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**Evaluation of the Metabolic Fate of
Munitions Material (TNT & RDX) in Plant Systems
and Initial Assessment of Material Interaction with
Plant Genetic Material**

**Initial Assessment of Plant DNA Adducts as
Biomarkers**

**S. D. Harvey
T. W. Clauss
R. J. Fellows
D. A. Cataldo**

**August 1995
Project Order No. 93MM3548**

**Prepared for
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**

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with U.S. Department of Energy
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**Pacific Northwest Laboratory
Operated for the U.S. Department of Energy
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Richland, Washington 99352**



Pacific Northwest Laboratory

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EVALUATION OF THE METABOLIC FATE OF
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INITIAL ASSESSMENT OF MATERIAL INTERACTION WITH
PLANT GENETIC MATERIAL (DNA)

INITIAL ASSESSMENT OF PLANT DNA ADDUCTS AS
BIOMARKERS

Scott D. Harvey
Therese W. Clauss
Robert J. Fellows
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August 1995
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REPORT DOCUMENTATION PAGE

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EXECUTIVE SUMMARY

Genetic damage to deoxyribonucleic acid (DNA) has long been suspected of being a fundamental event leading to cancer. A variety of causal factors can result in DNA damage including photodimerization of base pairs, ionizing radiation, specific reaction of DNA with environmental pollutants, and nonspecific oxidative damage caused by the action of highly reactive oxidizing agents produced by metabolism. Studies described here assess the possibility for utilizing adducts formed between DNA and metabolically activated trinitrotoluene (TNT) intermediates as biomarkers of environmental contamination. Our research strategy has been to adduct calf thymus DNA with TNT *in vitro* using an established hypoxanthine/xanthine oxidase enzyme system to produce nitroreduction reaction intermediates. Once adducted, DNA was purified and enzymatically cleaved to the 5'-monophosphate nucleotide level. Subsequent high pressure liquid chromatographic (HPLC) profiling of the nucleotides was used to identify suspected TNT adducts.

Chromatographic comparisons between control and TNT-adducted hydrolysates allowed the tentative identification of two TNT-adducted nucleotides. Chromatographic profiles of TNT-adducted DNA hydrolysates featured these compounds eluting in the 21.4- to 22.6-min retention window. The suspect adducts were not observed in all TNT adduct reactions performed, perhaps because of the formation at concentrations below the analytical detection limit. These studies can be added to the mounting evidence that specific TNT-DNA adducts may occur but definitive proof was not obtained.

Further work is clearly needed to understand the variables leading towards reproducible high yield TNT adduction reactions. Studies with TNT have been considerably more difficult than DNA adduct studies of carcinogens that form fluorescent adducts. Fluorescent adducts are far easier to identify and quantitate because of the enhanced selectivity and sensitivity possible with fluorescence detection. A viable approach for extending the present studies would be to perform *in vitro* adductions with radiolabeled TNT. Adducted nucleotides could then be identified by radiochromatographic means.

HPLC isolation of adducted nucleotides is essential to collect enough material of sufficient purity for identification. Although TNT adducts have been visualized with postlabeling techniques, insufficient amounts of material were used to provide structural information. Similarly, capillary separation techniques can be expected to be ideal for trace analysis and detection; however, the capacity of the technique is incapable of providing sufficient material for complete structural characterization.

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1.0 INTRODUCTION

Genetic damage to deoxyribonucleic acid (DNA) has long been suspected of being a fundamental event leading to cancer. A variety of causal factors can result in DNA damage including photodimerization of base pairs, ionizing radiation, specific reaction of DNA with environmental pollutants, and nonspecific oxidative damage caused by the action of highly reactive oxidizing agents produced by metabolism. Because organisms depend on an unadulterated DNA template for reproduction, DNA repair mechanisms are an important defense for maintaining genomic integrity.

The objective of this exploratory project was to evaluate the potential for TNT to form DNA adducts in plants. These adducts, if they exist in sufficient quantities, could be potential biomarkers of munitions exposure. The ultimate goal is to develop a simple analytical assay for the determination of biomarkers that is indicative of munitions contamination. DNA repair exists in dynamic equilibrium with DNA damage. Repair mechanisms are capable of keeping DNA damage at remarkably low concentrations provided that the repair capacity is not overwhelmed. Typically, DNA aberrations are maintained at very low levels of approximately 1 damaged/10⁹ normal bases (Everson et al. 1986).

