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# Methods Development Toward the Measurement of Polyaromatic Hydrocarbon–DNA Adducts by Mass Spectrometry

Roger W. Giese and Paul Vouros
*Departments of Pharmaceutical Sciences and Chemistry, and Barnett Institute, Northeastern University, Boston, MA*

**Includes the Commentary of the Institute's Health Review Committee**

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# HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

HEI receives half its funds from the Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the U.S. However, the Institute exercises complete autonomy in setting its research priorities and in disbursing its funds. An independent Board of Directors governs the Institute. The Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the investigator's report and the Review Committee's evaluation of the work's scientific and regulatory relevance.

# HEI Statement

**Synopsis of Research Report Number 61**

# Detection of Carcinogen-DNA Adducts: Development of New Methods

## BACKGROUND

Each year more than a half-million Americans die from cancer, and twice that many are diagnosed with the disease. Both environmental and genetic factors are believed to contribute to the multistage process that results in carcinogenesis. Therefore, determining the health risks associated with exposure to known and suspected carcinogenic chemicals is essential for informed decision-making by regulatory agencies. However, the process of assessing cancer risk is hampered by the limited information regarding carcinogen exposures.

Traditional methods of exposure assessment rely on monitoring levels of the chemical of interest in air, water, or food. Such data provide rough estimates of potential exposures of the general population but do not take into account individual differences in actual exposures or responses. Recently, there have been major advances in the development of sensitive biomarkers for measuring human exposures to chemical carcinogens. One such method is based on the fact that when carcinogens (or their metabolites) react with DNA, they form structures called DNA adducts. Because such adducts can be detected in tissues and blood cells, scientists are testing their usefulness as molecular biomarkers of human exposures to chemical carcinogens. The HEI has supported research to develop sensitive and specific techniques for measuring polyaromatic hydrocarbon (PAH)–DNA adducts, a class of DNA adducts associated with exposure to constituents of diesel emissions and other combustion products.

## APPROACH

Several different procedures are currently available for measuring DNA adducts, including immunoassays, fluorescence spectroscopy, mass spectrometry (MS), and $^{32}$P-postlabeling. Each method has its advantages and disadvantages. Mass spectrometry, the technique used in this study, is usually linked to a chromatographic system, such as gas chromatography (GC). Mass spectrometry is a powerful technique because it can distinguish among different carcinogen-DNA adducts, and it provides information about chemical structure. Despite its advantages, MS has not been widely used for analyzing human DNA adducts because it is generally less sensitive than some of the other procedures, and it is difficult to convert bulky DNA adducts into the volatile derivatives that are required for GC-MS analysis. In this study, the investigators set out to develop derivatives of PAH-DNA adducts that are better than those that currently exist, and to improve the overall sensitivity of the MS technique.

## RESULTS AND INTERPRETATION

Drs. Giese and Vouros developed three new chemical techniques that show promise for converting PAH-DNA adducts into forms suitable for GC-MS analysis. For one of these methods (superoxide oxidation), they achieved marked improvement in sensitivity in detecting a model PAH-DNA adduct. However, the applicability of the procedure to actual DNA adducts in biological samples is not clear because the investigators conducted most of their work with model compounds, as opposed to DNA isolated from tissues from exposed animals or human subjects. The investigators also significantly improved the sensitivity of a newer, simpler technique (fast-atom-bombardment MS), which should allow qualitative and quantitative information to be obtained from the same sample.

A major consideration in evaluating MS strategies for measuring carcinogen-DNA adducts is the issue of sensitivity for biological samples. At present, MS procedures are not sufficiently sensitive to detect and quantify DNA adducts from small tissue samples. The development of novel procedures for sample preparation, combined with the increased instrument sensitivity, indicates a potential for MS techniques to play an important role in the detection and characterization of PAH-DNA adducts. However, current MS methods are still less sensitive than other available technologies, and more work needs to be done to explore their suitability for biological samples.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1989 to 1991. This study was conducted by Drs. Roger Giese and Paul Vouros at Northeastern University. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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The Statement is a nontechnical summary, prepared by the HEI and approved by the Board of Directors, of the Investigators' Report and the Health Review Committee's Commentary.

**II. INVESTIGATORS' REPORT Roger W. Giese and Paul Vouros** ..... 1

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee, and, if necessary, revise their report.

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# Methods Development Toward the Measurement of Polyaromatic Hydrocarbon–DNA Adducts by Mass Spectrometry

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## ABSTRACT

The measurement of DNA adducts in human samples is at an early stage. The accuracy of some of the current measurements is not defined, the structures are unknown for a significant number of the adducts that have been detected, and there is little information about how many adducts remain to be discovered. This is due largely to the trace amounts of human DNA adducts in any sample. A consequence of this is that the true potential of DNA adducts as indicators of exposure and risk in human toxicology is far from realized.

Mass spectrometry, a powerful technique for organic analysis, is the key to exploiting fully the usefulness of human DNA adducts as biomarkers of human exposure and risk. Mass spectrometry can make accurate measurements, discover unknown compounds, and determine the structures of these unknown compounds. However, the trace (very small) amounts of human DNA adducts have limited mass spectrometry's usefulness in analyzing such samples.

This project focused on increasing the sensitivity of mass spectrometry for measuring human DNA adducts. Advances in sensitivity have been achieved for two modes of mass spectrometry applied to standards related to DNA adducts: gas chromatography with electron-capture negative ion mass spectrometry, and fast-atom-bombardment mass spectrometry. These advances involve both sample preparation and instrument conditions.

For sample preparation, three new chemical techniques, each targeted toward a class of PAH-DNA adducts, show promise for measuring DNA adducts with increased sensitivity or scope by mass spectrometry: superoxide oxidation, hydrazinolysis, and hydrogenation. The three classes are diolepoxide polyaromatic hydrocarbon–DNA adducts (superoxide oxidation), amino-PAH– and nitro-PAH–DNA adducts (hydrazinolysis), and general PAH-DNA adducts (hydrogenation). The general concept for each of these new techniques involves the chemical transformation of a selected class of PAH-DNA adducts into secondary products possessing improved recovery and sensitivity characteristics for detection by mass spectrometry. Most attention was given in this project to the superoxide oxidation technique, which was brought to the femtomole level for the measurement of a standard of benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol as a model analyte. Also, as described briefly in Appendix A, this compound was detected in a lymphocyte cell culture exposed to benzo[*a*]pyrene. A standard of the terminal, chemical transformation product for this tetrahydrotetrol, 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate, was detected at the attomole level.

Improved instrument conditions increased the sensitivity for measuring polyaromatic hydrocarbon-DNA adducts by fast-atom-bombardment mass spectrometry. This work began as static fast-atom bombardment and was continued as continuous-flow fast-atom bombardment, which was then coupled to capillary electrophoresis and capillary liquid chromatography. The major model analytes for the fast-atom bombardment studies were *N*-(deoxyguanosin-8-yl)-2-aminofluorene and the corresponding *N*-acetyl-2-aminofluorene adduct. Optimization of conditions led to detecting at the femtomole level a standard with selected reaction monitoring. We found that 270 pmol of a completely unknown adduct of this type, isolated, for example, as a liquid chromatography peak, could be thoroughly studied by fast-atom-bombardment mass spectrometry. This is because 1/8 of the sample could be used first to identify a molecular ion based on the diagnostic appearance of this ion and a corresponding aglycone ion. The remaining sample then could be used to obtain daughter ion spectra by tandem mass spectrometry.

Appendix A summarizes our use of capillary liquid chro-

A list of abbreviations appears at the end of the Investigators' Report for your reference.

This Investigators' Report is one part of Health Effects Institute Research Report Number 61, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Roger Giese, Department of Medicinal Chemistry, Northeastern University, 360 Huntington Avenue, 110-MU, Boston, MA 02115.

Although this document was produced with partial funding by the United States Environmental Protection Agency under assistance agreement X-816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

matography and continuous-flow fast-atom-bombardment mass spectrometry in tandem to provide the first direct evidence for the existence of *N*-acetyl-2-aminofluorene–deoxyadenosine adducts, obtained through the reaction of calf thymus DNA and *N*-acetoxy-*N*-acetyl-2-aminofluorene.

## INTRODUCTION

### DETECTING AND MEASURING DNA ADDUCTS

DNA adducts play an important role in carcinogenesis and mutagenesis caused by exposure to chemicals and radiation (Singer and Grunberger 1983; Garner et al. 1991). Thus, it important to measure DNA adducts accurately and comprehensively. The rationale behind this project was that mass spectrometry (MS) must play a major role in such measurement of DNA adducts in human fluid and tissue samples. In turn, the definitive measurement of human DNA adducts would allow their unrealized potential to be achieved as molecular biomarkers to help define both exposure and effects in risk assessment.

DNA adducts can be measured in cellular samples, such as blood, and also in urine. The interpretation of urinary DNA adducts can be confounded by the possibility that they were derived from the diet (Shuker 1991). Thus, urinary DNA adducts can reflect prior reactions of chemicals with the DNA of animals or plants that furnished the diet. Therefore, these adducts probably do not represent toxic exposure for the individual. Measurements of urinary DNA adducts, nevertheless, can be important, especially because urine samples can be collected without trauma. However, the primary, long-term concern in this project was the measurement of cellular DNA adducts in blood and tissue samples as a more well-defined test of human exposure to toxic chemicals.

Studies are underway in other laboratories on other molecular biomarkers to improve our understanding of the health effects from exposure to toxic chemicals. These other molecular biomarkers include protein adducts and chemically induced somatic mutations and clastogenic effects. How useful are these other biomarkers of toxic chemical exposure compared with DNA adducts? This question cannot be answered definitively at the present time, but a few comments can be made to help put the usefulness of DNA adduct measurements into perspective.

The relation between DNA and protein adducts was reviewed by Watson and Farmer (1991). Hemoglobin has been the principal protein studied for this purpose because of its high level and long half-life in the blood; albumin also has been studied. Chemicals that form covalent DNA adducts tend to be electrophilic, and both DNA and proteins are rich in nucleophilic sites. The much higher levels of hemoglobin and albumin than DNA in blood and the apparent lack of repair of protein adducts (unlike DNA adducts) currently make protein adducts much easier to measure. Measurements of protein adducts provide important information about human exposure to toxic chemicals (Day et al. 1991). However, all of this does not diminish interest in measuring DNA adducts, for several reasons. For example, some DNA-reactive chemicals may not react with hemoglobin or albumin, or to the same degree with phenotypic variations of these proteins in different individuals. DNA adduct levels also can vary depending on the cell type, apparently because of differences in repair; this, in turn, can correlate with the incidence of tumors, as was observed when $O^6$-methylguanine was measured in samples from rats exposed to 1,2-dimethylhydrazine (Bedell et al. 1982). Such valuable information is difficult to obtain from measurements of protein adducts. Finally, our understanding of the details by which DNA adducts lead to mutations depends, in part, on our ability to measure DNA adducts. Taking all of this into account, as well as other considerations (Garner et al. 1991), it is clear that measurements of both DNA and protein adducts are both valuable and complementary.

Chemicals that damage DNA produce specific patterns of point mutations, which are called mutational spectra (Marx 1989; Thilly 1990). Until recently, mutational spectra have been obtained largely by a tedious process of isolating individual mutants for characterization via cloning. However, more powerful methodology is emerging that takes advantage of recent tools such as denaturing gradient gel electrophoresis and the polymerase chain reaction to speed up and extend the analysis (Thilly 1990; Cariello et al. 1991; Keohavong et al. 1991). Mutational spectra can provide information about the environmental causes of human point mutations (Cariello and Thilly 1986). The further development and application of this technology will be helped by measurements of DNA adducts.

The existing methods of $^{32}$P-postlabeling thin-layer chromatography (TLC), immunoassay, and fluorescence for human DNA adducts (Bartsch et al. 1988) have been only partly successful, both individually and collectively, in providing accurate and comprehensive measurements of human cellular DNA adducts. Why is this? Let us first consider the strengths and weaknesses of the major, current technique for human DNA adducts, $^{32}$P-postlabeling TLC. With the basic form of this technique, the DNA is purified from a biological sample and digested enzymatically to deoxynucleotides. Then, the adducts, sometimes after separation from the bulk of the normal deoxynucleotides by solvent extraction or chromatography, are radioenzymati-

cally labeled with $^{32}P$, separated by TLC, and detected as black spots by autoradiography. The technique is extremely sensitive (the attomole level has been reported for at least some bulky, known adducts), utilizes techniques and instrumentation that are commonly available, and is used in many laboratories to detect DNA adducts in human or animal blood, tissue, and fluid samples (reviewed by Randerath and Randerath 1991).

Like all analytical methodology, $^{32}P$-postlabeling TLC also has some shortcomings. In its basic form, the technique only measures bulky, nonpolar DNA adducts. This is because polar adducts are necessarily washed off the TLC plate along with normal deoxynucleotides during one step of the method. Although the technique can be extended to nonbulky adducts (e.g., 7-methylguanine) (Mustonen et al. 1991), each adduct of this type tends to require a special procedure and needs to be a known adduct beforehand. Also, it can be more difficult to purify nonbulky adducts from normal DNA constituents because of the small structural differences. This raises the detection limit or can increase the complexity of the assay procedure.

A second problem with $^{32}P$-postlabeling TLC is its low resolution. For example, a smear of spots that is apparently the result of numerous, incompletely resolved DNA adducts is observed when human lung tissue from smokers is analyzed by $^{32}P$-postlabeling TLC (Geneste et al. 1991). This limited ability to separate different DNA adducts arises not only from the poor chromatographic characteristics of bulky, nonpolar deoxynucleotides, but from the considerable spreading of solute bands in ordinary TLC as well. Thus, a given spot may represent more than one adduct.

The reliance on enzymes in $^{32}P$-postlabeling TLC is both a strength and a weakness. This provides specificity, although the radioenzymatic step is not absolutely specific for nucleotide substrates (Masento et al. 1989). Not all adducts are labeled to the same degree; in fact, some adducts can undergo labeling at least 50-fold less efficiently than others (Shields et al. 1993; Vodicka and Hemminki 1991; Koivisto and Hemminki 1990). Related problems in achieving quantitative results by $^{32}P$-postlabeling have been presented (Vaca et al. 1992).

Immunoassays also have contributed to the measurement of DNA adducts in human samples (Zhang et al. 1991). As with $^{32}P$-postlabeling TLC, these assays rely on techniques and equipment that are available in many laboratories. High sensitivity and convenience are strengths, whereas application only to known adducts and cross-reactivity of antibodies with related adducts (Weston et al. 1989) are shortcomings. The signal from an immunoassay looks the same whether an interference or analyte is detected, which is why it is important to confirm results from such an assay with an independent method.

Fluorescence has been employed to measure DNA adducts in physiological samples (Manchester et al. 1990). Although the sensitivity of this technique falls short of that provided by $^{32}P$-postlabeling TLC, different adducts can have different fluorescent spectra that add to the specificity of the detection and can provide clues to the structures of some unknown adducts that are similar to adducts characterized previously. However, only a limited number of adducts are inherently fluorescent, and even those that are can differ significantly in their sensitivity. Fluorescent DNA adducts also tend to emit light in the low ultraviolet (UV) range, where interfering signals and quenching effects can be encountered. This increases the demands on sample cleanup to achieve reliable signals on trace amounts of such adducts.

Several summaries discuss how different results are sometimes obtained when the current methods of $^{32}P$-postlabeling TLC, immunoassay, and fluorescence are used to analyze the same samples (Weston et al. 1989; van Schooten et al. 1990; Weston and Bowman 1991). For example, putative benzo[*a*]pyrene-DNA adducts were detected in placental DNA samples by immunoassay, but no such adducts or any known polyaromatic hydrocarbon (PAH)-DNA adducts were found in these samples by $^{32}P$-postlabeling TLC (Everson et al. 1986). When three leading laboratories in the use of $^{32}P$-postlabeling TLC analyzed the same samples by this technique, correlation coefficients for total adduct levels were in the range of 0.45 to 0.62 (Savela et al. 1989). Although these different techniques have provided many interesting findings and have given consistent results individually within single laboratories, there are still opportunities for improving some aspects of their performance (Manchester at al. 1990).

## ANALYSIS OF DNA ADDUCTS BY MASS SPECTROMETRY

Although methods exist for detecting DNA adducts in human samples, it is not always clear that the adducts are being measured accurately. Furthermore, difficulties have been encountered in determining the structures of many adducts that have been detected. For example, $^{32}P$-postlabeling TLC does not inherently provide structural information about unknown adducts that it detects. This obviously invites MS to play a role. This technique, via the use of stable isotope internal standards, can more directly provide accurate quantitative measurements. It is well-established as a powerful method for the qualitative analysis (structural elucidation) of unknown trace organic compounds. This technique also potentially can join the other techniques (aside from conventional immunoassays) in detecting previously unknown DNA adducts. (This latter capability is discussed in more detail later.)

Qualitative and quantitative MS are two somewhat different techniques. Identifying an unknown compound by MS generally requires from $10^3$ to $10^5$ more of a sample than is necessary for detecting a known compound. Furthermore, chemical derivatization of the compound can often help to increase its sensitivity for detection by quantitative MS, but it may complicate the compound's structural elucidation by qualitative MS. Finally, different techniques or conditions are commonly selected for volatilizing and ionizing a sample for qualitative, as opposed to quantitative, MS measurements.

Mass spectrometry has begun to be employed for the quantitative detection of certain known DNA adducts in large samples (one gram or more) of human tissue, such as placenta (Manchester et al. 1988), and in lymphocytes from individuals who have experienced a high level of chemical exposure (Weston et al. 1989). Significant qualitative work also has been conducted by MS on the measurement of DNA components, including DNA adducts, from nonhuman sources (reviewed by Burlingame et al. 1983, by Mitchum et al. 1985, and by McCloskey 1990, and partially summarized by Annan et al. 1990). Unfortunately, nanomole amounts of DNA components and adducts were required for the qualitative analyses by MS.

