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Development of Methods for Measuring Biological Markers of Formaldehyde Exposure

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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.

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HEI Statement

Synopsis of Research Report Number 67

Detection of Formaldehyde–DNA Adducts: Development of New Methods

BACKGROUND

Formaldehyde is a small organic molecule that has generated great public controversy because of uncertainties about its adverse human health effects. Although a link has been documented between chronic exposure to inhaled formaldehyde and cancer development in rodents, a similar link has not been established for humans. Formaldehyde exposures occur during the manufacture of many consumer products. Furthermore, during their subsequent use in the home, formaldehyde vapors are released from these products. Formaldehyde is also an outdoor air pollutant produced by fuel combustion. If the use of methanol as an alternative fuel increases, future outdoor formaldehyde levels may also increase because formaldehyde is a methanol combustion product.

Determining the amount, or dose, of formaldehyde actually received by exposed humans is important in determining the health risk of formaldehyde exposures. Formaldehyde can damage cells by binding to DNA and thereby forming formaldehyde-DNA adducts; this process may interfere with accurate DNA replication and lead to mutations and cancerous tumors. Although formaldehyde-DNA adducts have the potential to serve as biomarkers for measuring formaldehyde dose, they are very unstable and many current analytical methods are not sensitive enough to detect them. The Health Effects Institute sponsored this study to develop methods for improving the detection of formaldehyde-DNA adducts in exposed cells and tissues.

APPROACH

Dr. Fennell treated formaldehyde-DNA adducts with sodium bisulfite, a compound that reacts with these adducts and traps them as stable compounds, and then tested different analytical techniques for separating and detecting the adducts. He exposed pure DNA, cell nuclei, and cells in culture to formaldehyde and treated them with sodium bisulfite under a variety of experimental conditions. When the use of radiolabeled formaldehyde proved to be unsuitable for experiments on cells or tissues, he evaluated several different modifications of ^{32}P-postlabeling (a sensitive technique for detecting DNA adducts) to determine if he could isolate the adducts from formaldehyde-treated preparations. Finally, he explored alternative methods for modifying formaldehyde adducts to forms suitable for detection by either gas chromatography and mass spectrometry or immunoassays.

RESULTS AND IMPLICATIONS

Dr. Fennell developed optimal experimental conditions for stabilizing formaldehyde-DNA adducts with sodium bisulfite. However, based on his results with adduct standards, he concluded that neither the ^{32}P-postlabeling method nor modifications thereof were sufficiently sensitive to detect the bisulfite-stabilized adducts in cells and tissues. A fundamental problem was that normal bases migrate along with the modified bases and he was unable to separate the two adequately. The investigator then explored alternative approaches and was able to prepare synthetic adducts, which, when coupled with proteins, could possibly be used to produce antibodies that could be used in immunoassay detection procedures.

Developing methods for quantifying formaldehyde-DNA adducts remains a worthwhile goal because these adducts could serve as biomarkers of formaldehyde exposure. Although this study did not achieve all of its original aims, Dr. Fennell provided new directions for other investigators, such as using immunoassays to detect such adducts. Innovative approaches like these have the potential to expand our understanding of human health risks associated with exposure to formaldehyde.

This Statement, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, is a summary of a research project sponsored by HEI from 1989 to 1991. This study was conducted by Dr. Timothy R. Fennell at the Chemical Industry Institute of Toxicology. The following Research Report contains both the detailed Investigator's Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

Formaldehyde, a widely used industrial chemical that is also present in automobile exhaust, causes nasal tumors in rats and mice after prolonged inhalation exposure to high concentrations. The induction of squamous cell carcinomas in rats by formaldehyde displayed a highly nonlinear dose response with a disproportionately large number of tumors at higher exposure concentrations. A sufficient amount of formaldehyde reaching target cells, and the saturation of formaldehyde metabolism to formate can increase the covalent binding of formaldehyde to DNA. The carcinogenicity of formaldehyde may result from its ability to induce DNA-protein cross-links and/or hydroxymethyl adducts in DNA. Measuring these products can indicate the dose of this carcinogen at a critical target site, and such assessment has been conducted for formaldehyde by measuring DNA-protein cross-links. The objective of this study was to develop methods for measuring hydroxymethyl adducts in DNA that do not require the use of radiolabeled formaldehyde.

The detection of N^6-hydroxymethyldeoxyadenosine and N^2-hydroxymethyldeoxyguanosine, the major adducts formed by the reaction of formaldehyde with DNA in vitro, is complicated by their instability. The stabilization of hydroxymethyl adducts by reaction with sodium bisulfite in aqueous solution at 4°C before isolating DNA from homogenates was investigated. On treatment of calf thymus DNA or isolated rat liver nuclei with [^{14}C]formaldehyde, followed by reaction with bisulfite and isolation of DNA, radioactive peaks corresponding in retention time to N^6-sulfomethyldeoxyadenosine and N^2-sulfomethyldeoxyguanosine were detected by high-performance liquid chromatography of nucleoside digests. However, on treatment of cultured lymphoblasts with [^{14}C]formaldehyde, extensive metabolic incorporation of radioactivity into normal nucleosides precluded the detection of the derivatives.

Methods for detecting these derivatives that do not involve the use of radiolabeled formaldehyde, such as ^{32}P-postlabeling and electrophore postlabeling, were investigated. For electrophore postlabeling, several reactions for preparing a derivative suitable for analysis by gas chromatography with mass spectrometry were investigated unsuccessfully. For ^{32}P-postlabeling, a method was developed for detecting sulfomethyldeoxyadenosine 3′,5′-diphosphate that involved separating sulfomethyldeoxyadenosine 3′-monophosphate from normal nucleotides by reverse-phase high-performance liquid chromatography using two columns with column switching. The purified adduct fractions were subjected to ^{32}P-postlabeling, and the labeled adduct was separated by two-dimensional thin-layer chromatography on polyethyleneimine-cellulose plates. The adduct spots were quantitated by comparing them with standards labeled directly or mixed with normal nucleotide 3′-monophosphates and separated by high-performance liquid chromatography. The level of sulfomethyldeoxyadenosine 3′,5′-diphosphate detected in DNA from isolated rat liver nuclei treated with formaldehyde and sodium bisulfite was similar to that in control nuclei. With TK6 human lymphoblasts in culture exposed to formaldehyde, the presence of additional radioactive spots that comigrated with the sulfomethyldeoxyadenosine 3′,5′-diphosphate spots precluded its detection. The low levels of sulfomethyldeoxyadenosine 3′,5′-diphosphate in isolated nuclei treated with formaldehyde suggest that N^6-hydroxymethyldeoxyadenosine is not formed to a significant extent in systems that contain protein together with DNA. Under similar conditions, high levels of DNA-protein cross-links have been measured. The results of this study suggest that measurement of hydroxymethyldeoxyadenosine would not provide a useful measure of exposure.

INTRODUCTION

Formaldehyde (HCHO)*, a widely used industrial chemical that is also present in automobile exhaust, causes nasal tumors in rats and mice after prolonged inhalation exposure to high concentrations (Kerns et al. 1983). The induction of squamous cell carcinomas in rats by HCHO displayed a highly nonlinear dose response with a disproportionately large number of tumors at higher exposure concentrations. A sufficient amount of HCHO reaching target cells, and saturation of its metabolism to formate can increase the covalent binding of HCHO to DNA.

A central hypothesis of how chemical carcinogens act is that the agent or its metabolites are electrophilic and react with DNA to form covalently modified bases (adducts) (Miller and Miller 1981). The altered bases cause mutations by mispairing on replication, and cells with mutations in

This Investigator's Report is one part of Health Effects Institute Research Report Number 67, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigator's Report may be addressed to Dr. Timothy R. Fennell, Chemical Industry Institute of Toxicology, P.O. Box 12137, 6 Davis Drive, Research Triangle Park, NC 27709

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* A list of abbreviations appears at the end of the Investigator's Report.

critical genes can multiply and eventually give rise to tumors.

A considerable limitation of animal bioassays is their lack of sensitivity at low levels of exposure. Since DNA adducts are thought to be an important determinant of the relation between dose and carcinogenic response, measuring DNA adducts over a wide range of carcinogen doses may provide a better measure for estimating the risk associated with a range of doses than external exposure does. The level of DNA adducts can indicate the dose of an active chemical that reaches the critical target site. Measurement of adducts in exposed individuals may provide a useful biomarker of exposure, as well as a better measure of dose than that obtained by monitoring exposure concentrations (Perera and Weinstein 1982; Harris et al. 1987; Perera 1987a,b).

Several different methods have been developed for analyzing DNA adducts. Many investigations in laboratory animals have been conducted using radiolabeled carcinogens with detection of radioactive adducts. This type of approach is not applicable to studies in humans, and limits the utility of adduct determinations in laboratory animals, particularly in studies involving repeated exposures. More widely applicable approaches involve the measurement of fluorescent adducts, immunoassays, and analysis by gas chromatography with mass spectrometry (GC/MS) (Farmer et al. 1987). A method that has been widely used to determine the presence of adducts of unknown structure is the ^{32}P-postlabeling assay (Randerath et al. 1981; Gupta et al. 1982; Reddy et al. 1984).

Three types of covalent interaction of HCHO with DNA have been described: formation of *N*-hydroxymethyl adducts with the exocyclic amino groups of adenine, guanine, and cytosine (McGhee and von Hippel 1975a,b; Beland et al. 1984); DNA-DNA cross-link formation (Chaw et al. 1980); and DNA-protein cross-link formation (Ross and Shipley 1980; Bedford and Fox 1981; Casanova-Schmitz et al. 1984; Casanova et al. 1989, 1991). Of these, DNA-protein cross-links have been described in cells or tissues treated with HCHO (Casanova-Schmitz et al. 1984) and may constitute lesions capable of inducing the carcinogenic process.

Hydroxymethyl adducts may contribute to the induction of mutations and cancer; however, the measurement of their formation in DNA by conventional techniques using radioactive HCHO is complicated by their instability (Beland et al. 1984), by the metabolic incorporation of HCHO into nucleic acids (Beland et al. 1984; Casanova-Schmitz et al. 1984), and by an isotope effect that limits the oxidation of ^{3}H-formaldehyde to formate (Heck and Casanova 1987). The instability of HCHO-induced DNA-protein cross-links further complicates the detection of hydroxymethyl adducts by releasing free HCHO during DNA digestion. This released HCHO is capable of reacting with digested nucleotides to generate artifactual hydroxymethyl adducts (Casanova et al. 1989).

The nonlinearity between exposure concentration and carcinogenic response in the rat prompted studies investigating the dosimetry of HCHO in rat nasal tissue using DNA-protein cross-links as an indicator of the tissue dose of HCHO (Casanova-Schmitz et al. 1984; Casanova et al. 1989, 1991). A nonlinear relation was found between exposure concentration and the extent of HCHO bound by DNA-protein cross-linking. These studies were conducted using radiolabeled HCHO and, until recently, were restricted to a single exposure (Heck et al. 1992). The relevance of DNA-protein cross-links in the carcinogenic process has been under debate. An important question is whether hydroxymethyl adducts are formed in cells and tissues in which DNA-protein cross-links can be formed (Heck et al. 1990).

AIMS

The objective of this study was to develop methods for measuring specific HCHO adducts in DNA that do not require the use of radiolabeled HCHO. Hydroxymethyl adducts were selected as the type of adduct to be measured. A method of stabilizing hydroxymethyl adducts by reaction with sodium bisulfite to form sulfomethyl adducts (Hayatsu et al. 1982) was investigated. Reaction of HCHO-treated DNA with sodium bisulfite (Figure 1) was used to convert the unstable hydroxymethyl adducts to stable sulfomethyl derivatives of adenine and guanine in DNA (Hayatsu et al. 1982). The use of ^{14}C-formaldehyde and nonradioactive techniques (^{32}P-postlabeling and electrophore postlabeling) to detect these adducts was investigated.

METHODS

CHEMICALS

Adenosine 5′-[γ-^{32}P]triphosphate (triethylammonium salt, specific activity approximately 3,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [^{14}C]Formaldehyde was purchased from DuPont New England Nuclear (Wilmington, DE). T4-Polynucleotide kinase, deoxyadenosine 5′-monophosphate, deoxycytidine 5′-monophosphate, deoxyguanosine 5′-monophosphate, thymidine 5′-monophosphate, deoxyadenosine 3′,5′-diphosphate, deoxycytidine 3′,5′-diphosphate, deoxyguanosine 3′,5′-diphosphate, thymidine 3′,5′-diphosphate, and deoxyade-

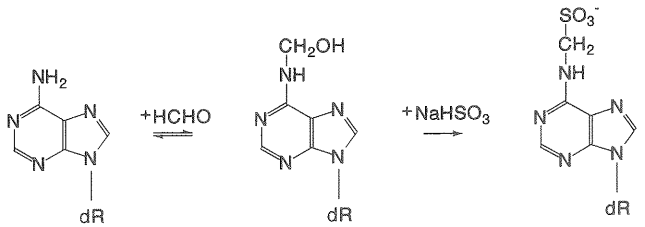

Figure 1. Reaction of deoxyadenosine with HCHO to form N^6-hydroxymethyldeoxyadenosine and subsequent reaction with sodium bisulfite ($NaHSO_3$) to form N^6-sulfomethyldeoxyadenosine.

nosine, deoxycytidine, deoxyguanosine, and thymidine purified by high-performance liquid chromatography (HPLC) were obtained from Pharmacia (Piscataway, NJ). Spleen phosphodiesterase, obtained from Boehringer Mannheim (Indianapolis, IN), and micrococcal endonuclease, obtained from Sigma Chemical Co. (St. Louis, MO), were dialyzed against distilled water before use. Deoxyadenosine 3′-monophosphate, deoxycytidine 3′-monophosphate, deoxyguanosine 3′-monophosphate, thymidine 3′-monophosphate, proteinase K, RNaseA, RNase T_1, nuclease P_1, potato apyrase, and adenosine 5′-triphosphate were obtained from Sigma Chemical Co. Calf thymus DNA (Sigma Chemical Co.) was either treated with alkali, dialyzed, and precipitated before use, or treated with RNase A and RNase T_1, and precipitated by phenol and ethanol before use. Phenol redistilled for molecular biology was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Sodium bisulfite and deuterium oxide (D_2O), were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methanol of HPLC grade was obtained from J.T. Baker (Phillipsburg, NJ). Formaldehyde (37% solution), HPLC-grade ammonium acetate, and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). Plastic-backed polyethyleneimine-cellulose (PEI-cellulose) thin-layer chromatography (TLC) plates with fluorescent indicator (EM Science, 20 × 20 cm), and aluminum-backed silica gel 60 plates with fluorescent indicator (EM Science, 20 × 20 cm) were obtained from Alltech Associates (Deerfield, IL). Other reagents were obtained from Fisher Scientific, Aldrich Chemical Co., or Pierce (Rockford, IL).

ANIMALS

Male F344 rats (200 to 300 g), obtained from Charles River Breeding Laboratories (Raleigh, NC), were housed in a 12-hour light and 12-hour dark cycle, at a temperature of 22° ± 2°C and humidity of 55% ± 5%, and were provided with food (NIH07 diet, Ziegler Brothers, Gardners, PA) and water ad libitum.

CELL CULTURE

The origin of the human TK6 lymphoblastoid cell line was described previously (Skopek et al. 1978). Tissue culture medium RPMI 1640 and horse serum were purchased from Gibco (Grand Island, NY). The stock culture cells were maintained in a humidified incubator with 6% carbon dioxide at 37°C in RPMI 1640 medium supplemented with 15% heat-inactivated horse serum (Craft et al. 1987).

