF ETHYLENE OXIDE IN MICE

R. A. Popp, R. E. Hand, Jr., R. C. Mann and D. M. Popp

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

Because exposure to ethylene oxide (EtO) has been reported to adversely affect the hemopoietic system,[1] studies were designed to identify and quantify EtO-induced perturbations in peripheral blood and bone marrow of adult mice and in hemopoietic progenitor cells of fetal mice. C57BL mice were exposed in 1.5m^3 inhalation chambers to 255 ppm EtO for 6 hours a day and 5 days a week. Analyses were made after 1, 2, 4, 8 and 14 days and after 4, 6, 8 and 20 weeks of exposure to EtO. Hematology as done manually at first and in later experiments an automatic hematology analyzer, Ortho EL-15 that had been modified to facilitate analysis of nucleated cells, was used to obtain rapid analysis of 15 blood parameters. Bone marrow cells were flushed from femurs and tibias and were counted. Aliquots were used for CFU-S[2] (stem cell) and mutation assays and an aliquot was used for flow cytometry (FCM) analysis.[3]

Perturbations of peripheral leukocytes occurred within the first 6 hours of exposure and there was a shift in differential toward granulocytes, which persisted through the fourth exposure and resulted in severely depressed numbers of lymphocytes in the peripheral blood. After the fourth exposure, hematocrit, red cell number and hemoglobin were generally depressed, and bone marrow cellularity and number of CFU-S were below normal. Bone marrow also responds quickly and often specifically to toxic stress, but many cell populations make up the marrow and it has been difficult to characterize the response of specific populations of cells. FCM analyzes single cells and can be used to identify changes in subpopulations of cells. Aliquots of bone

marrow were analyzed on an Ortho 50H Cytofluorograf. Computer analysis of scattergrams and histograms[4] showed that EtO exposure caused a rapid loss of granulocytes which was most severe by day 2, followed by a deficit of lymphocytes which was most pronounced at 8-10 weeks. The results showed that FCM analysis of mixed cell populations in bone marrow was useful in identifying the effect of EtO on specific cells.[5]

Gas sterilizer operators are more likely to be exposed to short bursts of high levels of EtO so we have exposed C57BL and SEC mice to 1500 ppm EtO for 10 minutes twice a day for 4 days and studied its effect at daily intervals on the hematology of exposed mice. Significant depression in lymphocytes and granulocytes occurred in both strains after 2 and 4 exposures. The effects of EtO on the developing hemopoietic system in the fetal liver of embryos were also examined.[6] C57BL females were mated and pregnant females at 13.5 and 14.5 days post copulation were exposed to 255 ppm EtO, 6 hours a day for 4 days. The fetuses were removed and weighed, and single cell suspensions of fetal liver were prepared in phosphate buffered saline. Nucleated cell counts were made and aliquots were injected into lethally irradiated recipients to assess the CFU-S number. At these time periods exposure of C57BL mice to EtO caused a 60% reduction of fetal weight, 65% reduction in cellularity, 68% reduction in incidence of CFU-S and a 43% reduction of hemopoietic stem cells. These results show that the fetal mouse is a sensitive indicator of the cytostatic effects of EtO.

Acknowledgments: Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.

REFERENCES

1. Hogstedt, C., Malmqvist, N., and Wedman, B. JAMA 241, 1132-1133, 1979.
2. Till, J. E., and McCulloch, E. A. Radiat. Res. 14, 213-222, 1961.
3. van den Engh, G., Visser, J., and Trask, B. Experimental Hematology Today 1979, eds. S. J. Baum and G. D. Ledney, p. 19, New York: Springer Verlag, 1979.
4. Mann, R., Popp, D. M., and Hand, R. E., Jr. Cytometry 5, 304-307, 1984.
5. Popp, D. M., Popp, R. A., Lock, S., Mann, R. C., and Hand, R. E., Jr. J. Toxicol. Environ. Health 18, 543-565, 1986.
6. Popp, D. M., Lock, S., and Popp, R. A. Toxicologist 5, 89, 1985.

CELL KINETICS IN THE LUNG AS A PREDICTIVE TOOL IN THE ASSESSMENT OF FIBROGENESIS AND TUMORIGENESIS

Hanspeter Witschi

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

Up to the late 1960's, there was only a limited amount of information available on cytodynamics in the lung parenchyma. Morphometry provided insights on qualitative and quantitative changes in lung injury and repair, and the role of the alveolar type II epithelial cell in repairing damaged pulmonary epithelium became apparent. Autoradiography allows the study of cell renewal in airways and lung parenchyma. With the advent of plastic sections, the technique became a powerful tool. Embedding lung tissue in glycol methacrylate enabled one to cut sections thin enough (1 μm or less) to permit identification of individual cell types with much more precision than can be done with conventional paraffin sections. As a result of these developments there is now a considerable body of data available on cell injury and tissue repair in lungs damaged by toxic inhalants and by bloodborne agents.

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

In order to examine whether such a scenerio would apply to a variety of lung-toxic agents, mice were treated with three cytostatic drugs: cyclophosphamide, busulfan, or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). The alveolar labeling index was measured following drug administration with a pulse of ^{3}H-labeled thymidine and autoradiography. In cyclophosphamide treated animals, peak alveolar cell proliferation was seen 5 days after injection of the drug. In animals treated with busulfan or BCNU proliferation was even more

delayed (occuring 2 to 3 wks after administration). In contrast, with oleic acid, the highest alveolar cell labeling was found 2 days after intravenous administration. In animals exposed to a cytostatic drug, proliferation of type II alveolar cells was never a prominent feature; whereas, in animals treated with oleic acid there was an initial burst of type II cell proliferation. It was concluded that the patterns of pulmonary repair vary between chemicals designed to interfere with DNA replication as compared to agents which produce acute lung damage such as oleic acid (1).

A detailed knowledge of cell kinetics might also be of some use in assessing the likelihood of tumor development or of tumor promotion. Certain mouse strains have a high spontaneous incidence of pulmonary tumors. If mice from such a susceptible strain are treated with a carcinogen, they will develop within a few months an additional number of tumors. Knowing that many lung tumors in mice were type II cell tumors, having read that all agents active as skin tumor promoters were capable of producing a hyperplasia of the target cell population and having shown that BHT would produce a proliferation of type II alveolar cells, we reasoned that BHT should promote tumor development in mouse lung.

This prediction was fully borne out in a series of experiments, the first one published in 1977 (2). In several mouse strains, BHT enhances the development of lung tumors, provided exposure to BHT is begun after exposure to the carcinogen. However, the generalization made originally - that any agent capable of producing cell hyperplasia would enhance lung tumor development in mouse lung - could later on not be corroborated in experiments involving such other agents which would produce alveolar cell hyperplasia, such as MMT, oxygen or ozone.

Originally it was also assumed that after each BHT injection the alveolar cells would undergo a cycle of division; later it was found that cell proliferation occurs only after the first two BHT injections. We then documented that in animals made "tolerant" to BHT enhanced tumor development can be found even in the absence of any detectable hyperplasia in the alveolar zone. Available evidence thus suggests that cell hyperplasia is not a prerequisite for tumor promotion in mouse lung (3).

Conclusion

Detailed analysis of cell kinetics following acute lung injury has given us some insight into possible pathogeneic mechanisms underlying chronic lung disease. Possible correlations between defective reepithelialisation and development of fibrotic changes need to be explored in future work. On the other hand certain concepts generally thought to apply to tumor promotion have been found not be be correct in mouse lung. It will be challenging to see, whether other experimental model systems of two stage carcinogenesis follow the mouse lung or the mouse skin paradigms.

References

1) Witschi HP, Godfrey G, Frome E, and Lindenschmidt RC. Fundam. Appl. Toxicol. 8, 253-262, 1987.

2) Witschi HP, Williamson D, and Lock S. JNCI 58, 301-306, 1977.

3) Witschi HP. Cancer Res. 46, 2675-2679, 1986.

LOCAL IMMUNE RESPONSES IN THE LUNG AND IMMUNE CELLS AT RISK TO INHALED CHEMICALS

D. E. Bice and B. A. Muggenburg

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

The inhalation of pollutants in the environment and in certain occupations appear to be important in the etiology of human infectious diseases, cancer, and allergic diseases. These increased lung disease may be due to altered immune defenses in the lungs. To design experiments to evaluate the effects of inhaled chemicals on pulmonary immune responses, it is necessary to first obtain an understanding of the tissues and cells responsible for the development of immune responses in the lower respiratory tract.

The evaluation of immunity induced by the localized deposition of antigen into selected airways of the lungs of dogs and primates has provided details of the mechanisms involved in development of pulmonary immunity. The size of these animals allows the instillation of antigens into selected lung lobes by the use of a fiberoptic bronchoscope, while saline or other control materials are instilled into lung lobes of the same animal. At intervals after immunization (e.g. 3, 5, 7, 10, 12, and 14 days), the animals are anesthetized and the control and immunized lung lobes are lavaged by the instillation and withdrawal of saline through the fiberoptic bronchoscope. The immune responses which develop in lymph nodes, blood, and in control and immunized lung lobes are then evaluated.

In primary antibody responses, antigen is cleared from the lung to the lung-associated lymph nodes, possibly within neutrophils and/or alveolar macrophages. There are several lung-associated lymph nodes in the thoracic cavity, and large numbers of antibody-forming cells (AFC) are found only in

those lymph nodes which receive lymphatic drainage from the lung lobe instilled with antigen (1). Large numbers of specific AFC and antibody are released via the efferent lymphatics into the blood (2). Although the mechanisms are not understood, antibody and AFC present in the blood accumulate predominantly in the immunized lung lobes (2). This accumulation of immune cells into the lung is not antigen specific, and cells produced in any lymphoid tissue can enter into immunized lung lobes (3, 4). Lymphocytes recruited into the lung mature to plasma cells and produce antibody which increases the titer above that entering the lung by transudation from the vasculature (5, 6). In addition, memory cells are present, and additional antigen challenges result in intense antibody responses only in the immunized and challenged lung lobes (7, 8).

The identification of the tissues and cells responsible for the development of lung immunity helps identify immune cells at risk to inhaled chemicals. Because the lung-associated lymph nodes are the tissues responsible for the induction of primary immune responses, studies have evaluated the effects of inhaled pollutants on their immune function. In addition to being responsible for the induction of immunity, the lung-associated lymph nodes also are a site for the metabolism of chemicals which leave the lung in the lymphatics, and they also retain insoluble particulates which clear from the lung. Studies using rats indicate that immune responses in the lung-associated lymph nodes can be altered by inhaled pollutants (9, 10).

The inhalation of chemicals could alter not only the accumulation of immune cells into the lung, but also the functions of cells which enter the lung from the blood. Because antigen-specific lymphocytes and mature plasma cells are found in interstitial lung tissues and in the alveoli, these immune cells are at risk to inhaled chemicals. Based on preliminary data,

memory cells are mainly in interstitial lung tissues. Additional data are needed to determine if inhaled chemicals will decrease local antibody production in the lung, and if immune memory is altered by inhaled materials.