However, there is still a big gap between the existing accomplishments of MS in the analysis of DNA adducts (which require nanomole levels for qualitative analysis, and at least femtomole levels for quantitative work on biological samples) and the challenge of measuring DNA adducts by this technique both quantitatively and qualitatively in routine human samples, or from routine chemical exposure, for which attomole level measurements may be needed. Potentially, the development of improved MS techniques, including both sample preparation and detection, could at least partially bridge these sensitivity gaps of at least 1,000-fold. In the latter case (as an initial or intermediate goal), the remainder of the gap might be filled in by obtaining a larger sample of human fluid or tissue. For example, if one knew that qualitative MS could provide a definitive, sensitive analysis of a significant (based on epidemiology studies) human DNA adduct, then it could be appropriate to obtain and test a very large sample (e.g., one kilogram of tissue) to identify the adduct. An example of this type of strategy for the quantitative detection of a known adduct is the pooling of human placenta to afford 15-mg samples of DNA for the determination of a benzo[*a*]pyrene-DNA adduct by MS (Manchester et al. 1988). Alternatively, the significant, unknown adduct sometimes might be available in large quantity from a cell culture or animal. Of course, this strategy involving large samples for qualitative analysis is compromised by the fact that MS is not always successful, on its own, in fully elucidating the structure of an unknown compound.

With regard to the quantitative analysis of a known DNA adduct (intended as a biomarker to monitor a given exposure) in human samples by MS, the sensitivity gap is more likely to be closed without resorting to nonroutine samples of human fluids. In part because of the work reported here, MS now can detect attomole amounts of purified, diluted standards of some derivatized DNA adducts (Abdel-Baky and Giese 1991). Although progressing from this stage to the practical detection of attomole amounts of DNA adducts in biological samples remains an unsolved problem, we believe that it can be done.

The goal of our project was to advance the ability of MS to measure PAH-DNA adducts both qualitatively and quantitatively. These adducts were chosen because of their relevance to vehicle emissions. Two modes of MS were selected for this work: gas chromatography electron-capture negative ion MS (GC-ECNI-MS) for quantitative measurements, and fast-atom-bombardment MS (FAB-MS) for qualitative analysis. A brief description of these two MS methods and an explanation of why we selected them is presented next.

Gas chromatography electron-capture negative ion MS was selected because it is one of the most sensitive forms of quantitative MS. With this technique, the sample is dissolved in an organic solvent and injected into a gas chromatograph, where the analyte is separated in the gas phase from many of the impurities in the sample. The analyte then elutes from the gas chromatograph and encounters a cloud of low-energy electrons (and other reactive species) in the ion source of the MS. If the analyte is able to capture an electron efficiently under such conditions (few compounds can), it will ionize by electron capture, and the resulting anionic product will be detected with high sensitivity in the MS. Sometimes, the ionized analyte fragments after it captures an electron; in such cases, an anionic fragment product (or more than one) is detected.

At the outset of our project, GC-ECNI-MS had not been applied to the detection of PAH-DNA adducts. Basically, this was because such adducts are nonvolatile, thermally unstable (preventing their separation by GC), and lack the structural features necessary for efficient electron capture. Furthermore, these essential properties cannot be conferred by conventional derivatization because PAH-DNA adducts are too large and have too many functional groups. We proposed, at the outset of this project, to develop novel chemical transformation techniques to bring PAH-DNA adducts within the scope of GC-ECNI-MS. Three such techniques, which will be described later, were introduced in this project: superoxide oxidation, hydrazinolysis, and hydrogenation. These techniques all potentially provide convenient and effective sample preparation.

Gas chromatography with electron capture detection (GC-

ECD) also was used in this project as an ancillary technique to help develop methodology based on GC-ECNI-MS. As with ECNI-MS, detection relies on electron capture, but the gross change in electron capture current, rather than the appearance of a specific analyte ion, is monitored. This reduces the specificity of the detection step, making interferences more likely. However, GC-ECD equipment is much less expensive than GC-ECNI-MS equipment, and it also is useful for the early development of methodology intended ultimately for GC-ECNI-MS.

In FAB-MS, the sample usually is introduced into the ion source of the MS as a small liquid puddle (static FAB) or as a dynamic (continually regenerating) liquid droplet (continuous-flow FAB). With the latter technique, a flowing sample stream emerges slowly from the end of a capillary tube, where it forms a steady-state droplet, which, in turn, is bombarded by fast atoms as it evaporates in the ion source. Upon bombardment under high vacuum with a beam of fast atoms (e.g., xenon atoms), the analyte, even if it is already ionized, along with the liquid (e.g., glycerol), is transferred into the vacuum. A common event for a neutral but polar molecule is to gain or lose a proton, thereby acquiring a charge. The matrix or solvent for the sample plays a positive and negative role simultaneously. On the positive side, the matrix contributes to the desorption and ionization of the sample. The price for this is that the matrix itself gives rise to ions, which manifest as chemical noise, as well as adduct ions with the analyte, which can split the analyte signal. This raises the detection limit. The overall process is a relatively reliable, sensitive, and gentle way to volatilize and ionize polar compounds. Because thermally unstable compounds, such as deoxynucleosides, are readily measured without any chemical pretreatment, the method is quite different from GC-ECNI-MS. Although it has not yet provided the detection limits achieved by the latter technique for standards, FAB-MS can simplify sample preparation, and it is much better suited for the qualitative analysis of polar compounds.

Although FAB-MS had proved to be useful in other laboratories for the qualitative analysis of DNA adducts, large amounts of samples (nanomole range) were required. Thus, there was a great gap between the amount of sample used for this technique and the DNA adduct levels present in human samples. We worked on improving the sensitivity of this technique for PAH-DNA adducts and found that the femtomole level can be reached for such compounds, at least for standards of amino-PAH–DNA adducts detected in the selected ion monitoring mode. (For complete tandem MS qualitative analysis, however, taking into account practical sample preparation, an amount of a completely unknown adduct in the midpicomole range is currently required in our laboratory, as explained in more detail later.)

### NEW MASS SPECTROMETRIC TECHNIQUES

The area of MS is currently undergoing a revolution in its techniques, as well as its instrumentation. Some of the buzzwords in this area are "particle beam," "electrospray," "ion traps," and "matrix-assisted laser desorption." These techniques collectively have two major impacts on the practice of MS: large molecules, such as proteins and DNA oligomers, are being analyzed directly, and the ease of coupling MS to liquid-flow techniques for sample introduction, such as liquid chromatography or capillary zone electrophoresis, is enhanced. It is too early to predict what impact these and related developments will have on the measurement of DNA adducts, but there are some exciting possibilities. For example, potentially intact DNA fragments (containing a DNA adduct) could be detected and also sequenced directly by MS with high sensitivity (e.g., ion-trap MS might eventually achieve this).

Nevertheless, it is essential to purify a sample thoroughly before valid MS measurements of any type can be obtained on a trace amount of an analyte. Thus, we have given primary attention in this project to sample preparation, particularly the late steps, because this stage of sample preparation has been the least developed for the analysis of DNA adducts by MS. It is not trivial to find a combination of chemical and physical steps that both recover and purify a trace analyte for MS analysis. Trace analytes in general tend to undergo losses in sample preparation procedures, and improper selection of reagents and techniques simply can exchange one set of interferences for another. This largely explains why MS is being held back currently in the area of human DNA adducts, and why the new MS techniques may not change the situation. The future of MS in the measurement of human DNA adducts greatly depends on advances being made in sample preparation.

## EXPERIMENTAL METHODS

### CHEMICALS AND REAGENTS

Potassium superoxide ($KO_2$), 18-crown-6, 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one, pentafluorobenzyl bromide, triethylamine, succinic anhydride (99%), aluminum chloride (anhydrous, 99.99%), zinc (20 mesh), mercury(II) chloride (99.5%), phosphorus pentachloride, stannic chloride, high-performance liquid chromatography (HPLC)-grade dichloromethane, HPLC-grade benzene, nitrobenzene (99+%), HPLC-grade *p*-xylene, HPLC grade *N*,*N*-dimethylformamide (DMF), and HPLC-grade glacial acetic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,3,5,6-Tetrafluorobenzyl bromide was obtained from Alfa Products

(Danvers, MA). Benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol (referred to as "Tetrol") was purchased from the NCI Chemical Carcinogen Repository (Kansas City, MO). Pyrene-$d_{10}$ was purchased from Icon Services (Summit, NJ), and acetonitrile (for UV detection) and hexane (for GC) were purchased from American Scientific Products (Boston, MA). Distilled water was purified to an HPLC grade with a Nanopure/Organicpure System (Barnstead Thermolyne Corp., Boston, MA). For the reactions, DMF and benzene were dried with type 4A molecular sieves (Aldrich). The molecular sieves were washed with hexane three times and then activated overnight at 250 °C before use. United States Pharmacopeia–grade carbon dioxide (99.9%), ultra-high–purity helium (99.999%), high-purity helium, ultra-high–purity nitrogen (99.999%), high-purity nitrogen, chemically pure–grade methane (99.998%) and Oxisorb-LP cartridges were purchased from Medical Technical Gases (Medford, MA). Anhydrous hydrazine (Aldrich) was treated with 20% (w:w) of potassium hydroxide overnight and then distilled under anhydrous conditions. The fraction that distilled at 110 °C was collected. 2-Aminofluorene (2-AF) (98%) was purchased from Aldrich. High-performance liquid chromatography solvents were obtained from American Burdick and Jackson (American Scientific). *N*-(Deoxyguanosin-8-yl)-2-aminofluorene (*N*-(dG-8-yl)-2-AF) and *N*-acetyl-*N*-(deoxyguanosin-8-yl)-2-aminofluorene (*N*-acetyl-*N*-(dG-8-yl)-2-AF) were kindly provided by Dr. F.A. Beland (National Center for Toxicological Research, Jefferson, AR).

## EQUIPMENT AND TECHNIQUES

### High-Performance Liquid Chromatography

The HPLC consisted of a Series-4 Liquid Chromatograph from Perkin-Elmer Corp. (Norwalk, CT), a Rheodyne 7125 injector from Rainin Instruments (Woburn, MA), a Spectroflow 773 UV detector from Kratos Analytical (Ramsey, NJ), and an SP-4270 integrator from Spectra Physics (Piscataway, NJ). The solvents were degassed and the solvent chamber pressurized to 7 psi with helium. The analytical HPLC cleanup was carried out on a Microsorb $C_{18}$ column (150 × 4.6 mm, 5-mm diameter particles) (Rainin Instruments). Detection was at 263 nm for 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate.

### Gas Chromatography with Electron-Capture Detection

A model 3740 gas chromatograph from Varian Analytical Instruments (Walnut Creek, CA), was fitted with a $^{63}Ni$ electro-capture detector, a model 1095 on-column capillary injector, and a Waters 840 data system (Millipore-Waters, Milford, MA). The quantification of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate was done on a PH-1, cross-linked methyl silicone gum, capillary column (10-m long, 0.32-mm i.d., 0.17-μm film thickness) (Hewlett-Packard Co., Palo Alto, CA). The flow of the carrier gas, helium, was 4 mL/min at 250 °C. The flow of the make-up gas, nitrogen, was 26 mL/min at 250 °C. The initial injector and column temperatures for GC-ECD were 90 °C and 120 °C, respectively. Immediately after injection, the injector temperature was programmed to 280 °C at a setting of 180 °C/minute. The column, after a three-minute hold, was programmed to 290 °C at 10 °C/minute. The detector temperature was kept constant at 340 °C.

### Gas Chromatography with Electron-Capture Negative Ion Mass Spectrometry

The equipment consisted of a Hewlett-Packard 5988A mass spectrometer equipped with an HP-5890 gas chromatograph. The mass spectrometer was interfaced to an HP-59970C (Rev. 3.2) MS ChemStation computer and an HP-7957B disk drive (with a formatted disk storage capacity of 81 megabytes). The GC separations were carried out on an Ultra-1 cross-linked methyl silicone capillary column (12-m long, 0.20-mm i.d., 0.11-μm film thickness) (Hewlett-Packard). The column was interfaced directly to the mass spectrometer, using an interface temperature of 290 °C. Helium (ultra-high purity) at a column head pressure of 20 psi was used as a carrier gas. Methane (ultra-high purity) was used as the reagent gas with a source temperature of 250 °C and a source pressure of 2.0 torr. The instrument was tuned manually daily for maximum sensitivity. For the quantification of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate, the oven was programmed to increase from 160 °C to 290 °C at 70 °C/minute. The on-column injector temperature was set at 300 °C.

### Fast-Atom-Bombardment Mass Spectrometry

Positive ion FAB-MS spectra (including tandem MS spectra) were recorded on a VG/Fisons Instruments (Danvers, MA) 70–250SE forward geometry, double-focusing mass spectrometer. An Ion Tech (Fort Collins, CO) FAB gun was operated at 8 kV (1.8 mA) with xenon. The static FAB probe tips supplied by VG/Fisons Instruments were modified, to lower the detection limit, by removing two-thirds of the sample deposit area with a Dremmel (Racine, WI) tool. This yielded a thicker film of sample when a limited sample volume was applied. The mass spectrometer was operated at a resolution of 1,500 with an accelerating voltage of 10 kV. The instrument was scanned at 10 sec/decade for FAB-MS experiments and 15 sec/decade for tandem MS experiments.

The tandem/spectra (metastable ion and collision-induced dissociation [CID]) were acquired using scans with linked magnetic and electric fields (B/E-linked). These were generated under computer control, with the product ions formed from a selected precursor in the first-field free region of the

instrument observed at the detector. Spectra were acquired in the multichannel analyzer mode, with 7 to 10 scans collected and added. The metastable ion spectra were acquired by collecting the unimolecular decomposition products of precursor ions. For FAB-MS, the CID spectra were generated by colliding the ion beam (10 kV) with a helium gas in a collision cell located between the source and the electric sector, and then collecting the product ions. The collision cell was filled with helium until the precursor ion beam was attenuated to 30%. For reasons that are not clear, this gave a higher signal-to-noise ratio than was provided by higher or low attenuations.

To obtain the FAB-MS and FAB-tandem MS spectra, the amino-PAH-adducts were dissolved in a mixture of methanol and water (50:50, v:v). Samples (0.5 to 1.0 μL) were applied to the probe tip, and the solvent was evaporated. Between 0.5 and 1.0 μL of matrix then was applied to the tip, and the probe was inserted into the mass spectrometer. This procedure was used because of the low solubility of some of the samples in the matrix. The trimethylsilyl derivatives were prepared by dissolving the samples in 10 to 20 μL of bis-(trimethylsilyl)trifluoroacetamide in 0.5-mL Reacti-Vials (Pierce, Rockford, IL) and sealing the vials with Teflon-lined caps. The samples were heated at 80°C for one hour. After cooling, 0.5 to 1.0 μL of the reaction mixture was applied directly to the probe tip and the bis-(trimethylsilyl)-trifluoroacetamide was evaporated.

Thioglycerol and 3-nitrobenzyl alcohol, respectively, were used as matrices to record the FAB-MS spectra of the underivatized and derivatized adducts. A mixture of thioglycerol and glycerol (9:1, v:v) was used to record FAB-tandem MS spectra of the underivatized adducts. The FAB tandem MS spectra of the derivatized adducts were acquired in 3-nitrobenzyl alcohol.

### Capillary Electrophoresis Continuous-Flow Fast-Atom-Bombardment Mass Spectrometry

An FAB-MS system with the added dynamic of capillary electrophoresis was assembled with a homemade liquid junction interface (Wolf et al. 1991). Capillary electrophoresis was conducted using a buffer that consisted of a 0.01 M ammonium acetate ($NH_4OAc$) solution adjusted to pH 9.5 with ammonium hydroxide ($NH_4OH$) and a capillary electrophoresis column (50- to 70-cm long, 75-μm i.d., fused-silica capillary with a polyimide coating). Injections were made by siphoning 7.5 nL of the sample dissolved in methanol at the anode. The capillary electrophoresis analyses were conducted at 10 to 15 kV (7 to 11 μA). The dynamic FAB matrix consisted of 1% glycerol, 5% acetonitrile, and 19% methanol in water that flowed at a vacuum-induced rate of about 1 μL/min. A 65-cm, 50-μm i.d., fused-silica capillary with a polyimide coating was employed as the dynamic FAB capillary. A VG/Fisons Instruments Quattro triple quadrupole mass spectrometer fitted with a cesium ion FAB gun operated at 10 kV (2.7 A) was used. (Initially, the capillary electrophoresis continuous-flow FAB interface was tried on the VG70–250SE magnetic mass spectrometer. However, significant improvements in performance were achieved recently through its coupling to the VG/Fisons Instruments Quattro mass spectrometer.)

### Other Techniques

Fluorescent indicator plates from Analtech (Newark, DE) were used for analytical and preparative separations by TLC. $^1H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-300 spectrometer. All the glassware was cleaned by a procedure described by Fisher and Giese (1988).

## CHEMICAL SYNTHESES

For 2,3-pyrenedicarboxylic acid, finely powdered $KO_2$ (657 mg, 9.2 mmol), 18-crown-6 (611 mg, 2.3 mmol), and 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one (50 mg, 0.18 mmol) were suspended in dry benzene (20 mL). The mixture was stirred vigorously for 20 hours at room temperature in the dark, then 40 mL of water was added. The benzene layer was separated, and the water layer was filtered through a filter paper (Whatman, Clifton, NJ). The filtrate was acidified to pH of approximately 2 (pH paper) with concentrated hydrochloric acid under stirring, and extracted with ethyl acetate. After the ethyl acetate was evaporated on a rotary evaporator, the product was purified on a Dynamax $C_{18}$ semipreparative HPLC column (Rainin Microsorb, 250-mm × 10-mm i.d., 5-μm particles) with a Rainin $C_{18}$ guard column (50-mm × 10-mm i.d., 5-μm particles). The detection was at 264 nm. The mobile-phase program was: (1) a 15-minute equilibrium period with acetonitrile:0.1 M acetic acid (50:50) at a flow rate of 5 mL/min; (2) a linear increase of acetonitrile:0.1 M acetic acid to 58:42 over an eight-minute period; (3) a linear increase over one minute to 100% acetonitrile, which was maintained for 10 minutes; and (4) a linear change in a 10-minute period back to the initial composition. The time window for peak collection was 5.7 to 6.7 minutes. After the evaporation, the product gave: $^1H$ NMR (acetone $d_6$), d 8.21–8.45 (m, 7H, Ar), 8.90 (s, 1H, $H_1$), 11.94 (br s, 2H, COOH); MS(EI), 290 (M, 4%), 272 (M-water, 50%), 200 (M-$2CO_2H$, 100%). Anal. calcd for $C_{18}H_{10}O_4$: C, 74.47; H, 3.47. Found: C, 74.37: H, 3.41.