INSTRUMENTATION

The system used for HPLC consisted of two Waters 510 pumps, a Waters 680 gradient controller, and a Waters 490 absorbance detector (Waters Associates, Milford, MA) interfaced to an IBM PC XT equipped with Maxima software (Dynamic Solutions, Ventura, CA), and a Rheodyne (Cotati, CA) 7125 injector. For some experiments, two automated switching valves (Waters 60057), operated by compressed air and programmed via the Waters 680 gradient controller, were used to switch the effluent from one column either to a detector or to a second column. Alternatively, a second system was used, consisting of two Waters 6000A pumps, a 680 gradient controller, a Rheodyne 7125 injector, a Hewlett-Packard (Palo Alto, CA) 1040 diode array detector, and a Ramona radioactivity detector (Raytest GmbH, Straubenharbt, Germany) or a Flo-One radioactivity detector (Radiomatics, Tampa, FL).

Nuclear magnetic resonance (NMR) spectra were acquired on a Varian (Palo Alto, CA) VXR 300 spectrometer equipped with a dual proton-multinuclear 5-mm probe. Ultraviolet (UV) spectra were measured on a Varian Cary 219 spectrophotometer. Mass spectra were acquired using a Kratos (Ramsey, NJ) MS 890 MS spectrometer operating at a resolution of approximately 1,000. Electron ionization spectra and chemical ionization spectra were acquired with solid probe insertion. Isobutane was the reagent gas used for chemical ionization. Fast atom bombardment was conducted using xenon, a beam of 0.5 mA at 8 kV, and a matrix of glycerol or glycerol with 5% trifluoroacetic acid.

Radioactivity was measured by scintillation counting (^{14}C) with external standardization using a Packard (Downers Grove, IL) Tricarb 1900CA counter, or by Cerenkov counting (^{32}P) in a Packard Tricarb 460-CD counter. Ecolume scintillant was obtained from ICN Biomedicals Inc. (Irvine, CA), and Flo-Scint II was from Radiomatics.

PREPARATION OF SULFOMETHYL ADDUCT STANDARDS

N^6-Sulfomethyladenine (SOMeA) and N^2-sulfomethylguanine (SOMeG) were synthesized by the method of Hayatsu and colleagues (1982). Nucleoside standards were synthesized by incubating deoxyadenosine or deoxyguanosine (10 mM) with 100 mM HCHO at 37°C for 4 hours, followed by treatment with 1.0 M sodium bisulfite at 4°C for 12 hours.

Sulfomethyldeoxyadenosine 3′-monophosphate (SOMedAp) was prepared by reaction of 40 mM deoxyadenosine 3′-monophosphate with 100 mM HCHO at 37°C for 4 hours, followed by treatment with 1.0 M sodium bisulfite (pH 5.0) at 4°C for 48 hours. The products of the reaction were separated by chromatography on a Beckman (San Ramon, CA) C_{18} column eluted with 0.25 M ammonium acetate (pH 6.0), and a gradient of 5% to 20% methanol over 10 minutes. Fractions were collected, the solvent was removed by lyophilization, and the product was used for analysis by NMR spectroscopy or ^{32}P-postlabeling.

Sulfomethyldeoxyguanosine 3′-monophosphate (SOMedGp) was synthesized similarly by reaction of 40 mM deoxyguanosine 3′-monophosphate with 200 mM HCHO at 37°C for 4 hours, followed by reaction with 1.0 M bisulfite (pH 7.0) at 4°C for 48 hours. The products of the reaction were separated by chromatography on a Beckman C_{18} column

eluted with 0.25 M ammonium acetate (pH 6.0) and 5% methanol. Fractions were collected, the solvent was removed by lyophilization, and the product was used for analysis by NMR spectroscopy or by ^{32}P-postlabeling.

N^2-Sulfomethylguanosine and N^6-sulfomethyladenosine were prepared by reaction of guanosine or adenosine with HCHO at pH 9.5 and treatment with sodium bisulfite at pH 2.5. The products formed were purified by chromatography on activated charcoal/celite eluted with ethanol/water (Hayatsu et al. 1982), and by preparative HPLC on a Beckman Ultrasphere ODS column (1 × 25 cm) eluted with 0.25 M ammmonium acetate (pH 6.0) and methanol. Fractions were collected, and the solvent was removed under vacuum.

MODIFICATION OF CALF THYMUS DNA

Calf thymus DNA was incubated with 0.4 mM ^{14}C-formaldehyde (56 mCi/mmol) for four hours at 37°C, followed by treatment with sodium bisulfite at 4°C.

MODIFICATION OF RAT LIVER NUCLEAR DNA

Nuclei were isolated from rat liver DNA by the method of Blobel and Potter (1966), using *N,N*-bis(2-hydroxyethyl)glycine (bicine)–sodium hydroxide instead of 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–hydrochloric acid (HCl) buffer. Incubation was carried out with 0.40 mM ^{14}C-formaldehyde in 50 mM bicine (pH 7.4), 25 mM potassium chloride, and 5 mM magnesium chloride for four hours at 37°C, followed by treatment with sodium bisulfite.

EXPOSURE OF LYMPHOBLASTS TO FORMALDEHYDE

Human TK6 lymphoblast cells in 15-mL centrifuge tubes at a density of 1×10^7/mL in 10 mL of RPMI 1640 medium with 25 mM N^2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) were treated with 0, 50, 100, or 400 μM ^{14}C-formaldehyde for 1.5 or 2 hours at 37°C. After cooling to 4°C and homogenizing, sodium bisulfite (1.0 M, pH 5.0 or 7.0) was added and the samples were kept at 4°C for 12 hours.

ISOLATION AND SEPARATION OF SULFOMETHYL ADDUCTS

After dialysis against distilled water to remove bisulfite, DNA was isolated from treated nuclei or cells by proteinase K treatment, phenol-chloroform extraction, and incubation with RNase A and RNase T_1 (Gupta 1985). DNA was quantitated by measuring absorbance at 260 nm (20 absorbance units = 1 mg).

DIGESTION OF DNA TO NUCLEOSIDES

DNA samples treated with ^{14}C-formaldehyde and bisulfite were dialyzed against distilled water. Digestion of calf thymus DNA to nucleosides was carried out by incubation (1 mg/mL) with DNase I (400 U/mL) in 10 mM 2-bis(2-hydroxyethyl)-amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris, pH 7.1), and 10 mM magnesium chloride with incubation at 37°C for 30 minutes. This was followed by addition of 100 mM Tris-HCl (pH 8.3), bacterial alkaline phosphatase, and snake venom phosphodiesterase (both 2.5 U/mL), and incubation was continued for 30 minutes at 37°C. DNA from nuclei or TK6 cells was digested similarly, but each incubation lasted two hours at 37°C. The digested samples were analyzed by HPLC using a C_{18} column eluted with 0.25 M ammonium acetate (pH 6.0), and a gradient of 5% to 20% methanol over 20 minutes. Elution was monitored using UV absorbance at 254 nm, by collecting samples for scintillation counting, or by using a radioactivity detector.

^{32}P-POSTLABELING

DNA samples were digested to nucleotide 3′-monophosphates using micrococcal endonuclease and spleen phosphodiesterase, essentially as described by Gupta and associates (1982). Both of these enzymes were dialyzed against distilled water at 4°C before use.

Samples were ^{32}P-postlabeled essentially as described by Gupta and coworkers (1982). For samples purified by HPLC before labeling, the solvent was removed in a centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY), and the samples were stored at –20°C before labeling. Water (5 μL) was added to each sample in a 1.5-mL microcentrifuge tube. The tube was capped, vortexed, centrifuged, and incubated at 37°C for 30 minutes. A mixture was prepared containing 2.5 μL of 0.8 M bicine–sodium hydroxide (pH 9.5), 2.5 μL of 0.4 M magnesium chloride, 2.5 μL of 0.4 M dithiothreitol, 2.5 μL of 40 mM spermidine, 20 μL of [γ-^{32}P]adenosine triphosphate (ATP) (200 μCi, 3,000 Ci/mmol), 5 μL of T4-polynucleotide kinase, 2 μL of 0.5 mM ATP, and 13 μL of water. The mixture was vortexed and centrifuged, and 5-μL aliquots were added to each adduct sample. Each tube was vortexed and centrifuged, and then incubated for 30 minutes at 37°C. To each tube was then added 1 μL of a solution of apyrase (40 mU/mL). After mixing and centrifuging, the samples were incubated at 37°C for 30 minutes. For analysis of postlabeled samples as nucleotide 5′-monophosphates, incubation with apyrase was omitted, and nuclease P_1 was added as described by Reddy and associates (1984) with further incubation at 37°C for one hour.

For adduct enrichment before postlabeling, several systems were evaluated. In system 1, samples of nucleotides were chromatographed on a C_{18} column (Beckman 5-μm Ultrasphere ODS, 0.46 × 25 cm), eluted with 0.25 M ammonium acetate (pH 6.0), and a gradient of 5% to 20% metha-

nol over 10 minutes. In system 2, two C_{18} columns were used in a column-switching system, in which the effluent from the first column (5-μm Ultrasphere ODS, 0.46 × 15 cm) was switched to collect the eluting adduct onto a second C_{18} column (5-μm Ultrasphere ODS, 0.46 × 25 cm). The columns were eluted with 0.25 M ammonium acetate (pH 6.0) and methanol at a flow rate of 1.0 mL/min. Elution was conducted with 4% methanol from 0 to 6 minutes, a linear gradient from 4% to 20% methanol from 6 to 16 minutes, and with 20% methanol to 30 minutes. The initial flow of solvent was through column 1 until 5.6 minutes, when the effluent from column 1 was switched onto column 2. At 6.8 minutes, the solvent flow was switched entirely onto column 2 for resolution of SOMedAp. After 23 minutes, the solvent flow was switched to column 1 for cleaning, and at 30 minutes to both columns 1 and 2. In system 3, nucleotides were chromatographed on a Chromegabond RPSAX (reverse-phase strong anion exchange) mixed functionality column (0.46 × 25 cm; ES Industries, Marlton, NJ) eluted with 0.05 M ammonium formate (pH 2.67) and a linear gradient of 0% to 30% methanol. Nucleotides and adduct standards were detected by measuring absorbance at 254 nm.

The eluted adducts were collected and concentrated under vacuum using a Savant centrifugal concentrator. Samples were redissolved in 5 μL of water, mixed, centrifuged, and incubated at 37°C for 30 minutes. ^{32}P-Postlabeling was carried out as described above by adding 5 μL of a solution of 40 mM bicine (pH 9.5), 20 mM magnesium chloride, 20 mM dithiothreitol, 0.2 mM spermidine, 20 μCi of [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 5 U of T4 - polynucleotide kinase. After incubating for 30 minutes at 37°C, 1 μL of a solution of apyrase (10 mU) was added, and incubation was continued for one hour at 37°C.

Samples were analyzed by two-dimensional TLC using PEI-cellulose plates. Before use, the TLC plates were washed in 500 mL of methanol and twice in 500 mL of distilled water and then dried. Dried plates were stored at 4°C before use. Samples were analyzed for the presence of labeled normal nucleotides using a one-dimensional TLC system with development in water to the origin (2 cm from the bottom of the plate) and development in 0.27 M ammonium sulfate to the top of the plate.

For analysis of nucleotide 3′,5′-diphosphate samples, the plates were soaked in 0.15 M ammonium formate (pH 3.5) and dried. Aliquots (1 to 4 μL) of labeled samples were applied to 10- × 10-cm TLC plates 2 cm from the bottom left corner. Development in the first dimension was with 1.5 M ammonium formate (pH 3.5). The plate was dried, washed with 0.01 M Tris base followed by distilled water, and dried. The plate was rotated through 90° and was developed in the second dimension with 0.5 M ammonium sulfate (pH 8.0).

Nucleotide 5′-monophosphates were analyzed by two-dimensional chromatography on 10- × 10-cm PEI-cellulose TLC plates using 0.15 M ammonium formate (pH 3.5) for development in the first dimension and 0.1 M ammonium sulfate (pH 8.0) for development in the second dimension. Between the first and second dimension, each plate was washed in methanol and dried.

Radioactivity was detected by autoradiography using Kodak XAR-5 film with intensifying screens (Lighting Plus). Chromatograms were mounted on a sheet of light cardboard (8 × 10 in) and were labeled using Rad Tape (Diversified Biotech, Newton Centre, MA) and radioactive ink. The chromatograms were then wrapped in Saran Wrap, and x-ray film was placed between the chromatogram and the intensifier screen. Exposures were conducted for 30 minutes to 24 hours at room temperature. Exposed films were developed in a Kodak M-35A X-OMAT automatic film processor. Radioactive spots on PEI-cellulose chromatograms were quantitated by excision and measurement of the radioactivity by Cerenkov counting.

DERIVATIZATION REACTIONS FOR N^6-SULFOMETHYLADENINE AND N^2-SULFOMETHYLGUANINE

N^2-Sulfomethylguanine (0.75 mg) was reacted with diazomethane in diethyl ether (0.5 mL) at room temperature for two hours. The solvent was removed under nitrogen.

Synthesis of a trimethylsilyl ester was attempted with 0.75 mg of SOMeG or SOMeA, 0.27 mL of acetonitrile, and 50 μL of bis(trimethylsilyl)trifluoroacetamide. The mixture was vortexed and heated to 80°C for two hours. The solvent was removed under nitrogen. Similarly, reactions were conducted with 100 μL of acetonitrile or pyridine, and 50 μl of bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane with heating for two hours at 80°C.

Synthesis of a pentafluorobenzyl ester was attempted with 3.2 mg of SOMeA in 230 μL of acetonitrile, 15 mg of sodium carbonate, and 3.2 μL of α-bromo-2,3,4,5,6-pentafluorotoluene. The reaction was stirred and heated to 80°C for 20 hours, and further aliquots of acetonitrile and α-bromo-2,3,4,5,6-pentafluorotoluene were added. The reaction continued for 20 hours. The solid was removed by filtration, and the acetonitrile phase was analyzed by TLC.

Reaction of SOMeA (10 mg) with sodium methoxide (12 mg) in methanol (8 mL) was attempted. After two days at room temperature, the formation of reaction products was evaluated by TLC. Reaction of SOMeA (10 mg) with diethyl malonate (10 mg) in water (80 μL) and piperidine (4 μL) was carried out for four hours at room temperature. Treatment of SOMeA (2.5 mg) with potassium cyanide (3 mg) in 0.01 M potassium hydroxide was conducted at room temperature for 18 hours.

Reactions of SOMeA or SOMeG with derivatizing agents were monitored by TLC of aliquots of reaction mixtures on silica gel 60 TLC plates with fluorescent indicator developed in methanol, or 10% methanol in methylene chloride. Alternatively, HPLC using essentially system 1 described

above, with a 15-minute linear gradient of 5% to 20% methanol, was used.

RESULTS

REACTION OF FORMALDEHYDE WITH DNA IN VITRO

The optimization of the reaction of bisulfite with HCHO and nucleosides has been investigated using a range of pH, reagent concentrations, and reaction times. The original report by Hayatsu and associates (1982) described the synthesis of SOMeA, SOMeG, N^4-sulfomethylcytosine, and various nucleoside and nucleotide derivatives, as well as the formation of N^2-sulfomethyldeoxyguanosine 5′-monophosphate (SOMedpA) in DNA treated with HCHO followed by sodium bisulfite at pH 7.0. However, limited data were reported for the effects of pH, reaction time, and reagent concentrations. In the current work, products were analyzed by separation on reverse-phase HPLC, using an Ultrasphere ODS column with 0.25 M ammonium acetate (pH 6.0), with a methanol gradient for elution of the products (the percentage of methanol used depended on the nucleoside under investigation). Reactions of deoxyadenosine and deoxyguanosine with HCHO and bisulfite at low pH produced extensive depurination of the resulting sulfomethyl adducts, giving both sulfomethyl nucleoside and base adducts. The optimum yield of sulfomethyldeoxyguanosine (SOMedG) was obtained at pH 7.0, with little formation of sulfomethyldeoxyadenosine (SOMedA) at this pH. The optimum yield of SOMedA was found at pH 5.0, but at this pH depurination of SOMedG was extensive. Sodium bisulfite concentrations of 0.5, 1.0, 1.5, and 2.0 M were investigated for the reaction yield. After 12 hours, the reaction at 0.5 M concentration was incomplete, but the reaction at 1.0 M and above was essentially complete, with small amounts of hydroxymethyl adducts remaining.