Acknowledgements: Research sponsored by the U.S. Department of Energy Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

REFERENCES

1. Bice D.E., Harris D.L., and Muggenburg B.A. Exp. Lung Res. 1, 33-40; 1980.
2. Bice D.E., Harris D.L., Hill J.O., Muggenburg B.A., and Wolff R.K. Am. Rev. Respir. Dis. 122, 755-60; 1980.
3. Bice D.E., Degen M.A., Harris D.L., and Muggenburg B.A. Am. Rev. Respir. Dis. 126, 635-9; 1982.
4. Hillam R.P., Bice D.E., and Muggenburg B.A. Immunology 55, 257-61; 1985.
5. Bice D.E., Gray R.H., Evans M.J., and Muggenburg B.A. J. Leukocyte Biol. 41, 1-7; 1987.
6. Harmsen A.G., Bice D.E., and Muggenburg B.A. J. Leukocyte Biol. 37, 483-92; 1985.
7. Mason M.J., Bice D.E., and Muggenburg B.A. Am. Rev. Respir. Dis. 132, 657-60; 1985.
8. Bice D.E., and Muggenburg B.A. Am. Rev. Respir. Dis. 133, A97; 1986;.
9. Bice, D.E., Mauderly, J.L., Jones, R.K., and McClellan, R.O. Toxicol. Appl. Pharmacol. 5, 1075-86; 1985.
10. Bice, D.E., Hahn, F.F., Benson, J., Carpenter, R.L., and Hobbs, C.H. Environ Res. (in press).

IN VIVO EFFECTS OF TRANSIENT NEUTROPHIL INFLUX ON NASAL EPITHELIAL MUCOSUBSTANCES: QUANTITATIVE HISTOCHEMISTRY.

J. R. Harkema, J. A. Hotchkiss, A. G. Harmsen, and R. F. Henderson

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Certain inhaled toxicants are known to induce mucous hypersecretion by the respiratory epithelium. It is not known whether this secretory change is a direct effect of the toxicant or an indirect effect of the host's concomitant inflammatory response.

The present study was designed to determine by quantitative histochemistry if an in vivo influx of neutrophils through the nasal mucosa would induce significant quantitative changes in the amount of stored mucosubstance in the nasal respiratory epithelium.

We specifically wanted to answer the following questions: 1) Does a short-term influx of neutrophils within the respiratory epithelium change the amount and character of the stored epithelial mucosubstance?; 2) If such a change occurs, is it transient or persistent?; and 3) Is there neutrophil-associated morphologic damage to the epithelial cell population that can be detected by light microscopy?

The study was divided into two parts. The first part morphometrically characterized the temporal post-exposure (24 hour period) response of the rat nasal mucosa to a single intranasal instillation of endotoxin (E), a chemotoxinogen for neutrophils. The second part examined the direct effects of E and indirect effects of infiltrating neutrophils on nasal intraepithelial mucosubstances by preventing the neutrophilic influx into the E-exposed nasal mucosa of the rat.

In the first experiment, rats were intranasally instilled with either 0.1 ml of saline (S) containing 0.5 mg of E or with S alone. Rats were

killed immediately after, or 0.5, 1, 3, 6, or 24 hours (hrs) after instillation. Few neutrophils were evident in nasal epithelium of rats killed immediately, at 0.5, or 1 hour after E instillation, or at any time after instillation of S. A marked neutrophil infiltration in the nasal mucosa was observed 6 hrs after E instillation. Intraepithelial neutrophils were also evident at 3 and 24 hrs after E, but in numbers significantly less than at 6 hrs. The quantity of stored mucosubstance was inversely related to neutrophil number. Six hrs after instillation, E instilled rats had 5 - 8 times less stored mucosubstance than rats instilled with S alone. At 24 hrs post instillation, the amount of mucosubstance in nasal epithelium of E treated rats had increased to approximately 4.5 times of that at 6 hrs and returned to a level which was not significantly different from that of saline instilled controls.

In the second experiment, rats were divided into three groups. Animals in the first group were both intratracheally and intranasally instilled with E. The second group was instilled intratracheally with S and intranasally with E. The third group were both intranasally and intratracheally instilled with S. Rats in all three groups were killed 6 hrs after instillation.

Intratracheal instillation of E induced a marked neutrophilic bronchopneumonia and prevented an E-induced neutrophilic influx into the nasal epithelium. The number of neutrophils within the nasal epithelium of rats intratracheally and intranasally instilled with E were not significantly different from that of S-instilled rats, while rats receiving only an intranasal instillation of E had a marked neutrophilic inflammatory response in the nasal mucosa.

Concomitantly, the amount of stored mucosubstance within the epithelium of the nasal septum in rats both intranasally and intratracheally instilled with E was significantly greater than that in rats that were instilled only intranasally with E. The amount of mucosubstance in the intranasally and intratracheally E-instilled rats was not significantly different from that in S-instilled rats.

These results indicate that an endotoxin-induced intraepithelial influx of neutrophils into the nasal mucosa can decrease the amount of stored epithelial mucosubstance. The change in the quantity of mucosubstance is not persistent, but returns to pre-neutrophilic infiltration levels by 24 hrs after E-instillation, a time when intraepithelial neutrophil numbers have diminished.

The observation that rats in which neutrophils were prevented from migrating into E-exposed nasal epithelium had no change in the quantity of stored mucosubstance additionally supports the role of the neutrophil (inflammation) in diminishing the storage of mucosubstances, rather than a direct effect of E. Since there was no evidence of secretory cell necrosis or exfoliation, and assuming mucosubstance synthesis was unaltered, the depletion of stored mucosubstance in the nasal epithelium was most likely the result of increased secretion. Although the mechanism of this hypersecretion is unknown, the results of this study indicate that neutrophilic infiltration provides a stimulus for increased mucous secretion from respiratory epithelium. A similar neutrophilic infiltration induced by an inhaled chemical irritant could be responsible in part for the characteristic hypersecretion of mucus in respiratory airways.

Acknowledgements: Research sponsored by the U.S. Department of Energy's Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

PULMONARY TRANSLOCATION OF PARTICLES BY PHAGOCYTIC CELLS: MECHANISMS AND SPECIES COMPARISONS

M. B. Snipes, D. E. Bice, N. A. Gillett, R. A. Guilmette, A. G. Harmsen, H. L. Mueller, and B. A. Muggenburg

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Understanding mechanisms involved in retention and clearance of inhaled particles is vital to evaluating exposure-dose-response relationships for the lung and other tissues effected by inhalation exposures, such as lung-associated lymph nodes (LALN). We are conducting studies to define the roles of phagocytic cells in lung clearance (and retention), with emphasis on translocation of particles to the LALN. Interspecies comparisons are an important aspect of these studies, since species similarities and differences in biological responses to inhaled materials appear to be related to similarities and differences in retention and clearance of inhaled particles.

To study the role of phagocytic cells in the translocation of particles from alveoli to LALN, red and green fluorescent polystyrene microspheres (1.3 μm diameter) were instilled into separate, but adjacent lung lobes of dogs. Lavage fluid and LALN were analyzed. Macrophages in LALN contained either all red or all green microspheres, and less than 5% of the macrophages contained a combination of both colors. As a control, a mixture of red and green microspheres was instilled into one lung lobe of dogs. Most of the microsphere-containing macrophages in LALN contained both colors. These results indicated that individual macrophages phagocytized microspheres in the lung and transported them to the LALN.

In another study, macrophages were allowed to phagocytize either 1.3 μm red microspheres or 1.3 μm green microspheres *in vivo*. The macrophages

were collected by lavage, mixed and instilled back into another lung lobe of the same dog (autologous instillation). After three days, the majority of the macrophages in the LALN contained either red or green microspheres. This further supported the hypothesis that individual particle-containing macrophages had migrated from the instillation site to the lymph node.

The role of neutrophils in particle translocation from lung to LALN was examined in another group of dogs. Autologous neutrophils that had phagocytized 1.3 μm red or green microspheres were instilled into a previously unexposed lung lobe. After 24 hours, the LALN had numerous neutrophils, 99% of which contained either red or green microspheres, but not both. Other dogs had their peripheral blood neutrophils depleted by injection of hydroxyurea, then the dogs were instilled with fluorescent microspheres and killed 40 hours later. Hydroxyurea reduced neutrophil accumulation in the lung by 79% and reduced particle translocation to the LALN by 80%. These results indicated that neutrophils, like macrophages, phagocytize particles in the lung, then migrate to the LALN.

In another study with rats, 1.3 μm microspheres were intratracheally instilled. Groups of rats were killed after 1, 2, 4, and 8 days. About 90% of the microspheres in the LALN were within macrophages at each sampling time. We instilled mixtures of macrophages containing either red or green microspheres into the lungs of other rats. After three days, the LALN contained numerous macrophages with microspheres of only one color, consistent with results from dogs. In both species, the microspheres were translocated from the lung to LALN within phagocytic cells.

To determine the role of phagocytic cells in the clearance of particles by the mucociliary apparatus, lungs of dogs were instilled with 0.9 and 3.9 μm microspheres. The dog's tracheas were lavaged at 2 hours, 1, 3,

7, 14, 21, 35, and 49 days. At two hours, about 12% of the microspheres in the trachea were associated with phagocytes. By one day after instillation and for the remainder of the experiment, about 70% of the microspheres in the lavage fluids were within phagocytes. During the first seven days, most of the cells containing microspheres in the trachea were neutrophils. At 14 days and for the remainder of the experiment, most of the microspheres were within macrophages. The two different microsphere sizes were cleared from the lung at similar rates.

The studies conducted to date have used instillation of particles rather than inhalation. These studies are subject to criticism that exposures were not by inhalation. Thus, the numbers of particles per unit of lung were generally larger than they would be after an inhalation exposure, and instillation probably produced inflammation. Regardless, these results provide useful insights into mechanisms of clearance. For example, clearance by neutrophils may be a consequence of a local inflammatory response. After a few days, the inflammatory response resolves and the pulmonary alveolar macrophages dominate particle translocation. The same results might be expected for inhalation exposures that produce an inflammatory response.

These studies demonstrate that physical transport of inhaled particles clearly involves phagocytic cells, and that the cells phagocytizing particles in the airspaces appear to be the same cells that transport the particles into the interstitium, up the mucociliary escalator, or to LALN.

Acknowledgements: This research was supported by the U. S. Department of Energy's Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

GENOTOXICITY AND CARCINOGENICITY OF DIESEL SOOT AND OIL SHALE DUST, TWO MARKEDLY DIFFERENT PARTICLES WITH ASSOCIATED ORGANIC CONTENT

J. L. Mauderly[1], E. B. Barr[1], W. E. Bechtold[1], J. A. Bond[1], A. E. Brooks[1], A. F. Eidson[1], J. R. Harkema[1], R. F. Henderson[1], R. K. Jones[1], R. O. McClellan[1], K. Randerath[2], and R. K. Wolff[1]

[1]Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185
[2]Baylor College of Medicine

Increased lung DNA adduct levels and an increased prevalence of lung tumors among rats exposed chronically by inhalation to diesel exhaust suggested that DNA adduct formation signals an important step in carcinogenesis from particle-associated organic compounds (1,2). Recent results give additional insight into adduct-tumor relationships by providing a comparison of genetic injury and carcinogenicity from inhaled diesel exhaust and oil shale dust, materials with quite different physical and chemical compositions.

The carcinogenicity of diesel exhaust is known to be related to the soot particles, 0.3 μm chain aggregate carbonaceous particles having approximately 12% (by mass) methylene chloride-extractable organic content. The extracted organic material has both direct and indirect mutagenic activity in the Ames Salmonella assay. In contrast, the unretorted (raw) oil shale dust used in these studies consisted of 3.1 μm monolithic mineral particles with only 1.4% extractable organic content. The shale extract had negligible mutagenic activity in Salmonella.