For 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate-1,2,-3,4,5,6,11,12,-$d_8$, finely powdered potassium carbonate (210 mg, 1.52 mmol, dried at 250°C) in 20 mL of acetonitrile was

allowed to stir at room temperature overnight. 2,3-Pyrenedicarboxylic acid (32 mg, 0.11 mmol) and pentafluorobenzyl bromide (1.4 mL, 9.27 mmol) were added. The derivatization was performed at 60°C (water condenser), with stirring until no more starting material could be seen on silica TLC (hexane:ethyl acetate was 9:1). The reaction mixture was filtered, and the acetonitrile was evaporated with a rotary evaporator. The product was purified twice by preparative silica TLC (20-cm × 200-cm, 1,000 μm particles) with hexane and ethyl acetate (9:1, v:v). This led to a light yellow powder, which produced a single peak on analytical HPLC. $^1$H NMR ($CDCl_3$), d 5.53, 5.64 (s, 2H each, $CH_2$), 8.05–8.26 (m, 7H, Ar), 8.67 (s, 1H, $H_1$); MS(EI), *m/z* 650 (M, 11%), 181 ($C_6F_5CH_2$, 100%).

2,3-Bis(tetrafluorobenzyl)pyrenedicarboxylate was synthesized and purified using the same procedure as for 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate, except that 2,3,-5,6-tetrafluorobenzyl bromide was substituted for pentafluorobenzyl bromide. $^1$H NMR ($CDCl_3$), d 5.58, 5.67 (s, 2H each, $CH_2$), 7.08–7.14 (m, 2H, $C_6F_4H$), 8.03–8.23 (m, 7H, Ar), 8.67 (s, 1H, $H_1$); MS(EI), 614 (M, 13%), 163 ($C_6F_4HCH_2$, 100%).

For β-3-pyrenoyl-$d_9$-propanoic acid (Bachmann et al. 1941), anhydrous aluminum chloride (1.20 g, 9 mmol) was added slowly, with cooling (ice-bath) and stirring, to a solution of succinic anhydride (0.48 g, 4.8 mmol) in nitrobenzene (20 mL). Pyrene-$d_{10}$ (1 g, 4.7 mmol) was then gradually introduced, and the color of the solution (yellow) immediately turned to deep red. The mixture was stirred at 0°C for three hours, then hydrolyzed by the careful addition of diluted hydrochloric acid (3 mL of concentrated hydrochloric acid in 9 mL of water). The yellow precipitate was filtered and washed with water. The filter cake then was redissolved in hot aqueous sodium carbonate (12 g of sodium carbonate in 100 mL of water) and filtered immediately in order to remove aluminum hydroxide. The filtrate, upon cooling to room temperature, produced a solid, which was the sodium salt of β-3-pyrenoyl-$d_9$-propanoic acid. This was filtered and recrystallized from acetic acid to yield yellow needles (1.12 g, 82%). $^1$H NMR (DMSO), d 2.77, 3.49 (t, 2H each, $CH_2$), 12.26 (br s, 1H, $CO_2H$).

For γ-pyrenyl-$d_9$-butyric acid (Bachmann et al. 1941), zinc (10 g, 20 mesh) was cleaned by stirring in 10% hydrochloric acid (2.6 mL of concentrated hydrochloric acid in 7.4 mL of water) for two minutes, and then washing it with water (3 × 10 mL). The zinc then was amalgamated with mercury(II) chloride (1 g). The amalgamated zinc was placed in a 100-mL round-bottom flask. Then, 7.5 mL of water, 10 mL of concentrated hydrochloric acid, 10 mL of *p*-xylene, and 1 g of β-3-pyrenoyl-$d_9$-propanoic acid were added. The mixture was refluxed for six hours, and concentrated hydrochloric acid (2 × 5 mL) was added during this time. The reaction mixture was allowed to cool until the crystallization was completed. The crystals, together with the zinc, were filtered, washed with water, and dried under a vacuum. The product, with the zinc, was digested with a hot sodium hydroxide solution (3 g of sodium hydroxide in 150 mL of water) to separate the reduced acid from the insoluble colorless by-product and zinc. Upon acidifying the alkaline solution with concentrated hydrochloric acid, the product was obtained as a chalk-white solid (0.87 g, 91%), which was washed (water, then a small amount of benzene) and dried under vacuum. $^1$H NMR (DMSO), d 2.02 (m, 2H, $CH_2$), 2.40, 3.34 (t, 2H each, $CH_2$), 12.10 (br s, 1H, $CO_2H$).

For 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one-1,2,3,4,5,6,11,12-$d_8$ (Bachmann et al. 1941), phosphorus pentachloride (460 mg, 2.20 mmol) was added in portions to a stirred solution of γ-pyrenyl-$d_9$-butyric acid (600 mg, 2.02 mmol) in 50 mL of dry benzene. The mixture was stirred at room temperature under nitrogen for 1.5 hours. Stannic chloride (0.3 mL, 2.55 mmol) was added to the clear, dark yellow solution, and the mixture was stirred at room temperature under nitrogen for eight hours. The purple complex was decomposed by adding ice and concentrated hydrochloric acid with vigorous stirring until a clear, yellow two-phase solution was formed. The benzene layer was separated, washed twice with water, and dried with anhydrous sodium sulfate. The solvent then was removed under vacuum. The residual solid was recrystallized from dichloromethane and methanol to render a yellow product (260 mg, 46%). $^1$H NMR ($CDCl_3$) d 2.24 (m, 2H, $CH_2$), 2.74, 3.30 (t, 2H, each, $CH_2$); $^{13}$C NMR ($CDCl_3$) d 22.9, 25.8, 38.7, 122.8, 124.0, 124.8, 125.0, 126.7, 128.0, 128.9, 129.3, 131.1, 137.3, 199.0 (CO).

For 2,3,-bis(pentafluorobenzyl)pyrene dicarboxylate-1,2,3,4,5,6,11,12-$d_8$, a solution of 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one-1,2,3,4,5,6,11,12-$d_8$ (100 mg, 0.36 mmol) in 10 mL of DMF was added to a suspension of $KO_2$ (767 mg, 10.80 mmol) and 18-crown-6 (1.14 g, 4.30 mmol) in 10 mL of DMF in a 100-mL round-bottom flask. The oxidation was conducted at room temperature, in the dark, with stirring. The reaction was stopped after 24 hours by adding water (5 mL), and the mixture was carefully acidified with concentrated hydrochloric acid until a precipitate formed. The reaction mixture then was extracted with ethyl acetate (3 × 20 mL), dried over sodium sulfate, and evaporated to dryness. The crude product was esterified directly with pentafluorobenzyl bromide (1 mL) and triethylamine (0.5 mL) in acetonitrile (20 mL) at 60°C for four hours. The product was purified by preparative TLC using a 1:1 mixture of hexane and dichloromethane and recrystallized from dichloromethane and methanol to produce white crystals (144 mg, overall yield of 61% from 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one-1,2,3,4,5,6,11,12-$d_8$). $^1$H NMR ($CDCl_3$), d 5.53 (s, 2H each, $CH_2$).

## CHEMICAL TRANSFORMATION TECHNIQUES

### Superoxide Oxidation

For oxidation, 70 μL of a Tetrol stock solution (1.56 pg/μL in methanol) was added in triplicate to a 1-mL clear silanized Micro-V vial (American Scientific Products). The sample was dried at 40°C under nitrogen; this was followed by the addition of 25 μL of DMF containing 200 μg of $KO_2$ and 190 μg of 18-crown-6 ether. The vial was capped with a Teflon-lined screw-cap, and kept at room temperature, in the dark, for 20 hours, with vortexing for 30 seconds every 30 minutes for the first six hours. Water (25 μL) was added, and then two drops of acetic acid. The solvent was removed at 70°C under nitrogen.

The 2,3-pyrenedicarboxylic acid formed was derivatized in the same vial by adding 30 μL of acetonitrile containing 0.8 μL of pentafluorobenzyl bromide and 0.15 μL of triethylamine. The vial was capped and kept at 60°C for six hours with vortexing for 30 seconds every 30 minutes. After cooling and the addition of 70 μL of acetonitrile, the sample was kept at 4°C overnight before the postderivatization cleanup by HPLC.

The mobile-phase program for the quantification of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate included a 10-minute equilibrium period with a mixture of water and acetonitrile (15:85) at 1 mL/min. Upon injection, the mixture of water and acetonitrile was changed linearly to 5:95 in 10 minutes. This composition was kept constant for five minutes. Then, 100 μL of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate standard (36.05 pg/μL in acetonitrile) was injected to establish the retention time. The injector was washed with hot acetonitrile (2 × 1 mL). Acetonitrile (3 × 100 μL) was then injected. After 10.4 to 11.6 minutes, a fraction was collected from the third acetonitrile injection, evaporated, and analyzed by GC-ECD to assure that the HPLC system was clean.

The sample was removed from the refrigerator, left at room temperature (30 minutes), vortexed well, and allowed to sit for 5 to 10 minutes. The entire volume then was injected onto an HPLC column. Three blanks were injected first, and then the three samples were injected. A fraction was collected from 10.4 to 11.6 minutes for each sample into a 2-mL silanized Reacti-Vial. The injector was washed with hot acetonitrile (2 × 1 mL) after each injection. After evaporation at 60°C under nitrogen, the residue was redissolved in 100 μL of acetonitrile, and the entire volume was again purified as before on the same HPLC column. Thirty microliters of GC internal standard, 2,3-bis(tetrafluorobenzyl)pyrenedicarboxylate, was added to each of the second collection vials prior to evaporation.

For GC-ECD, a standard curve was established by injecting 1 μL of the three GC standards containing 1.2 to 12.1 fmol/μL of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate (A) and 6.6 fmol/μL 2,3-bis(tetrafluorobenzyl)pyrenedicarboxylate (B) in acetonitrile. The ratio of the concentration of A:B was plotted against the ratio of the peak areas, using the Least Square Quantitation Equation (Waters Chromatograph, [Milford, MA] 840 data system), along with the correlation coefficient. Isooctane was injected before and after each standard injection. After the HPLC solvent was evaporated at 60°C under nitrogen, the residue was redissolved in 30 μL of isooctane and vortexed. Then, 1 μL of each sample was injected into the GC-ECD system for quantification. The peak area was obtained by manual integration using the 840 data system.

After the GC-ECD quantification, 100 μL of a 524.6 fg/μL solution of deuterated internal standard in acetonitrile was added to both blanks and samples and evaporated at 60°C under nitrogen. The residue was redissolved in 100 μL of isooctane, and 1 μL was injected into the GC-ECNI-MS system.

### Hydrazinolysis Procedure

A methanolic solution (5 μL from a more concentrated stock solution) of *N*-(dG-8-yl)-2-AF (116.5 ng) was evaporated in a 1-mL ampule, and 20 μL of anhydrous hydrazine was added. The ampule was flame-sealed and heated to 180°C for 24 hours in a Reacti-Block aluminum block E-1 (13-ampule capacity) (Pierce). This heating was achieved by placing the block in a Pyrex crystallizing dish (50- × 75-mm i.d.) that had been filled to a depth of 3.5 cm with silicone oil. Each hole in the heating block had been filled to a depth of 1.5 cm with sea sand in order to heat primarily the bottom of the ampule. The ampule was cooled to room temperature, its top was removed, and the excess reagent was evaporated to dryness using a Speed-Vac concentrator (Sarant Instruments, Farmingdale, NY). After the addition of 100 μL of an acetonitrile and water mixture (2:1, v:v) 10 μL was injected into an HPLC system.

## STUDY DESIGN

The goal of the project was to develop new and improved analytical methodology for the measurement of PAH-DNA adducts by both qualitative and quantitative MS. Because of the small amounts of DNA adducts encountered, the key challenge was to improve the sensitivity. This goal was the driving force behind all of the experimental work conducted in this project. Ultimately, the more sensitive MS methodology will be used both in combination with other methods for DNA adducts, and also independently. For example, an unknown adduct discovered by $^{32}P$-postlabeling TLC potentially could be structurally elucidated by qualita-

tive MS, if the amounts of the adduct could be scaled up enormously and the sensitivity of MS greatly improved. The best way to proceed, obviously, is first to test known DNA adducts as a way to improve the sensitivity and provide a knowledge base for improved MS techniques, so that unknown, trace adducts can be tested.

The chemical transformation of PAH-DNA adducts for quantitative detection by GC-ECNI-MS, as proposed, is a two-step process in which the PAH moiety first is released from the DNA, and then electrophore-derivatized before the MS detection. Ideally, all of the PAH adducts of a given class (e.g., all diolepoxide PAH-DNA adducts) release at a given step and also release the PAH moiety in a form that is easy to electrophore-derivatize. With these features, chemical transformation could go beyond determining known PAH-DNA adducts and discover individual unknown adducts of a given class. The latter capability would require that the unknown adducts be detected initially under scanning conditions, which could compromise the sensitivity of a conventional GC-ECNI-MS instrument by a factor of about 1,000 relative to the detection limit it can achieve by selected ion monitoring. However, once an ion (a particular $m/z$ value) is discovered, the corresponding compound subsequently could be detected with high sensitivity by relying on selected ion monitoring. Alternatively, this loss in sensitivity in the scanning mode potentially could be reduced by using an array detector (Evans 1990). An advantage of the proposed, novel chemical transformation and GC-ECNI-MS concept is that many PAH-DNA adducts within a given class can be detected, in principle, by a single method.

Another important advantage of chemical transformation and GC-ECNI-MS is that the adducts, by design, are converted into products with good recovery characteristics. This means that the products are stable, structurally homogeneous, and very soluble in common solvents. One of the general difficulties of trace analysis is that analytes tend to undergo losses during sample preparation. Analytes with good recovery characteristics are easier to purify without losses.

The price to be paid for these advantages of chemical transformation is the loss of structural information about the parent analyte (the adduct as it exists on DNA), and the burden of subjecting the analyte to an additional step before its detection. But the loss of structural information eventually can be overcome by scaling up the amount of the discovered, unknown adduct into the range of FAB-MS or a related MS technique. Potentially, the burden of the chemical transformation step can be minimized, and also balanced, by the specificity that it adds to the overall procedure. Unfortunately, because the chemical transformation product may be formed from more than one precursor compound, this technique, in general, will be more susceptible to interferences.

## RESULTS

### CHEMICAL TRANSFORMATION TECHNIQUES

Three chemical transformation techniques in conjunction with quantitative MS were introduced in this project for the potential measurement of PAH-DNA adducts: superoxide oxidation, hydrazinolysis, and hydrogenation, as summarized in Figure 1. These techniques will be presented first, followed by our results on the direct detection of standards of PAH-deoxynucleoside adducts by FAB-MS.

#### Superoxide Oxidation for Diolepoxide PAH-DNA Adducts

We worked toward a goal of establishing a general assay, based on GC-ECNI-MS, for diolepoxide PAH-DNA adducts. One such adduct, as a prototype, has been studied previously by several investigators (reviewed by Singer and Grunberger 1983). This adduct arises from the reaction of *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene onto the $N^2$ position of guanine residues in DNA. The resulting adduct, similar to related adducts at this site, can be hydrolyzed by mild acid hydrolysis of the DNA (Vahakangas et al. 1985). For the prototype, this releases Tetrol. Thus, as a first step toward our goal, we undertook the development of an assay for the latter compound.

By reaction with pentafluorobenzyl bromide, carboxylic acids can be converted into corresponding esters that are sensitive for detection by GC-ECD (Gyllenhaal 1978) and GC-ECNI-MS (Hofmann et al. 1990). These esters undergo dissociative electron capture to form a structurally characteristic carboxylate anion in high yield. Thus, the detection of such esters by GC-ECNI-MS can be specific. This makes

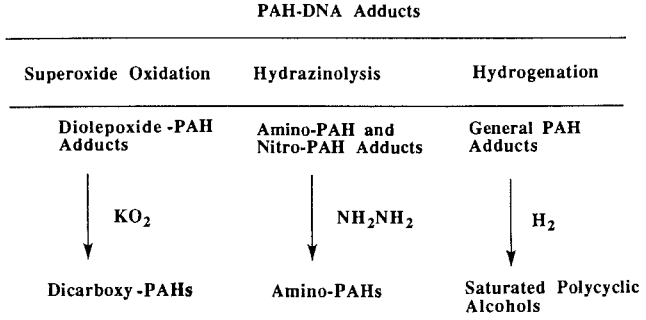
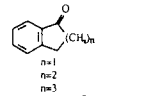
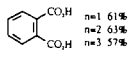
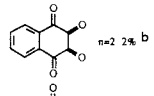
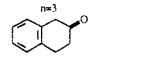
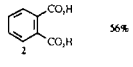
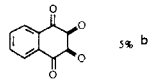
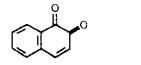
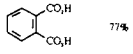
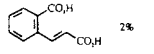
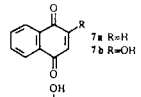
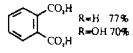
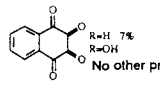
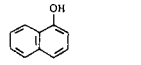
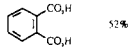
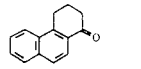
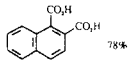
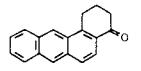
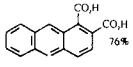
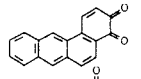
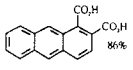
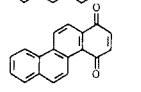
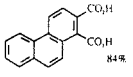
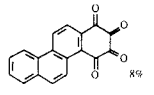
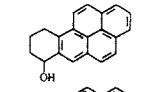
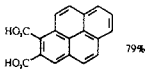
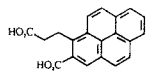
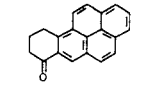
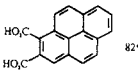
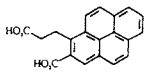
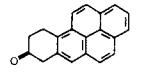
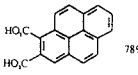
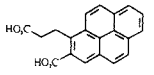
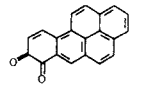
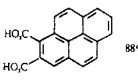
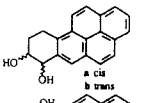
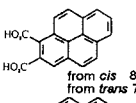
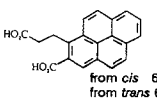
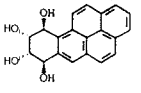
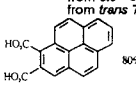
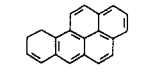



**Figure 1. Chemical transformation techniques initiated in this project for PAH-DNA adducts.**

**Table 1.** Potassium Superoxide Oxidation of Various Derivatives of Aromatic Hydrocarbons[a]

| Compound | Major Product, Yield (%) | Minor Product, Yield (%) |
|---|---|---|
|  |  n=1 61% n=2 63% n=3 57% |  n=2 2% [b] |
|  |  56% |  5% [b] |
|  |  77% |  2% |
|  7a R=H 7b R=OH |  R=H 77% R=OH 70% |  R=H 7% R=OH No other product |
|  |  52% | No other product |
|  |  78% | Not isolated |
|  |  76% | Not isolated |
|  |  86% | Not isolated |
|  |  84% |  8% |
|  |  79% |  11% |
|  |  82% |  9% |
|  |  78% |  15% |
|  |  88% | Not isolated |
|  a cis b trans |  from *cis* 83% from *trans* 79% |  from *cis* 6% from *trans* 6% |
|  |  80% | No other product |
|  |  86% | No other product |

[a] The oxidations were carried out with 1 mg of starting material, a 100-fold molar excess of $KO_2$, and a 40-fold excess of 18-crown-6-ether in 1 mL of DMF for 20 hours at room temperature. Product yields were obtained by HPLC.