The reaction of ^{14}C-formaldehyde with calf thymus DNA and then bisulfite was also investigated. After treatment with bisulfite, the reaction mixture was dialyzed against distilled water and then digested enzymatically to nucleosides for separation and analysis by HPLC. The pH of bisulfite treatment was varied, with investigations carried out at pH 5.0, 5.5, 6.0, 6.5, and 7.0. Figure 2 shows the chromatograms of the DNA hydrolysates. At pH 7.0, peaks were present due to HCHO, N^6-hydroxymethyldeoxyadenosine (HOMedA), SOMedG and SOMeG. As the pH was decreased, the percentage of radioactivity associated with the SOMedG decreased, and the percentage of SOMeG increased, indicating depurination of the adduct was taking place. The percentage of radioactivity associated with the SOMedA peak increased with decreasing pH. However, a significant amount of HOMedA remained at pH 5.0, suggesting either that the reaction was incomplete, or that the reaction had occurred following the bisulfite pretreatment with residual HCHO.

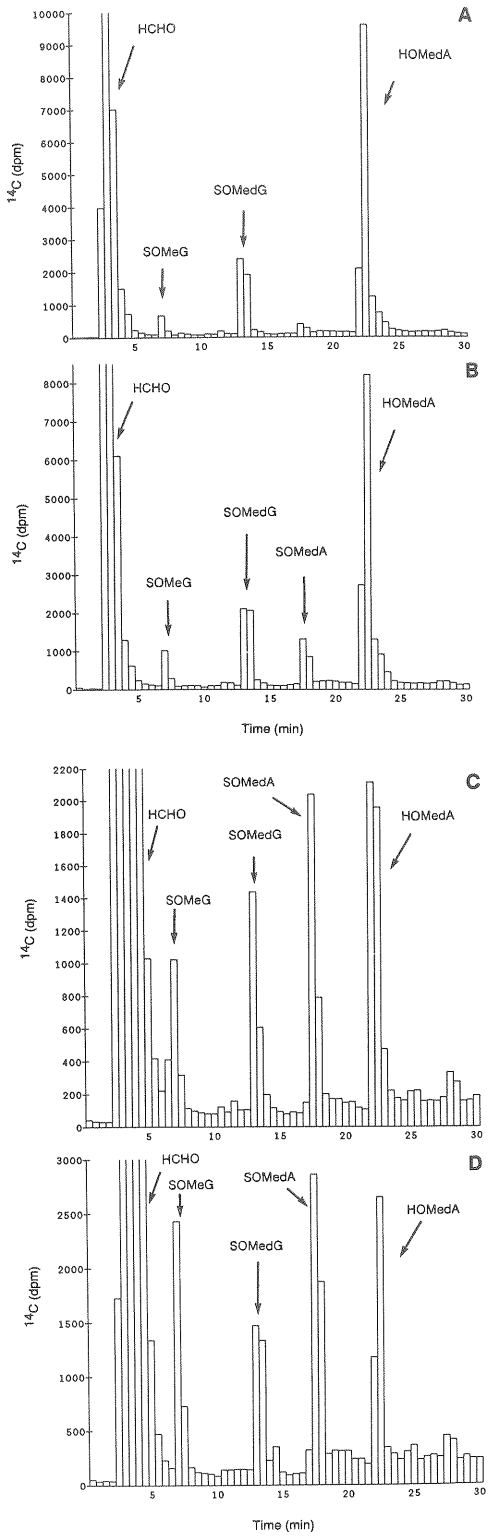

Figure 2. Reverse-phase HPLC chromatogram of nucleoside digests of calf thymus DNA after treatment with 500 μM ^{14}C-formaldehyde for four hours at 37°C and then with 1.0 M sodium bisulfite at 4°C. The sodium bisulfite solution was (A) pH 7.0, (B) pH 6.0, (C) pH 5.5, or (D) pH 5.0. Fractions of the eluate were collected for monitoring of radioactivity.

The removal of residual HCHO from calf thymus DNA presented difficulties in another study (Beland et al. 1984). Extensive precipitation and gel permeation chromatography did not remove all the "noncovalently" bound HCHO. Extensive dialysis and purification of DNA reacted with HCHO and bisulfite in vitro did not remove entirely the early eluting material, presumed to be HCHO or HCHO-bisulfite complex. Hydroxymethyl adducts can be formed by reaction of HCHO with nucleosides during the digestion of DNA (Casanova et al. 1989). In calf thymus DNA at pH 5.0 (Figure 2), the SOMedA:HOMedA ratio was 1.3:1. Although lower pH, or increased reaction times, or bisulfite concentrations could have increased the yield of SOMedA, more extreme conditions were avoided because of concern about decomposition of adducts or DNA-protein cross-links in biological samples. For subsequent treatments, 1.0 M bisulfite at either pH 5.0 or 7.0 was used.

To ensure that the HCHO-bisulfite complex was not reacting with nucleosides to form sulfomethyl adducts, HCHO and bisulfite were mixed together and then added to a solution of DNA. No sulfomethyl adducts were detected following dialysis and digestion of the DNA.

REACTION OF FORMALDEHYDE WITH DNA IN NUCLEI AND CELLS IN CULTURE

Formaldehyde reacts extensively with DNA in isolated systems to produce hydroxymethyl adducts; however, its reaction with DNA in cells in which other nucleophiles such as glutathione and proteins preferentially react with HCHO has been the subject of controversy. The reaction of HCHO in DNA with isolated nuclei was investigated as a model for biological systems. Isolated nuclei obtained from rat hepatocytes by density gradient centrifugation were incubated with 400 μM ^{14}C-formaldehyde (50 mCi/mmol). The suspensions then were treated with sodium bisulfite at 4°C, followed by dialysis and isolation of the nuclear DNA. On chromatography of nucleoside digests of the DNA, two radioactive peaks were found that comigrated with added SOMedG and SOMedA (Figure 3). The level of adduct formation was variable and resulted in low levels of radioactivity associated with the eluting peaks. In turn, the quantitation of these peaks is inherently uncertain because of the low level of activity above background. For the peak comigrating with SOMedA, radioactivity equivalent to 4.1 ± 0.5 pmol/mg was detected (mean ± SD, n = 3).

To extend detection of these adducts to isolated cells, experiments similar to those described above were carried out in human TK6 lymphoblasts. Mutagenesis data for HCHO in this system have already been obtained (Craft et al. 1987). The cells were treated with 50 μM or 400 μM ^{14}C-formaldehyde for two hours at 37°C. After cooling to 4°C and disruption of the cells, sodium bisulfite (1.0 M, pH 5.0 or 7.0) was added, and the samples were kept at 4°C for 12 hours. Following dialysis and isolation of DNA, hydrolysis to nucleosides, and chromatography on reverse-phase HPLC, high levels of radioactivity were found associated with the UV-absorbing peaks for deoxyadenosine, deoxyguanosine, and deoxythymidine (Figure 4). This would result from oxidation of the HCHO to formate and incorporation into the normal nucleosides in DNA. In addition, other UV-absorbing and radioactive peaks were found at approximately 13 and 19 minutes, which also interfered with the detection of SOMedG andSOMedA. The large extent of metabolic incorporation limited the sensitivity of detection for the sulfomethyl adducts because they elute close to the normal nucleosides in this chromatography system. These data indicate that other methods are required to detect sulfomethyl adducts in intact biological systems such as cells or tissues from whole animals.

^{32}P-POSTLABELING

Previous work suggested that the sulfomethyl adducts could be detected by ^{32}P-postlabeling (Fennell et al. 1987). Sulfomethyldeoxyadenosine 3′-monophosphate (SOMedpA) was a substrate for T_4-polynucleotide kinase and could

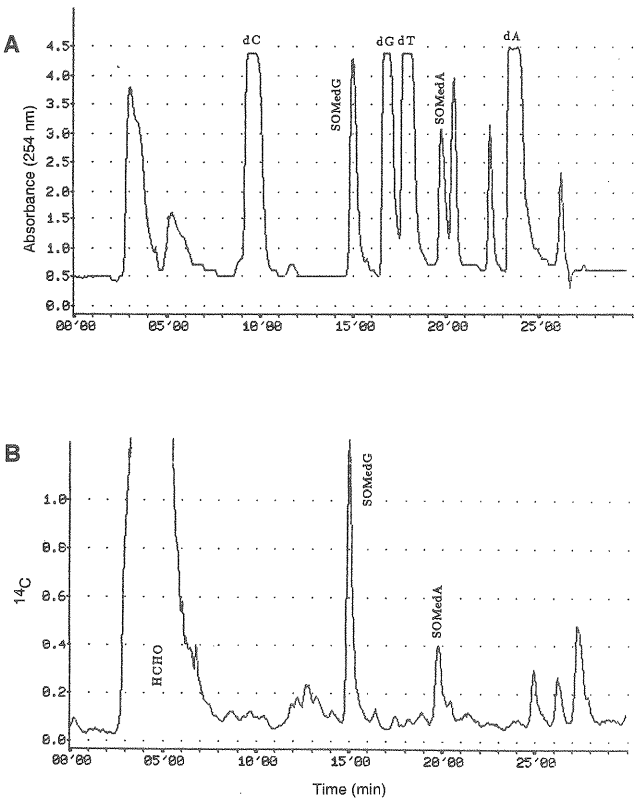

Figure 3. Reverse-phase HPLC chromatogram of a nucleoside digest of DNA isolated from rat liver nuclei after treatment with 400 μM ^{14}C-formaldehyde. After incubation with HCHO, the nuclei were treated with 1.0 M sodium bisulfite at 4°C. Following dialysis and isolation of DNA with digestion to nucleosides, HPLC was carried out, with monitoring of (A) UV absorbance and (B) radioactivity. The scales on the y axis represent counts per second (× 10) for radioactivity and an arbitrary scale for absorbance. dC = deoxycytodine, dG = deoxyguanosine, dT = deoxythymidine, dA = deoxyadenosine.

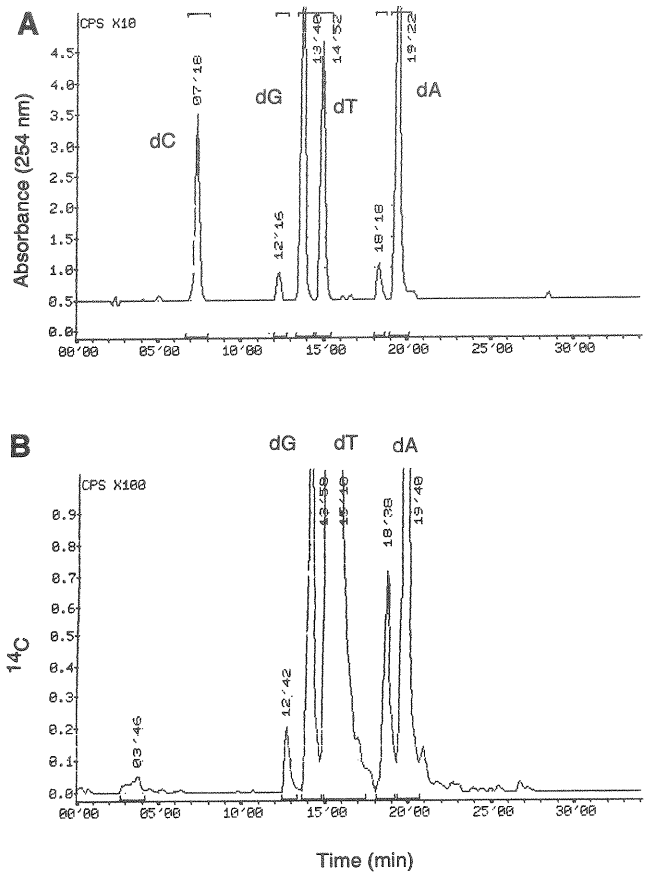

Figure 4. Reverse-phase HPLC chromatogram of a nucleoside digest of DNA isolated from human TK6 lymphoblasts treated with 400 μM ^{14}C-formaldehyde. Following treatment with HCHO at 37°C, the cells were treated with 1.0 M sodium bisulfite (pH 7.0) at 4°C. The suspension was then dialyzed, and the DNA was isolated and digested to nucleosides. (A) The UV absorbance at 254 nm and (B) the radioactivity were recorded. Two large UV-absorbing and radioactive peaks (retention times of approximately 13 and 19 minutes) obscure the regions were SOMedG and SOMedA elute. The scales on the *y* axis represent counts per second (× 100) for radioactivity and an arbitrary scale for absorbance. dC = deoxycytodine, dG = deoxyguanosine, dT = deoxythymidine, dA=deoxyadenosine, CPS = counts per second.

be detected in calf thymus DNA following treatment with HCHO and sodium bisulfite.

On reaction of HCHO with deoxyadenosine 3′-monophosphate followed by treatment with sodium bisulfite, a product was formed that was isolated and characterized by NMR spectroscopy (Figure 5). At room temperature, broad peaks were obtained and resolution was poor, but heating the sample to 45°C improved the resolution. This suggests that sample aggregation or intermolecular hydrogen bonding is occurring. Similarly, broad lines that sharpened on heating were observed for HOMedA (Beland et al. 1984). The expected signals for the deoxyadenosine 3′-monophosphate protons were detected, and in addition, a two-proton singlet at 4.9 parts per million (ppm) assigned to the CH_2 protons derived from HCHO was observed. This adduct has been assigned the structure of SOMedAp.

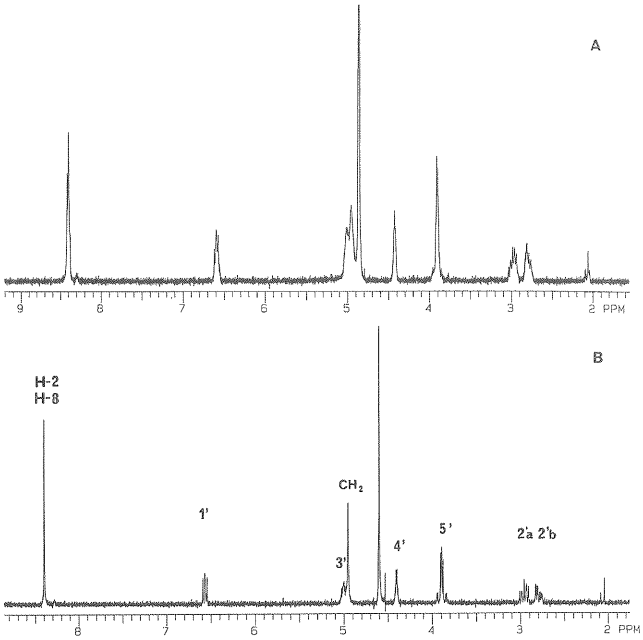

Figure 5. 300 MHz ^{1}H Nuclear magnetic resonance spectrum of SOMedAp in D_2O. Spectra were obtained (A) at room temperature and (B) at 45°C.

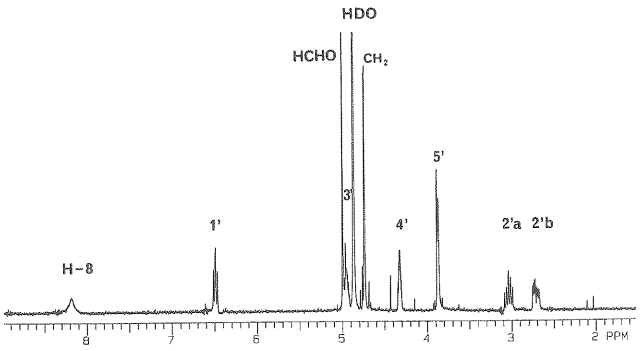

Figure 6. 300 MHz ^{1}H Nuclear magnetic resonance spectrum of SOMedGp in D_2O. HDO indicates signals arising from the low concentration of protons in the deuterated water solvent.