1.1 POLYCYCLIC AROMATIC HYDROCARBON STUDIES

Studies on the carcinogenic properties of polycyclic aromatic hydrocarbons (PAHs) have formed the cornerstone of DNA adduct research. Much of the PAH work has focused on metabolic activation and subsequent reaction of benzo[a]pyrene (B[a]P) with DNA. B[a]P has been extensively used as a model because adduction proceeds by a defined mechanism that results in one principal DNA adduct. Additionally, the B[a]P DNA adducts are fluorescent which greatly simplifies trace detection during subsequent analytical studies. The B[a]P studies show that B[a]P first undergoes hepatic activation to the biologically active electrophile 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P diol epoxide) (Ashurst and Cohen 1982). The reaction of B[a]P diol epoxide with DNA proceeds by electrophilic attack by the epoxide C-10 on the N² amino group of guanine. The reaction results in a covalent bond forming through opening of the epoxide ring. Several minor adducts of B[a]P, which involve reaction with different bases, have also been reported. The principal B[a]P adduct formed *in vitro* has been demonstrated to be identical to the adduct formed during *in vivo* studies (Ashurst and Cohen 1982).

1.1.1 Fluorescence Line Narrowing and Postlabelling Analytical Techniques

Researchers have sought to develop analytical approaches capable of quantifying B[a]P adducts in DNA samples at concentrations that result from environmental exposure. Such a capability would be extremely useful for measuring baseline adduct levels in various populations. Health risks associated with occupational (coke workers) or lifestyle (smoking) exposures could then be assessed by noting increased levels of B[a]P adducts in the DNA. Only a handful of analytical techniques are capable of quantifying DNA adducts at the necessary sensitivity; however, these techniques all lack the desired selectivity. One technique for quantification of B[a]P adducts in intact DNA is fluorescence line narrowing. Sensitivities on the order of 3 adducts/ 10^8 bases have been reported using this low temperature fluorescence technique (Jankowiak et al. 1988). However, methods based on fluorescence are not generally applicable because the majority of DNA adducts are not fluorescent. Additionally, possible interference from other fluorescent species is another shortcoming of this technique. DNA postlabeling is an alternate technique that has gained wide acceptance (Randerath et al. 1981; Gupta et al. 1982). The initial step in postlabeling is DNA isolation and enzymatic hydrolysis to the 3'-nucleotides. Next, nucleotides are labeled at the 5' position by treatment with inorganic ^{32}P -phosphate and T4 polynucleotide kinase. The resulting [5'- P^{32}]-3',5'-biphosphate nucleotides are then fractionated by two-dimensional thin layer chromatography (TLC), detected by autoradiography, and quantitated by liquid scintillation spectrometry. The postlabeling technique is extremely sensitive; however, this method is not selective. Poor resolution on the polyethyleneimine cellulose TLC plate along with limited availability of DNA adduct standards make identification with this technique difficult at best. However, postlabeling is capable of differentiating between adducted nucleotides and the preponderance of normal nucleotide bases. Subsequent autoradiography of the adducted fraction allows determination of unknown adducts at impressive sensitivities. Gupta and Randerath have reported sensitivities of 1 adduct/ 10^9 to 10^{10} bases using the postlabeling technique (Randerath et al. 1981; Gupta et al. 1982).

1.1.2 High-Resolution Capillary Separations

Several research groups have begun to explore high resolution capillary separations for DNA adduct studies to improve the low separation efficiency and lack of specificity encountered with DNA postlabeling. Separations conducted with capillaries offer an inherent detection advantage (provided concentration-dependent detection is used) because of the very narrow concentrated analyte bands that elute from the column. These capillary columns are ideally suited for interfacing with selective and sensitive mass spectrometric detectors (electrospray ionization or fast atom bombardment). Jackim and Norwood (1990) were the first to report separation by capillary zone electrophoresis (CZE) of the 5'-mononucleotides resulting from an *in vitro* adduction of calf thymus DNA with B[a]P diol epoxide. After adduction, the DNA was enzymatically hydrolyzed to the

5'-mononucleotides. This hydrolysis mixture was directly analyzed by CZE with ultraviolet (UV) absorption detection. The principal adduct was easily resolved from the normal bases and hydrolysis enzymes. In more recent work, Harvey et al. (1992) examined a variety of high-resolution capillary separation techniques combined with fluorescence detection for the analysis of B[a]P adducts. One approach involved liberation of B[a]P tetrahydrotetrols from adducted DNA by acid treatment followed by analysis of the tetrahydrotetrols. Tetrahydrotetrols were analyzed either directly by microcolumn HPLC or as the negatively-charged borate complexes by CZE. Alternatively, DNA was enzymatically hydrolyzed to the 5'-mononucleotides and the adducted nucleotides separated from normal nucleotides by chromatography on Sephadex LH-20. The adducted fraction was then subjected to CZE with either fluorescence or on-line electrospray ionization mass spectrometric detection. Further studies examined micellar electrokinetic capillary chromatography (MECC) with fluorescence detection for sensitive analysis of the B[a]P-adducted nucleotides. With these approaches, high separation efficiency along with the possibility of sensitive and selective mass spectrometric detection offers distinct advantages over postlabeling methods.