[b] Traces of 3-(2-carboxyphenyl)-propionic acid were detectable in <1% yield.

it potentially attractive to oxidize the Tetrol to 2,3-pyrenedicarboxylic acid.

We tested several oxidation reagents for their ability to achieve this kind of oxidation (data not shown). We discovered that such a conversion can take place with $KO_2$. In fact, we found that high yields of aromatic *ortho*-dicarboxylic acids can be obtained in general by the reaction of $KO_2$ with quinones, cyclic alcohols and ketones fused to various aromatic hydrocarbons (Sotiriou-Leventis et al. 1990). Table 1 summarizes representative reactions that we have conducted with $KO_2$. Related substrates also have been studied (Li et al. 1993). It is important to employ a balanced molar ratio of $KO_2$:crown ether:substrate (such as 100:40:1) in an aprotic solvent such as DMF, under oxygen or air. The identification of minor side products and a study of reaction conditions led to mechanistic insights that are reported elsewhere (Sotiriou-Leventis et al. 1990). 1-Nitropyrene-*cis*-dihydrodiols were found in this project to give corresponding lactones when subjected to $KO_2$ oxidation (Abdel Baky et al. 1991a).

Not only did $KO_2$ oxidation of the Tetrol give a good yield of 2,3-pyrenedicarboxylic acid, but the characteristics of the reaction made it attractive for use at the trace level. The reaction takes place at room temperature and yields the product dissolved in a clear, aqueous-organic solution when quenched, at its conclusion, with aqueous acetic acid. When chemical reactions are required in trace organic analysis, it can be important to select reactions that yield soluble side products (including residual reagents and their decomposition products) to enhance recovery of the desired product.

We established that the product, 2,3-pyrenedicarboxylic acid, could be smoothly converted into a corresponding bis-pentafluorobenzyl diester by its reaction with pentafluorobenzyl bromide. As anticipated, the latter compound, as a diluted standard, could be detected by GC-ECNI-MS at the attomole level. This is shown in Figure 2. In order to achieve this detection limit, we used unusual conditions in the GC-ECNI-MS. The column head pressure was increased, as were the settings for the pressure, temperature, electron energy, and emission current (Abdel-Baky and Giese 1991).

We prepared a stable isotope internal standard: 9,10-dihydrobenzo[*a*]pyrene-7[8H]-one-1,2,3,4,5,6,11,12-$d_8$. This compound also is oxidized by $KO_2$ to a corresponding dicarboxylic acid. Compounds 2 and 3, shown in Figure 3, were prepared as other standards to help develop the method.

Initially, the $KO_2$ oxidation and pentafluorobenzylation reactions were conducted at the millimole level. Then, we began the progressive extension of the two reactions, as a

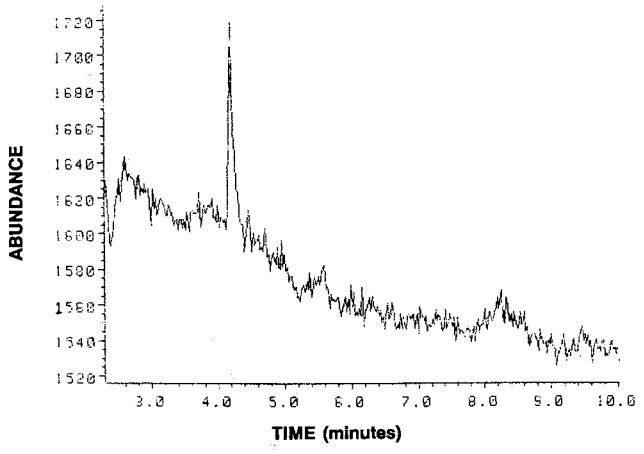



**Figure 2. Detection of 14 amol of a standard of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate by GC-ECNI-MS** (Hewlett-Packard 5890 gas chromatograph, 5988A mass spectrometer).

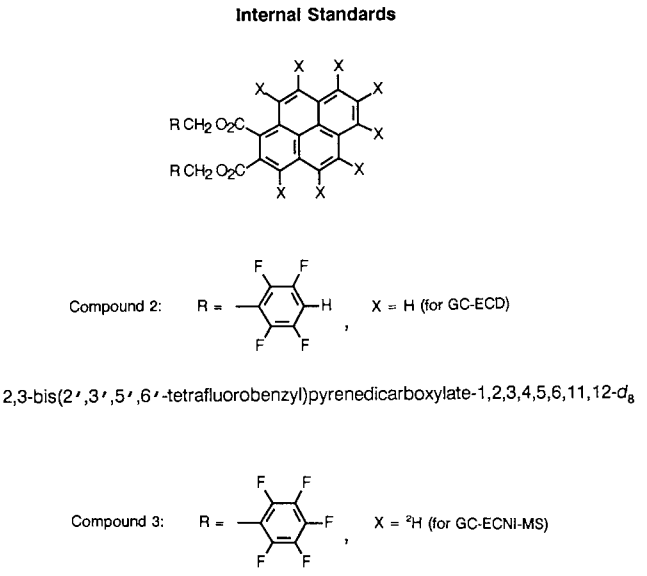



**Figure 3. Internal standards for the scheme shown in Figure 4.**

two-step reaction sequence, to lower levels. Our goal was to find the level, as close as possible to the attomole level, at which we could detect the final diester as a diluted standard. Initially, the reactions were monitored by HPLC, with UV detection, because both products could be detected in this way down to the low nanomole level (data not shown). This strategy was convenient for the optimization of the reaction conditions, for the coupling of the reactions, and for the post-reaction cleanup. Subsequent work, below the nanomole level, was monitored by GC-ECD and GC-ECNI-MS.

This work led to the scheme shown in Figure 4, which was successful when applied to as little as 313 fmol of the Tetrol. In this scheme, the Tetrol was first oxidized with $KO_2$ in the presence of 18-crown-6 in DMF at room temperature in the dark for 20 hours (Figure 4, step 1). The 2,3-pyrenedicarboxylic acid product then was derivatized with pentafluorobenzyl bromide in the presence of triethylamine in acetonitrile, at 60 °C for six hours (Figure 4, step 2). The reaction volume was only 30 μL, thereby allowing the entire sample to be diluted simply with acetonitrile (70 μL) and injected into an HPLC column for postderivatization cleanup. Vortexing was used instead of magnetic stirring because a Teflon-coated stirring bar caused an adsorption loss of the analyte, and a glass-coated stirring bar was fragile.

Postderivatization sample cleanup by HPLC is attractive because it is reproducible, convenient, and allows a high recovery. For example, a quantitative recovery was obtained when 0.9 fmol of standard bis-pentafluorobenzyldiester was injected into the HPLC column, then collected, evaporated, redissolved, and detected by GC-ECNI-MS (data not shown). The potential to automate the HPLC separation for this method is also important. Because interferences were en-

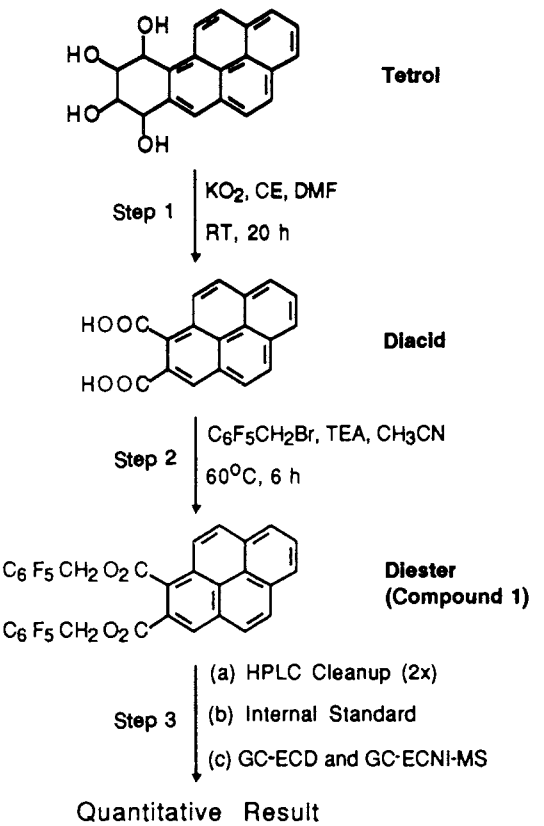



**Figure 4. Scheme for the measurement of Tetrol by superoxide oxidation, electrophore derivatization, GC-ECD, and GC-ECNI-MS.** CE = 18-crown-6 ether; RT = room temperature; $C_6F_5CH_2Br$ = pentafluorobenzyl bromide; TEA = triethylamine; $CH_3CN$ = acetonitrile.

countered in the subsequent GC-ECD step after a single passage of the sample through the HPLC column, the HPLC purification was done twice (step 3a in Figure 4). The same column was used twice for each sample, and the column was carefully washed between the injections to prevent analyte carryover.

After the second HPLC separation, the first internal standard, 2,3-bis(2′,3′,5′,6′-tetrafluorobenzyl)-pyrenedicarboxylate-1,2,3,4,5,6,11,12-$d_8$ (compound 2 in Figure 3) (for GC-ECD purposes; see below), was added to each sample (step 3b in Figure 4); this was followed by evaporation. The sample was redissolved in 30 μL of isooctane, and 1 μL was injected into the GC-ECD for quantification (step 3c in Figure 4). This resulted in the GC-ECD chromatograms shown in Figure 5, in which A is the reaction mixture (starting from 313 fmol of Tetrol), and B is the blank (entire procedure starting from 0 fmol of Tetrol). As shown in Figure 5, the chromatogram for the reaction blank was reasonably clean at this level of sensitivity in the elution region of interest. The overall, absolute average yield for the triplicate sample was 69% (77%, 68%, and 62%), based on a standard curve constructed by measuring samples containing increasing amounts of the analyte compound 1 and a constant amount of compound 2.

After the GC-ECD quantification, the second internal standard, 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate-1,2,3,4,5,6,11,12-$d_8$ (compound 3 in Figure 3), was added to all of the same samples. The subsequent GC-ECNI-MS provided the chromatograms shown in Figure 6. Chromatogram A is from the reaction mixture, and B is the blank. In this case, the yield was 60% (80%, 50%, 49%), which agrees with the GC-ECD value.

As is apparent from comparing Figures 5 (GC-ECD) and 6 (GC-ECNI-MS), only the latter method can be trusted at lower analyte levels. This reaffirms our earlier observation that GC-ECD tends to be useful in procedures like this down to only the midfemtomole level (Fisher and Giese 1988). Because GC-ECD was compared with GC-ECNI-MS in this experiment, internal standard compounds 2 and 3 were added late in the current procedure just to monitor the GC step.

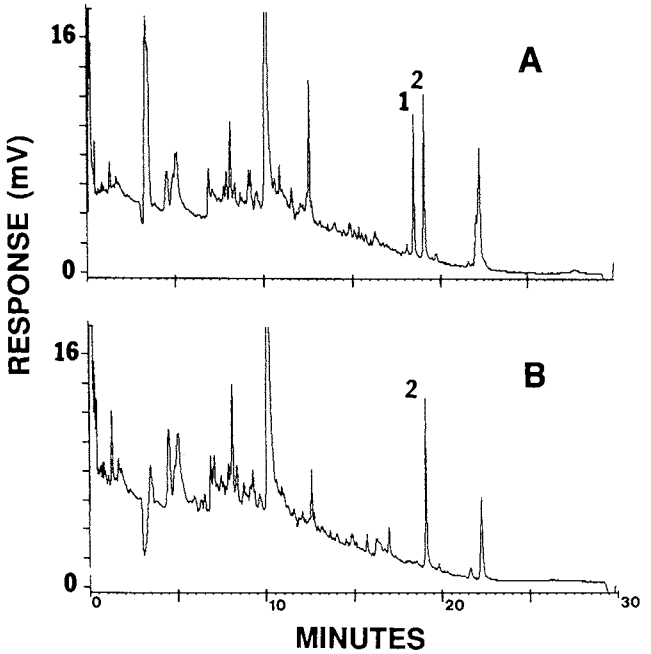



**Figure 5. The GC-ECD chromatograms for the oxidation and derivatization of 313 fmol of Tetrol.** After the second HPLC cleanup, 30 μL of an internal standard solution of compound 2 (6.6 fmol/μL in acetonitrile shown in Figure 3) was added to blanks and samples, and evaporated at 60°C under nitrogen. The residue was redissolved in 30 μL, and 1 μL was injected. A: reaction mixture. B: reaction blank. Peak 1 = 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate; and peak 2 = 2,3-bis(tetrafluorobenzyl)pyrenedicarboxylate.

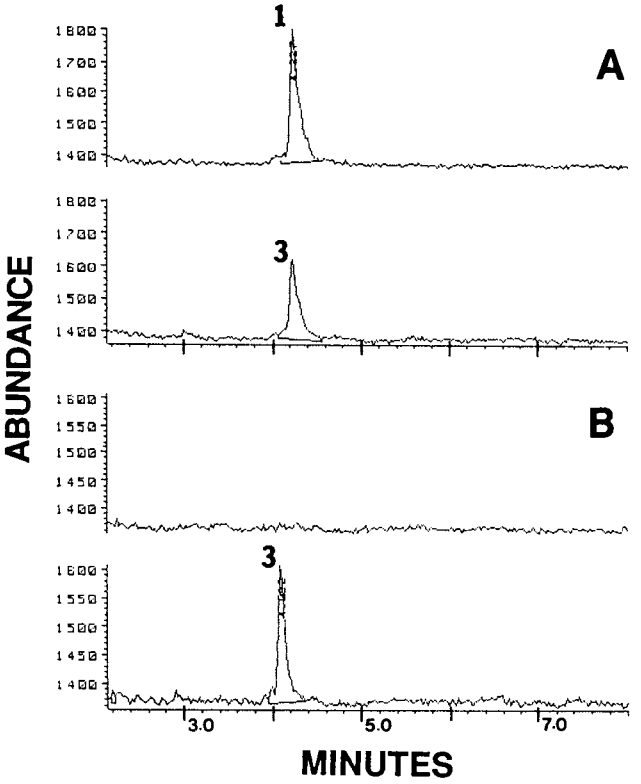



**Figure 6. The GC-ECNI-MS chromatograms for the oxidation and derivatization of 313 fmol of Tetrol.** After GC-ECD quantification, 100 μL of an internal standard solution of compound 3 (52.6 fg/μL in isooctane shown in Figure 3) was added to both blanks and samples, and evaporated at 60°C under nitrogen. The residue was redissolved in 100 μL of isooctane, and 1 μL was injected. A: reaction mixture. B: reaction blank. Peak 1 = 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate; and peak 3 = 2,3-bis(pentafluorobenzyl)pyrene- $d_8$-dicarboxylate.

In parallel work, the superoxide oxidation procedure was extended to nitro-dihydrodiol PAHs by applying it to two model compounds: *cis*-4,5-dihydro-4,5-dihydroxy-3-nitropyrene (compound 4), and *cis*-4,5-dihydrodiol-4,5-dihydroxy-1-nitropyrene (compound 5) (Abdel-Baky et al. 1991a). The $KO_2$ oxidation of compound 4 produced the known lactone, 3-nitro-5H-phenanthro[4,5-*bcd*]pyran-5-one (compound 6). Similarly, the analogous 4,5-dihydrodiol (compound 5) produced the known lactone, 1-nitro-5H-phenanthro[4,5-*bcd*]-pyran-5-one (compound 7). Although the yield of compound 6 was 80%, it was only 16% for compound 7. A study of the latter oxidation, relying especially on the use of HPLC, led to a change in the conditions that increased the yield of compound 7 from 16% to 88%. The change was to quench the reaction with hydrogen peroxide shortly after it began instead of letting it proceed, as usual, for several hours before quenching it with water. This change was based on the identification of an α-ketoacid intermediate, which is known to undergo oxidative decarboxylation under basic conditions by hydrogen peroxide (House 1965).