The reaction of HCHO with deoxyguanosine 3′-monophosphate (dGp) followed by sodium bisulfite yielded a product that has been isolated by reverse-phase HPLC and characterized by NMR spectroscopy (Figure 6). This sample also showed broad signals, especially of the H-8 proton. Couplings have been assigned with ^{1}H-^{1}H-correlation spectroscopy (Figure 7). The expected signals for the deoxyguanosine 3′-monophosphate residue were observed. In addition, a two-proton multiplet (AB quartet, J = 14.5 Hz) was observed at 4.72 ppm for the CH_2 signal derived from the HCHO group. The presence of spin-spin coupling between these protons indicates that they are nonidentical. In addition, a peak due to residual HCHO (5.0 ppm) was observed in this sample. This adduct has been assigned the structure of SOMedGp.

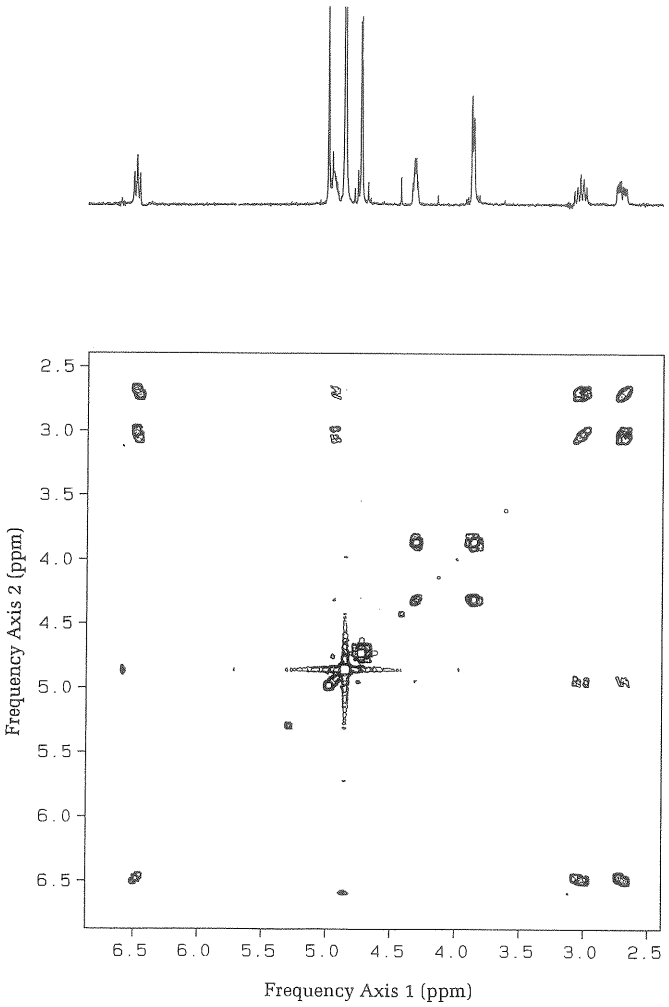

Figure 7. 300 MHz ^{1}H-^{1}H Correlation spectroscopy of SOMedGp in D_2O.

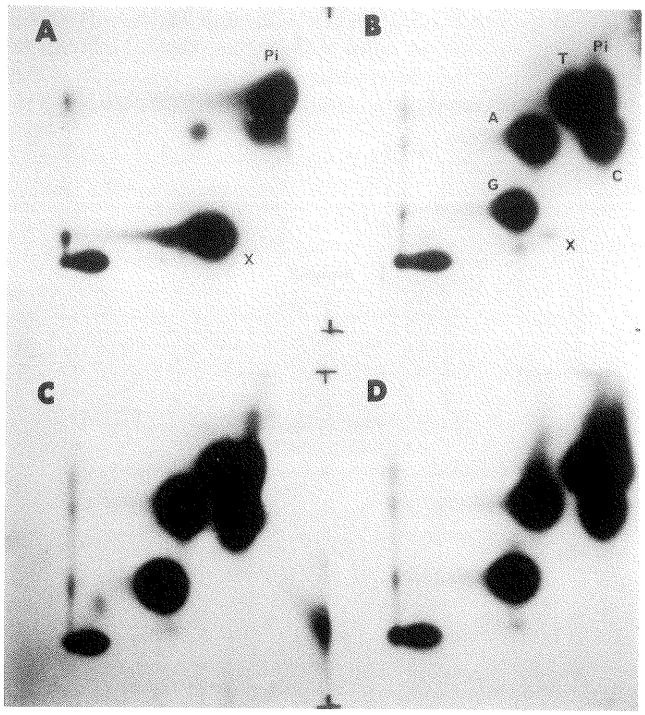

Figure 8. Polyethyleneimine-cellulose chromatograms of ^{32}P-postlabeled samples of SOMedAp. Samples consisted of (A) pure SOMedAp, (B) SOMedAp, 1 adduct in 10^3 normal nucleotides, (C) SOMedAp, 1 adduct in 10^4 normal nucleotides, and (D) normal nucleotides without adduct. A = deoxyadenosine 3′,5′-diphosphate, C = deoxycytodine 3′,5′-diphosphate, G = deoxyguanosine 3′,5′-diphosphate, T = deoxythymidine 3′,5′-diphosphate, X = SOMedpAp, Pi = phosphate. Labeling was carried out using the method of Gupta and associates (1982), and samples (2 µL containing approximately 8 µCi of ^{32}P) were chromatographed from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). The origin is in the lower left corner of each plate.

SENSITIVITY OF DETECTION BY ^{32}P-POSTLABELING

For experiments to determine the sensitivity of the ^{32}P-postlabeling assay, standard nucleotide 3′-monophosphate solutions were prepared using UV absorbance to determine concentration. A solution of SOMedAp was prepared, also using UV absorbance to determine concentration. Standard solutions were then prepared with final concentrations of 0.3 mM total nucleotides, with ratios of adduct to total nucleotides ranging from 1 in 10 to 1 in 10^6. The sensitivity of detection of SOMedAp was investigated using a modification of the method of Gupta and coworkers (1982). For the studies on postlabeling described below, SOMedAp was investigated. SOMedAp was detected in samples with adduct ratios down to 1 adduct in 10^3 normal nucleotides (Figure 8B). In samples containing 1 adduct in 10^4 normal nucleotides, it was not possible to detect SOMedAp (Figure 8C). The sensitivity of detection of the adducts was poor because of the difficulty in separating the adducts from the labeled normal nucleotides and other contaminating spots on the chromatograms.

In general, three approaches have been utilized by others for the enhancement of sensitivity of detection of adducts by ^{32}P-postlabeling, and involve enrichment of the adduct with removal of normal nucleotides prior to the postlabeling step. The approaches include the use of nuclease P_1 (Reddy and Randerath 1986), butanol extraction (Gupta 1985), and HPLC enrichment (Dunn and San 1988; Wilson et al. 1988; Shields et al. 1990; Watson et al. 1992).

Treatment of digested DNA samples containing both normal and adduct nucleotide 3′-monophosphates with nuclease P_1 can cause enrichment of adducts for postlabeling when the adducts are resistant to the actions of this enzyme, which hydrolyzes the 3′-phosphate group of normal nucleotides, resulting in the formation of nucleosides (Reddy and Randerath 1986). The resulting nucleosides do not act as substrates in the kinase reaction, which can considerably enhance the sensitivity of detection of ad-

ducts. However, for SOMedAp, although hydrolysis to the nucleoside proceeded more slowly than the hydrolysis of normal nucleotides, the adduct was not resistant to the effects of nuclease P_1 (Figure 9). This rules out nuclease P_1 enrichment as a method of improving the sensitivity of detection for SOMedA.

Extraction with butanol has been used to enhance the sensitivity of ^{32}P-postlabeling for aromatic adducts derived from compounds such as acetylaminofluorene and aminofluorene, in which the adducts are hydrophobic and possess solubility characteristics that are significantly different from those of normal nucleotides (Gupta 1985). However, the sulfomethyl adducts are not expected to be extracted preferentially using this type of approach.

High-performance liquid chromatography has been used to enrich adduct fractions of a number of adducts prior to postlabeling, including large hydrophobic adducts and adducts derived from alkylating agents that have chroma-

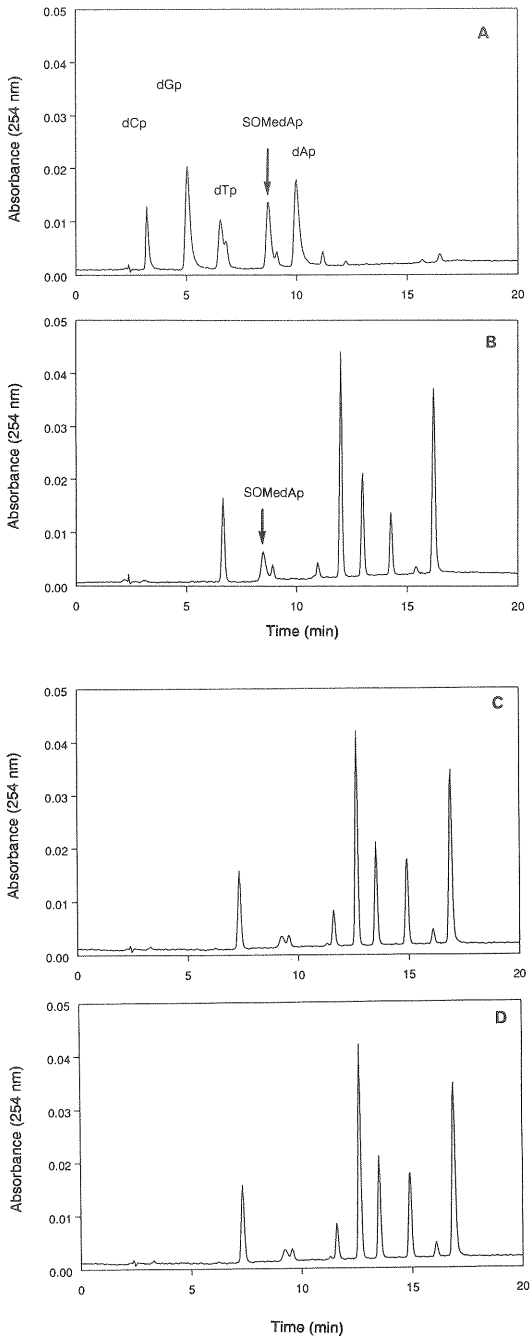

Figure 9. Reverse-phase HPLC separation of deoxyadenosine 3′-monophosphate (dAp), deoxycytodine 3′-monophosphate (dCp), deoxyguanosine 3′-monophosphate (dGp), deoxythymidine 3′-monophosphate (dTp), and SOMedAp (A) before incubation; and after incubation with nuclease P_1 for (B) 15 minutes, (C) 30 minutes, and (D) 45 minutes. Elution was carried out with a 10-minute gradient of 5% to 20% methanol in 0.25 M ammonium acetate (pH 6.0) with measurement of absorbance at 254 nm.

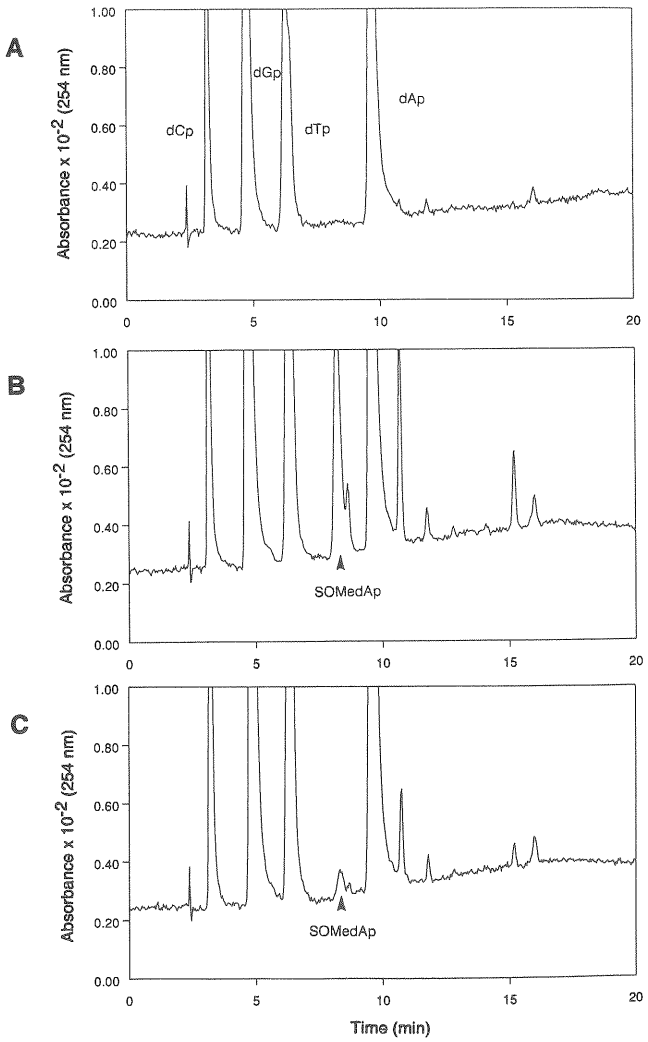

Figure 10. Separation of SOMedAp from normal nucleotide 3′-monophosphates by reverse-phase HPLC. Aliquots (10-μL) of solutions of 0.3 mM nucleotides containing (A) no adducts, (B) SOMedAp, 1 adduct in 10^2 normal nucleotides, or (C) SOMedAp, 1 adduct in 10^3 normal nucleotides. HPLC system 1 was used (see the Methods section). dCp = deoxycytodine, 3′-monophosphate, dGp = deoxyguanosine 3′-monophosphate, dTp = deoxythymidine 3′-monophosphate, and dAp = deoxyadenosine 3′-monophosphate.

tographic properties similar to those of normal nucleotides (Dunn and San 1988; Wilson et al. 1988; Shields et al. 1990; Watson et al. 1992). Reverse-phase HPLC using an Ultrasphere C_{18} column was used to separate the normal nucleotides from SOMedAp. Elution was carried out with an ammonium acetate buffer, which was required for retention of the nucleotides on the column and could be removed under vacuum. Figure 10 shows a chromatogram of normal nucleotides and SOMedAp with detection by measurement of UV absorbance. A UV-absorbing peak could be detected for SOMedAp at adduct levels of 1 in 10^3. Fractions corresponding to the adduct were collected, the solvent was removed under vacuum, and the residue was redissolved and subjected to ^{32}P-postlabeling. In initial experiments with HPLC enrichment of SOMedAp, recovery of adducts was poor, frequently with complete disappearance of the adduct. This could result from absorption to the plastic tube, or from degradation during the process of solvent removal, reconstitution, postlabeling, and separation, and may involve depurination, loss of the 3′-monophosphate group, or loss of the sulfomethyl group. Incomplete removal of salts from the HPLC buffer system could also inhibit the T4-polynucleotide kinase reaction.

To minimize possible losses, some changes were made to the procedure for purifying of adducts. The microfuge tubes were washed with distilled water and methanol, then dried before use. The sample was concentrated using a Savant centrifugal concentrator. It was reconstituted by addition of 5 µL of distilled water, vortex mixing, centrifuging, and incubation at 37°C for 30 minutes. The recovery of adduct was investigated using 3 nmol of SOMedAp added to 1 mL of mobile phase (10% methanol in 0.25 M ammonium acetate, pH 6.0). The samples were dried in a Savant concentrator, reconstituted, and subjected to HPLC. Figure 11 shows chromatograms of standards of equivalent concentration to the starting sample, and the samples recovered from drying. The recovery of the adducts after drying was 79%. No additional products were detected.