Data from two studies are used in the present comparison. In the first study, rats were exposed 7 hr/day, 5 days/wk for up to 30 mo to diesel exhaust diluted to 0.35, 3.5, or 7.0 mg soot/m^3, or to clean air as controls. A progressive, dose-related chronic lung disease, with inflammation and focal fibrosis resulted from the exposures. Lung tumor prevalence was significantly increased at the two highest levels. Lung tumor prevalence among rats exposed to exhaust at 0, 0.35, 3.5, and 7.0 mg soot/m^3 was 0.9%, 1.3%, 3.6%, and 12.8%, respectively.

The second study compared effects of diesel and shale. Rats were exposed 7 hr/day, 5 days/wk for up to 30 mo to several atmospheres, including raw Paraho oil shale dust at 5.1 mg/m^3, diesel exhaust at 3.5 mg $soot/m^3$ (identical to the preceeding mid-level), and clean air as controls. DNA was extracted from whole lungs after 24 mo of exposure and analyzed for adducts by the ^{32}P-postlabeling technique.

Both particles accumulated in lungs during the 24 mo exposure, with mean values of 11.9 and 3.9 mg per lung for soot and shale, respectively. Both materials accumulated in macrophages which aggregated and caused focal chronic inflammation, epithelial cell proliferation and fibrosis; thus, both exposures provided opportunity for tumor promotion. These responses were greater in the exhaust-exposed rats.

Whole-lung DNA adduct levels were increased similarly by both exposures. The exhaust, shale-exposed and control rats had mean adduct levels of 12.6, 13.8, and 8.8 per 10^9 bases, respectively. Control rats had a reproducible chromatographic pattern of adducts that was also observed in both exposed groups. The chromatographic patterns of the adducts resulting from the two exposures overlapped, but differed. A distinctive pattern of "abnormal" adducts occurred in both the exhaust and shale-exposed rats. Additional abnormal adducts were found only in shale-exposed rats.

The prevalence of lung tumors was 0.9% among both the shale-exposed and control rats in the second study (identical to that of controls in the first study). Oil shale dust, therefore, was not carcinogenic. Histopathology of the exhaust-exposed rats in the second study is incomplete. However, the reproducibility of carcinogenicity among rats in chronic diesel exhaust studies at several laboratories suggests that the increased incidence observed at the same (3.5 mg/m^3) level in the first study provides a useful interim comparison.

These preliminary results suggest that whole-lung DNA adduct levels are not predictive for carcinogenicity from chronic inhalation exposure to particles having associated organic compounds. Carcinogenicity was correlated to the mutagenicity of extracts and severity of epithelial proliferation. The adducts produced by the exposures have not been identified; it is possible that specific adducts might be predictive for carcinogenicity. Adducts in specific cell types might correlate with carcinogenicity more closely than whole-lung adduct levels. The finding of substantial adduct levels in controls raises important questions about accumulation of DNA damage with age. The overlapping of adduct patterns in the two exposed groups raises questions about the range of materials which might increase adduct levels, and the source of the molecules forming adducts. It is clear that the relationship between lung DNA adducts and tumor formation is complex.

Acknowledgements: Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

REFERENCES

1. Wong, D., Mitchell, C., Wolff, R., Mauderly, J., and Jeffrey, A. Carcinogenesis. 7, 1595-97; 1986.

2. Mauderly, J., Jones, R., Griffith, W., Henderson, R., and McClellan, R. Fund. Appl. Toxicol. (In Press).

SESSION 6

Gene Regulation

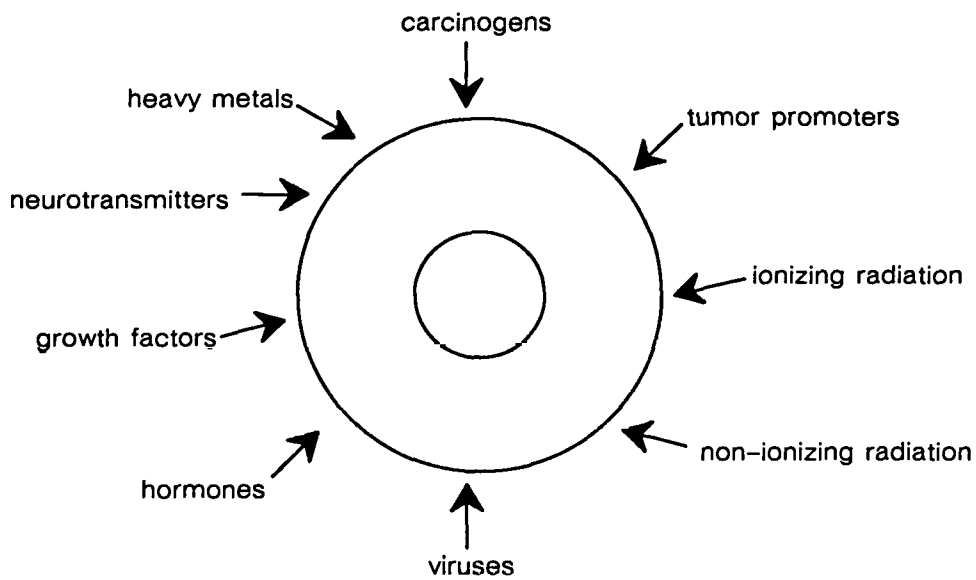
Gene Regulation

Summary of Session 6

H. R. Herschman

University of California, Los Angeles

Transcriptional regulation of gene expression occurs in cells in response to a variety of both endogenous agents and xenobiotic agents, as illustrated below:



Transcriptional responses of the genome to heavy metals, carcinogens, tumor promoters and other xenobiotic agents must utilize either (1) pathways and molecules evolved for responses to normal transcriptional regulatory agents, or (2) unique pathways evolved for cellular protection in the face of a hostile environment. Several speakers at this meeting addressed the mechanisms by which such agents modulate gene expression. Heavy-metal effects on gene expression were described in presen-

tations on the modulation of chromatin structure of the metallothionein gene during cadmium challenge (Andersen *et al.* UCLA; Metals Toxicity, Session 7) and the accumulation of metallothionein during lactation (Bhattacharyya *et al.*, ANL; Session 3). Carcinogen modulation of gene expression was described for the aryl hydrocarbon hydroxylases (Hankinson *et al.*, UCLA; Metabolism and Genotoxicity, Session 4), the H-ras oncogene (Wood, Roche Research Center, Keynote Address), and electrophoretically defined mutations and quantitative inductions (Giometti and Taylor, ANL). Autocrine production of growth factors by tumor cells was also considered in a murine lymphoma model (Hays *et al.*, UCLA). Regulation of gene expression by tumor promoters as agents that (1) modify mitogenic responses (Herschman *et al.*, UCLA), (2) differentiation responses (Huberman, ANL; Biological Mechanisms, Session 8), or (3) induction of P450 metabolic enzymes (Wen and Fulco, UCLA; Metabolism and Genotoxicity, Session 4) was a topic of considerable interest.

The presentations and discussions emphasized the importance of obtaining fundamental knowledge in the areas of mechanisms of regulation of transcription in normal systems as a key prerequisite in understanding how metals and chemicals alter these controls to produce pathologic events. However, it also became clear that the intensive efforts to identify the specific sites of interactions of heavy metals, carcinogens, tumor promoters, and other types of environmental agents have lead to fundamental insights into mechanisms of gene regulation that will have important practical consequences in medicine and epidemiology.

Hays *et al.* (UCLA) described the production by a virally induced murine T-cell lymphoma of a growth factor, termed Lymphoma Derived Growth Factor (LDGF). The factor is produced by high-density cells adapted to grow in serum-free medium. The test system is a lymphoma cell line that does not grow in serum-free medium, but responds to the conditioned medium factor. Preliminary fractionation data suggests a protein fraction in the range of M.W. 31,000. A wide variety of known hematopoietic growth factors were tested for LDGF activity; only IL-1α was found to stimulate the T-cell growth in a fashion analogous to LDGF. LDGF appears to be a specific growth factor produced by a restricted population of cells. LDGF is, however, produced by a variety of T-cell tumors, including a human T-cell line and a murine T-cell line (W7) induced by a chemical carcinogen.

Giometti and Taylor (ANL) used sophisticated two-dimensional gel analyses and computer evaluation to monitor individual mice for quantitative and qualitative changes induced by exposure to chemicals. Four distinct heritable protein variants have been identified in the murine livers of offspring of ENU-treated sires. In a second application of this procedure, Giometti described protein changes observed in livers of animals exposed to the hepatocellular peroxisomal proliferation agents (and carcinogens) Wy-14,643 and DEHP. In particular, the *de novo* synthesis of a new protein by these agents was demonstrated and quantitated.

Tetradecanoyl phorbol acetate (TPA) is among the most potent tumor promoters known. It is also a potent mitogen for some cells, and has profound effects on

expression of differentiation function, on gene amplification, and on cellular communication. Herschman *et al.* (UCLA) described the isolation of TPA non-proliferative variants of 3T3 cells. The variants retained normal TPA-stimulated protein kinase C levels and phosphorylation responses. The major deficit identified in the variants was a TPA-specific inability to induce ornithine decarboxylase. In a second, more molecular, approach a collection of cDNA's was isolated that represents 8 distinct genes that are rapidly and transiently induced by TPA. All the "TIS genes" are also expressed in 3T3 cells exposed to TGF, FGF, or serum. One gene is the c-fos oncogene; the other seven are newly described sequences. The TPA non-proliferative variants, when treated with TPA, express all these messages; their defect(s) must be at a distal point, or on an independent pathway. PC12 pleochromocytoma cells, when treated with Nerve Growth Factor, stop dividing and differentiate. Seven of the eight TIS genes are rapidly and transiently expressed in this differentiation response.

The reader is also referred to papers by Andersen (Session 7), Bhattacharyya (Session 3), Huberman (Session 8), Fulco (Session 4) and Hankinson (Session 4) for additional discussions of the Gene Regulation.

MODULATION OF GENE EXPRESSION BY ONCOGENIC RETROVIRUS.

Esther F. Hays, Shinichi Kitada, and Jeanne Merrill

Laboratory of Biomedical and Environmental Sciences, 900 Veteran Avenue, Los Angeles, CA 90024-1786

The initial transformation event in murine T cell lymphoma is in the thymus. It is believed to result from oncogenic retrovirus interaction with the genome of the thymocyte. However, similar mouse lymphomas are known to occur after exposure to ionizing radiation or chemical carcinogens. Lymphoma cells, regardless of their etiology have the capacity for continuous proliferation in vivo. They spread throughout the body and eventually kill the host. These cells also have property of immortality and can be established as continuous lines in vitro. It is not well understood what alteration of gene expression is responsible for this continuous proliferation resulting in malignant phenotype of these cells. Oncogenes and growth factors related to them have been implicated. Probably multiple factors, both intrinsic and extrinsic, are involved in maintaining the proliferative state of the malignant cell. The present study reports the effects of two peptide factors, leukemia-derived growth factor (LDGF) and interleukin 1 (IL-1) on the growth of murine lymphoma cells in vitro. LDGF is an autocrine growth factor produced by and acting on murine T lymphoma cells of immature phenotype (1). IL-1 is a molecule produced primarily by monocytes on other accessory cells. It is a co-activating molecule to make cells ready for growth (2,3,4).