### Hydrazinolysis Procedure for Amino-PAH– and Nitro-PAH–DNA Adducts

Along with our development of chemical transformation and GC-ECNI-MS methodology to measure diolepoxide-PAH–DNA adducts, we also set out to bring amino-PAH– and nitro-PAH–DNA adducts within the scope of GC-ECNI-MS. We found that appropriate chemical transformation for this can be achieved by high temperature hydrazinolysis (Bakthavachalam et al. 1991). For example, as shown in Figure 7, the DNA adduct *N*-(dG-8-yl)-2-AF yields 2-AF by this reaction.

In the optimized, overall procedure that we developed, the parent DNA adduct was subjected to hydrazinolysis at 160°C for 24 hours. The reaction shows promise for being applied to a trace amount of analyte. For example, the yield of 2-AF, starting from 115 ng of the adduct, was 62% ± 2%. The related adduct, *N*-acetyl-*N*-(dG-8-yl)-2-AF, also yielded the same amount of 2-AF. As shown in Figure 8, aside from nonretained, polar compounds, analysis of the crude reaction mixture by HPLC shows only a single peak, which is the product.

The hydrazinolysis reaction was tested on nitrobenzene, 1-nitropyrene, and 2,7-dinitrofluorene as models for DNA adducts containing a nitro-PAH moiety (Abdel-Baky et al. 1991b). Yields of corresponding amines ranged from good to excellent, as summarized in Table 2. The cited yields were achieved by heating these compounds in hydrazine for two hours or less in a sealed ampule at 160°C.

The speed of the reaction was examined with nitrobenzene. When this compound was heated in hydrazine for 5, 15, and 120 minutes at 160°C, the yields of aniline were 80%, 95%, and 95%, respectively. Aside from a minor, early-eluting side product, only peaks for starting material and the product were observed in the HPLC chromatograms, and mass balance was maintained. This shows that the intermediates in the reaction mixture were quickly converted to the product, which is stable under the reaction conditions.

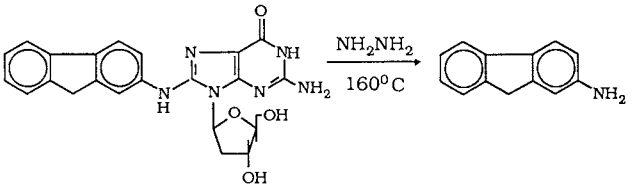



**Figure 7. Chemical transformation of *N*-(dG-8-yl)-2-AF by hydrazinolysis.**

Two other types of compounds were tested in an initial effort to examine the scope of the reaction. Bromobenzene

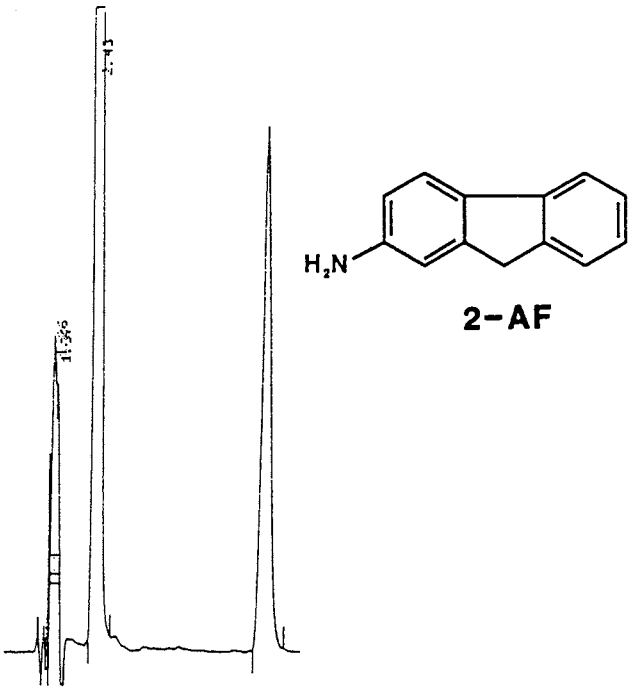
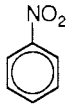
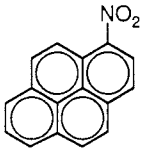
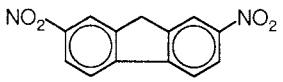



**Figure 8. The HPLC separation of the hydrazinolysis reaction products.** *N*-(dG-8-yl)-2-AF (13 nmol) was subjected to hydrazinolysis in 150 µL of hydrazine. The reaction mixture was treated with 100 µL of water and 150 µL of methanol; then 10 µL of this solution was injected into the HPLC column (Brownlee, Rainin Instruments, analytical cartridge, RP-8 reverse-phase, 10-cm × 4.6-mm i.d., 5-mm particle size). The mobile phase used acetonitrile (10 mM) potassium dihydrogen phosphate adjusted to pH 6.6 with 5 mM triethylamine (45:55, v:v) at 1 mL/min. Detection was at 280 nm, and retention time was 6.8 minutes.

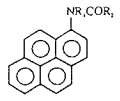
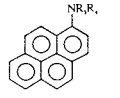
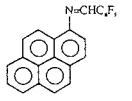
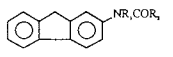
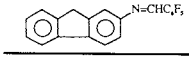
**Table 2.** Hydrazinolysis of Nitroaromatic Compounds

| Starting Material[a] | Product | Yield |
|---|---|---|
|  | Aniline | 95% |
|  | 1- Aminopyrene | 90% |
|  | 2,7-Diaminofluorene | 62% |

[a] The starting materials, from top to bottom, are nitrobenzene, 1-nitropyrene, and 2,7-dinitrofluorene.

[b] The yields were determined by reverse-phase HPLC calibrated with external standards. Column: Brownlee 4.6- × 220-mm R-18, 5-μm particles, operated isocratically at 1 mL/min with NaOAc (0.1 M, pH 6.5) and methanol as solvents. For aniline, retention time was 4.6 minutes in 65:35 NaOAc:methanol. For 1-aminopyrene, retention time was 7.3 minutes in 40:60 NaOAc:methanol. For 2,7-diaminofluorene, retention time was 7.0 minutes in 65:35 NaOAc:methanol.

**Table 3.** Electrophoric Derivatives of 1-Aminopyrene and 2-Aminoflourene

| Structure[a] | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
|  | H | $C_3F_7$ | | |
| | $CH_3$ | $C_3F_7$ | | |
| | $CH_2C_6F_5$ | $C_3F_7$ | | |
| | H | $C_6F_5$ | | |
| | $CH_3$ | $C_6F_5$ | | |
|  | | | H | $CH_2C_6F_5$ |
| | | | $CH_2C_6F_5$ | $CH_2C_6F_5$ |
|  | | | | |
|  | H | $C_3F_7$ | | |
| | $CH_3$ | $C_3F_7$ | | |
| | $CH_2C_6F_5$ | $C_3F_7$ | | |
|  | | | | |

[a] The first three base structures in this column are pyrene; the last two are flourene. A given pair of the $R_1$, $R_2$ or $R_3$, $R_4$ groups, as appropriate, further defines the structures of these compounds by being inserted at the indicated positions.

was not affected by the reaction conditions, whereas benzoic acid was converted quantitatively into benzoylhydrazine.

Because we were able to transform chemically amino-PAH– and nitro-PAH–DNA adducts into corresponding amino-PAHs by high temperature hydrazinolysis, it was appropriate to optimize the electrophoric derivatization of the latter class of compounds for detection by GC-ECNI-MS (Bakthavachalam et al. 1991). 1-Aminopyrene and 2-AF were studied as model compounds, and the derivatives shown in Table 3 were synthesized.

Taking into account the ease of formation, yield, stability, and fragmentation characteristics by GC-ECNI-MS, the best derivative that we formed was the pentafluorobenzylidene product. It forms rapidly in high yield, is hydrolytically stable above pH 6, and gives an intense molecular anion by GC-ECNI-MS. For example, Figure 9 shows the detection of 28 amol, at a signal-to-noise ratio of 40, of the pentafluorobenzylidene derivative of 2-AF. An *N*-heptafluorobutyryl-*N*-pentafluorobenzyl derivative also was worthy of further study.

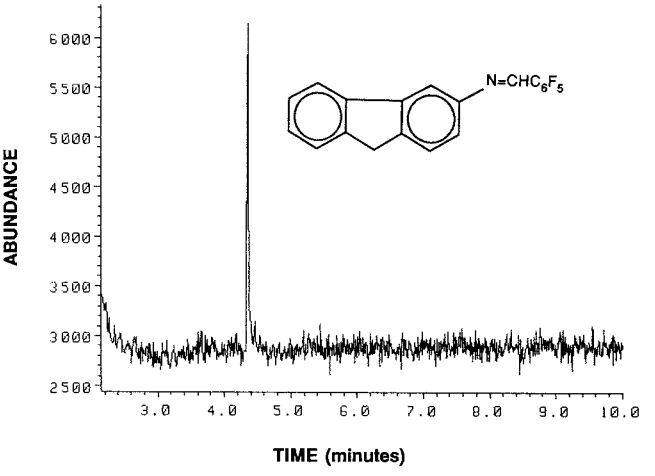



**Figure 9. Detection of *N*-pentafluorobenzylidenyl-2-AF (N=CHC$_6$F$_5$) by GC-ECNI-MS.** Toluene (1 μL) containing 28 amol of analyte was injected via splitless mode with splitless valve open after 0.8 minutes into a Hewlett-Packard (model 5890/5988A) GC column (12-m × 0.2-mm i.d., 0.33-mm film, Ultra-1 [Hewlett-Packard] fused-silica capillary, with 20 psi helium carrier gas). The following conditions were used: 2 torr of methane in the source, electron energy 240 eV, source temperature 200°C, emission current 300 mA, interface between GC and MS at 280°C, initial GC oven temperature 100°C, then increased to 280°C at 60°C/min and held for five minutes. Signal-to-noise ratio was 40.

## Hydrogenation Procedure for Unknown Bulky DNA Adducts

To the best of our knowledge, DNA has never been subjected to a high pressure and high temperature hydrogena-

tion reaction. What would one anticipate to happen? In theory, the DNA should largely "disappear" because volatile products such as methanol and ammonia should form from the nucleobases. This is due to the high content of nitrogen and oxygen atoms in these bases. This would constitute, in a sense, a reversal of the biosynthesis of DNA, because the bases of DNA are formed from simple molecules such as ammonia. How might such a reaction be used? Potentially, it could facilitate the detection of many bulky adducts on DNA, because the remnants of the adducts would tend to remain behind at the conclusion of the hydrogenation. This is because many bulky DNA adducts are either not heteroaromatic, or only partly so, and thereby would not disintegrate, as would DNA nucleobases, under such conditions. An analogy is that detecting a needle in a haystack could be made easier by first burning the haystack.

We have made a start on high pressure and high temperature hydrogenation as a novel chemical transformation technique to detect unknown bulky DNA adducts. First, we designed and built a low-cost apparatus that allowed a medium pressure and high temperature hydrogenation reaction to be conducted in a glass vessel on a small scale (Kresbach et al. 1991). Using this apparatus, we found that both the DNA adduct *N*-acetyl-*N*-(dG-8-yl)-2-AF and 2-AF yielded 2-hydroxyperhydrofluorene. The reaction of the former compound is summarized in Figure 10. The product was obtained as a mixture of at least two isomers by TLC. Completion of the reaction (under 250 psi hydrogen, at 180°C) required 10 days. A minor product from the reaction appeared to be an ether or amine dimer of 2-AF.

Encouraged by these results, we obtained a high-pressure autoclave from Berghof/America (Concord, CA) and adapted it for a high-pressure and high-temperature hydrogenation reaction. DNA (20 mg) suspended in isopropanol was hydrogenated (under 1,000 psi of hydrogen, at 150°C, with palladium and carbon, for 24 hours). Although DNA was initially visible at the start of the reaction, no DNA was visible when the reaction vessel was opened, and no organic-soluble products were apparent either. All that remained in the evaporated, aqueous extract was a white, water-soluble salt that tested positive for phosphorous. (Apparently, the salt is sodium phosphate.) As before, both *N*-acetyl-*N*-(dG-8-yl)-2-AF and 2-AF yielded 2-hydroxyperhydrofluorene;

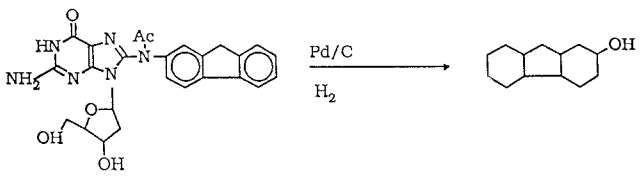



**Figure 10. Chemical transformation of *N*-acetyl-*N*-(dG-8-yl)-2-AF by hydrogenation.**

however, the minor side product was no longer present. The preparative yield of product was nearly 80% from 2-AF. See Appendix A for an update.

## MASS SPECTROPHOTOMETRIC TECHNIQUES FOR ANALYSIS OF PAH-DNA ADDUCTS

### Static Fast-Atom-Bombardment Mass Spectrometry

In order to extend the sensitivity of detecting and characterizing amino-PAH–DNA adducts by FAB-MS, four adducts, as deoxynucleosides, were studied by static FAB-MS (Annan et al. 1990). The matrix was optimized for sensitivity, and a number of additives were investigated to increase the ionization efficiency. The use of trimethylsilyl derivatives and metastable ion analysis, to improve both the level of detection and the information content of the spectra, was investigated. The CID spectra of both the protonated molecule ($[MH]^+$) and the protonated aglycone ($[AH_2]^+$) ions of derivatized and underivatized adducts were studied.

The positive ion FAB spectra of these adducts exhibited an $[MH]^+$ ion and a more abundant aglycone fragment ion, $[AH_2]^+$, which resulted from the loss of the deoxyribose sugar. High-quality full-scan spectra were obtained on less than 200 pmol of the derivatized adducts without signal averaging. With linked scans (linked magnetic and electric fields) of the $[MH]^+$ ion for the $(trimethylsilyl)_2$ species, these same adducts were detected by examining their metastable ion spectra at levels as low as 8 to 10 pmol (signal-to-noise ratio greater than 10). The CID of the $[MH]^+$ ion yielded the aglycone fragment and an $S_1$ ion, which resulted from cleavage through the sugar. The CID spectrum of the aglycone $[AH_2]^+$ ion was much more useful, providing structural information relating to the base, the PAH, and possibly the site of covalent attachment. Differentiation of isomeric aminophenanthrene-guanine adducts was demonstrated on the basis of the CID spectra of their respective $[AH_2]^+$ ions.

### Continuous-Flow Fast-Atom-Bombardment Mass Spectrometry

A variety of mobile-phase solutions were examined in order to establish optimum conditions for continuous-flow FAB-MS using the VG70 250SE instrument. An order of magnitude of improvement was achieved over the detection limits provided by static FAB for underivatized samples. For example, as little as 450 fmol of underivatized *N*-acetyl-*N*-(dG-8-yl)-2-AF was detected by this technique in the selected reaction monitoring mode by monitoring the intense metastable ion at *m/z* 373 for the aglycone (data not shown).

To estimate the ability of our continuous-flow FAB-MS technique to obtain structural data on a completely unknown

PAH deoxynucleoside adduct of the amino-PAH type, we did a simulation experiment. We added 270 picomoles (100 ng) of *N*-acetyl-*N*-(dG-8-yl)-2-AF to 3 mL of methanol, simulating a collected HPLC peak. The volume was reduced to 4 μL by sequential evaporations into smaller vials, and 0.5 μL was injected into the continuous-flow FAB-MS under full-scan conditions. Even some of the single scans revealed the presence and molecular weight of the adduct by the characteristic sugar loss from the $[MH]^+$ at *m/z* 489, leading to the $[AH_2]^+$ at *m/z* 373, as shown in Figure 11. The recovery of the analyte was 50% by external standardization. In an actual application of the method to an unknown adduct, the residual sample could be used for tandem MS experiments to reveal additional structural features. However, to obtain 270 pmol of an adduct that is present in DNA at a level, say, of 1 in $10^8$ base pairs, would require nearly 2 g of DNA, an unrealistic amount. More realistic is a 2.0-mg DNA sample (see Introduction). Thus, this method, in its current form, requires that a completely unknown adduct fall into the range of 1 in $10^5$ or higher in a DNA sample in order to make a complete study of its structure by tandem MS realistic. This, in turn, means that the method generally will be confined to the analysis of nonphysiological samples such as calf thymus DNA reacted in a test tube with a high amount of a chemical, or, similarly, DNA obtained from a heavily exposed cell culture. A sensitive technique such as $^{32}P$-postlabeling, or, potentially, our electrophore labeling GC-ECNI-MS technique, then could be used to test whether the adduct appears to correspond to one that exists in a physiological sample.

A homemade liquid junction interface connecting capillary electrophoresis to continuous-flow FAB-MS was set up and used to detect standards of PAH-DNA adducts on a VG Quattro Instrument (Wolf et al. 1991). The most sensitive measurements made were of the aglycone ion in the selected reaction monitoring mode. Shown in Figure 12 is the detection of 140 fmol of some underivatized amino-PAH deoxy-

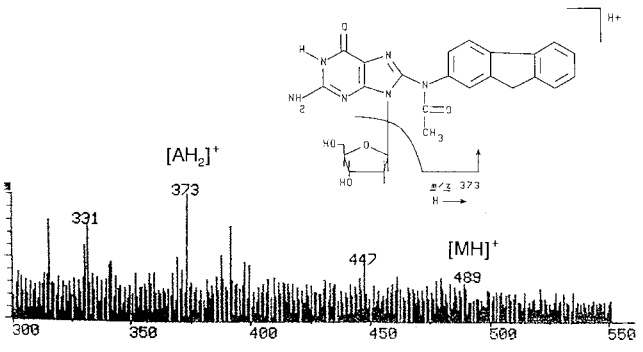



**Figure 11. Single-scan detection of *N*-(dG-8-yl)-2-AF by continuous-flow FAB-MS.** Approximately 17 pmol was injected.