1.1.3 Persistence of DNA Adducts

Whereas B[a]P-DNA adducts have been the most extensively characterized, there exists an entire spectrum of environmental pollutants that form electrophilic metabolic intermediates capable of covalently bonding with DNA or other biological macromolecules. Although the potential to form a DNA adduct of a given carcinogen is directly related to the exposure concentration (Pereira et al. 1981; Dunn 1983), the relative amounts of the various adducts in the genome may be quite different than the corresponding ratios of the parent pollutants in the environment. The full range of DNA adducts present in an organism's genetic material represents a historical repository of past genetic insults. Therefore, analytical characterization of DNA aberrations has the potential to reveal an exposure history of the individual.

DNA adducts, once formed, tend to persist for extended periods. Although data are sparse, some studies performed with B[a]P adducts in mouse skin tend to indicate that DNA adducts are repaired at approximately the same rate as normal DNA turnover. One study indicated that maximal binding occurred 24 h after exposure and 25% of the adducts remained after 7 days (Shugart 1985). Similar investigations have implicated the existence of a cellular subset that contains adducts that are highly resistant to repair (adduct concentration significantly higher than predicted by the DNA turnover rate) (DiGiovanni et al. 1985). These studies found 15% of the initial B[a]P adducts remained in mouse skin 30 days after exposure. Relevant studies performed with other chemical adducts are scarce, and studies addressing adduct persistence in plants are nonexistent.

1.2 NITRO-SUBSTITUTED POLYCYCLIC AROMATIC HYDROCARBON STUDIES

One class of compounds that have been extensively studied are the nitro-substituted PAHs (nitro-PAH). Initial studies with nitro-PAH were conducted with 1-nitropyrene. Unlike metabolic activation to reactive diol epoxides (as occurs with PAH), bioactivation of nitro-PAH depends on nitro-reduction pathways. The normal sequence of nitro-PAH metabolism in bacteria involves a series of stepwise nitro reductions leading from nitro-PAH to an acylated amino PAH. Specific intermediates identified in the nitro reduction pathway, in order of their appearance, are nitroso, hydroxylamino, hydroxamic acid ester, amino, and finally the acylated amino form (Rosenkranz and Mermelstein 1985). Neither nitro- nor nitroso-PAH species maintain full mutagenic activity in bacterial mutants that lack the full complement of nitroreductase enzymes. Therefore, hydroxyl amino or hydroxamic acid esters are believed to be more closely related to the active adducting species. A hypothesis exists that the nitrenium ion, formed directly from either hydroxylamino or hydroxamic acid ester, is the penultimate carcinogen derived from nitro-PAH (Rosenkranz and Mermelstein 1985).

The ability of 1-nitropyrene to form DNA adducts with calf thymus DNA has been studied *in vitro*. To promote the formation of bioreactive intermediates, an anaerobic system that uses xanthine oxidase has been developed (this system is discussed in detail below) (Howard et al. 1983). As with B[a]P adducts, 1-nitropyrene forms one principal adduct under *in vitro* conditions that is identical with that formed *in vivo*. The principal 1-nitropyrene DNA adduct is N-(deoxyguanosin-8-yl)-1-amino pyrene.

Many nitroaromatic explosives are known to be mutagenic and, further, are known to undergo reductive pathways as part of their environmental fate. Although electron withdrawing properties of nitro-substituents can lead to unusual reactivities, bioreactive intermediates formed in association with metabolic reduction may be responsible for mutagenic activity of these compounds. Provided that DNA adduction proceeds in a manner similar to 1-nitropyrene, two principal adducts should be formed depending on which nitro position undergoes initial reduction. The structure of these hypothetical TNT adducts is shown in Figure 1.1. A variety of other DNA adducts may form depending on the site of reaction, reaction with bases other than guanosine, and the degree of reduction in the remaining ring substituents. The hypoxanthine/xanthine oxidase system that was developed for studying 1-nitropyrene should also be applicable to studying nitroaromatic explosives as well.

Note, other, more complex metabolic mechanisms exist that may result in reactive intermediates capable of inflicting damage on DNA. For example, reduction of nitroaromatic substrates correlating with superoxide production has been demonstrated (Middlebrook et al. 1993). Superoxide production is linked to microsomal nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 (c) reductase and cystolic

xanthine oxidase. The initial species formed by nitro reduction is a nitro radical anion. This anion can undergo further reduction, disproportionate to other nitro compounds, donate electrons to oxidizing agents such as oxygen, or directly react with macromolecular species to form adducts. Electron donation to oxygen results in the formation of superoxide that quickly reacts in the aqueous environment to produce highly reactive species such as peroxide and hydroxyl radicals. These species may indiscriminately react with DNA or proteins resulting in nonspecific oxidative cellular damage. Therefore, the presence of nitroaromatic pollutants may also facilitate oxidative DNA damage in addition to the formation of specific TNT adducts (Middlebrook et al. 1993).