Growth promotion of lymphoma cell targets by LDGF. The source of LDGF for these experiments was conditioned medium from SL 12.4 murine T lymphoma cells growing continuously for 1 year in serum-free medium (SL 12.4 LT cells) the conditioned medium does not have interleukin 2 (IL-2) activity. The serum-free medium was Iscoves Modified Dulbecco's

medium (IMDM) plus 2 μg/ml transferrin and BSA. Figure 1 summarizes seven experiments in which the CM was harvested from exponentially growing cells and tested on SL 12.4 targets which were cells in first passage in serum-free medium from medium containing 10% fetal bovine serum (FBS). They were seeded at 50 cells per well in 96-well plates, in 200 μg total volume. Figure 1 shows a consistent marked growth stimulation in a dose responsive manner when cells were studied 6 days after addition of CM.

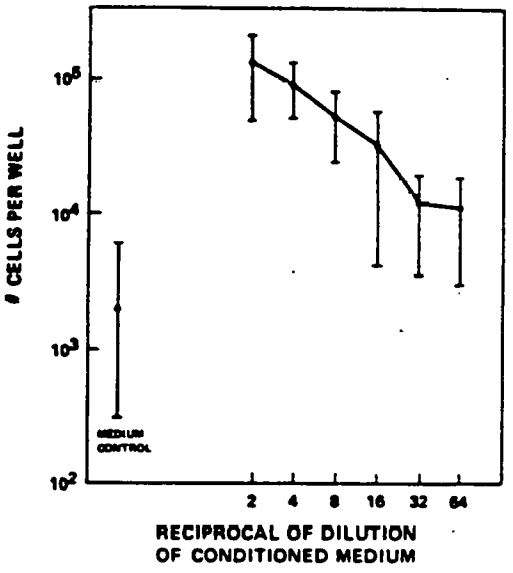


Figure 1. The proliferation of 50 SL 12.4 cells after 6 days incubation with serial dilution of SL 12.4 LT conditioned medium containing LDGF. The error bars show the 95% confidence level for mean of seven individual experiments.

In a second series of experiments, the kinetics of growth were studied comparing 10% FBS, SL 12.4 LT conditioned medium from exponentially growing cells at 1:4 dilution and medium controls. 10% FBS stimulated growth to maximum which was 10-fold higher than growth achieved by the addition of CM. Control cultures showed proliferation at a much more reduced rate. This is in contrast to a similar kinetic study using SL 12.4 LT cells. These cells proliferated in a similar manner in the presence of serum, CM or medium alone reaching maximum at 6 days. Thus, we have shown that the murine T lymphoma cells secrete a factor into the surrounding medium which stimulates their growth and that they adapt to serum-free conditions by a constitutitive production of this factor.

Evaluation of growth-stimulatory properties of purified growth factors using SL 12.4 lymphoma cell targets in serum-free medium. These experiments were initiated to determine if LDGF activity might be due to expression of a known hemopoietic growth factor. To do this, several purified hemopoietic growth factors at varying dilutions on SL 12.4 targets. The positive control in all experiments was FBS which consistently stimulated the cells to confluent growth in 6 days. The following factors produced no growth stimulation over control: human GM-CSF; murine GM-CSF; murine G-CSF; murine CSF 1; murine IL-3; IL-4; and IL5; human IL-2, human TNF and human IL-1β. Purified recombinant human IL-1α was found to stimulate lymphoma cell growth.

Because of this observation, we tested supernatants from a cloned glial cell line established from a human brain tumor (5) which produces IL-1α. This molecule stimulates growth of the glial cells. Interestingly, supernatants harvested from glial cells in serum-free media consistently stimulated the growth of first passage SL 12.4 cells. At a 1:10 dilution of glial CM, SL 12.4 cells were stimulated to 190,000 viable cells in 6 days. SL 12.4 LT CM was then tested for IL-1 activity. No IL-1 activity was found suggesting that LDGF is not IL-1.

Test for molecular weight of growth stimulatory activities from SL 12.4 LT and human glial cell line conditioned media. CM from both of these sources was concentrated by lyophylization and then analyzed by gel permeation chromatography using an FPLC unit (Superose 12 column). Growth promoting activity from lymphoma cell CM was found at a sharp peak at approximately 31 Kd. IL-1 activity was not detected in any fraction. Chromatography of conditioned medium from the human glial cell line revealed a peak of growth promoting activity for both glial cells and lymphoma cells at approximately 33 Kd. IL-1 activity co-eluted with

this peak. These results show that the IL-1 produced by and stimulating the glial line was also a growth stimulating material for the lymphoma cells. The finding of IL-1 growth promoting activity suggests that the lymphoma cells have constitutive expression of IL-1 receptors which may be another example of altered gene expression by the retrovirus and respond to the molecule by inducing cell proliferation. Thus, the cells can be driven to proliferation by at least two mechanisms: LDGF resulting from an altered expression of a putative LDGF gene by an autocrine manner and by IL-1 produced by accessory cells in the thymus and acting on thymocytes as in paracrine factor. Since IL-1 is a growth facilitating molecule, its activity on the lymphoma cells may be making the cells produce more or be more responsive to LDGF.

REFERENCES

1. Hays, E. F., D. Goodrum, M. Bessho, S. Kitada, and C. H. Uittenbogaart. Proc. Natl. Acad. Sci. 81, 7807-7811, 1984.
2. Mizel, S. B. Immunol. Rev. 63, 51, 1982.
3. Oppenheim, J. J., E. J. Kovacs, K. Matsushima and S. K. Durum. Immunol. Today 7, 45-46, 1986.
4. Mosley, B., et al. J. Biol. Chem. 262, 2941-2944, 1987.
5. Manuelidis, L., R. K. Yu, E. E. Manuelidis. Acta Neuropathologica 38, 129-135, 1977.

MONITORING THE EFFECTS OF TOXIC CHEMICALS ON PROTEIN EXPRESSION

Carol S. Giometti and John Taylor.

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439

We are using two-dimensional gel electrophoresis (2DE) [1], coupled with computer-assisted image and data analysis [2,3], to monitor protein populations for both qualitative and quantitative changes induced by exposure to chemicals. For mutagenesis studies designed to screen for heritable mutations, we use a computer-assisted search of the optical density data from 2DE patterns to look for (a) new protein spots, (b) missing protein spots and/or (c) altered expression of normal protein spots. Using this approach, we have screened 320 mice for mutations induced by treatment of sires with 150 mg/kg body weight of ethylnitrosourea (ENU) and have identified four different mutations (Fig. 1). As shown in Table 1, each of the four mutations resulted in the appearance of a new protein with an approximately 50% decrease in the intensity of a normal neighboring protein spot. The heritability of the protein variants has been demonstrated by test-crosses, and stocks of homozygous carriers are being maintained for further analysis of the mutations. Protein patterns from 105 offspring from untreated male mice (controls) and 369 offspring from irradiated male mice (3 Gy gamma) were also screened. No heritable mutations were found in those data sets, however. These results demonstrate that chemically-induced heritable mutations can be detected as alterations in protein expression by using 2DE.

The 2DE system also has potential for monitoring altered protein expression in exposed individuals [4]. The data requirements for assessing multiple quantitative and qualitative protein changes in sets of exposed individuals, however, are far different (and less stringent) than those for

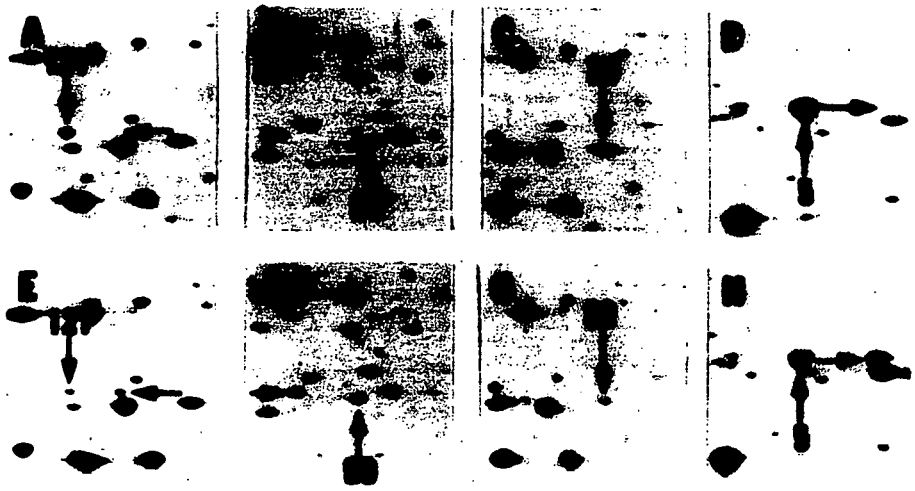


Figure 1. Two-dimensional electrophoresis patterns of mouse liver proteins. A-D, control patterns showing the location of normal proteins (identification numbers and vertical arrows); E-H, patterns showing appearance of ENU-induced protein variants, ENU1, 2, 4, and 8, respectively (horizontal arrows).

Table 1. Integrated density values of ENU-induced protein variants and their normal protein counterparts

Normal Protein	Variant Protein	Normal Protein Integrated Density[a] in Absence of Variant	Normal Protein Integrated Density[b] in Presence of Variant
Spot 5	ENU8	26448	16698
Spot 39	ENU4	5864	3556
Spot 99	ENU2	6681	3100
Spot 121	ENU1	2515	1308

[a]The integrated spot densities from 2D Gaussian models of actual data averaged from all of the patterns in the data set (159 female or 164 male offspring from ENU-treated sires.

[b]The integrated spot densities from the models of data from individual patterns showing each protein variant.

mutation screening. Studies to detect heritable mutations require a search for rare changes, usually in a single protein, and therefore necessitate extremely precise measurements on a large number of samples. In contrast, studies to detect effects in exposed individuals require a method for identification of significant protein differences between groups of samples. Such studies require smaller sample sizes than required for mutation studies, for example 20-30 individuals per treatment group compared to the hundreds needed for rare-event screening.

We are currently designing analysis strategies to separate nonspecific from specific effects of chemical exposures, and to permit correlation of alterations in protein expression with known biological changes. These strategies are being tested with data from 2DE patterns of liver proteins from mice fed normal chow or chow containing either clofibrate, ciprofibrate, [4-cloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643), or di-(2-ethylhexyl)-phthalate (DEHP). All of these chemicals are known to cause hepatomegaly, peroxisome proliferation, and, upon prolonged administration, hepatocellular carcinoma [5]. Figure 2 shows a comparison of one section from representative 2DE patterns of liver from each of the treatment groups, indicating one of the most significant of the more than 50 protein changes found in this experiment. The protein indicated was not expressed by control animals, but was expressed to varying extents by animals in each of the treatment groups. Our preliminary results indicate that specific and nonspecific effects can be identified in studies of this type and thus suggest that 2DE can be used to monitor exposed individuals for significant alterations in protein expression.