The limit of detection of pure SOMedAp in the postlabeling assay was investigated by labeling known amounts of pure adduct (1.5 fmol to 1.5 pmol) using ^{32}P-ATP of high specific activity of either 3,000 or 350 Ci/mmol. At the lower specific activity, the adduct was readily detectable at 150 fmol (Figure 12). At the higher specific activity, the adduct was readily detectable at 15 fmol (Figure 13). With a longer period of autoradiography, the adduct spots could be detected at 1.5 fmol with both the high and low specific activity ATP (Figure 14).

The detection of SOMedAp following HPLC separation from mixtures of normal nucleotides was investigated using standard nucleotide mixtures containing dilutions of adduct. Samples containing 3 nmol of total nucleotides (equivalent to approximately 1 µg of DNA) in 10 µL of water were chromatographed by HPLC (as in Figure 10), and the fractions corresponding to SOMedAp were collected, dried,

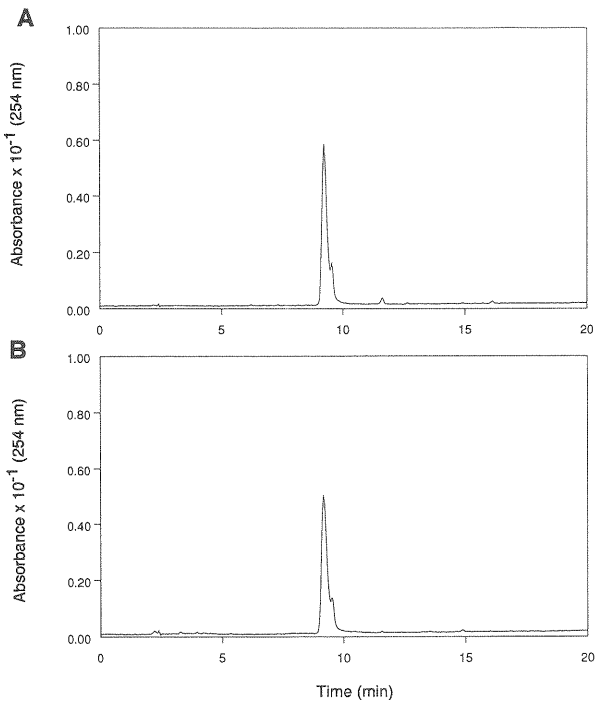

Figure 11. Recovery of SOMedAp after removal of solvent. HPLC analysis was conducted using system 1 on (A) a standard of SOMedAp, and (B) a sample of SOMedAp after recovery from 1 mL of 0.25 M ammonium acetate (pH 6.0), containing 10% methanol, by drying in a Savant centrifugal concentrator.

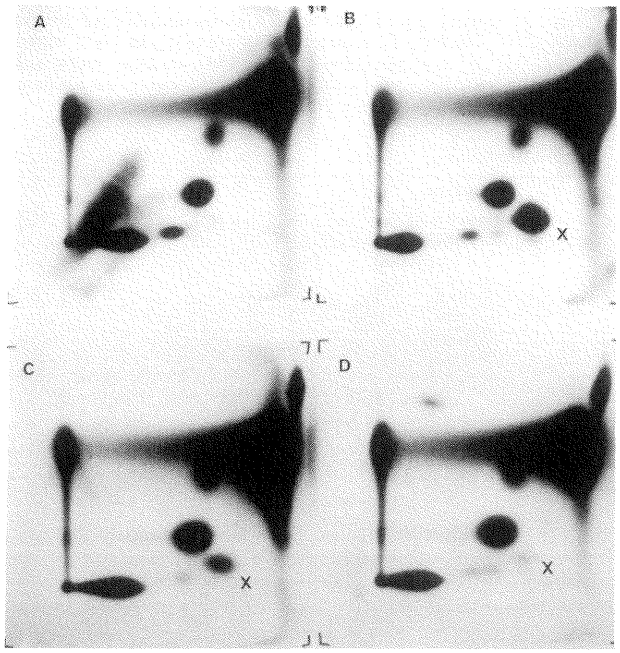

Figure 12. Detection of adduct standards following ^{32}P-postlabeling with ^{32}P-ATP (specific activity 350 Ci/mmol). Serial dilutions of standard SOMedAp were prepared for labeling. The amounts of adduct labeled with each sample (total volume of 10 µL) were (A) 0, (B) 1.5 pmol SOMedAp, (C)150 fmol SOMedAp, and (D) 15 fmol SOMedAp. Aliquots of 2 µL were chromatographed on PEI-cellulose TLC plates from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). Autoradiography was carried out with intensifying screens for one hour. The origin is in the lower left corner of each plate. X = SOMedpAp.

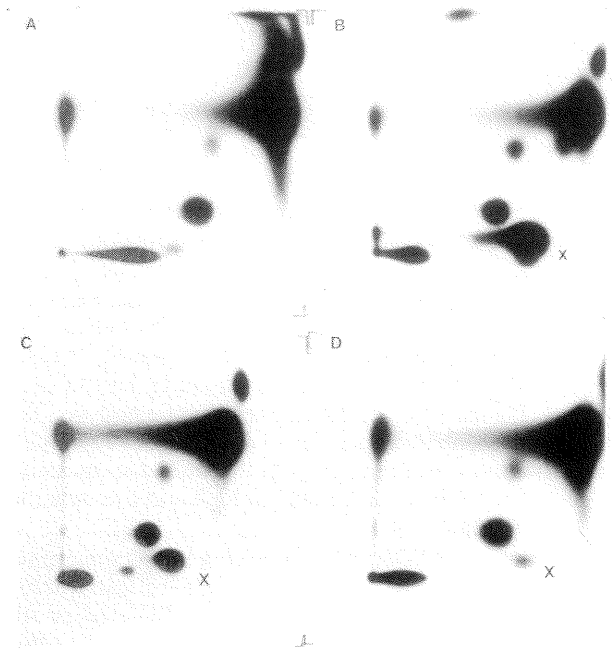

Figure 13. Detection of adduct standards following ^{32}P-postlabeling with ^{32}P-ATP (specific activity 3,000 Ci/mmol). Serial dilutions of standard SOMedAp were prepared for labeling. The amounts of adduct labeled in each sample (total volume of 10 µL) were (A) 0, (B) 1.5 pmol SOMedAp, (C) 140 fmol SOMedAp, and (D) 14 fmol SOMedAp. Aliquots of 2 µL were chromatographed on PEI-cellulose TLC plates from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). Autoradiography was carried out with intensifying screens for one hour. The origin is in the lower left corner of each plate. X = SOMedpAp.

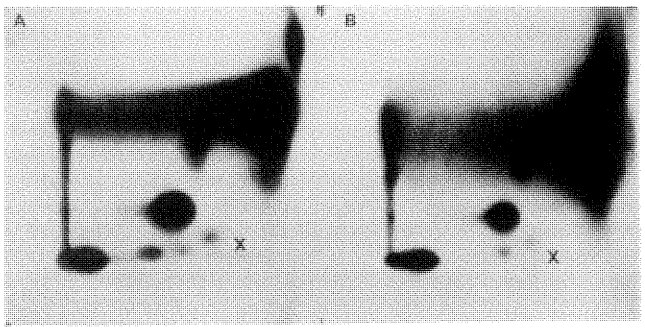

Figure 14. Detection of SOMedAp standard by ^{32}P-postlabeling. Samples containing 1.5 fmol of SOMedAp were labeled with [γ-^{32}P]ATP with specific activity of (A) 3,000 Ci/mmol or (B) 350 Ci/mmol. Aliquots of 2 µL were chromatographed on PEI-cellulose TLC plates from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). Autoradiography was carried out with intensifying screens for 24 hours. The origin is in the lower left corner of each plate. X = SOMedpAp.

redissolved, and postlabeled. The resulting sulfomethyldeoxyadenosine 3′,5′-diphosphate (SOMedpAp) was detected by PEI-cellulose TLC (Figure 15). The sensitivity achieved with this system was with samples containing 30 to 300 fmol of SOMedAp, or one adduct in 10^4 to 10^5 normal nucleotides.

The sensitivity of this method was limited by the carryover of normal nucleotides and by the presence of low levels of what appeared to be SOMedAp contributing to a background in the system. To circumvent these problems, several avenues were explored. Postlabeling analysis was investigated with conversion of the labeled nucleotide 3′,5′-diphosphates to 5′-monophosphates, with PEI-cellulose TLC for resolution of the labeled adducts. The chromatograms contained diffuse areas of radioactivity in the region of the SOMedpA. Further analyses were conducted with chromatography of the nucleotide 3′,5′-diphosphates.

Ion exchange with reverse-phase HPLC was investigated for sample purification prior to labeling on a mixed functionality RPSAX column with a variety of solvent systems for elution. The system chosen for further investigation consisted of 50 mM ammonium formate (pH 2.7), with a 20-minute linear gradient of 0% to 30% methanol. The

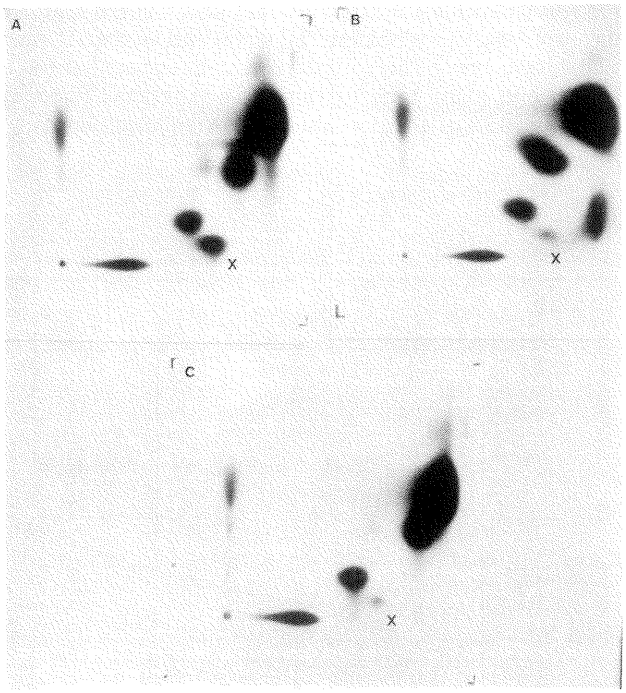

Figure 15. ^{32}P-Postlabeling of SOMedAp separated from mixtures of adduct and normal nucleotide 3′-monophosphates by reverse-phase HPLC. Elution was carried out with a 10-minute gradient of 5% to 20% methanol in 0.25 M ammonium acetate (pH 6.0). Adduct samples were concentrated using a Savant centrifugal concentrator and were labeled with [γ-^{32}P]ATP with specific activity of 350 Ci/mmol and chromatographed from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). The origin is in the lower left corner of each plate. The original samples (10-µL aliquots) contained SOMedAp and normal nucleotides (total concentration 0.3 mM) in ratios of (A) 1 adduct in 10^3 nucleotides, (B) 1 adduct in 10^4 nucleotides, and (C) 1 adduct in 10^5 nucleotides. X = SOMedpAp.

normal nucleotides eluted prior to 10 minutes, and SOMedAp eluted at approximately 16 minutes. The baseline UV absorbance decreased slowly following the elution of the normal nucleotides. Samples collected from the nucleotide standards containing SOMedAp for postlabeling analysis were found to contain high levels of normal nucleotides. This method was not investigated further because of the high degree of contamination from normal nucleotides.

A purification method recently applied to the separation of adducts from normal nucleotides for ^{32}P-postlabeling is HPLC with column switching, enabling purification by chromatography of the adduct using two columns (Watson et al. 1992). A similar method was devised for the separation of SOMedAp from normal nucleotides, using two Ultrasphere ODS columns. The first column (0.46 × 15 cm) was used for initial separation, with the column effluent passing through the UV detector to waste as the normal nucleotides eluted, and onto the second column (0.46 × 25 cm) as SOMedAp eluted from the first column. The flow was switched entirely to the second column for chromatography of SOMedAp, and fractions were collected for ^{32}P-postlabeling. Figure 16 shows a chromatogram of a mixture of normal nucleotides and SOMedAp. Samples of nucleotides and SOMedAp were chromatographed using this method, and fractions were postlabeled. The two-column system allowed the chromatography of larger samples of nucleotides, with 100-μL samples of 3 mM nucleotide solutions used for analysis (corresponding to approximately 100 μg of DNA). The concentration of ATP used in the reaction was titrated to ensure that complete labeling of the nucleotides present was accomplished. For further analysis, 20 μCi of [γ-^{32}P]ATP with a specific activity of 330 Ci/mmol, corresponding to 60 pmol of ATP per sample, was used for postlabeling.

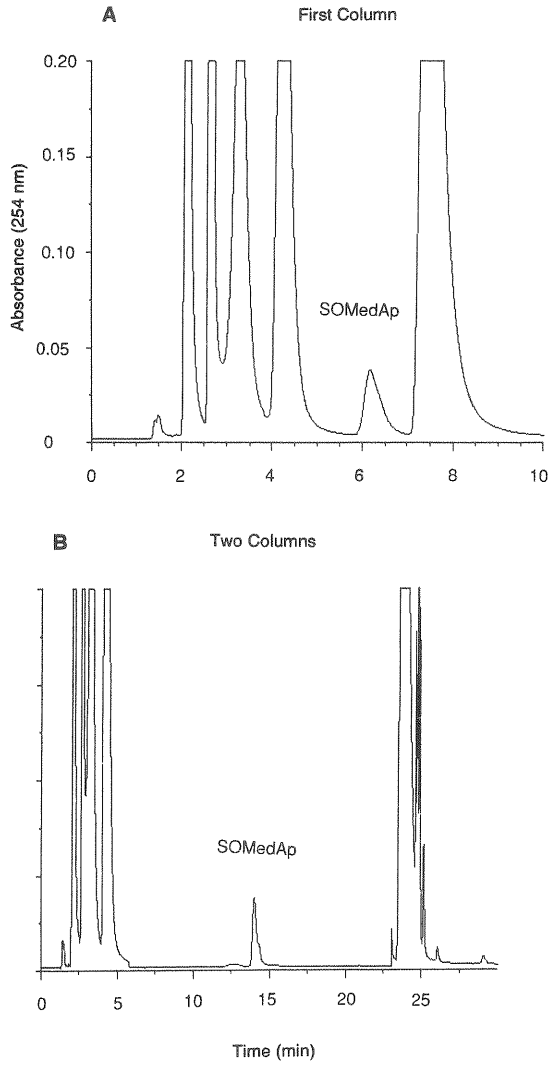

Figure 16. HPLC separation of SOMedAp from normal nucleotides using column switching. A 100-μL sample of normal nucleotides (final concentration 3.0 mM) and SOMedAp (0.015 mM) was chromatographed by HPLC using column switching. (A) Elution of UV-absorbing material from the first column (0.4 × 15 cm); (B) separation of SOMedAp on two columns.