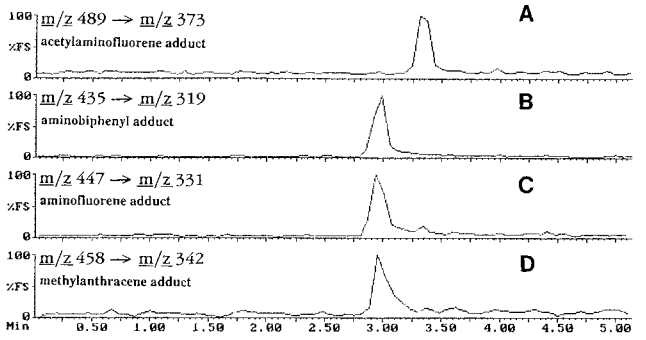



**Figure 12. Single transition monitoring of the loss of deoxyribose from DNA adducts by capillary electrophoresis continuous-flow FAB-MS system with selected ion monitoring**: A: *m/z* 373 from *N*-acetyl-*N*-(dG-8-yl)-2-AF, 140 fmol. B: *m/z* 319 from *N*-(dG-8-yl)-4-aminobiphenyl, 140 fmol. C: *m/z* 331 from *N*-(dG-8-yl)-2-AF, quantity unknown. D: *m/z* 342 from $N^2$-(anthracen-9-ylmethyl)-deoxyguanosine, 140 fmol. The acquisition was started three minutes after mixture injection. % FS = percent full scale.

nucleoside DNA adducts. The full-scan spectrum shown in Figure 13 was obtained by injecting 2.8 pmol. The spectrum shows the characteristic presence of $[MH]^+$ and $[AH_2]^+$ (loss of deoxyribose) ions, as well as ions resulting from the loss of the acetyl group (*m/z* 330/1 and *m/z* 446/7). As we have reported (Annan et al. 1990), the ions appearing in the range of 150 to 225 *m/z* provide information about the PAH and deoxynucleoside identities and about the site of their covalent linkage. Difficulties with sample handling currently limit the usefulness of this technique, however, because injections were made by siphoning a 5-nL volume of a microliter-size methanol solution of each compound. Work is currently in progress to reduce the absolute amount of sample required and to enrich the analyte during injection. See Appendix A for an update.

It was established that the use of tandem MS could provide significant structural information about an amino-PAH

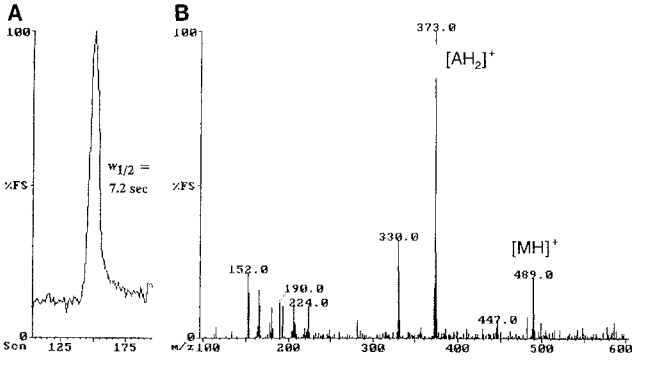



**Figure 13. Spectrum obtained from sampling 2.8 pmol of *N*-acetyl-*N*-(dG-8-yl)-2-AF into a capillary electrophoresis continuous-flow FAB-MS system.** A: the trace of the *m/z* 373 ion (peak width at half height = 7.2 seconds). B: the spectrum obtained by the summation of scans across the peak with background subtraction. Scn = scan number; % FS = percent full scale.

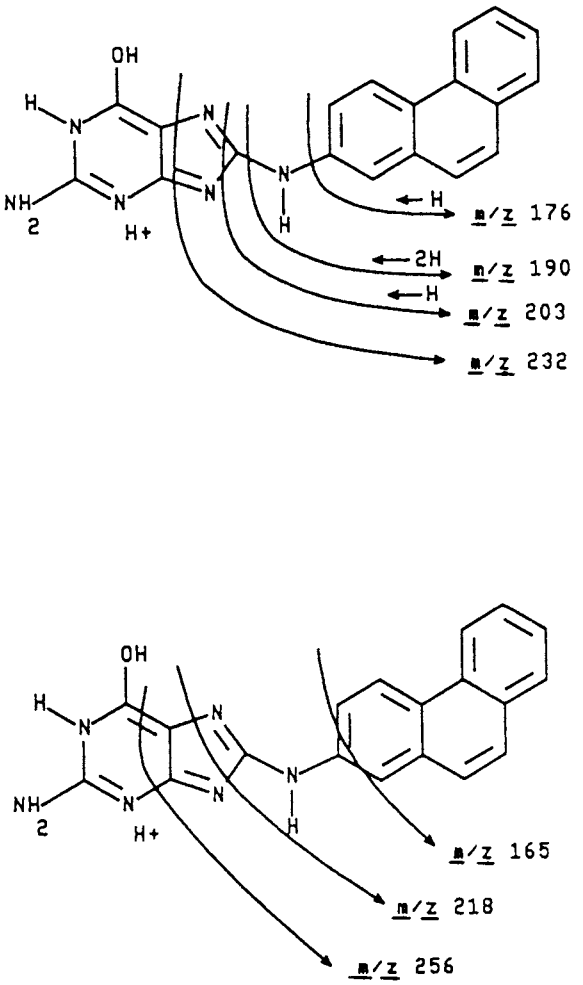



**Figure 14. Some of the fragmentation of the aglycone ion of *N*-(dG-8-yl)-2-aminophenanthrene by tandem MS.**

deoxynucleoside adduct. For example, Figure 14 shows the identity of the more informative daughter ions that were obtained when the aglycone ion of *N*-(dG-8-yl)-2-aminophenanthrene was subjected to CID. Nevertheless, there is no substitute for a broad knowledge base of data on the fragmentation patterns of known DNA adducts to guide the interpretation of related, unknown adducts.

## DISCUSSION

This project has focused on working toward the long-term goal of measuring DNA adducts in human samples. Such measurements are important in several respects, three of which are discussed here. The first reason is that humans are exposed to a diversity of chemicals. It is difficult to know which chemicals, or combinations of chemicals, pose the greatest risk to health. While the chemicals that form the most DNA adducts in people are not necessarily the worst ones, until data on their biological effects dictates otherwise, they are certainly the ones to worry about first. Animal exposure studies and other techniques also are used to identify the most dangerous chemicals to which humans are exposed, but neither the exposure conditions nor the biochemical aspects can be made the same for animal versus human exposure to chemicals.

The quantitative measurement of DNA adducts in human samples can serve as a useful tool for epidemiology studies. For example, if a human population with an elevated incidence of some cancer also has an elevation of a distinctive DNA adduct, then the chemical (or physical exposure condition) giving rise to this adduct may be a biomarker or even the cause of the cancer. This application of DNA adducts would fail, however, if the critical exposure had taken place at a much earlier point in time, so that none of the DNA adducts remained when the samples were collected. Nevertheless, measurement of DNA adducts today can help select which people should be monitored for future cancer incidence. For people who develop cancer, it might be helpful to have earlier blood samples, taken from before the onset of cancer.

The third application of human DNA adducts concerns the wide variation in both genetic makeup and lifestyle of individuals. It is possible that some of the human disease burden of cancer and genetic disease is a consequence of the "wrong people" (genetically susceptible to a given chemical) living the "wrong way" (lifestyle that increases exposure to the special chemical or chemical mixture). The measurement of DNA adducts in these individuals (along with appropriate genetic evaluation) might be a good way to test this hypothesis.

How much sensitivity is needed to make useful quantitative measurements of DNA adducts in human samples? This question cannot be answered very well at the present time, because neither the types nor amounts of human DNA adducts that reflect an unacceptable risk to health are well defined. Nevertheless, there are reasons to believe that extraordinary sensitivity in measurements would be useful. Samples such as blood contain little DNA and are necessarily limited in availability and size. DNA adducts are also subject to repair, and the sample might only contain a fraction of the DNA adduct level that is present in the target tissue (e.g., lung). Thus, only tiny amounts of DNA adducts are available for measurement. For example, Jahnke and associates (1990) reported that the lymphocytes of smokers contain a total bulky adduct concentration of approximately 1 in $10^8$ to $10^{10}$ DNA nucleotides. A midrange value (1 in $10^9$, which corresponds to about one adduct per cell) is

equivalent to about 3 amol of adduct per microgram of DNA, and 1 mL of blood contains about 45 μg of DNA. Thus, a 50-mL sample of such blood would contain, collectively, only about 7 fmol of multiple bulky adducts. Even patients on cisplatin chemotherapy have only low femtomole amounts of platinum-DNA adducts per microgram of DNA (Parker et al. 1991). Given this scenario, the best initial course of action, which is taking place currently, is to use the most sensitive, valid methods available for DNA adducts and apply them to cases in which significant chemical exposure has taken place, larger physiological samples can be obtained, or both. For example, benzo[*a*]pyrene-diolepoxide-DNA adducts have been detected by fluorescence in 50-mL blood samples from highly exposed coke oven workers (Weston et al. 1989) and in 40-g placental samples from women who smoke (Manchester et al. 1988). As our methods and knowledge improve, the sensitivity needed to make meaningful measurements of DNA adducts should become clearer.

In this project we have worked toward a goal of increasing the sensitivity of both qualitative and quantitative MS for measuring DNA adducts. As summarized in the Results section, significant advances in sensitivity have been achieved in both cases for standards. To the best of our knowledge, the detection limits that we have obtained for standards of DNA adducts and chemical transformation products from such adducts, by GC-ECNI-MS (attomole level) and FAB-MS (femtomole level), are equivalent or somewhat lower than detection limits reported by these techniques for any types of compounds.

We believe that much of the future of qualitative MS in the analysis of human DNA adducts will involve very large DNA samples (e.g., pooled fluid or tissue samples), in spite of the ongoing development of new MS techniques. Based on findings from epidemiology studies for an important unknown adduct, a pooled fluid or tissue sample may be needed. The most attractive route, however, is to duplicate an unknown human DNA adduct in another type of sample, such as an exposed cell culture or animal, making a scale-up of the adduct easier.

Although MS is an expensive and complex technique, its application to DNA adducts will increase. Largely, this will be motivated by the ability of MS not only to measure the entire spectrum of DNA adducts, but to provide definitive quantitative and qualitative analysis as well. The other techniques currently being used to measure DNA adducts are limited in one or the other of these aspects. It is sometimes difficult to know how accurate the measurements of DNA adducts are by the other techniques, because interferences may not always be apparent. With MS, however, the general appearance of the data and the opportunity to measure the analyte in different ways (e.g., by the preferred detection of multiple ions) can help to determine when an interference is present. Fortunately, the number of laboratories applying and developing MS methods for DNA adducts is increasing, which will speed up realization of the potential of DNA adducts as an important tool in advancing our understanding of human toxicology.

Much of the future of MS in the analysis of human DNA adducts will be tied to its use in combination with the existing techniques of $^{32}$P-postlabeling TLC, immunoassay, and fluorescence, including the various reagents employed. This is just an extension of the ongoing combined use of these latter techniques, which serves to improve their performance. For example, an antibody column was employed to facilitate the purification of a benzo[*a*]pyrene DNA adduct so that a low detection limit could be achieved by fluorescence (Weston and Bowman 1991). $^{32}$P-Postlabeling TLC will be an important procedure for guiding sample purification of unknown DNA adducts before their analysis by MS. Ultimately, the problem of measuring DNA adducts accurately and comprehensively in human samples is so difficult that all applicable techniques need to be employed. Related to this, accuracy in chemical analysis most definitively is established when independently validated, different techniques come up with the same value.

Three new techniques for sample preparation by chemical transformation of model compounds relevant to DNA adducts were introduced in this project: superoxide oxidation, hydrazinolysis, and hydrogenation. For the superoxide oxidation, we initially screened a variety of oxidation reactions before this one emerged as a new reaction, and the best one, for the kind of transformation desired. Other reactions studied had less scope, produced more side products, were unattractive for trace work because insoluble products formed from the reagents, or had a combination of these problems. Optimizing the reaction conditions, which included mechanistic studies, and testing the scope of the superoxide reaction, were the next tasks accomplished. Finally, we worked out methodology that allowed us to conduct the reaction on a model analyte at the femtomole level.

A shortcoming of the superoxide oxidation technique is that some diolepoxide PAH moieties will be transformed into the same final product, or into positional isomers that may coelute from the GC column. Generally, however, HPLC or an antibody column can be used to resolve different DNA adducts, and GC is a powerful technique for separating positional isomers. For example, GC is commonly used to resolve the multiple isomers of chlorinated aromatics (Clement and Tosine 1989; Lang 1992).

Recently, Weston and Bowman (1991) reported that, using fluorescence detection, 6 out of 25 samples of human lung

(biopsy samples from people who had died due to trauma) tested positive for benzo[*a*]pyrene-diolepoxide-DNA adducts. The level of the adducts was in the range of 1 to 40 in $10^8$ nucleotides. As in the superoxide method that we are developing, the adducts were hydrolyzed to a corresponding tetrahydrotetrol of benzo[*a*]pyrene before final detection. Evidence was cited for the presence of other, unidentified, structurally related PAHs in these samples. It will be interesting to subject samples such as this to our superoxide oxidation technique and MS once this technique more fully matures.

With regard to the chemical transformation achieved by the hydrazinolysis reaction, initially we investigated a variety of acidic and basic hydrolysis conditions to release 2-AF from the parent adduct, *N*-(dG-8-yL)-2-AF. We decided to test hydrazinolysis because the known transformation of arginine to ornithine by hydrazinolysis was similar to what we wanted to achieve. Whether or not there are actually any mechanistic overlaps in the two reactions is unknown. Using conventional conditions for the hydrazinolysis of this DNA adduct produced only a moderate yield of the desired product. It then was observed that the reaction proceeded best at a considerably higher temperature than that ordinarily used for hydrazinolysis. Although the reagent itself is toxic and potentially explosive, the small amount of it needed for a trace analytical reaction makes its use acceptable for this purpose. Others have continued to explore the analytical usefulness of alkaline chemical transformation of aminopolyaromatic and related DNA adducts (F. Kadlubar, personal communication).

It is well-established that nitroaromatics can be reduced to corresponding amines by hydrazine in the presence of a metal (e.g., palladium-carbon, platinum-carbon, or Raney nickel). We showed that high temperature can be employed instead of the metal (Abdel-Baky et al. 1991b). This is potentially important for the chemical transformation of amino-PAH– and nitro-PAH–DNA adducts by hydrazinolysis because the metal might cause adsorption or chemical losses of the trace analyte.

Chemical transformation of susceptible DNA adducts by high-temperature hydrazinolysis was elaborated in a third respect in this project: optimization of electrophoric derivatization of the aminopolyaromatic product (Bakthavachalam et al. 1991). Two derivatives emerged that were superior to the others investigated: pentafluorobenzylidene and *N*-heptafluorobutyryl-*N*-pentafluorobenzyl.

Successful ultratrace analysis by GC-ECNI-MS depends critically on working with a derivative that forms readily, is chemically and physically stable, and has good detection characteristics by GC-ECNI-MS. There is a tendency in the literature to compromise one or more of these aspects, thereby leading to GC-ECNI-MS methods that may be successful at moderately trace levels, but are handicapped for ultratrace applications. For example, two electrophoric derivatives may perform similarly on a new GC column, or at the femtomole level, but only the more stable, less polar of the two may continue to perform well on an aged GC column, at lower levels, or both. Workers should not just proceed with the first electrophoric derivative that emerges for an analyte; an optimum derivative should be selected.

Chemical transformation of DNA adducts by high temperature and high pressure hydrogenation potentially casts a bigger net for catching unknown adducts than the superoxide and hydrazinolysis techniques. This is both a strength and a weakness. Each of the latter procedures is designed to test for a well-defined class of DNA adducts: diolepoxide PAH, and amino-PAH and nitro-PAH adducts, respectively. This is attractive because of the structural information imparted by a positive result. Both classes, as well as other classes of adducts, potentially would be detected by the hydrogenation technique. Such detection might be done on the transformed chemical directly, or after it is electrophore-derivatized. This novel hydrogenation methodology is at an early stage of development but may become a useful tool for DNA adduct detection. This is particularly true because this hydrogenation tends to "erase" the organic component of intact DNA, potentially making it easier to detect the presence of unknown DNA adducts. However, its usefulness for measuring DNA adducts depends on how sensitive it can become for this purpose.

It is useful first to establish stoichiometry when a total unknown is under study. The investigation of DNA adducts by reacting DNA in the test tube with a radioactive chemical, or via an exposed animal, is advantageous in this respect. The total radioactivity on the DNA provides a measure of the total number of DNA adducts. This makes it easier to know when all of the adducts have been collected. The chemical transformation techniques promise to make a similar kind of assessment for the classes of adducts that they detect.

All three of these new chemical transformation techniques may be useful for studying not only DNA adducts, but also some protein adducts. This latter possibility was not explored, however, because it is outside the scope of this project.

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## APPENDIX A. Additional Progress

Since the completion of our HEI project, we have been able to conduct some additional experiments that extend some of those presented in this Investigators' Report. Here we provide a brief synopsis of this additional progress.

A capillary liquid chromatography, continuous-flow FAB, and tandem MS system was set up and used to provide the first direct evidence for the formation of 2-acetylaminofluorene–adenine adducts (two were observed) when *N*-acetoxy-2-acetylaminofluorene reacts in vitro with DNA. Detection was done in the constant neutral loss mode for the loss of the sugar from $[MH]^+$. Also, capillary electrophoresis was interfaced to continuous-flow FAB–tandem MS and used to detect *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene-5′-monophosphate at the low femtomole level as 3.5 μL of a $6.3 \times 10^{-8}$ M solution via polarity switching injection. Thus, the technology has been extended from deoxynucleoside adducts to deoxynucleotide adducts, and, when the sample is limited, from an impractical nanoliter injection volume to a realistic microliter injection volume.

The $KO_2$ oxidation, electrophore labeling, and GC-ECNI-MS procedure was simplified by substituting a solid-phase extraction step for the two HPLC separations indicated in Figure 4. The procedure then was used to detect acid-labile benzo[*a*]pyrene-DNA adducts formed when a human lymphocyte cell culture ($5 \times 10^5$ cells/mL) was exposed to this compound (1 μg/mL) for 28 hours at 37°C. The exposed sample was obtained from Gentest (Woburn, MA). We subjected amounts of DNA in the range of 50 to 100 μg to our procedure, and found the DNA from the exposed cells to contain 5 adducts in $10^7$ deoxynucleotides. The potential of the method to detect diolepoxide-PAH–DNA adducts in general was demonstrated by applying it to chrysene-1,4-quinone as a model compound possessing a different PAH moiety (Allam et al. 1993).