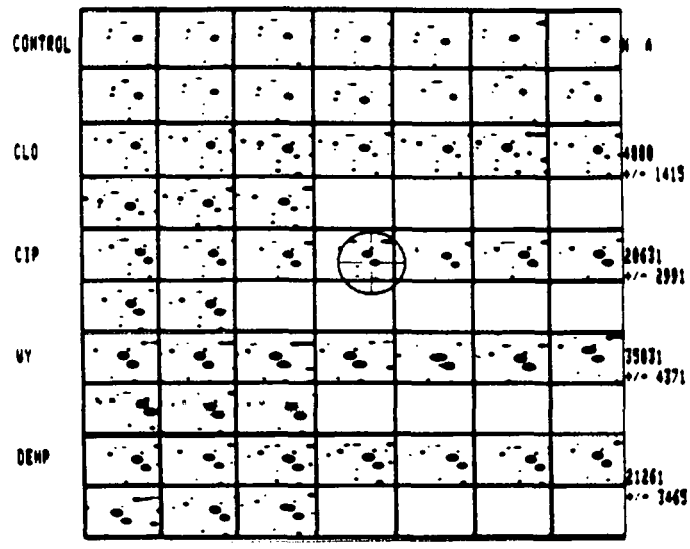


Figure 2. Identical region of spot models from 2DE patterns of different mouse liver samples. Rows 1 and 2, controls; 3 and 4, mice treated with 0.25% clofibrate, 5 and 6, mice treated with 0.025% ciprofibrate; 7 and 8, mice treated with 0.1% WY-14643; 9 and 10, mice treated with 2.0% DEHP. Spot indicated by the cursor (circle in center of figure) is induced to varying degrees by each of the treatments, but is not visible in control patterns.

Acknowledgments: This research was supported by the U. S. Department of Energy, Office of Health and Environmental Research, under contract No. W-31-109-ENG-38.

REFERENCES

1. O'Farrell, P. H. J. Biol. Chem. 250, 4007-4021; 1975.

2. Taylor, J., Anderson, N. L., Anderson, N. G., Gemmell, A., Giometti, C. S., Nance, S. L., and Tollaksen, S. L. in Electrophoresis '86, VCH Verlagsgesellschaft, Weinheim, pp. 583-587; 1986.

3. Giometti, C. S., Gemmell, M. A., Nance, S. L., Tollaksen, S. L., and Taylor, J. J. Biol. Chem. 1987 (in press).

4. Anderson, N. L., Giere, F. G., Nance, S. L., Gemmell, M. A., Tollaksen, S. L., and Anderson, N. G. Fund. Appl. Toxicol. 8, 39-50; 1987.

5. Reddy, J. K. and Lalwani, N. D. CRC Crit. Rev. Toxicol. 12, 1-58; 1983.

MODULATION OF GENE EXPRESSION BY TUMOR PROMOTERS: RAPID, TRANSIENT INDUCTION BY TETRADECANOYL PHORBOL ACETATE OF A GROUP OF GENES THAT INCLUDES THE C-FOS ONCOGENE. **H.R. Herschman, R. Lim, B. Varnum, D. Kujubu. Laboratory of Biomedical and Environmental Sciences, UCLA, Los Angeles, California.**

Our laboratory has been interested in both normal and abnormal regulation of cellular proliferation, as reflected in the mode of action of mitogens and in the process of carcinogenesis. Tetradecanoyl phorbol acetate (TPA) is both a potent mitogen for quiescent murine 3T3 cells and the most active tumor promoter known. We have previously used a selective procedure to isolate TPA nonproliferative variants of 3T3 cells (1). These variants have normal levels of protein kinase C (the molecular target of TPA-induced cellular activation) and phosphorylate appropriate substrates (2), despite their inability to divide when challenged with TPA. They do not, however, show the characteristic transient elevation in ornithine decarboxylase (ODC) activity associated eith TPA-induced proliferation, although serum -- a competent mitogen -- does induces this activity (3). TPA-induced transcription of quiescent genes is not universally turned off in these TPA nonproliferative cells; TPA can induced MEP (major excreted protein) mRNA in this variant as well as it can in parental cells (4). To approach the molecular basis for the action of this potent modulator of biological responses, we have now cloned a family of "primary response" genes (i.e. genes whose induction is independent of concurrent protein synthesis) whose expression is induced by TPA (5).

Quiescent, non-dividing Swiss 3T3 cells were treated with TPA plus cycloheximide (CHX). After 3 hours cells were harvested, poly A^+ RNA was isolated, and a cDNA library was prepared in a λgt10 cloning vector. Fifty thousand clones were screened by comparing hybridization of cDNA made either from poly A^+ RNA isolated from TPA+CHX treated cells, or cDNA prepared from cells treated with CHX alone. Fifty clones from this library were further

characterized by cross-hybridization and mRNA sizing; we find cDNAs identifying seven distinct mRNAs. The seven TPA induced sequences, which we refer to as TIS genes, all show the following characteristics: (i) they are rapidly induced by TPA; (ii) they are primary response genes, (iii) in the absence of CHX they are present only transiently, peaking at 30-90 min, and returning to baseline by three to four hours, (iv) they are superinduced in the presence of CHX, and (v) they are induced by other mitogens, such as epidermal growth factor and fibroblast growth factor.

The five characteristics discussed above are shared by the cellular fos oncogene. Hybridization studies demonstrated that TIS28 is, indeed, the c-fos gene. C-fos apparently is one representative of a class of rapidly and transiently induced genes; we have now identified six additional members of the family. Expression of the c-fos oncogene is also induced in response to the activation of differentiation pathways as diverse as neuronal and lymphocyte maturation. We find that specific subsets of the TIS genes are also induced as a consequence of nerve growth factor induced neuronal differentiation and the induced maturation of B-lymphocytes (6,7).

References

1. Butler-Gralla, E. and Herschman, H.R. (1981) Variants of 3T3 cells lacking mitogenic response to the tumor promoter tetradecanoyl-phorbol-acetate. J. Cell. Physiol. 107:59-68.
2. Bishop, R., Martinez, R., Weber, M.J., Blackshear, P.J., Beatty, S., Lim, R., and Herschman, H.R. (1985) Protein phosphorylation in a TPA non-proliferative variant of 3T3 cells. Mol. Cell. Biol. 5:2231-2237.
3. Butler-Gralla, E. and Herschman, H.R. (1983) Glucose uptake and ornithine decarboxylase activity in tetradecanoyl phorbol acetate nonproliferative variants. J. Cell. Physiol. 114:317-320.
4. Herschman, H.R. (In Press) Mitogen-specific nonresponsive mutants and the initiation of cellular proliferation. In BioEssays.
5. Lim, R.W., Varnum, B.C., and Herschman, H.R. (In Press) Cloning of tetradecanoyl phorbol ester induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. Oncogene.
6. Kujubu, D.A., Lim, R.W., Varnum, B.C., and Herschman, H.R. (In Press) Induction of transiently expressed genes in PC-12 pheochromocytoma cells.

Oncogene.
7. Law, R., Hermanson, G., Wall, R., Lim, R.W., Varnum, B.C., and Herschman, H.R. (In Preparation).

SESSION 7

Metal Toxicity

Metals Toxicity

Summary of Session 7

M. Vanderlaan

Lawrence Livermore National Laboratory

This session dealt with diverse aspects of the toxicology of metals; these papers illustrated a few of the many biological effects of metals. Progress in this area requires both development of appropriate animal models for observed human diseases and an understanding of the endogenous defense systems that have evolved to cope with metal exposure.

Dr. Bhattacharyya *et al.* (ANL) reported on a developmental model in the mouse for the effects of cadmium (Cd) on bones in postmenopausal women. This appears to be a good model for exploring the role of Cd in human osteoporosis, a disease that affects 15 to 20 million Americans. In this study, the combined effects of Cd and ovariectomy were synergistic for inducing weight loss in bones. In addition, the combination of dietary Cd and multiple pregnancies was shown to depress bone calcification. In an *in vitro* incubation of the bone with Cd, Cd was shown to cause bone resorption directly. Particularly significant were findings of adverse affects of cadmium at very low levels, 5 ppm in the feed and 10 nM in the *in vitro* test. Dr. Bhattacharyya emphasized that systemic effects, such as those that might be mediated by kidney failure, were not needed to explain the effects of Cd on bones. In a related postor by

the same group, metallothionein, a Cd binding protein, was shown to be elevated during lactation in mice.

The metallothionein gene, and its induction by heavy metals, has provided molecular geneticists with a unique example for studying the regulation of inducible genes. Anderson *et al.* (UCLA) reported on the use of "*in vivo* footprinting" to identify changes in chromatin structure in the metallothionein (MT-1) gene promoter in response to Cd in intact rat hepatoma cells. This method allows one to study the interactions between DNA promoter sequences and regulatory proteins in intact cells. "Genomic footprinting" allows one to infer the exact DNA sequence bound by a protein by the ability of that protein to shield G residues in the DNA from methylation by dimethyl sulfate. This method could have wide application in studies of gene regulation, cellular differentiation, and enzyme induction by xenobiotic agents.

In this study, the difference in methylation pattern between unexposed cells and cells exposed to Cd provide strong suggestive evidence for metal-dependent binding of transcription regulating factor(s) to the regulatory elements of the MT-1 promoter. In addition, in both untreated and Cd-treated cells, a combination of methylation protection and enhancement was also seen over a "GC box" region of the MT-1 promoter in a pattern identical to that observed in experiments *in vitro* with SP1 transcription factor binding. The role of the "GC box" in constitutive MT-1 transcription and the co-operativity of metal regulatory sequences in the MT-1 promoter were

confirmed by analysis of expression of transfected fusion gene constructs of MT-1 promoter deletions and the chloramphenicol acetyl transferase (CAT) gene.

Haley *et al.* (ITRI) reported on a dog model for the induction of granulomatous lung disease (GLD) by inhaled beryllium. The prevalence of GLD has been greatly reduced in recent years by effective industrial hygiene programs that reduce occupational exposures, although some accidental exposures do occur. GLD is characterized by focal histiocytic infiltrations of the lung and an immunological hypersensitivity to beryllium in both humans and dogs. In the exposed beagles, the lesions and immunological sensitization was heterogeneous, but did follow a pattern related to dose and solubility of the beryllium particles.

CADMIUM AND POSTMENOPAUSAL BONE LOSS

Maryka H. Bhattacharyya°, Bartlett D. Whelton+, Paula H. Stern*, David P. Peterson°, Elizabeth S. Moretti°, and Heidi A. Dare°

°Biological and Medical Research Division, Argonne National Laboratory, Argonne, IL 60439-4833

+Chemistry Department, Eastern Washington University, Cheney, WA 99004

*Pharmacology Department, Northwestern University Medical and Dental School, Chicago, IL 60611

Osteoporosis, which affects 15-20 million people in the United States[1], is a major cause of bone fractures in older persons, particularly postmenopausal women. Bone loss has occurred among postmenopausal women in Toyama prefecture, Japan, in the form of Itai-Itai (Ouch-Ouch) disease, which is characterized by severe osteoporosis/osteomalacia and renal tubular dysfunction[2-5]. Both diet and drinking water of women with Itai-Itai disease contain elevated levels of cadmium (Cd), the result of using Jintsu River water containing effluent from an upstream zinc-lead mine for rice paddy irrigation and direct consumption. On the basis of epidemiological data and the finding of high Cd levels in tissues taken at autopsy from women with Itai-Itai disease, the Japanese Ministry of Health in May, 1968, declared Cd to be one of the causative factors in the etiology of the disease[4,6].