This method was applied to DNA digests of nuclei and TK6 cells treated with 400 μM HCHO and bisulfite. The PEI-cellulose TLCs in Figure 17 are from the major fraction containing SOMedAp. Standards containing the equivalent of one adduct in 10^5 nucleotides and one adduct in 10^6

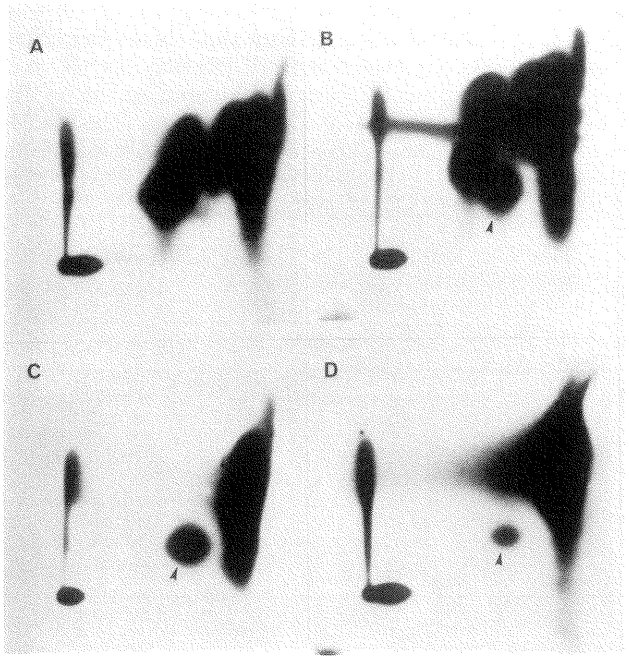

Figure 17. ^{32}P-Postlabeling analysis of SOMedAp in mixtures of normal nucleotides. (A) control nucleotides or (B) SOMedAp (equivalent to one adduct in 10^5 nucleotides) were chromatographed prior to postlabeling by HPLC with column switching (100-μL sample volume). After labeling and incubation with apyrase, the adduct samples (4 μL), were separated by two-dimensional PEI-cellulose TLC. Samples of pure SOMedAp standard equivalent to (C) 1 adduct in 10^5 nucleotides and (D) 1 adduct in 10^6 nucleotides were labeled and chromatographed on TLC, without separation by HPLC. Each sample was incubated with approximately 20 μCi of [γ -^{32}P]ATP (specific activity 330 Ci/mmol). The arrows indicate the SOMedpAp spots.

nucleotides were included in the labeling reaction (Figure 17). A standard corresponding to one adduct in 10^5 nucleotides was chromatographed on the HPLC system, and fractions were collected for ^{32}P-postlabeling (Figure 17). Comparison of the amount of radioactivity associated with SOMedpAp for the adduct standards indicated a 10-fold increase in activity between one adduct in 10^6 and one adduct in 10^5 nucleotides. Chromatography of the standard recovered 78% of the adduct as calculated from the radioactivity recovered in SOMedpAp. The labeled sample from the isolated nuclei treated with HCHO and bisulfite contained low levels of radioactivity in the region of the SOMedpAp, but they were only 25% above control levels. The radioactivity recovered in the control samples was equivalent to a background of approximately two adducts in 10^7 nucleotides. The radioactivity recovered from this region in samples from isolated nuclei treated with HCHO was not elevated above the background level of control nuclei (Figure 18).

A limited number of samples of DNA obtained from TK6 lymphoblast cells treated with HCHO and bisulfite were digested to nucleotide 3′-monophosphates, chromatographed on HPLC using the column-switching system, and ^{32}P-postlabeled as described for the isolated nuclei. The chromatogram for the control sample treated with bisulfite alone (Figure 19A) was similar to that obtained from nuclei. One of the samples treated with HCHO and bisulfite was similar to the control sample (Figure 19B). In the two other samples of TK6 cells treated with HCHO (Figures 19C and 19D), the region in which SOMedpAp would appear on the chromatogram is obscured by additional spots. The identities of these additional spots are not known. At this time, whether or not SOMedpAp is formed in the DNA of TK6 cells treated with HCHO is not known.

ALTERNATIVE METHODS FOR DETECTING ADDUCTS

As a result of the problems encountered initially with enrichment of adducts for detection by ^{32}P-postlabeling, two alternative approaches to the sensitive detection of sulfomethyl adducts have been investigated. These are electrophore postlabeling and the synthesis of ribonucleoside adducts to produce antibodies for use in immunoassays.

Electrophore Postlabeling

The feasibility of developing a method that detects the sulfomethyl adducts as the bases, rather than the nucleosides or nucleotides, has been investigated. The strategy is to derivatize the adducts for detection by GC/MS. Considerable sensitivity and specificity can be obtained by developing suitable derivatives and using selected ion monitoring in mass spectrometry. Methods have been developed for detecting several adducts using electrophore postlabeling in which an electrophoric derivative of the adduct is used to detect the adduct in DNA (Nazareth et al. 1984; Adams et al. 1986; Fedtke et al. 1990). Detection of

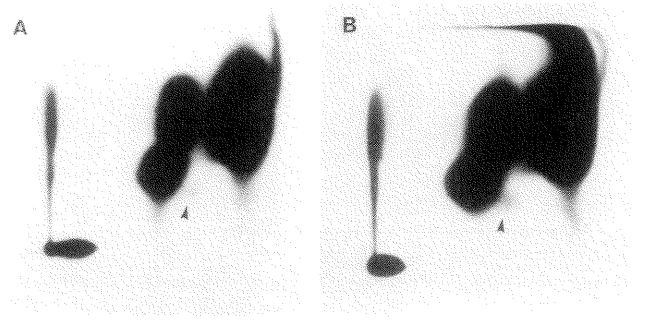

Figure 18. ^{32}P-Postlabeling analysis of DNA from isolated nuclei for detection of SOMedAp. DNA (100 µg) from isolated nuclei reacted with (A) 400 µM HCHO followed by sodium bisulfite, or (B) sodium bisulfite alone, was digested to nucleotides and separated by HPLC with column switching. After ^{32}P-postlabeling, 4-µL aliquots of the labeled digests (containing 8 µCi of ^{32}P) were chromatographed from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). The origin is in the lower left corner of each plate. The arrows indicate the areas where SOMedpAp would be expected to appear.

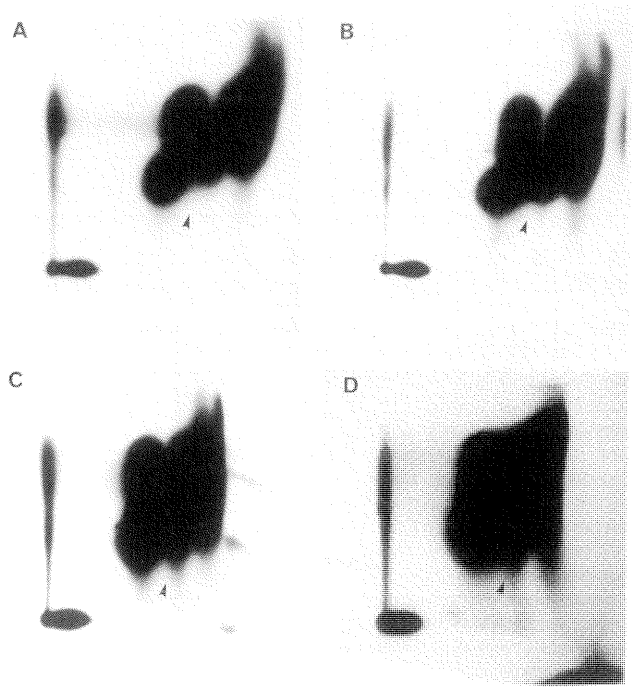

Figure 19. ^{32}P-Postlabeling analysis of DNA from TK6 cells incubated with HCHO for detection of SOMedAp. DNA was obtained from TK6 cells treated with (A) no HCHO, (B) and (D) 400 µM HCHO, or (C) 100 µM HCHO for two hours and with sodium bisulfite at pH 5.0. After digestion to nucleotide 3′-monophosphates, HPLC purification, and postlabeling, samples were chromatographed on PEI-cellulose TLC plates from bottom to top in 1.5 M ammonium formate (pH 3.5), and from left to right in 0.5 M ammonium sulfate (pH 8.0). The origin is in the lower left corner of each plate. The arrows indicate the areas where SOMedpAp would be expected to appear.

sulfomethyl adducts by GC/MS requires a volatile derivative. Since the sulfonic acid group is a major polar group on these adducts, either derivatization or replacement is required to make the adduct volatile.

N^6-Sulfomethyladenine and SOMeG have been synthesized and purified for subsequent synthesis of derivatives. The NMR spectrum of SOMeA is shown in Figure 20 and that of SOMeG in Figure 21. Preliminary reactions for the synthesis of derivatives were carried out on a small scale (milligram quantities), and derivatives were analyzed by TLC and by mass spectrometry. The solubilities of SOMeA and SOMeG were examined in a range of solvents and found to be slightly soluble in acetonitrile, dimethylsulfoxide, and dimethylformamide, and insoluble in tetrahydrofuran, diethyl ether, and 10% methanol in diethyl ether. Basic solvents such as pyridine were avoided because of the instability of the sulfomethyl adducts under basic conditions (Hayatsu et al. 1982).

The formation of trimethylsilyl derivatives was investigated with acetonitrile, dimethylsulfoxide, dimethylformamide, or tetrahydrofuran as solvent using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, or using bis(trimeth-ylsilyl)trifluoroacetamide alone, or with 1% trimethylchlorosilane. Thin-layer chromatography of the reaction mixtures indicated the formation of a product with these derivatizing reagents in tetrahydrofuran, dimethylformamide, and acetonitrile. The products were analyzed by mass spectrometry using a direct probe and electron impact, chemical ionization, and fast atom bombardment. Ions indicative of the products were not detected, which suggests that the products are not volatile.

Methylation of the sulfonic acid group is another potential method for generating volatile derivatives. In general, acids can be methylated by reaction with diazomethane or with methanolic HCl. Neither of these procedures generated volatile derivatives with the expected mass spectra.

Preparation of a pentafluorobenzyl ester of SOMeA was attempted using α-bromo-2,3,4,5,6-pentafluorotoluene in dry acetonitrile and sodium bicarbonate. Analysis by TLC demonstrated that no reaction occurred.

Hydrolysis of the sulfomethyl adducts at alkaline pH was described by Hayatsu and colleagues (1982). This occurs presumably by a nucleophilic substitution reaction, with displacement of the sulfonic acid moiety by an hydroxyl ion and subsequent breakdown of the resulting hydroxymethyl product. Reactions of α-aminoalkanesulfonic acids with a variety of nucleophiles have been described (Neelakantan and Hartung 1959). Therefore, nucleophilic substitution is a potential mechanism for replacing the polar sulfonic acid group with a group that would confer more volatility, yet retain the HCHO-derived carbon atom. Replacement of the sulfonic acid group of SOMeA with a methoxy group was attempted with sodium methoxide in methanol and in dimethylsulfoxide. Only SOMeA was detected in these reactions by TLC, suggesting that reaction did not occur.

In reactions of sulfomethyl adducts with diethyl malonate in aqueous piperidine, diethyl malonate should displace the sulfonic acid group. Reaction of SOMeA or SOMeG under these conditions produced several spots on TLC that were not present in control reactions. However, these products were difficult to resolve from starting material, so they were subjected to analytical HPLC using a reverse-phase C_{18} column. For both adducts, a peak was formed that represented more than 90% of the UV absorbance and was not formed in control samples. However, isolation of the peaks and characterization by NMR and mass spectrometry showed that they had retained the sulfonic acid groups and were not volatile.

Synthesis of Ribonucleoside Adducts

The synthesis of ribonucleoside adducts was investigated to enable coupling to proteins for raising antibodies against SOMeA and SOMeG. These antibodies would enable purification of adducts by immunoaffinity methods and detection of adducts by immunoassays.

Sulfomethyladenosine was synthesized by the reaction of HCHO with adenosine and subsequent treatment with sodium bisulfite at pH 5.0. The reaction products were purified by reverse-phase HPLC, and sulfomethyladenosine was identified by its UV and NMR spectra (Figure 22).

Sulfomethylguanosine was synthesized similarly by the reaction of guanosine with HCHO and subsequent treatment with sodium bisulfite at pH 7.0. Figure 23 shows the NMR spectrum of the isolated product. Coupling between resonances was established by analysis with ^1H-^1H correlation spectroscopy (Figure 24).

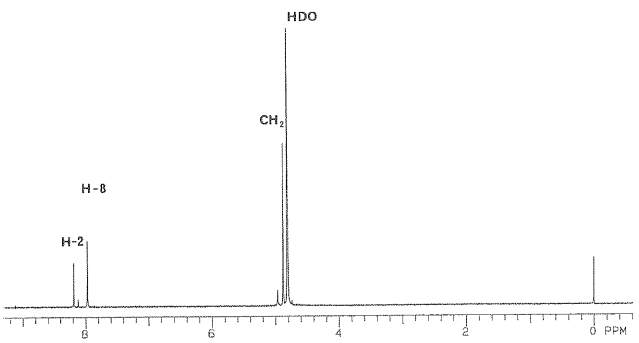

Figure 20. 300 MHz ^1H Nuclear magnetic resonance spectrum of sulfomethyladenine in D_2O with potassium deuteroxide.

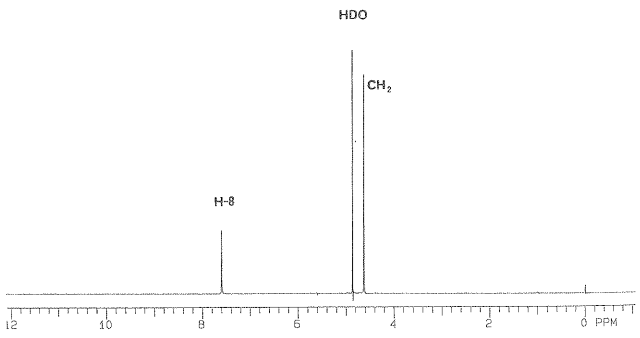

Figure 21. 300 MHz ^1H Nuclear magnetic resonance spectrum of sulfomethylguanine in D_2O with potassium deuteroxide.

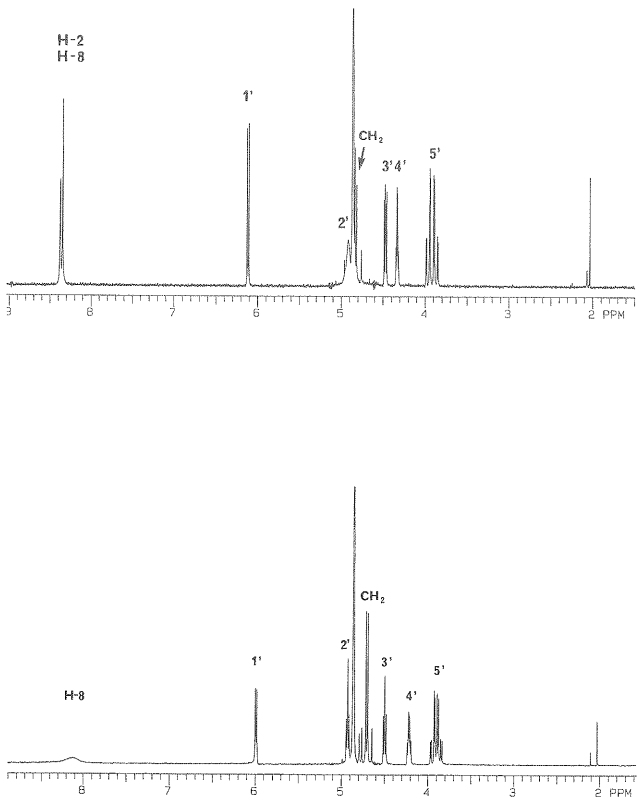

Figure 23. 300 MHz ^{1}H Nuclear magnetic resonance spectrum of sulfomethylguanosine in D_2O.