The hydrogenation and GC work (with a flame ionization detector) was extended to detect 100 μg of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene spiked into 1 mg of DNA, and 1 mg of 4-aminobiphenyl spiked into 150 mg of hemoglobin. Lower levels of detection can be anticipated with additional work.

## ABOUT THE AUTHORS

**Roger W. Giese** received a Ph.D. in organic chemistry from the Massachusetts Institute of Technology in 1969 and was a post-doctoral fellow at Harvard Medical School until 1973. He is currently a Professor of Chemistry and Biomedical Science in the Bouve College of Pharmacy and Health Sciences, and the Barnett Institute at Northeastern University. Dr. Giese's primary research interest is trace organic analysis.

**Paul Vouros** received a Ph.D. in chemistry from the Massachusetts Institute of Technology in 1965. He was an Assistant Professor of Chemistry at Baylor College of Medicine until 1974. Currently, he is a Professor of Chemistry in the Department of Chemistry and the Barnett Institute at Northeastern University. His primary research interest is mass spectrometry.

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## ABBREVIATIONS AND METRIC UNITS

| | |
|---|---|
| 2-AF | 2-aminofluorene |
| $[AH_2]^+$ | protonated aglycone |
| $C_6F_5CH_2Br$ | pentafluorobenzyl bromide |
| $CH_3CN$ | acetonitrile |
| CID | collision-induced dissociation |
| dG | deoxyguanosin |
| DMF | *N*,*N*-dimethylformamide |
| DMSO | dimethylsulfoxide |
| FAB-MS | fast-atom-bombardment mass spectrometry |
| GC | gas chromatography |
| GC-ECD | gas chromatography with electron-capture detection |
| GC-ECNI-MS | gas chromatography with electron-capture negative ion mass spectrometry |
| HEI | Health Effects Institute |
| HPLC | high-performance liquid chromatograhy |
| $KO_2$ | potassium superoxide |
| $[MH]^+$ | protonated molecule |
| MS | mass spectrometry |
| *m/z* | mass-to-charge ratio |
| *N*-(dG-8-yl)-2-AF | *N*-(deoxyguanosin-8-yL)-2-aminofluorene |
| *N*-acetyl-*N*-(dG-8-yl)-2-AF | *N*-acetyl-*N*-(deoxyguanosin-8-yL)-2-aminofluorene |
| NaOAc | sodium acetate |
| $NH_4OH$ | ammonium hydroxide |
| $^{63}Ni$ | nickel-63 |

| | |
|---|---|
| NMR | nuclear magnetic resonance |
| $NH_4OAc$ | ammonium acetate |
| PAH | polyaromatic hydrocarbon |
| SIMS | secondary ion mass spectrometry |
| Tetrol | benzo[*a*]pyrene-*r*-7,8,9,*c*-10-tetrahydrotetrol |
| TLC | thin-layer chromatography |
| UV | ultraviolet |

**Metric Units**

| | |
|---|---|
| milli | $\times 10^{-3}$ |
| micro | $\times 10^{-6}$ |
| nano | $\times 10^{-9}$ |
| pico | $\times 10^{-12}$ |
| femto | $\times 10^{-15}$ |
| atto | $\times 10^{-18}$ |
| zepto | $\times 10^{-21}$ |
| yocto | $\times 10^{-24}$ |

## INTRODUCTION

Humans are exposed on a daily basis to a wide variety of mutagenic and carcinogenic chemicals, including polycyclic aromatic hydrocarbons (PAHs)*. Persons living in industrialized regions receive the highest PAH exposures, generally through occupational exposures to coal tar, creosote, coke oven emissions, and the products of incomplete combustion of fossil fuels. Diesel emissions are of particular concern with regard to exposures because of their high PAH content. Many of the exposure sources mentioned above have been shown to increase the risk of developing certain cancers (Bos et al. 1984; van Schooten et al. 1990). The involvement of PAHs in cancer development is also supported by laboratory studies that demonstrate tumor formation in a variety of tissues in animals exposed to PAHs, including the liver, lungs, skin, and mammary glands (International Agency for Research on Cancer 1983, 1989).

Since 1983, the Health Effects Institute (HEI) has conducted a multidisciplinary research program to improve our understanding of the biological effects of some of the PAHs found in diesel exhaust, with the ultimate goal of providing regulators with the necessary information to conduct sound risk assessments. Because a major weakness in most cancer risk assessments is a lack of information regarding human exposures to suspected and known carcinogens, part of the HEI program has been directed toward developing better methods for assessing exposure to PAHs and related compounds. The study described in this report was funded to address that need.

In 1985, HEI issued a Request for Preliminary Applications (RFPA 85-2) soliciting proposals for studies of "New Methods for Assessing Exposure and Health Risks from Automotive Emissions." In response to this RFPA, Dr. Roger W. Giese of Northeastern University, Boston, MA, submitted a letter of intent for a project entitled "Electrophore-GC [Gas Chromatography] Determination of Automobile-Emission DNA Adducts." At the request of the HEI Health Research Committee, the investigator submitted a full proposal, "Determination of Nitro-PAH Adducts by GC-MS [Gas Chromatography–Mass Spectrometry]" in February 1986. The approved project began on April 1, 1987 and was completed in 1991. Total expenditures were $402,651. The Investigators' Report was received at HEI on October 1, 1991 and was accepted by the Health Review Committee in July 1992. During the review process, Dr. Giese submitted a brief description of his further progress in developing some of the more promising procedures described in his report (Appendix A of the Investigators' Report). Because these experiments were conducted after the conclusion of the study and only limited experimental details were provided, the material in Appendix A was not subjected to the customary review processes and are not evaluated in this Commentary. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The following Commentary is intended to serve as an aid to the sponsors of HEI and to the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

## REGULATORY BACKGROUND

Diesel engines, which have an efficiency advantage over spark-ignited engines, are used primarily in heavy-duty applications such as trucks, buses, construction equipment, locomotives, and ships. Light-duty diesel engines are also used in passenger cars and trucks, especially in Europe, but these applications account for less than 2% of the vehicle sales in the United States at the present time. In addition to fuel efficiency, diesel engines emit 10% to 20% less carbon dioxide than gasoline engines. Both factors account for the renewed interest in diesel engines in this country.

Despite the advantages of diesel engines, their use has potential environmental and health consequences. Diesel engines operating without emission control technology emit higher levels of nitrogen oxides than gasoline engines, as well as a highly complex mixture of gases and carbonaceous particles. The particles in diesel exhaust are particularly troublesome because they are of respirable size and have hundreds of toxic chemicals, including PAHs, adsorbed onto their surfaces.

Polycyclic aromatic hydrocarbons and their chemical derivatives are well-known to be mutagenic in bacteria and mammalian cells and carcinogenic in laboratory animals (International Agency for Research on Cancer 1983, 1989). Moreover, several long-term animal bioassays have shown that chronic inhalation of high concentrations of diesel exhaust causes lung tumors in rats, some evidence suggests that workers exposed to high concentrations of diesel exhaust have a higher incidence of lung cancer than their nonexposed counterparts (reviewed by Mauderly 1992). Because of concerns about the potential health consequences

of inhaling diesel exhaust, the U.S. Environmental Protection Agency (EPA) sets emissions standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990.

Section 202(a)(1) of the Act directs the Administrator of the EPA to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) of the Act specifically directs the Administrator to set standards for the "emissions of carbon monoxide, hydrocarbons, oxides of nitrogen and particulate matter from classes of heavy-duty vehicles and engines . . . ."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and (a)(3)(A)(i). For example, the EPA has set emission standards for hydrocarbons, carbon monoxide, nitrogen oxides, and particulate matter for heavy-duty and light-duty trucks. These emission standards are initially made applicable to all engines and vehicles produced in a given model year. Engines and vehicles of the same class that are produced in succeeding years must also comply with these existing standards, unless the EPA establishes a new set of standards.

Determining appropriate emission standards for particles and hydrocarbons depends, in part, on assessing the health risks they present. That process requires a better understanding of the levels of pollutants to which individuals have been exposed than that which currently exists. Dr. Giese and coworkers were funded by HEI to improve the sensitivity and specificity of analytical techniques used to measure individual exposures to PAHs. Such research is essential to the informed regulatory decision-making required by the Clean Air Act.

## SCIENTIFIC BACKGROUND

Human cancers encompass a wide range of diseases that are caused by chemical, physical, or microbial carcinogens. The presently accepted paradigm for chemical carcinogenesis is that it is a multistage process that includes initiation, promotion and progression (reviewed by Harris 1991). DNA mutations can occur in all of these stages. During initiation, the covalent binding of chemical carcinogens or their metabolites to DNA causes structural alterations in the genetic material, which can lead to subsequent mutagenic and carcinogenic processes. These structural alterations are called DNA adducts.

The qualitative and quantitative analysis of DNA adducts is only useful for understanding the mechanisms of carcinogenesis, but also has potential for determining human exposures to carcinogenic chemicals. The focus of this report is the development of new gas chromatographic–mass spectrometric (GC-MS) techniques to measure carcinogen-DNA adducts. This section will discuss why such measurements are important for risk assessment. It will provide a brief overview of the diverse methods that are currently available for measuring DNA adducts and will focus on MS procedures in order to provide the reader with the basic principles underlying the techniques used by the investigators.

### DNA ADDUCTS AS BIOMARKERS OF CARCINOGEN EXPOSURE

DNA (and other) adducts are produced by covalent modification of macromolecules, either by electrophilic chemicals or by their metabolites that have been converted into electrophiles. A large proportion of chemical carcinogens, that is, genotoxic carcinogens, or their metabolites, form adducts with various nucleophilic sites on deoxynucleosides and the phosphate groups. Thus, it is not surprising that the term "DNA adduct" encompasses a wide range of chemical structures, including simple methylated bases, as well as the bulky PAH-DNA adducts studied by Dr. Giese.

A major reason for identifying and quantifying DNA adducts is to determine exposure to genotoxic carcinogens. Such information has obvious applications to epidemiological investigations and risk assessment and has been the subject of recent books (Groopman and Skipper 1991), monographs (Bartsch et al. 1988; U.S. Department of Health and Human Services 1993), and reviews (Schut and Shiverick 1992). The goal of any cancer risk assessment is to determine the nature and magnitude of risk associated with exposure to a given chemical. Therefore, a quantitative estimate of the dose of carcinogen (or the biologically relevant metabolite) delivered to the target tissue under different exposure scenarios is essential to that assessment. At present, this dose can only be estimated from limited information on ambient exposures, data from animal bioassays, and pharmacokinetic modeling. Measurements of molecular markers of exposure, such as adducts, mutational spectra, and other genetic alterations could reduce the uncertainty inherent in the traditional risk assessment process.

Two types of covalent adducts have been used as molecular dosimeters of exposure to chemical carcinogens: DNA adducts and protein adducts. Initially, researchers focused their attention on developing methods to measure DNA adducts because their formation is thought to reflect mutagenic processes in the target tissues. The mutagenic processes

generally are assumed to be pivotal events in chemical carcinogenesis. In animals, the levels of persistent DNA adducts in tissues reflect the steady-state levels that are produced by the competing steps of DNA adduct formation and repair and are generally well-correlated with the susceptibility of those tissues to tumor development. However, for ethical and practical reasons, it is difficult to measure DNA adducts in target tissues in human subjects. Therefore, nontarget cells, such as blood lymphocytes, or cells shed into body fluids, have been used as sources of samples for human DNA adduct measurements. Despite the problems with obtaining an adequate amount of sample, there has been rapid progress over the last few years in developing methods to detect and quantify DNA adducts. Such data should provide a more pertinent measurement of an individual's exposure to a carcinogen than assessment by environmental monitoring because DNA adduct levels reflect individual differences in absorption, distribution, metabolism, adduct repair, and excretion. Quantifying DNA adducts can also provide mechanistic information about which specific cell type is most affected by environmental agents and their electrophilic metabolites.

A major difficulty in using DNA adducts as biomarkers is that it is usually not possible to obtain sufficient quantities of DNA adducts from human tissue samples, or even from laboratory animals that have been dosed with low amounts of carcinogens, to allow elucidation of the adduct structure. For this reason, the major stable DNA adducts formed by many carcinogens have been identified by reactions of parent compounds or preformed (synthetic) metabolic electrophiles, or by generating reactive metabolites generated in situ with microsomal systems and mammalian DNA. These types of experiments provide information about the chromatographic and spectral properties of the major adducts and have been essential in developing analytical techniques that can be applied to the analysis of human samples. However, even these approaches do not guarantee success. Metabolic differences between rodents and humans is a confounding problem, as evidenced by the recent findings of El-Bayoumy and coworkers (1993), who reported that 1-nitropyrene hemoglobin adducts formed in vitro had a different structure than those found in vivo.

Although protein adducts are not the subject of this report, it should be noted that they are also potentially attractive molecular dosimeters (Skipper and Tannenbaum 1990; Farmer 1991). Hemoglobin and serum albumin have proven to be the two proteins that are most useful for dosimetry in humans. They each reside in the body for relatively long and predictable periods of time (human hemoglobin has a lifetime of 120 days, and human serum albumin has a half-life of 20 to 25 days) and are available in relatively large quantities from human body fluids by minimally invasive techniques. Human hemoglobin has been successfully used as a molecular dosimeter for exposure to PAHs (Day et al. 1990a,b) and work on PAH-serum albumin has been conducted (Day et al. 1991a, 1992).

## METHODS FOR MEASURING DNA ADDUCTS

Early approaches for measuring DNA adducts in tissues from laboratory animals relied on physicochemical or radiochemical methods of detection. These methods have limited utility for human studies, either because they are relatively insensitive or because they require the use of radiolabeled metabolites. Recently, exquisitely sensitive and selective techniques have been developed to measure DNA and protein adducts from human tissues, blood, and urine. These methods include immunoassays, $^{32}$P-postlabeling, fluorescence spectrophotometry, and chromatographic procedures such as GC or liquid chromatography coupled with MS (Lohman 1988; reviewed by Talaska and Roh 1992). Although GC-MS techniques are the focus of Dr. Giese's report, the other procedures will be described briefly here because they are widely used and are the benchmarks for evaluating the sensitivity and specificity of new approaches. All of the methods described below allow analysis of intact adducts or their hydrolysis products (that is, the cleaved carcinogen residue) from samples ranging in size from the midfemtomole to attomole levels.

Highly sensitive immunoassays can detect and quantify DNA adducts in the femtomole range (Poirier 1991). Researchers have developed polyclonal and monoclonal antibodies to specific DNA adducts and have used these reagents in enzyme-linked immunoassays and ultrasensitive enzymatic radioimmunoassays on small samples of DNA (greater than 50 μg) (Santella 1991). These immunological methods, however, lack specificity because of the presence of cross-reacting materials, and thus require prior knowledge of the adduct structure in order to develop the appropriate antibody.

A more sensitive assay, which requires only 1 to 50 μg of DNA for detecting DNA adducts, is the $^{32}$P-postlabeling assay (Randerath et al. 1981). There have been several modifications of this procedure, but the original serves as a good outline for understanding the technique. DNA samples with covalently modified nucleotides (adducts) are digested by endo- and exonucleases to 3′-monophosphates. The modified nucleotides are selectively labeled at the 5′ position using a polynucleotide kinase. With limiting amounts of $^{32}$P-adenosine triphosphate, the relative labeling of some bulky adducts to unmodified nucleotides is increased. Separating normal nucleotides from the labeled adducted nucleotides by chromatographic methods and Cerenkov counting of the fractions (for high-performance

liquid chromatography) or autoradiography of the spots (for thin-layer chromatography) allows detection of bulky adducts in the attomole range (Randerath et al. 1981; Wilson et al. 1989). The $^{32}$P-postlabeling assay is quite sensitive for detecting known DNA adducts of this type with detection levels of approximately one adduct per $10^9$ nucleotides (Kadlubar 1992). However, quantification of unknown adducts or mixtures of adducts is limited because of differences in enzymatic digestion labeling efficiency, recovery, and the requirement for synthetic standards.

Detecting DNA adducts by fluorescence spectroscopy relies on the presence of a chromophore associated with the DNA adduct. For example, the PAH-DNA adducts that Giese studied contain the chromophore pyrene. Pyrene chromophores absorb light of a higher wavelength than normal biological constituents, and thus can be selectively excited to higher electronic states. The sensitivity of this method has been increased by using sophisticated techniques such as room temperature synchronous scanning (Day et al. 1990a; Weston et al. 1991; Bowman et al. 1992) and cryogenic Shpol'skii or line-narrowing fluorescence spectroscopies (Jankowiak et al. 1990; Jankowiak and Small 1991). The synchronous scanning method probes adducts at high wavelengths and allows detection down to the high femtomole range. However, this method is often labor intensive and suffers from interferences by other chromophores and quenching effects. Fluorescence techniques also require specialized instrumentation and are difficult to standardize in a quantitative fashion.

Mass spectrometry is a powerful tool for studying DNA adducts and has the potential to play an important role in molecular dosimetry. For readers who are not familiar with MS, a brief introduction to the basic principles and terminology may be helpful. Mass spectrometry is a spectrometric technique that produces charged molecules and molecular fragments and measures their mass (or mass-to-charge) ratio (Anderegg 1990). It is a powerful tool for obtaining molecular weights and structural information. The latter characteristic, the ability to determine the structure of small amounts of chemical complexes such as DNA adducts, and high selectivity in low-level detection sets MS apart from other methods that are used to analyze DNA adducts. Mass spectrometry techniques have been widely used for the qualitative analysis of model DNA adducts; more recent efforts have been directed at quantification (Schram 1990; Fedtke and Swenberg 1991; Talaska and Roh 1992).