Because more than 95% of cases of Itai-Itai disease have occurred in postmenopausal women, we studied the effect of dietary Cd on loss of bone mineral in mice after ovariectomy, which simulates conditions of human postmenopausal hormone depletion. We exposed female CF1 mice to a purified diet containing $CdCl_2$ at either 0.25, 5.0, or 50 ppm Cd. Cadmium at 0.25 ppm is present in most stock rations; mice exposed to this concentration constituted our environmental exposure group. Cadmium at 5 ppm was chosen to provide an exposure equivalent to that of the Japanese women suffering from Itai-Itai disease. Cadmium at 50 ppm was chosen as a 10-fold higher level to allow for potential differences in species sensitivity between mice and humans. We introduced the mice to their experimental diets starting at 70 days of age. After 12 months of exposure, mice were ovariectomized (OV) or sham-operated (SO). After surgery, they remained on their respective diets for an additional six months before sacrifice.

Neither ovariectomy alone nor dietary Cd exposure alone caused statistically significant decreases in the mean calcium contents and calcium/dry weight ratios of the femurs and lumbar vertebrae (Fig. 1A-1D; compare OV/0.25 vs. SO/0.25 bars for effects of OV alone; compare SO/50 vs. SO/0.25 bars for effects of Cd alone). Exposure to 50 ppm dietary Cd,

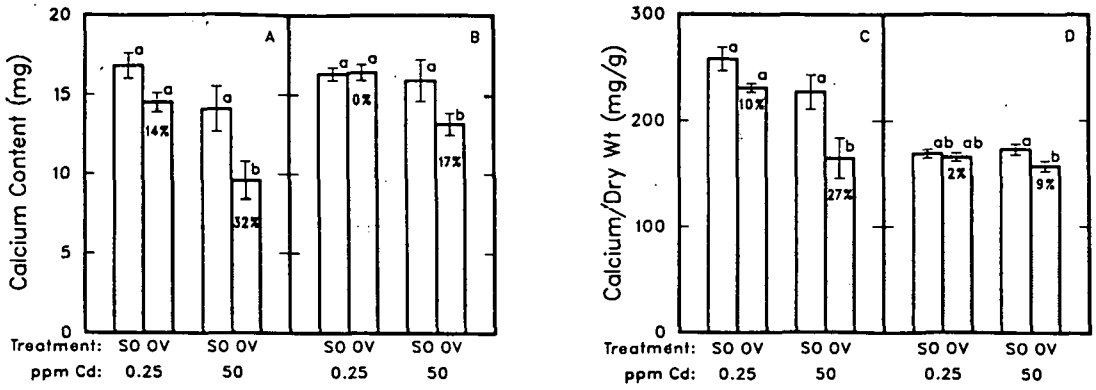


Fig. 1. Bone values from femurs (A & C) and lumbar vertebrae (B & D) from ovariectomized (OV) and sham-operated (SO) mice exposed to cadmium. Bars show means ± SE (n = 6-7). Percentages on shaded bars show % decrease for OV vs SO mice at a given Cd exposure level. Bars with same letters are not significantly different ($p < .05$) by ANOVA and least significant difference (LSD) t test.

however, caused a significant increase in the loss of bone calcium after ovariectomy such that the calcium contents and calcium-to-dry weight ratios of both femurs and lumbar vertebrae of the OV mice at 50 ppm dietary Cd were strikingly lower than those of all other groups (Fig. 2A-2D; OV/50, shaded bars). The femurs of OV mice receiving 50 ppm dietary Cd, for example, contained a mean of only 9.6 mg calcium, while femurs from the other groups contained 14-17 mg calcium (Fig. 1A). In addition, microradiographs of the cross sections of thoracic vertebrae showed that the calcium contents of the bones from OV mice exposed to 50 ppm dietary Cd were clearly lower than those of the other groups.

From reports of Cd levels in the blood of animals exposed to Cd[2], we estimated the Cd concentration in the serum of mice exposed to 50 ppm dietary Cd to be approximately 10 nM. To determine whether 10 nM Cd could directly increase bone resorption in organ culture, we exposed fetal rat limb bones (FRLB) prelabeled with ^{45}Ca to Cd in the presence and absence of parathyroid hormone (PTH). Cadmium at 10 nM produced a 68±6% (n=4) release of ^{45}Ca from

the FRLBs, similar to that elicited by 1 nM PTH (56±7%, n=4). Control bones released only 27±2% of their ^{45}Ca. Bone resorption in the presence of 10 nM Cd + 1 nM PTH was no greater than that in the presence of 10 nM Cd alone, perhaps because the rate of bone resorption was already maximal with 10 nM Cd. In contrast, Cd at a 100-fold higher concentration (1 μM Cd) had no significant effect alone and inhibited PTH-induced bone resorption (^{45}Ca release: Control = 28±4%; Cd = 34±3%; PTH = 73±5%; PTH + Cd = 46±4%).

It is clear that some women after the menopause lose mineral from their skeletons much faster than others[7]. We have identified an agent, Cd, that enhances the loss of bone mineral after ovariectomy in mice, and, at physiological concentrations *in vitro*, enhances bone resorption in organ culture. The *in vivo* results provide an animal model for research into at least one mechanism of increased postmenopausal bone loss. The *in vitro* results indicate that Cd may accelerate bone loss by a direct action on bone. Because cigarette smoking is a significant source of exposure to Cd[5], Cd-induced loss of bone mineral may contribute, along with smoking-induced decreases in serum estrogen levels[8], to the increased incidence of postmenopausal osteoporosis in women who are heavy smokers[1].

Acknowledgments: This research was supported by the Department of Energy and the National Institutes of Health.

REFERENCES

1. National Institutes of Health Consensus Development Conference Statement. *Osteoporosis*, 5 (3); 1984.
2. Friberg, L. T., Piscator, M., Nordberg, G. F., and Kjellström, T., *Cadmium in the Environment, 2nd Ed.*, C.R.C. Press, Cleveland, OH, 1974.
3. Lauwerys, R., in *The Chemistry, Biochemistry and Biology of Cadmium*, M. Webb, Ed., Elsevier/North Holland Biomedical Press, New York, NY, 1979.
4. Nogawa, K., in *Cadmium in the Environment*, J. O. Nriagu, Ed., John Wiley and Sons, New York, NY, 1981.
5. Friberg, L., C. Elinder, T. Kjellström, G. F. Nordberg, *Cadmium and Health: A Toxicological and Epidemiological Appraisal.* Volumes I and II. CRC Press, Inc., Boca Raton, FL, 1986.
6. Nomiyama, K., Science Total Environ. 14, 199-232; 1980.
7. Abbasi, R., and Hodgen, G. D., JAMA 255, 1600-1604; 1986.
8. Jensen, J., Christiansen, C., and Rodbro, P., N. Engl. J. Med. 313, 973-975; 1985.

MODULATION OF GENE EXPRESSION BY HEAVY METALS: ALTERATIONS OF THE STRUCTURE OF THE RAT METALLOTHIONEIN GENE THAT ACCOMPANY TRANSCRIPTION INDUCED BY CADMIUM.
R.D. Andersen, S. Taplitz, and H.R. Herschman. Laboratory of Biomedical and Environmental Sciences, UCLA, Los Angeles, California.

Metallothionein (MT) genes specify the synthesis of low molecular weight metal-binding proteins. The metallothionein genes are transcriptionally regulated by the metal ions cadmium and zinc. The metal ion regulation of metallothioneins is conferred by a short sequence element called the metal responsive element (MRE) or TGC box, which functions as a metal ion dependent enhancer. Deletion mutant analysis of the metallothionein promoter and introduction of synthetic DNA MREs into the promoters of other genes indicate that the presence of repeated copies of the MRE confers metal ion regulation of transcription. We have used the methodology of "genomic sequencing" (also termed "in vivo footprinting"), using the chemical reagent dimethyl sulfate (DMS) on intact cells, to observe changes in reactivity of the N-7 position of guanine residues in the promoter of the rat MT-1 gene in vivo following treatment of cells with cadmium ion. Transfection experiments with portions of the MT-1 promoter fused to a reporter gene were used to correlate the functional role of these sequences with the observed changes in their DMS reactivity.

We can demonstrate cadmium-inducible protection from DMS modification of guanine residues in vivo in five MREs in the promoter of the rat MT-1 gene. We also identify a site of extreme DMS hyperreactivity which, like the MRE protections, occurs only after metal-ion induction. With this hyperreactive site as an indicator, we find the kinetics of induction and deinduction to be rapid, indicating that changes in intracellular metal ion concentration are quickly reflected in alterations in the reactivity with DMS of guanine residues in the MT-1 gene promoter. Lastly, we observe DMS protections and enhancements, in both control and metal-induced cells, of a binding site (located 5' of the

distal MRE) which is a consensus sequence for the Sp1 transcription factor. Transfection experiments with fusion gene constructs indicate both that a sequence region which includes this GC box regulates the basal level of expression of the MT-1 gene, and that increasing the number of MRE elements in the promoter increases the induced level of transcription. Our genomic footprinting and transfection data together suggest that (i) a transcription factor, possibly Sp1, plays an important role in regulating the basal level of expression of the MT-1 gene, and (ii) cadmium induction involves the metal-dependent binding of a sequence-specific binding factor which responds rapidly to changes in intracellular metal ion levels.

We have also shown that nuclei from cells treated with cadmium retain their altered chromatin conformation. The enhancement of methylation at residue -110 bp is present if isolated nuclei from Cd-treated cells are subsequently subjected to the genomic sequencing procedure. Using isolated nuclei we can study the interactions of specific metal regulating transcription factors with the endogenous MT promoter in living experimental organisms, by first exposing them to metal, then isolating nuclei prior to genomic sequencing.

We have now been able to identify, in nuclear extracts, a factor that binds to the MT promoter region in a metal dependent manner and leads to retardation of the promoter-protein complex in a gel electrophoresis assay. This assay should permit us to isolate the protein factor(s) that bind to the MT promoter as a result of metal challenge and regulate MT expression.

GRANULOMATOUS LUNG DISEASE FROM INHALED BERYLLIUM

P. J. Haley, F. F. Hahn, G. L. Finch, and J. A. Mewhinney

Lovelace Inhalation Toxicology Research Institute, Albuquerque, NM 87185

Inhalation of beryllium compounds may result in the development of an unrelentlingly progressive granulomatous lung disease called chronic beryllium disease which has a number of different histological characteristics that include interstitial mononuclear cell infiltration, granuloma formation, and calicific inclusion formation. Each of these parameters may be present in varying degrees or entirely absent.

Despite the decline of documented cases of chronic beryllium disease there is still great concern for the potential occurrence of this disease because of the increasing uses of beryllium compounds in the space and nuclear industries. In addition, the unique pathogenic mechanisms involved in the induction of this disease may serve as a model of immunologically mediated chronic pulmonary disorders. Furthermore, the progression of chronic berylliosis is insidious and accurate diagnosis difficult. Confident separation of chronic berylliosis from other granulomatous lung diseases remains a continuing problem for clinicians.

Despite numerous attempts, induction of a disease in laboratory animals that accurately mimics the syndrome in man both histologically and immunologically has not been achieved. Only rats, dogs and monkeys have been exposed by inhalation to beryllium however these studies have demonstrated beryllium-induced granulomatous lung lesions without characterization of immunologic manifestations of the disease. From studies of humans, it appears that localized lung lymphocyte reactivity to beryllium may be the pivotal event in the development of berylliosis and that sampling

of lung lymphocytes is necessary for diagnosis as well as experimental manipulation.

In the present study, we exposed Beagle dogs to low and high concentrations of beryllium oxide calcined at two different temperatures (500°C, 1000°C). Dogs were then divided into two groups, one of which was sacrificed at intervals for characterization of lung histologic lesions, while the other group was maintained for sequential bronchoalveolar lavage and assessment of lung lymphocte hypersensitivity to beryllium. This report deals only with the histologic lesions observed during the first year after exposure.