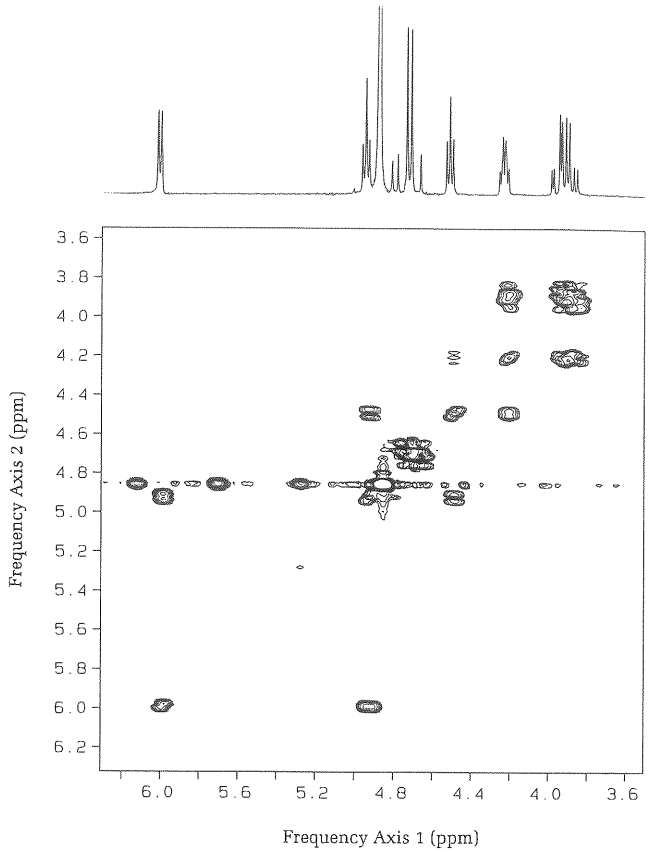

Figure 24. 300 MHz ^{1}H-^{1}H Correlation spectroscopy of sulfomethylguanosine in D_2O.

DISCUSSION

This study verified the observations of Hayatsu and coworkers (1982). Following reaction of HCHO with DNA, bases, nucleosides, and nucleotides to form unstable hydroxymethyl adducts, sodium bisulfite treatment converts these to sulfomethyl adducts. N^6-Hydroxymethyldeoxyadenosine was chosen for particular attention because it was expected to be the predominant adduct from consideration of previous work on its formation, stability (McGhee and von Hippel 1975a,b; Beland et al. 1984; Casanova et al. 1989), and possible role in HCHO mutagenicity (Craft et al. 1987). The study focused on developing a method for measuring SOMedA in DNA from biological systems exposed to HCHO, as an indicator of formation of HOMedA.

One of the difficulties in establishing a method for measurement of hydroxymethyl adducts in DNA was ensuring their complete conversion to sulfomethyl adducts. Parallel measurements of HOMedA and SOMedA in DNA could not be obtained because of the instability of the former; however, analysis of both in DNA treated with HCHO and bisulfite indicated extensive but not complete conversion of HOMedA to SOMedA.

The extensive metabolic incorporation of radioactivity restricted the use of ^{14}C-formaldehyde for detecting adducts to systems that were not capable of synthesizing DNA. Analysis of SOMedA in isolated nuclei by detection of ^{14}C-labeled adduct indicated the formation of approximately 4 pmol/mg DNA. The criterion for identification was comigration on HPLC with a synthetic standard. However, the low level of counts associated with the adduct peaks made quantitation difficult. Incubation of samples with higher concentrations of HCHO was precluded because DNA-protein cross-linking reduced the yield of DNA isolated. The specific activity of the ^{14}C-formaldehyde used was the highest commercially available. Little advantage would have been gained by using ^{3}H-formaldehyde, which is available in a similar range of specific activity. In addition, an isotope effect for oxidation of ^{3}H-formaldehyde limited its usefulness for measuring HCHO binding associated with DNA-protein cross-linking (Heck and Casanova 1987). To pursue these investigations, a technique with greater sensitivity and specificity that did not use labeled HCHO was required for analyses in biological systems. Much of the research conducted was to develop alternative methods for detecting sulfomethyl adducts, with emphasis on ^{32}P-postlabeling. Other methods involving derivatization for analysis by GC/MS were also investigated.

The strategy used in this study for ^{32}P-postlabeling involved development of a method for the analysis of a specific adduct. SOMedAp was synthesized, characterized,

and used as a standard in developing a postlabeling assay. The purified adduct was labeled on incubation with [γ-^{32}P]ATP. High-performance liquid chromatography purification of the adduct prior to labeling was required to increase the sensitivity of the detection method. Reverse-phase HPLC, using two columns with column switching to enhance sensitivity, produced a limit of detection of approximately four adducts in 10^7 nucleotides. In the samples purified by HPLC, the sensitivity was limited by a background of approximately two adducts in 10^7 nucleotides. In DNA from isolated nuclei treated with HCHO and bisulfite, the radioactivity associated with SOMedAp was approximately 25% above this background. However, on analysis of [^{14}C]SOMedA by HPLC in DNA from nuclei, radioactivity corresponding to approximately 4 pmol/mg (1.3 adducts in 10^6 nucleotides) was detected. The discrepancy in measurement suggests either that ^{32}P-postlabeling was underestimating the amount of adduct, or that the measurement of ^{14}C overestimated the amount of adduct. The requirement of comigration on two HPLC columns, labeling with ^{32}P-ATP, and comigration in two dimensions on TLC suggests that the ^{32}P-postlabeling assay has considerably more specificity than the detection of ^{14}C after resolution on a single HPLC column. With SOMedAp in cells treated with HCHO, other spots were observed in the postlabeled samples that overlapped with the expected adduct mobility. This precluded the detection of SOMedpAp by postlabeling in these samples. Further work on separation of the adduct on TLC may both lower the limit of detection and remove contaminating spots in the samples obtained from cells.

A major objective of this study was to establish whether hydroxymethyl adducts are formed in cells treated with HCHO under conditions that would stabilize these adducts and avoid interference from DNA-protein cross-links. This objective has not been met because of the interference of additional labeled spots in the detection of SOMedpAp on postlabeling. However, the experiments with isolated nuclei may serve as a useful model for the intact cell. The levels of SOMedA in nuclei reacted with 0.4 mM HCHO measured by the two different methods range from background (^{32}P-postlabeling) to 4 pmol/mg (^{14}C). The kinetics of reactions of HCHO with DNA and with protein suggest that DNA-protein cross-links would be the predominant reaction products (French and Edsall 1945; Feldman 1973; Ohba et al. 1979; Heck et al. 1990). Incubation of rat hepatic nuclei with 0.1 mM HCHO for 1.5 hours at 37°C resulted in the binding of HCHO at approximately 120 pmol/mg DNA as DNA-protein cross-links (Heck and Casanova 1987). Incubations of nuclei with 0.4 mM HCHO would be expected to produce higher levels of DNA-protein cross-linking. The combined data suggest that the hydroxymethyl adducts formed constitute a very small fraction of the total HCHO bound to DNA in systems containing protein.

Other study objectives were not attained. The evaluation of the ^{32}P-postlabeling method with calf thymus DNA treated with HCHO and bisulfite was not used for several reasons. At the low levels of sensitivity required for the postlabeling assay, a defined standard was difficult to generate. Mixtures of nucleotides with known levels of SOMedAp provided a more suitable standard for analysis. Although calf thymus DNA can be used for systems in which the level of modification is high, contaminating spots are a problem with low levels of adducts. The measurement of HOMedAp in DNA from nasal tissue of exposed rats was proposed if a method of detection could be successfully applied to cells exposed to HCHO. The incorporation of an internal standard into the postlabeling reaction was planned for determination of adduct levels. However, an external standard was used because of the high levels of contaminating nucleotides.

As with any assay to be used in biological systems, this investigation raises several issues with respect to the sensitivity and specificity of the method of analysis. Several scenarios are possible and may dictate the success of further efforts to develop more sensitive and specific methods of analysis. The limit of detection may be determined by interfering substances in the assay system or in the biological system under investigation. Cross-contamination between samples separated on the same HPLC column can impose constraints on sensitivity (Gorelick 1993). A background level of adducts in control samples may arise from endogenous sources. In this case, there appears to be a background contributed by the assay, a high level of interfering substances in the biological samples, and the potential for a background contributed by endogenous HCHO. Efforts to increase the sensitivity of the adduct assay will also depend on the level of adducts that exist within the sample. Currently, it is not clear that the hydroxymethyl adducts will constitute a significant fraction of the HCHO bound to DNA as DNA-protein cross-links.

A more sensitive method for detecting SOMedAp may be attainable with further work. Improving the separation of SOMedpAp from other labeled material on TLC could lower the limit of detection. An alternative would be to improve the selectivity of purification before labeling. A method of immunoaffinity purification and ^{32}P-postlabeling has been described for the measurement of O^6-methylguanine (Cooper et al. 1992). Use of several HPLC separations prior to postlabeling, as reported for *N*-7-alkyldeoxyguanosine adducts (Kato et al. 1993), could enhance sensitivity. With a sufficiently sensitive method for detecting the SOMedAp adduct, the conditions of bisulfite treatment necessary for its stabilization in cell systems or in isolated nuclei could be examined.

It has been assumed that sulfomethyl adducts detected in DNA arise from hydroxymethyl adducts. This appears to be the case in pure DNA samples treated with HCHO, in which a HCHO-bisulfite complex does not seem to be capable of reacting with DNA. However, in systems more representative of a cell in which HCHO can react with DNA and protein, it is possible that DNA-protein cross-links may react with bisulfite to form sulfomethyl adducts. The background level of SOMedAp in isolated nuclei reacted

with HCHO, in which the levels of DNA-protein cross-links would be several orders of magnitude higher, suggests that the DNA-protein cross-links do not react with bisulfite to any significant extent to form sulfomethyl adducts.

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Timothy R. Fennell received a Ph.D. in biochemistry from the University of Surrey in 1980. He was a postdoctoral fellow at the McArdle Laboratory at the University of Wisconsin. Initially employed as a visiting scientist at the Chemical Industry Institute of Toxicology, he was appointed to the position of scientist in 1988. Dr. Fennell's primary research interests are metabolic activation of chemical carcinogens and toxins and the use of DNA and protein adducts as indicators of exposure.

ABBREVIATIONS

ATP	adenosine triphosphate
bicine	*N,N*-bis(2-hydroxyethyl)glycine
CIIT	Chemical Industry Institute of Toxicology
D_2O	deuterium oxide (deuterated water)
dAp	deoxyadenosine 3′-monophosphate
dCp	deoxycytodine 3′-monophosphate
dGp	deoxyguanosine 3′-monophosphate
dpm	disintegrations per minute
dTp	deoxythymidine 3′-monophosphate
GC/MS	gas chromatography with mass spectrometry
HCHO	formaldehyde
HCl	hydrochloric acid
HEPES	N^2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HOMedA	N^6-hydroxymethyldeoxyadenosine
HOMedG	N^2-hydroxymethyldeoxyguanosine
HPLC	high-performance liquid chromatography
$NaHSO_3$	sodium bisulfite
NMR	nuclear magnetic resonance
PEI	polyethyleneimine
ppm	parts per million
RPSAX	reverse-phase strong anion exchange
SD	standard deviation
SOMeA	N^6-sulfomethyladenine
SOMeG	N^2-sulfomethylguanine
SOMedA	N^6-sulfomethyldeoxyadenosine
SOMedG	N^2-sulfomethyldeoxyguanosine
SOMedAp	N^6-sulfomethyldeoxyadenosine 3′-monophosphate
SOMedGp	N^2-sulfomethyldeoxyguanosine 3′-monophosphate
SOMedpA	N^6-sulfomethyldeoxyadenosine 5′-monophosphate
SOMedpAp	N^6-sulfomethyldeoxyadenosine 3′,5′-diphosphate
TLC	thin-layer chromatography
UV	ultraviolet

INTRODUCTION

In October 1987, Dr. Timothy R. Fennell of the Chemical Industry Institute of Toxicology (CIIT)* submitted a proposal to the Health Effects Institute (HEI) entitled "Development of Methods for Measuring Formaldehyde Exposure." Submission of this proposal resulted from discussions at a workshop sponsored by HEI for four investigators who had recently been funded under HEI's RFA 85-1, "Health Effects of Aldehydes." At that workshop, the participants agreed that no methods were available for accurately quantifying the doses of formaldehyde that reach tissues or cells exposed to formaldehyde in the different studies. In the absence of these techniques, direct comparisons of the results from the in vivo and in vitro studies would not be possible. As a result, the HEI Research Committee initiated discussions with CIIT investigators who were developing promising new methods for aldehyde dosimetry. Based on those discussions, Dr. Fennell submitted his proposal for developing methods to measure DNA adducts produced by reactions between DNA and formaldehyde. If his new methods proved successful, Dr. Fennell also agreed to analyze tissues and other samples from the four investigators funded by HEI.

Dr. Fennell's study began on January 1, 1989 and ended on August 31, 1991. Total expenditures were $120,000. The Investigator's Report was received at HEI in July 1992 and accepted by the Health Review Committee in June 1993. During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Committee's Commentary. The following Commentary is intended to aid the sponsors of HEI and to the public by highlighting both the strengths and limitations of the study and by placing the Investigator's Report into scientific and regulatory perspective.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for air pollutants under Section 202 of the Clean Air Act, as amended in 1977. Section 202 (a)(1) directs the Administrator to "prescribe (and from time to time revise). . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or 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." Sections 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and provide the EPA with limited discretion to modify those requirements.

Several changes in the Clean Air Act instituted by the 1990 Amendments to the Act deal with formaldehyde. Section 211(k) of the Act, as added by Section 219 of the 1990 Amendments, establishes a program for the use of reformulated gasoline. The program is designed, at least in part, to reduce the "emissions of toxic air pollutants," like formaldehyde. Similarly, the "clean-fuel vehicle" program emission standards set out in Section 243, as added by the 1990 Amendments, require that certain formaldehyde emission targets be met.

Section 202(l) of the Clean Air Act, as added by Section 206 of the 1990 Amendments, requires the EPA to "complete a study of the need for, and feasibility of, controlling emissions of [certain] toxic air pollutants . . . ." Section 202(l) states that the study "shall focus on those categories of emissions that pose the greatest risk to human health or about which significant uncertainties remain . . . ." Defined as a "hazardous air pollutant" under Section 112(b) of the Act, as amended by Section 301 of the 1990 Amendments, formaldehyde is one of the three emissions specifically mentioned in Section 202(l)(1).

SCIENTIFIC BACKGROUND

Formaldehyde is a small organic molecule, yet it has generated great public controversy due to uncertainties about adverse human health effects caused by exposures to this compound. Formaldehyde is a major product of the chemical industry and it is used in the manufacture of many plastics, chemicals, textiles, and home products (Feinman 1988). As a result, exposures to this toxic compound can occur in a variety of occupational and home settings due to the release of formaldehyde vapors from various products. Formaldehyde is also an outdoor air pollutant because it is produced during combustion of gasoline and diesel fuels by motor vehicles. In general, ambient outdoor levels of formaldehyde are low; for example, 0.005 ppm is an average value for the Los Angeles area (Grosjean 1991), whereas indoor levels in manufacturing and occupational settings can be 0.1 ppm and higher (Hart et al. 1984). However, ambient formaldehyde levels may increase in the future if the use of methanol as an automotive fuel increases because formaldehyde is a major component of emissions from methanol combustion.

The toxicity of formaldehyde has been the focus of numerous in vitro, animal, and human epidemiological studies (U.S. Environmental Protection Agency 1991). The extent of the effects produced by formaldehyde clearly depends on the exposure concentration as well as the duration of exposure. Acute exposure to levels of 0.1 to 0.3 ppm formaldehyde can produce eye and throat irritation in humans and animals, and numerous in vitro and in vivo studies have demonstrated that formaldehyde can produce genetic damage (U.S. Environmental Protection Agency 1987). Kerns and colleagues (1983) confirmed earlier observations (Swenberg et al. 1980; Albert et al. 1982) that formaldehyde is a carcinogen in animals when they ob-

* A list of abbreviations appears at the end of the Investigator's Report.

served squamous cell carcinomas in the nasal region of rats and mice that had inhaled 5.6 or 14 ppm formaldehyde for 24 months.