Mass spectrometry has the potential to combine the sensitivity and specificity required for analyzing submicrogram quantities of sample with highly informative structural information (Schram 1990). A number of different chromatographic procedures have been linked to MS to separate, detect, and quantify DNA adducts. Gas chromatography–mass spectrometry, the procedure used by Dr. Giese, employs GC to separate the adduct of interest from other impurities in the sample and uses a variety of conventional and novel MS techniques to identify the adduct.

Two basic considerations that must be met in conventional MS procedures have limited their application to biological samples: the sample must be in the gas phase, and it must be ionized. For low-molecular-weight samples that are easily volatilized, conversion to the gas phase is not problematic. However, for measurement by GC-MS, nucleotides and other high-molecular-weight compounds that are not volatile or thermally stable require special procedures, such as derivatization, prior to analysis. For example, nucleic acid components are often converted to trimethylsilyl derivatives, which are volatile and thus suitable for GC-MS analysis. Once the sample is in the gas phase, it must then be ionized, either by bombardment with electrons (electron ionization), which may damage the molecule, or by a softer ionization mode such as chemical ionization. Other methods of soft ionization, called desorptive ionization, have been developed to deal with nonvolatile samples. These include secondary ion MS (SIMS), wherein a solid surface is bombarded with a beam of high energy ions, causing the material on the surface to sputter into the gas phase. Related to SIMS is liquid SIMS, in which a sample is dissolved in a liquid matrix. Similar to liquid SIMS is fast-atom-bombardment (FAB)-MS, in which a liquid matrix is also employed, but the desorption/ionizing beam consists of energetic neutral atoms. The liquid sample in either case can be either in a small liquid puddle (e.g., static FAB-MS) or in a continuously regenerating liquid droplet (e.g., continuous-flow FAB-MS). An attractive feature of FAB-MS on liquid SIMS is that large biomolecules, which are not readily volatilized and thus not amenable to GC-MS analysis. A disadvantage is the loss of sensitivity usually associated with FAB-MS and liquid SIMS.

One of the most successful and widely used GC-MS methods for detecting DNA adducts utilizes capillary column GC coupled with an ionization technique called negative ion chemical ionization (alternatively called electron capture negative ion mass spectrometry [ECNI-MS]) (Day et al. 1991b; Foiles et al. 1991). In this process, the carcinogen-DNA adduct is cleaved from the DNA by chemical methods, derivatized in order to convert it to a volatile form, and separated from other analytes by GC. The volatile analytes are introduced into a mass spectrometer source, which is filled with a moderating bath gas (such as methane); then this is bombarded with a beam of high-energy electrons. The bath gas absorbs some of the energy of the electrons, thereby forming "thermal electrons," which can be captured by the analyte. Depending on the structure of the derivatized analyte, one of two events may then occur. One outcome is the

usually minimal fragmentation of the analyte; the other possibility, especially if the carcinogen residue has been derivatized to contain electrophoric substituents, is the formation of a negatively charged molecular ion. When negative ions are formed efficiently, the compound is detectable in the attomole range. Electron capture negative ion mass spectrometry has been used to analyze molecules with a high affinity for electrons, such as nitro-PAHs.

Recently, tandem mass spectrometers (tandem MS or MS-MS) have been developed that utilize one stage to separate ions and a second stage to create a secondary set of ions for additional structural information on reduced noise.

## JUSTIFICATION FOR THE STUDY

When Dr. Giese's proposal was submitted (1985), researchers were limited in their ability to measure trace amounts of PAH-DNA adducts. Some methods, although highly sensitive, had limited ability to distinguish among different adducts or to resolve specific chemical structures. Others techniques, such as MS, were limited by the sensitivity of the instrument and the need to develop specialized techniques to transform the adducts into a form suitable for analysis. In the last few years, there have been remarkable new developments in MS and expansion of the technique's applications into the study of nucleic acids and nucleotide adducts. In the early 1980s, Dr. Giese successfully developed derivitization and GC-MS detection procedures to measure trace amounts of pyrimidines in vitro, but he had not tested his approach with bulky adducts or with biological samples. Dr. Giese's HEI-funded study is a logical extension of his earlier work and has obvious relevance to the rapidly developing field of molecular dosimetry, and ultimately to risk assessment.

## GOALS AND OBJECTIVES

The overall goal of this project was to improve GC-MS techniques, thus allowing detection and quantification of minute amounts of PAH-DNA adducts in human tissues. There are two options for preparing derivatives of PAH-DNA adducts: direct analysis, whereby the adduct is labeled with the electrophore, and digestion analysis, whereby the electrophore reacts with a degradation product of the adduct. In either case, the parent chemical, prototype adducts, or degradation compounds are generally used to evaluate new transformation procedures.

Dr. Giese's original proposal for a three-year study included the following objectives:

1. Developing and optimizing procedures for producing stable electrophoric derivatives of PAH-DNA adducts that would be suitable for detection by GC-MS techniques by using model PAHs such as nitropyrene, aminopyrene, hydroxypyrene, hydroxynitropyrene, hydroxynaphthoquinone, and 1-nitropyrene-9,10-dihydrodiol;
2. Applying the derivatization and GC-MS techniques to detect (a) trace amounts of microbial metabolites of nitropyrene, dinitropyrene, and related compounds; (b) known PAH adducts; and (c) unknown PAH adducts; and
3. Applying the procedures to detect trace amounts of adducts in human or animal tissues.

Drs. Giese and Vouros originally proposed to analyze the adduct derivatives by gas chromatography using two GC-MS techniques: GC with electron capture negative ion mass spectrometry (GC-ECNI-MS) and GC with electron capture detection (GC-ECD). During the first year of the study, the investigators modified their MS procedures to include FAB-MS, an MS mode that generally lacks the sensitivity of GC-ECNI-MS but is technically simpler. As the project progressed, the original work plan was modified further to take advantage of new developments in MS instrumentation and to focus on the more promising methods for adduct transformation.

Dr. Giese requested, and was granted, a fourth year of funding. The work scope for the final year of the project was to encompass direct analysis of DNA adducts, with particular attention to variables that affect the technique's application to biological tissues. In the final year, Dr. Giese was to analyze biological samples provided by other investigators in HEI's diesel program.

## TECHNICAL EVALUATION

The Investigators' Report summarizes several years of work and presents an overview, rather than an in-depth treatment of most of the work. The authors refer to published journal articles as a means to shorten the discussions of their experimental and instrumental results. Readers who are interested in a more detailed description of the methodology and results are referred to the references in the Investigators' Report and those listed in the section entitled, "Publications Resulting from This Research."

### STUDY DESIGN AND ATTAINMENT OF STUDY OBJECTIVES

The overall objective of the project was to improve available GC-MS techniques to allow quantification of minute amounts of DNA adducts in tissues. Drs. Giese and Vouros

set out to test systematically specific approaches for using a variety of MS techniques and preparative methods to detect and identify ever decreasing amounts of amino-arenes and PAHs, and subsequently, amino-arene– and PAH-nucleoside adducts as well. The Investigators' Report describes the development of several methods designed to increase the sensitivity of MS analyses of PAH-DNA adducts, including:

- Developing three chemical transformation methods suitable for converting DNA adducts to compounds amenable to GC-ECNI-MS (Objective 1);
- Improved detection limits for FAB-MS analysis of nucleoside-PAH adducts (Objective 2b); and
- GC-ECD and continuous-flow FAB-MS with capillary electrophoresis and capillary high-performance liquid chromatography.

The three chemical transformation methods, superoxide oxidation, hydrazinolysis, and high-temperature and high-pressure hydrogenation, were designed specifically for diolepoxide PAH–DNA adducts, amino-PAH–DNA adducts or nitro-PAH–DNA adducts, and general PAH-DNA adducts, respectively. Although all three methods were examined, the most emphasis was placed on superoxide oxidation. The investigators screened a variety of oxidation reactions to identify the reaction that yielded the appropriate transformation and produced soluble products and minimal side reactions. They then optimized the reaction conditions and developed methodology to conduct the reaction at the femtomole level. Model carcinogens, rather than nucleoside or DNA adducts, were used almost exclusively for this part of the work.

The investigators' approach was well organized, and almost every possible variability and artifact was traced down and eliminated. The authors made appropriate changes and additions to the study design to accommodate unforeseen results such as unexpected interferences or the observed sensitivity of FAB-MS for detecting trimethylsilyl derivatives of nucleoside adducts. Because of both the rigor with which these technical problems were approached and a shortage of time, the methodology was ultimately not applied to biological samples, namely the PAH-DNA adducts derived from treated animals and exposed human populations; nor was it applied to PAH metabolites from in vitro experiments (Objectives 2a and 3). However, following submission of the final report, the investigators submitted a short report documenting their ability to detect a diolepoxide-PAH–DNA adduct derived from benzo[*a*]pyrene-treated cultured human lymphocytes. (See Appendix A of the Investigators' Report.)

Because the ultimate goal of the study was to use MS in analyzing trace amounts of PAH-DNA adducts, an alternative approach would have been to skip a number of intermediary steps (with the option of returning to them later) and determine early on the likelihood of achieving the goal of isolating adducts from the DNA of animals exposed to the chemicals of interest.

## RESULTS AND INTERPRETATION

### Chemical Transformation Methods

The major part of this report deals with the development of three chemical transformation techniques for prototype adducts or their degradation products that were to be applied prior to GC-ECNI-MS. An impressive body of work was performed to investigate one of the three methods, superoxide oxidation, in some detail. The authors review their extensive research on the use of GC-ECNI-MS to detect fluorinated, electrophoric derivatives of the superoxide oxidation products of standard PAH metabolites and their analogues. They determined the structures of products from the reaction of a variety of oxidized PAH derivatives with potassium superoxide, and they synthesized potential internal standards to be used in the quantitative analysis of biological samples for PAH-DNA adducts.

The superoxide method shows promise for analyzing transformed diolepoxide PAH adducts at least down to the low femtomole level. The authors suggest that the superoxide method may be applicable to any PAH diolepoxide-DNA adduct, and they have included their preliminary study of its applicability to nitro-PAHs bearing the dihydrodiol moiety. Two potential drawbacks to the technique that may compromise the usefulness of the procedure for routine laboratory testing are the large number of steps involved and the length of time required for analysis. It appears from the experiments described in Appendix A (which have not been critically reviewed), that the revised procedure is shorter.

Additional chemical transformation techniques included hydrazinolysis of adducts from aromatic amino and nitro compounds to yield the corresponding amines, and hydrogenation to yield saturated polycyclic hydrocarbons. The preliminary data on the hydrogenation method are particularly interesting because they indicate the procedure's potential to be used directly on isolated DNA, thus solubilizing it and leaving the hydrogenated form of the carcinogen as the major organic soluble component. If successful, this method would have a major impact because current methods for preparing samples for MS analysis are generally specific for one compound or, at best, one chemical class. The hydrogenation method would be suitable for analyzing a wide variety of carcinogens from various DNA adducts. There are some potential difficulties in determining the

structure of the original carcinogen (prior to hydrogenation); difficulties could also arise if some of the carcinogens were lost. Notwithstanding these problems, this method would be a very valuable tool for the analysis of known or suspected carcinogen-DNA adducts, and more importantly, unknown carcinogen adducts.

**Instrumentation**

The digression from the GC-MS work into the area of FAB-MS was an unexpected turn in the project, but a very fruitful one, for which the investigators are to be commended. Derivatization of amino-PAH model compounds to incorporate trimethylsilyl groups improved the sensitivity of analysis by static FAB-MS to the high picomole level. For compounds having known or observable protonated molecules, continuous-flow FAB-MS was used to further enhance the detection of adducts in the low picomole range. Finally, continuous-flow FAB-MS using collisional activation and scans linked with magnetic and electric fields (B/E-linked) were used to probe adduct structures. In a simulated experiment, the full range of static FAB-MS and continuous-flow FAB-MS experiments was performed on a model amino-PAH–nucleoside adduct at the 270-picomole level. The fragmentation of the $[AH_2]^+$ ion was reported to provide more characteristic fragmentation than the protonated molecule.

The investigators' mastery of the FAB-MS method of analysis should allow them to apply, sequentially, one method for the measuring total adduct levels for a specific carcinogen, and then use FAB-MS, FAB-MS-MS, or both, to identify the structures of specific adducts, including isomers. Usually, researchers are limited to pursing only one of these two methodologies.

Because the quantity of adducts that can be isolated from human subjects is very small, it is unlikely that structure elucidation for DNA adducts will be based on conventional techniques such as nuclear magnetic resonance or infrared spectroscopic analysis. Thus, the use of FAB-MS for the analysis of DNA adducts will continue to increase, especially with the trimethylsilyl derivatives reported in this study. The continuous-flow FAB-MS capillary electrophoresis work showed low detection limits and is an important area for additional work. The serious limitations in the introduction of nanoliter samples nullified much of this enhanced sensitivity; as indicated by the preliminary data in Appendix A, the investigators have addressed this problem.

Advances in the instrumentation and user friendliness of software interfaces are rapidly making GC-MS a routine analytical procedure. Research, such as that presented in this report, concerning the transformation of adducts to derivatives more amenable to isolation and detection by the above-mentioned methods, also helps to increase the sensitivity of analysis to levels well within the range of adduct levels found in humans, provided that enough tissue is available for DNA isolation. Obviously, the more sensitive the method of analysis, the smaller the tissue sample required.

No matter what methodology is employed, trace organic analysis is tedious and difficult, and the methods described in the Investigators' Report are no exception. Furthermore, the structural elucidation of unknown trace compounds, a special challenge in the area of DNA adducts in human samples, is particularly problematic. Dr. Giese's methods are labor intensive, and to a large extent, depend on elucidating the nature of the DNA adduct using electrophilic intermediates, model substances such as deoxynucleotides, and activating systems such as fortified microsomal preparations or cultured cells. In view of the known diversity in the metabolism of the chemicals of interest (PAHs and nitro-PAHs), which reflect species and even tissue-specificity, it needs to be ascertained whether the nature of the DNA adduct deduced from analyzing the reaction between model metabolites and deoxynucleotides reflects the nature of the DNA adduct found in exposed humans. This is, of course, a demanding criterion to satisfy because the identity of the adduct formed in humans is difficult to establish due to the low levels of exposure to most environmental chemicals. The problem is further complicated by the known genetic heterogeneity in the metabolism of xenobiotics (for example, slow and fast acetylators, debrisoquine metabolizers, and nonmetabolizers). These factors in turn impinge upon both the nature and level of the adduct formed.

## IMPLICATIONS FOR FUTURE RESEARCH

A major consideration in evaluating MS strategies for measuring carcinogen-DNA adducts is the fact that at this time, and for the foreseeable future, MS procedures are not sufficiently sensitive to detect and quantify PAH-DNA adducts from small tissue samples. Thus, applying MS techniques to the detection of DNA adducts in biological samples will probably require alternate approaches, such as the formation of DNA adducts in cultured cells exposed to a specific chemical, or pooling tissues from a number of individuals. The first approach assumes that cultured cells reflect the metabolism and adduct formation characteristic of humans; the second approach assumes (1) that the human donors are genetically homogeneous with respect to the metabolism of the chemical of interest; and (2) that they are exposed to similar levels of the environmental chemicals. The above conditions are difficult to fulfill. Thus, it would

seem that although the methods being developed by the present investigators are very promising, their level of sensitivity, especially when compared to other currently available methods such as $^{32}$P-postlabeling and immunoassays, does not suggest that they are ready for use in studying environmentally exposed human populations.

Newer MS methods exemplified by FAB, secondary ion–MS, and laser-assisted desorption-time-of-flight MS, and the liquid phase introduction techniques of electrospray and ion spray may reach or come close to the combination of selectivity and sensitivity afforded by GC-MS. These newer techniques offer an advantage over GC-MS by allowing the analysis of a great diversity of underivatized masses, easily up to 10,000 and even over 300,000 for some proteins. Of the newer MS techniques, FAB-MS has been the technique most frequently applied to PAH-DNA adducts (Yamamoto et al. 1990). However, the detection capabilities of these techniques, now in the low-picomole to midfemtomole range, do not yet allow analysis at the low absolute analyte levels that GC-ECNI-MS affords. Yet these techniques are rapidly approaching the levels of sensitivity possible with GC-ECNI-MS.

At present, only one detection technique is truly powerful enough for achieving partial or complete structural elucidation of unknown carcinogen-DNA adducts: MS in the "scanning" mode (used with quadruple and time-of-flight instruments, by which single ionization and the resulting fragments or molecular ion species are analyzed), or in the "$MS^{n>1}$" mode (used with triple quadruple, sector, and ion trap instruments, by which further fragmentation of selected ions is analyzed). Combining any two or more of the concentration and detection techniques discussed above with the quantitative measurement of DNA adducts, especially if one of involves an MS method, has great potential for accurately measuring DNA adducts in exposed individuals.

## CONCLUSIONS

Mass spectrometry is a powerful tool for elucidating the structure of modified nucleosides; however, its application to the qualitative and quantitative analysis of DNA adducts requires new approaches for preparing biological samples and increasing the sensitivity of the MS technique. The carefully designed series of experiments conducted by Drs. Giese and Vouros demonstrates the current strengths and limitations of using MS methods to detect environmentally derived DNA adducts. Using model compounds, the investigators developed three new chemical transformation techniques (superoxide oxidation, hydrazinolysis, and hydrogenation) that show promise for converting PAH-DNA adducts to a form suitable for MS analysis. They also improved the sensitivity of FAB-MS analysis of amino-PAH model compounds by using appropriate derivatives and optimizing FAB-MS methods. The combination of novel transformation procedures, the appropriate sample matrix, and improved FAB-MS instrumentation conditions markedly increased the sensitivity of MS procedures for detecting model amino-PAH compounds. However, it should be noted that the methodology was applied to standards and model compounds, not to samples from animals or humans exposed to carcinogens.

Although the investigators' findings clearly demonstrate the promise of MS methods, the study also reveals the problems inherent in applying these methods to a biological matrix, such as DNA, that has been modified by exposure to trace levels of environmental contaminants. Mass spectrometry methods are powerful only when a sufficient amount of adduct is available. At this time, the sensitivity of these methods is probably three orders of magnitude lower than other currently available techniques for measuring carcinogen DNA adducts, which also suffer from a number of limitations.

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