Lesions in these dogs were characterized by mononuclear inflammatory cell infiltrates of lymphocytes and macrophages within the interstitium around small blood vessels and terminal bronchioles, increasing progressively in intensity starting at eight days after exposure and reaching a peak response at 64 days. Lymphocytes were small and differentiated in early lesions but were larger and lymphoblastic at later times. These cells aggregated with large epithelioid macrophages to form lymphoid nodules and microgranulomas within the parenchyma. Microgranulomas consisted of large vacuolated and epithelioid macrophages surrounded by lymphocytes. Some cells had mitotic figures. Alveolar macrophages were frequently large, vacuolated and filled with abundant yellow-brown flocculant to granular material and were found in locally intense intra-alveolar aggregates as well as within the interstitium, resulting in marked thickening of the alveolar septa. Multinucleated giant cells were present infrequently. Areas of the most intense interstitial inflammation were accompanied by moderate interstitial fibrosis and moderate to marked epithelial hyperplasia.

Tracheobronchial lymph nodes had moderate to marked cortical and paracortical hyperplasia with distortion of the normal nodal architecture. Numerous large, well developed germinal centers were frequently present deep in the paracortex. Sinus histiocytosis was also prominent, with most of the macrophages being enlarged and filled with a material similar to that seen in alveolar macrophages. At 365 days, some nodes still displayed some degree of hyperplasia while others were characterized by moderate lymphoid depletion, marked congestion, and medullary fibrosis.

These results indicate that inhalation of beryllium aerosols results in granulomatous lung disease; however, the histologic lesions described above did not follow a distinct pattern attributable to dose or calcination temperature of the compound. There was a great deal of individual variation in the severity of lesions among exposed dogs, a phenomenon also observed in man. The observation that lung lesions appear to wane with time suggest that (a) the dogs sampled at the later times were not susceptible, (b) insufficient beryllium was administered in the single exposure to maintain the disease process or (c) some of the lesions resolved. Analysis of the burdens of beryllium indicated that most of the 500°C beryllium was cleared by 180 days after exposure. It was this more soluble form that produced the most consistent lesions. These data suggest that the dog provides a unique model to investigate the pathogenesis of chronic berylliosis, identify procedures for identifying susceptible individuals prior to exposure, and provide insight into other immunologically mediated chronic lung diseases.

Acknowledgements: Research supported by the U. S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

SESSION 8

Biological Mechanisms

Biological Mechanisms

Summary of Session 8

M. Vanderlaan

Lawrence Livermore National Laboratory

Two papers in this session dealt with reproductive effects of exposure to xenobiotic chemicals. Chemicals and radiation can influence embryogenesis either by mutations in parental gametes or by interfering directly with implantation and maturation. Both of these papers presented new animal models for evaluating reproductive consequences of exposures.

Dr. Goldstein *et al.* (UCSF) reported on the reproductive consequences of previous parental exposure to mutagenic chemotherapeutic agents. This work included mouse models for effects on both male and female germ cells, although the effects on sperm are likely to be most readily extrapolated to man. Goldstein stressed the potential of this system for exploring the possible role of genetic counseling for the survivors of childhood cancers who have been treated with mutagenic compounds. Mechlorethanime clearly lead to dominant lethal mutations, as did X-ray treatment. Multiple exposures were no more effective than single exposures.

Generoso (ORNL) reported on a model for detecting the effects of chemical exposure to mouse zygotes during the first 24 hours post-fertilization. His studies showed a remarkably high rate (up to 30%) of malformations occurring late in gesta-

tion following single exposures to either ethylmethane sulfonate or ethylene oxide. These effects were striking in that the timing of the exposure was a critical factor, and had to occur immediately following fertilization, prior to the first round of DNA synthesis. These birth defects were not associated with chromosomal abnormalities. The mechanism for these effects is not clear, and he speculated that they may be the result of the induced re-arrangement of endogenous transposable elements.

Two papers reported on *in vitro* models for studying the regulation of cell transformation and differentiation. *In vitro* systems allow for the precise control of exposure conditions, the selection of mutants with specific properties, and assay of multiple endpoints. Differentiation and carcinogenesis are linked, with cancer cells often presenting as normal cells that have been blocked in their normal differentiation.

Dr. McCormick (Michigan State University) reported on work by his group on the transformation of human fibroblasts. Prior work on transformation of human cells has been hampered by the fact that the transformed phenotype is not known, and that normal human fibroblasts have a finite lifetime in culture, so that slow tumor cell progression may not have time to occur. The major research advances reported at this meeting were (1) the demonstration of autocrine stimulation of growth in human fibrosarcoma cells, which both secrete and have a receptor for several growth factors, and (2) selection of an immortal human fibroblast cell line by transfection with a v-myc oncogene. The immortal cell line could be further transformed by other oncogenes to

fully malignant cells. Using these cell lines, Dr. McCormick expects to be able to define stages in the progression of cells from normal to cancerous phenotypes.

Two important methods were developed in the course of this work. First, a serum-free medium was developed that supported human fibroblast proliferation. Normal fibroblasts required either 1 mM Ca^{++} or 0.1 mM Ca^{++} and growth factors (e.g., EGF or PDGF). Second, a method for transforming human cells by the transfection of oncogene DNA was developed. Human cells do not readily take up calcium phosphate precipitated DNA as do NIH 3T3 cells, and require treatment with DMSO and polybrene. Both of these methods may have important consequences for other studies on human cell primary cultures and the insertion of genes into human cell lines.

Dr. Huberman (ANL) reported on studies in the differentiation of the human leukemia cell line HL-60. This cell line may be induced to differentiate by the tumor promoter TPA. Variant cell lines, without the ability to be stimulated by TPA, were selected and compared to the parent line in an effort to determine the critical pathway for TPA-induced differentiation. TPA binding to its receptor, a protein kinase C, was not modified, but the subcellular location of the receptor was. Dr. Huberman speculates that the kinase no longer phosphorylates regulatory proteins in the nucleus.

Finally, Dr. T'so (Johns Hopkins University) presented several speculative ideas about how future studies might link human tumors and animal models for carcinogenesis. These include the study of the same carcinogen in both species, such as

ultraviolet light, and the use of non-invasive monitoring techniques as is now possible by new developments in nuclear magnetic resonance.

Certain toxicological problems have an explicit link with energy production and usage. Electrical generation from burning coal, oil, diesel fuels, and/or municipal wastes are major sources of atmospheric pollution and toxic chemicals. Whatever changes in energy production occur in the next several decades, we will continue to depend heavily on burning fossil fuels and will therefore have a need to understand the toxicology of their pollutants. Energy-related pollutants include heavy metals, aromatic hydrocarbons, chlorinated hydrocarbons, and aromatic amines.

Toxicology is a diverse field, as illustrated by these sessions. The exposure to metals and organic chemicals can result in mutations, alterations in gene expression, carcinogenesis, and reproductive failure. All of these effects require development of *in vitro* and animal models where the biological mechanisms can be established and the likely consequences to human health explored.

DOMINANT LETHALITY IN EMBRYOS FROM PARENTS TREATED WITH CHEMOTHERAPEUTIC DRUGS

Lawrence S. Goldstein, G.Y. Ross, J.F. Florence and J. Charron-Vennari.

Department of Radiation Oncology, University of California, San Francisco CA.

There are few clinical data relating to the long-term heritable genetic consequences of successful cancer chemotherapy. In the young patient cured of his disease this question may significantly impact the quality of life and an understanding of the mutagenic hazards due to combinations of drugs would be useful in determining a reasonable course of treatment where more than one therapeutic approach is possible.

A consequence of "MOPP", the multidrug chemotherapy for Hodgkin's lymphoma is oligospermia, but in many cases the sperm count may return to levels that render the patient fertile. The presence of mutagenic lesions induced by the therapeutic drugs in the surviving spermatogonial stem cell population raises the possibility of diminished reproductive fidelity and therefore reduced fertility or high risk pregnancies. In order to examine the mutagenic hazards due to MOPP therapy and to investigate possible alternatives, male mice were treated with clinically relevant doses of drugs and their reproductive fitness assayed in an in vitro dominant lethal assay.

The results of the mutagenesis assay indicated that the drugs used, the timing and the order of the administration all impacted the yield of mutations. When combined with X-radiation, the mutant rate could be approximated by that determined for single treatments with either modality.

Analogous experiments were conducted with female mice in an approach where the in vitro methodology seems particularly appropriate. Mature oocytes were treated with x-radiation, methylmethanesulfonate or cis retinoic acid and the frequency of dominant lethal mutations determined. A 2-component dose-response curve was found following x-radiation and significant mutation induction was determined after methylmethanesulfonate. The results with cis-retinoic acid were equivocal and may have been negatively impacted by an unusual result in controls. However, despite the fact that cis retinoic acid is a potent teratogen in humans, the dominant lethal test does not clearly identify it as mutagenic.

In summary a variety of drugs used in cancer therapy as well as two known mutagens, radiation and methylmethanesulfonate, gave positive results in an analysis of the long-term mutagenic hazards in mammalian germ cells. The in vitro method, when specifically applied to complex mixtures of environmental hazards may prove to be a powerful predictor of mutagenic and carcinogenic hazards.

EXPOSURE OF MOUSE ZYGOTES TO CHEMICAL MUTAGENS: A SOURCE OF CONGENITAL ANOMALIES

W. M. Generoso, Biology Division, Oak Ridge National Laboratory*, P. O. Box Y, Oak Ridge, Tennessee 37830

On the basis of previous genetic, as well as teratological studies, it had been generally concluded that high incidences of congenital anomalies were experimentally inducible only by treatments after implantation of the embryo during the period of major organogenesis. Our recent findings change this long-standing belief. Exposure of early zygotic stages to ethylene oxide (EtO) was found to produce remarkable increases in the incidence of congenital abnormalities and death of fetuses[1]. Subsequently, similar results were observed with ethyl methanesulfonate (EMS).[2]

Effects of the mutagens were studied either before they were mated (treatment of preovulatory oocytes) or after mating (treatment of zygotes and two-cell embryos). EtO was given by inhalation and EMS by i.p. injection. In experiments in which the females were exposed after they had been mated, the duration of the mating period was restricted to 30 min. and the females were treated beginning either 1, 6, 9 or ca 25 hours after the end of the mating period. These postmating intervals correspond, respectively, to the time of sperm entry, early pronuclear stage (before DNA synthesis), pronuclear DNA synthesis, and early two-cell stage.[3] In the case of EtO, exposure time was 1.5 hours. Females were killed for uterine analysis on the 17th day of gestation. Dead embryos were classified into 3 categories: resorption body (embryo that died near the time of implantation), midgestation death (characterized by the presence of placenta and embryonic mass that lacked eye pigment), and late fetal death (death that occurred on day 11 postmating or later, with at least some eye pigment visible).

The major finding concerns midgestation and late fetal death and congenital defects among some of the surviving fetuses. High incidences of these endpoints were induced by both mutagens, but the time window during which the effects were inducible was narrow. Large effects were observed when female mice were exposed at the time of fertilization of their eggs or during early pronuclear stage of

the zygote (before DNA synthesis). The effects were absent or minimal when females were exposed either before copulation or after the fertilized egg had progressed to the stage of pronuclear DNA synthesis or had reached the two-cell stage.