Whether long-term human exposure to formaldehyde is associated with an increased risk of cancer for humans is not yet known (Blair et al. 1990). Based in part on the clear link between chronic exposures to inhaled formaldehyde and cancer development in rodents, and on limited epidemiological evidence in humans, the U.S. Environmental Protection Agency (1987, 1991) and the International Agency for Research on Cancer (1987) have categorized formaldehyde as a probable human carcinogen. In addition, the Clean Air Act Amendments of 1990 (U.S. Congress 1991) have defined formaldehyde as a toxic air pollutant and have specified further study regarding the need for and feasibility of controlling its emission from mobile sources.

METHODS FOR EVALUATING FORMALDEHYDE TOXICITY

An important component for assessing human health risks that may be associated with exposure to any chemical is determining the amount, or dose, of the chemical that is delivered to critical target tissues in an exposed individual. Precise measurement of the dose of any toxic compound is essential for accurately interpreting the response that is elicited in the exposed cell, animal, or human subject. This dose-response relationship is one critical component of the risk assessment process, a quantitative approach for estimating the likelihood that a given toxic substance will produce health effects in humans (National Research Council 1991). With regard to formaldehyde, several different approaches have been taken to measure the doses that reach tissues or cells in exposed animals or human subjects. The amounts of formaldehyde that react with DNA are of particular interest because of the recognized links between genetic damage and carcinogenesis.

Radiolabeled Formaldehyde

Radiolabeled markers have been used successfully in many metabolic studies, and in evaluating the binding of xenobiotics to cellular macromolecules. As a result, formaldehyde labeled with a radioactive isotope was considered as a potential biomarker for measuring formaldehyde's reactions with exposed tissues and cells. As expected, the use of ^{14}C-formaldehyde was not practical because formaldehyde was metabolized to formate, which subsequently entered into the cell's pool of one-carbon compounds (Casanova-Schmitz et al. 1984). From this pool, the labeled carbon could be incorporated de novo into DNA bases, resulting in the generation of radioactive DNA in which the radioactive label could not be solely attributed to a formaldehyde-induced adduct. This recognition, therefore, precluded the usefulness of radiolabeled formaldehyde associated with DNA as a biomarker of dose.

DNA-Protein Crosslinks

Within the past few years, DNA-protein crosslinks also have been proposed as a potential dosimeter for formaldehyde exposures. For example, after formaldehyde enters cells, it can react with DNA, histones, nonhistone chromosomal proteins, or other proteins in the nuclear matrix and form crosslinks with DNA (Heck et al. 1990). In theory, the numbers of crosslinks that form in tissues or cells exposed to formaldehyde should serve as a measure of dose. Although it is not yet clear whether DNA-protein crosslinks are involved in the carcinogenic processes associated with formaldehyde exposure, the crosslinks may provide a useful index for the presence of formaldehyde in target cells (U.S. Environmental Protection Agency 1991). However, because of isotope effects and other technical problems, there may not be a one-to-one relationship between administered dose of formaldehyde and the numbers of DNA-protein crosslinks (Casanova et al. 1991). Currently available analytical methods for measuring DNA-protein crosslinks provide an indirect measure of crosslinks released as formaldehyde; this is not as desirable as a dosimeter based on direct adduct measurements.

DNA Adducts

DNA adducts have been proposed not only as potential dosimeters for monitoring exposures to carcinogens, such as formaldehyde, but also as one of the initiators of the carcinogenic process (Harris 1985). After exposure to a carcinogen, DNA adducts can form through reactions in which the carcinogen or its metabolites bind to DNA in target cells. The longevity of these DNA adducts can vary; some adducts remain for long periods of time, and replication of this damaged DNA is thought to fix this DNA lesion as a mutation. Therefore, particular attention has been focused on DNA adducts because they are considered to be potential initiators of the events that lead to mutation and cancer. Thus, DNA adducts may serve as biomarkers not only of exposure but also of effect.

The formation of DNA adducts after exposure to formaldehyde has been recognized since the early 1960s (Haselkorn and Doty 1961). One significant drawback to using these adducts as dosimeters is that they are not stable during isolation procedures. Over the years, investigators have tried to develop methods that would improve the stability of these adducts and facilitate their use in quantifying dose-response relationships. In 1982, Hayatsu and colleagues (1982) reported that treating the formaldehyde-induced hydroxymethyl adducts with sodium bisulfite resulted in the formation of stable sulfomethyl adducts. As a result, the possibility existed that this treatment could be combined with other methods, such as ^{32}P-postlabeling and chromatography, and used in assays for DNA adducts within cells and tissues that had been exposed to formaldehyde.

^{32}P-Postlabeling is a highly sensitive assay that has often been used to quantify DNA adducts. The basic strategy

behind ^{32}P-postlabeling is (1) to break down a DNA sample containing adducts into its basic units (nucleotides or nucleosides) by digesting it with specific enzymes, (2) to label all of these modified units enzymatically with ^{32}P, and then, (3) based on their chemical differences, to separate the adducted bases from the normal bases by thin-layer chromatography. Currently, this technique is well suited to detecting the bulky adducts formed by compounds much larger than formaldehyde. Two problems that arise in applying this technique to analyze formaldehyde adducts are the instability of the adducted nucleotides, and the similarity between the biochemical properties of the adducted nucleotides and those of normal nucleotides.

Measuring of DNA adducts may prove to be a reliable formaldehyde dosimeter if the technical problems associated with isolating and quantifying the relatively small DNA adducts resulting from formaldehyde exposure can be resolved. Such an approach could improve risk assessment estimates of the human health effects associated with formaldehyde exposure. Although both DNA adducts and DNA-protein crosslinks have been proposed not only as potential formaldehyde dosimeters but also as mechanisms by which formaldehyde may initiate cancer, DNA adducts may be a more direct index of early carcinogenic processes. Because cell replication is an essential step in tumor development, it is possible that DNA adducts could lead to mutations, and potentially to tumor formation, interfering with accurate DNA replication. Therefore, methods for quantifying the DNA adducts formed after formaldehyde exposure could yield a relevant indicator of this compound's cytotoxicity and its potential to initiate cancer in an exposed animal or human.

JUSTIFICATION FOR THE STUDY

The study by Dr. Fennell was one component of HEI's research program to evaluate the health effects of aldehydes at concentrations relevant to those present in automotive emissions. This program was initiated with RFA 85-1, "Health Effects of Aldehydes," that was issued to support studies to determine the effects of single or combined exposures to aldehydes. The Research Committee issued this RFA because of concerns about projected increases in the use of methanol as a motor vehicle fuel. Although motor vehicles that burn gasoline and diesel fuels produce emissions containing several aldehydes, including formaldehyde, acrolein, and acetaldehyde, emissions from methanol-burning vehicles contain significantly higher levels of formaldehyde than emissions from conventional fuels. The rationale for the addition of Dr. Fennell's study to the HEI aldehyde research program was to include a project that would address questions about aldehyde dosimetry. This study was also intended to complement the animal and in vitro studies conducted by the other HEI investigators funded under RFA 85-1.

SPECIFIC AIMS AND STUDY DESIGN

The primary objective of the study conducted by Dr. Fennell was to develop methods for measuring DNA adducts produced by exposure to formaldehyde that did not require the use of radiolabeled formaldehyde. Formaldehyde can interact covalently with DNA nucleotides to produce hydroxymethyl adducts, such as N^6-hydroxymethyldeoxyadenosine and N^2-hydroxymethyldeoxyguanosine. However, these adducts can break down during preparation for quantitative analysis by high pressure liquid chromatography (HPLC) and ^{32}P-postlabeling. Based on results from earlier studies (Fennell et al. 1987), Dr. Fennell proposed that DNA adducts formed by formaldehyde exposure could be detected by ^{32}P-postlabeling. He planned to stabilize formaldehyde-DNA adducts with sodium bisulfite, a compound that reacts with hydroxymethyl complexes formed by formaldehyde exposure and traps them as stable sulfomethyl derivatives. He then wanted to evaluate different techniques for isolating the stabilized adducts.

As described in his proposal, Dr. Fennell wanted specifically to examine the formation of N^6-hydroxymethyldeoxyadenosine and N^2-hydroxymethyldeoxyguanosine, two hydroxymethyl adducts produced by formaldehyde exposure (Haselkorn and Doty 1961). As a first step, he treated deoxyadenosine and deoxyguanosine with sodium bisulfite in order to synthesize sulfomethyl adduct standards. Then he tested the sensitivity of his experimental procedures for detecting the adduct standards. For his in vitro experiments on DNA adduct formation, he selected three model systems that represented an increasing range of biological complexity. He exposed isolated calf thymus DNA, nuclei isolated from rat liver cells, and intact human TK6 lymphoblasts to ^{14}C-formaldehyde and then treated these preparations with sodium bisulfite. He also exposed the rat hepatic nuclei and human TK6 lymphoblasts to unlabeled formaldehyde before sodium bisulfite treatment, and used these cells to evaluate various modifications of the ^{32}P-postlabeling technique, a procedure that does not require the use of radiolabeled formaldehyde.

In his proposal, Dr. Fennell stated that if he found that the ^{32}P-postlabeling methods proved unsuitable for the adduct analyses, he would explore alternative methods for adduct stabilization and detection. Dr. Fennell also proposed that, if his new adduct analysis methods proved to be successful, he would assay DNA samples from tissues provided by the other investigators who had been funded under the HEI aldehydes research program.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Dr. Fennell completed a series of studies to determine the optimal conditions for stabilizing the DNA-formaldehyde adducts with sodium bisulfite using adduct standards and

the biological test systems described above. Based primarily on his limited success in detecting the adduct standards synthesized for these studies, he concluded that his modifications of the ^{32}P-postlabeling technique did not yield an approach sufficiently sensitive to detect in cells and tissues the adducts stabilized with sodium bisulfite.

STUDY DESIGN AND METHODS

Dr. Fennell clearly described the different methods that he used to prepare his DNA adduct standards and to assay his DNA and cell preparations for DNA adducts formed following formaldehyde exposure. The experiments and procedures documented in the report appear to have been performed in a rigorous manner. Dr. Fennell selected a systematic and thorough approach to optimize the experimental conditions for isolating the DNA adducts, including variations in the pH, reagent concentrations, and reaction times. This same attention to detail was extended toward evaluating the feasibility of using several different modifications of the ^{32}P-postlabeling method to quantify the formed adducts.

RESULTS AND INTERPRETATION

This report details the repeated attempts by Dr. Fennell to increase the sensitivity of his analytical procedures for isolating and measuring DNA adducts formed by formaldehyde exposure. Although his ultimate goal was to develop isolation methods not requiring radiolabeled formaldehyde, Dr. Fennell used this compound in the initial phases of his study to facilitate identification of the adducts that he wanted to isolate. He reported that metabolism of the radiolabeled formaldehyde by the cells interfered with his analyses of the DNA adducts produced by formaldehyde exposure. In his experiments with human TK6 lymphoblasts, Dr. Fennell observed high levels of radioactivity associated with the peaks for deoxyadenine, deoxyguanine, and deoxythymine. These results indicated that the ^{14}C-formaldehyde was oxidized to ^{14}C-formate and that this compound was subsequently incorporated into normal nucleosides via de novo synthesis pathways. Based on his conclusion that this reaction limited the detection of sulfomethyl adducts, he suggested that additional innovations in these methods would be needed to detect sulfomethyl adducts in intact biological systems, such as cells and tissues from whole animals.

Dr. Fennell carefully evaluated several different variations of ^{32}P-postlabeling, a procedure which, as noted earlier, does not require the use of radiolabeled formaldehyde. The goal of these experiments was to determine whether he could enhance the sensitivity of his methods for detecting formaldehyde-DNA adducts. Dr. Fennell first determined that the standard methods used to enhance the sensitivity of detecting adducts by ^{32}P-postlabeling, including the use of nuclease P_1, butanol extraction, and the usual HPLC procedures, were not suitable for isolating the sulfomethyl adducts produced by the sodium bisulfite treatment. When he recognized that the HPLC methods did not separate the modified nucleotides from the unmodified ones, he not only tried to optimize the reaction conditions and increase the product yield, but also attempted other separation and detection approaches, including gas chromatography and mass spectrometry.

Dr. Fennell also used an HPLC approach requiring two columns, called column switching. For this technique, the sample was run through the first column for an initial separation, and then its effluent was run through a second column. The effluent from the second column was evaluated by HPLC, and fractions also were collected for ^{32}P-postlabeling. The aim of this approach was to augment the recovery of the modified nucleotides by increasing the amount of adducted material loaded onto the second column and to improve the extent of adduct purification. Because many normal nucleotides came through the second column, comigration of the normal nucleotides with the sulfomethyl adducts complicated subsequent adduct separation by HPLC and limited the sensitivity of this approach.

In the latter phase of his study, Dr. Fennell evaluated electrophore postlabeling and synthesis of nucleoside adducts. To evaluate the electrophore labeling approach, Dr. Fennell tested several chemical treatments to determine whether they would increase the volatility of the adducts for detection by gas chromatography and mass spectrometry. Although these studies did not yield many positive results, Dr. Fennell was successful in his attempts to synthesize two sulfomethyl-nucleoside adducts, sulfomethyladenosine and sulfomethylguanosine. These model formaldehyde adducts could be coupled to appropriate proteins and used for synthesis of antibodies directed against specific DNA adducts.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Based on the findings in this report, isolation and quantification of DNA adducts formed by exposure to formaldehyde remain elusive, though worthwhile, goals. Dr. Fennell's successful preparation of sulfomethyl-nucleoside adducts may, however, provide a new approach for detecting DNA adducts in animals and humans. When coupled with proteins and injected into animals, they could induce the production of antibodies against the complexes. Alternatively, hybridoma technology could be used to produce monoclonal antibodies against the nucleosides in vitro. The resulting antibodies could be used to purify adducts by immunoaffinity methods or to detect adducts formed in vivo with immunoassays.

CONCLUSIONS

Quantifying formaldehyde-DNA adducts is a potentially useful approach for obtaining the dose-response data that are needed for formaldehyde risk assessments (Nelson et al. 1986). Although the formation of DNA adducts following exposure to formaldehyde has been recognized since the early 1960's (Haselkorn and Doty 1961), their use as biological dosimeters has been compromised by a lack of reliable methods for stabilizing and isolating DNA adducts formed by low-molecular-weight compounds. The challenge taken on by Dr. Fennell in this study was to evaluate whether combining a sodium bisulfite stabilization procedure with rigorous and innovative isolation procedures would provide a practical approach for quantifying formaldehyde-DNA adducts.

Despite Dr. Fennell's methodical and thorough approach to this difficult problem, he was unable to develop techniques that were sensitive enough to detect formaldehyde-DNA adducts. He did confirm earlier reports that sodium bisulfite treatment stabilizes DNA adducts; however, his modifications of the ^{32}P-postlabeling procedure proved to be insufficiently sensitive for isolating and identifying bisulfite-stabilized adducts in exposed cells. Nevertheless, it must be stressed that, despite the generally negative results of this study, the solution to the problem addressed by Dr. Fennell has eluded many other qualified investigators. Dr. Fennell deserves recognition for his persistence in pursuing a valuable objective and for using biological systems that represent an increasing range of complexity rather than limiting his studies to synthetic adduct standards alone.

Although this study did not achieve the aims that were originally proposed, it does provide guidance to other investigators who undoubtedly will pursue studies of formaldehyde-induced adducts. Because formaldehyde-DNA adducts may interfere with accurate DNA replication, quantifying their presence may provide a useful index of formaldehyde's carcinogenic potential. Dr. Fennell's studies provide new directions for research on DNA adduct formation, such as immunoassays that could detect sulfomethyl adducts with antibodies directed against these adducts. Innovative approaches like these will clarify mechanisms by which formaldehyde damages genetic material in cells, and they can further our understanding of human health risks associated with exposure to this and other air pollutants.

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