In investigating the mechanisms responsible for the dramatic effect in the early pronuclear stage, we conducted a study in which zygotes that were treated with EMS 6 hours after mating were transferred to untreated pseudopregnant females 3 hours after treatment.[4] The high incidences of fetal death and malformations were again observed. Cytogenetic analyses of pronuclear metaphases, early cleavage embryos, and abnormal midgestation fetuses were also carried out but failed to show structural or numerical chromosome aberrations.[4]

The transplantation experiment rules out the maternal environment as a factor in the fetal maldevelopment and, along with the strict stage specificity, points to a genetic cause for the abnormalities. The negative cytogenetic evidence, however, indicates that genetic lesions in the conceptus are not the ones one might have suspected by conventional criteria and may therefore be of a type not heretofore identified in experimental mutagenesis. Molecular techniques, are presently being used to investigate possible new genetic mechanisms responsible for the zygote-derived congenital defects.

(*Research Sponsored jointly by the National Toxicology Program under NIEHS Interagency Agreement Y01-ES-20085 and the Office of Health and Environmental Research, U. S. Department of Energy, under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.)

REFERENCES

1. Generoso, W. M., J. C. Rutledge, K. T. Cain, L. A. Hughes, and P. W. Braden. Mutat. Res. 176:269-274 (1987).
2. Generoso, W. M., J. C. Rutledge, K. T. Cain, L. A. Hughes, and M. D. Shelby. In preparation.
3. Krishna, M. and W. M. Generoso. J. Exp. Zool. 202, 245-252 (1977).
4. Katoh, M., N. L. A. Cacheiro, C. V. Cornett, and W. M. Generoso. In preparation.

IN VITRO TRANSFORMATION OF HUMAN FIBROBLASTS

Dennis G. Fry, Peter Hurlin, P. Ann Ryan, Daniel M. Wilson, Veronica M. Maher, and J. Justin McCormick

Carcinogenesis Laboratory, Michigan State University, East Lansing

In vitro transformation of cells in culture is a powerful method for the study of the cellular and molecular changes that result in cancer. Such transformation assays have been developed with cell cultures from various rodent species. However, despite intensive research by many workers, no one has developed a reproducible system for the transformation of diploid human cells. Since fibroblastic tumors (fibrosarcomas) are found in humans, our inability to malignantly transform human fibroblasts in vitro is probably an indication that the procedures used so far are inadequate. If, as is commonly conceived, the process of carcinogenesis involves the sequential, clonal selection of cells for various premalignant phenotypes, the failure to identify one or more of these critical phenotypes could be the problem. Some tumor cells grow when their normal counterparts do not. Our first approach was to determine whether human tumor cells of fibroblastic origin differ from their normal counterparts in their response to exogenous growth factors. To carry out this assay, we developed a serum-free medium which is capable of supporting clonal and long-term growth of human fibroblasts. Using it, we determined that to exhibit high

replication rates, normal human fibroblasts require either epidermal growth factor (EGF), platelet derived growth factor (PDGF), or fibroblast growth factor (FGF, or a medium lacking the above protein growth factors, but containing 1.0mM calcium. No growth was observed in 0.1mM calcium medium without the addition of protein growth factors. Fibrosarcoma-derived human cell lines, a spontaneously transformed human fibroblast cell line and SV40-transformed human fibroblast cell lines all grew in 0.1mM calcium medium without the addition of protein growth factors. Since normal diploid human fibroblasts do not synthesize EGF or PDGF, whereas one of the fibrosarcoma-derived cell lines tested is reported to synthesize both of these growth factors, this may explain the results. We are now determining whether the other transformed cells make specific growth factors.

Transfection of oncogenes is a second approach to the identification of cells that possess phenotypes intermediate between normal and tumorigenic. In our earliest attempts to transform diploid human cells, using the standard calcium phosphate co-precipitation technique, we found that we were able to obtain only a few transfectants per dish (10-20 per 10 cells). However, by modifying the DMSO-polybrene technique for use with human cells, we were able to find 400 transfectant/10 cells. Using this technique, we transfected normal human fibroblasts with DNA

sequences for the B chain of PDGF, as well as mutated N-ras and H-ras genes and the v-myc gene. The cells transfected with DNA coding for PDGF exhibited a fibroblastic morphology, formed foci, exhibited anchorage independent growth, and grew to 10x higher density than control cells. However, they did not acquire an unlimited lifespan in culture and did not form tumors in athymic mice. The ras transfected cells were morphologically transformed, formed foci, and grew to higher density than control cells, but these cells also did not acquire an unlimited lifespan or form tumors. The v-myc transfected cells had a normal morphology and went into crisis at the end of their lifespan. After many weeks, a single clonal population grew out and these cells contained the transfected v-myc gene. These cells have now gone 120 population doubling since crisis and are apparently immortal.

Using this apparently immortal cell as the host, we have transfected in the same oncogenes discussed above, as well as the mutated K-ras gene. The yield of transfectants using the gene for PDGF was low and these cells proved to be non-tumorigenic. However, all three mutated ras genes independently caused morphological transformation of the cells, focus formation, and anchorage independent growth. Furthermore, all the ras transfectants formed invasive tumors (fibrosarcomas) with a short latent period after injection with athymic mice.

Using the partially and malignantly transformed cells developed in these studies, as well as this serum-free medium, we are now carrying out reconstruction studies to develop suitable assays for isolation of premalignant and fully malignant human cells. Such studies offer the possibility that we will be able to measure quantitatively the ability of carcinogenes to cause the malignant transformation of human cells.

NEW APPROACHES TO THE STUDY OF DIFFERENTIATION AND CARCINOGENESIS

P. O.P. Ts'o

The Johns Hopkins University, Baltimore, MD 21205

One of the most important biological topics today is the interrelationship among aging, cancer and differentiation. While the significance of this topic is well-recognized, it is not easy to develop effective and efficient systems for investigation. In order to study this problem mechanistically we have to use *in vitro* systems and molecular biology and chemistry techniques. However, it also means we have to face the challenge of *in vitro*/*in vivo* extrapolation so that the studies done on molecules and cells are relevant to animals and man.

Various cellular studies on *in vitro* senescence, *in vitro* transformation and *in vitro* differentiation will be described, including the interrelationship among these systems and the factors which tend to effect these systems in a coordinated manner.

The focal point of this study is the properties and function of stem cells/progenitor cells in aging, cancer and differentiation.

Mechanistically, this study requires the understanding of the control of gene expression and the control of changes in the genome, such as DNA rearrangement and DNA methylation status, etc. While methods of studying point mutation or single base changes have been highly emphasized in the past, methods to study the methylation status of the entire genome or DNA rearrangement of unknown fragments of the genome remain to be developed. Our laboratory will propose some studies in this direction.

In addition, computer assisted microphotometry has now been highly developed for use with monoclonal antibodies to measure DNA insults and expression of genes through measurement of the copy numbers of mRNA through nucleic acid hybridization. Furthermore, control of gene expression can be

accomplished by the use of sequence-specific nonionic oligonucleotide analogs which can form specific duplexes with single stranded accessible target nucleic acids such as mRNA, thereby blocking the expression of a specific gene. Examples of the use of this approach to suppress the expression of viral genes will be given.

In summary, various cellular systems and molecular procedures will be used to study the control of gene expression and genome rearrangement, which serve as the basic mechanisms for cellular studies related to aging, differentiation, and cancer through extrapolation between *in vitro* and *in vivo* systems.

CONTROL OF MUTAGENESIS AND CELL DIFFERENTIATION IN CULTURED HUMAN CELLS

E. Huberman, Argonne National Laboratory, Argonne, Illinois 60462

Carcinogenesis is a multistage process that can be divided into three sequential steps: initiation, promotion, and progression. Initiation is thought to involve mutational events in genes that control cell growth and differentiation. Promotion, on the other hand, is considered to involve nonmutational alterations in gene expression that produce a reversible selective clonal expansion of the initiated cells. The last step, progression, probably also involves genetic changes that cause enhanced and uncontrolled growth of the promoted cells.

Using an *in vitro* cell-mediated mutagenicity assay with Chinese hamster V79 or human P3 cells as targets, we showed a relationship between the degree of mutagenesis by various chemical carcinogens in this assay and their tumor-producing potential in experimental animals. However, these types of studies usually do not involve growth control genes. To address this issue, we studied carcinogen-induced alterations in a gene that codes for an enzyme involved in the regulation of cell growth: inosine 5'-monophosphate dehydrogenase (IMPDH). An increase in the activity of this enzyme is also associated with development of malignancy and tumor growth progression. To obtain cells with altered IMPDH activity, we used mycophenolic acid (MPA), a specific, highly cytotoxic inhibitor of IMPDH, for selection. Treatment of V79 cells with a carcinogen produced variant cells that were resistant to MPA. By using a specific anti-IMPDH antiserum and a specific cDNA probe, which we isolated from a gt11 expression library, we showed an increase in the amount and activity of IMPDH in the MPA-resistant cells, which resulted from IMPDH gene amplification. On the basis of these experiments, we suggest that a carcinogen insult may affect tumor initiation and more likely tumor progression by amplifying various genes, including those that control cell growth.

Tumor-promoting phorbol diesters, teleocidins, and other related agents are usually devoid of mutagenic activity and cannot, therefore, be detected in mutagenesis assays. These agents, however, can alter differentiation processes in various cell types including the human promyelocytic HL-60 leukemia cells. To study the mechanism whereby tumor

promoters induce maturation processes, we isolated HL-60 cell variants that are either susceptible or resistant to phorbol diester-induced cell differentiation. After treatment with tumor-promoting phorbol diesters, these cell variants also differ in the expression of c-fos, and c-fms protooncogenes. Using these HL-60 cells, we established that induction of cell differentiation by the phorbol diesters begins when they bind to a high-affinity and saturable receptor, which is a calcium- and phospholipid-dependent kinase (protein kinase C). This binding activates protein kinase C, causing the phosphorylation of various cellular proteins, including some that reside in or around the nucleus. On the basis of these studies, we speculate that during tumor promotion, the phorbol diester causes the migration of protein kinase C to the vicinity of the nuclei of the treated cells. In the nucleus, through phosphorylation of regulatory proteins, the kinase causes the expression of genes that control cell growth, in a process similar to that occurring during cell maturation. The expression of these growth control genes may cause the selective clonal expansion of initiated cells and eventually tumor formation.

References

1. Huberman, E., McKeown, C. K., Jones, C. A., Hoffman, D. R. and Murao, S-i. Mutat. Res., 130, 127-137, 1984.
2. Murao, S-i., Epstein, A. L., Clevenger, C. V., and Huberman, E. Cancer Res., 45, 791-795, 1985.
3. Huberman, E., and Jones, C. A. In: The Role of Chemicals and Radiation in the Etiology of Cancer, E. Huberman and S. H. Barr, eds. Raven Press, New York, NY, pp. 263-273, 1985.
4. Anderson, N. L., Gemmell, M. A., Coussens, P. M., Murao, S.-i., and Huberman, E. Cancer Res., 45, 4955-4962, 1985.
5. Mitchell, R. L., Henning-Chubb, C., Huberman, E., and Verma, I. M. Cell, 45, 497-504, 1986.
6. Homma, Y., C. B. H. Chubb, and Huberman, E. Proc. Nat. Acad. Sci. 83, 7316-7319, 1986.

UNITED STATES
DEPARTMENT OF ENERGY
WASHINGTON, D.C. 20545

ER-72