1.3 FUTURE ANALYSES

Trace analytical methods for specific TNT DNA adducts will be pursued once the potential for TNT to adduct DNA is delineated. Progress in DNA adduct research has been slow because of the very low concentration and difficulty in purifying adducts from complex biological samples. As plants are nonambulatory, they suffer the cumulative effects of chronic high-level exposure. This chronic exposure may lead to exceptionally high DNA adduct concentrations. Analytical methods would be greatly simplified if adduct concentrations in plant tissues are sufficiently elevated.

1.4 OTHER STUDIES PERFORMED

At the time of this report, there have been no published accounts addressing the possibility of using plant DNA adducts biomarkers for munitions contamination. One abstract, that appeared concurrently with preparation of this report, addressed the general formation of TNT adducts (Kohan et al. 1995). This study examined *in vitro* adduction of TNT with calf thymus DNA and *in vivo* studies of orally dosed rats. These authors used postlabeling techniques to identify the TNT-DNA adducts produced. The results generated during the *in vitro* study indicated four distinct adducts were formed. Hepatic DNA isolated from orally dosed rats displayed three to four different TNT adducts. The concentration of these adducts was in the 7 adducts/10⁹ nucleotide range. Results of this study were consistent with metabolic activation by a nitro-reduction pathway.

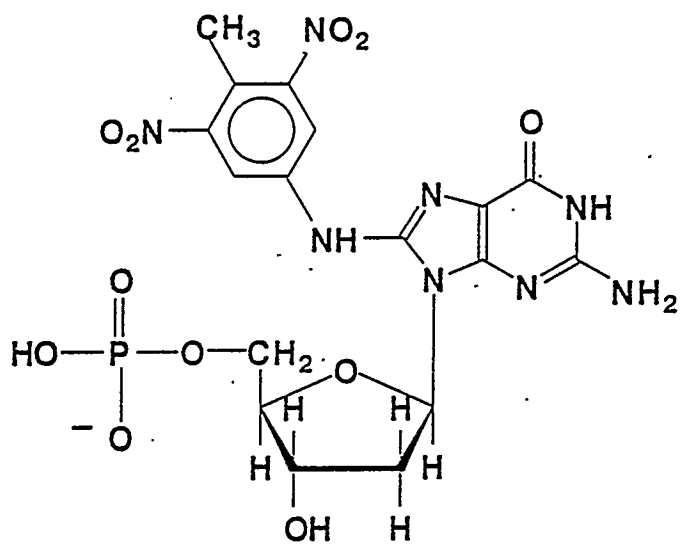
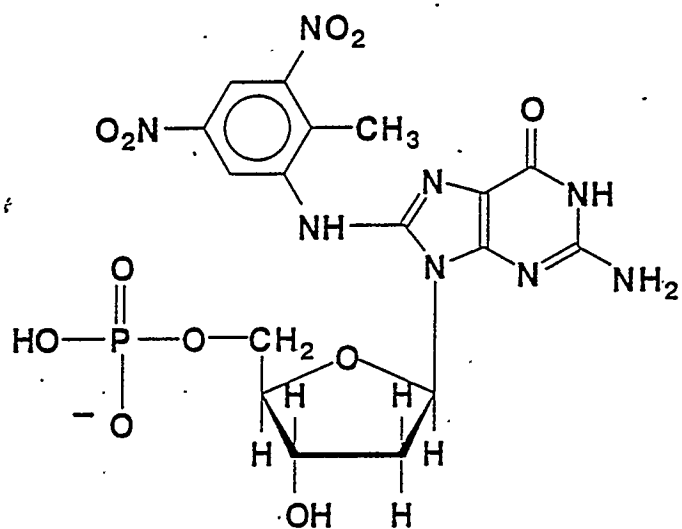


FIGURE 1.1. Structures of Hypothetical TNT-Adducts.

2.0 MATERIALS AND METHODS

2.1 GENERAL CONSIDERATIONS

TNT adduction studies are considerably more challenging than previous studies with B[a]P or 1-nitropyrene because neither TNT nor its expected DNA adducts are fluorescent. Therefore, by necessity, adducts were detected by UV absorption at 254 nm which is notoriously nonselective. Because of the nonselective detection, the study depends on careful comparisons with appropriate blanks. Because of these difficulties, separating and preconcentrating the adducted nucleotide fraction before analytical studies was desirable. Initially, pea sprouts were studied as the source of plant DNA; however, because unexpectedly complex nucleotide profiles resulted from this source, commercially available calf thymus DNA was ultimately substituted.

2.2 PLANT GROWTH AND DNA ISOLATION

Chemical adduction studies with TNT required isolation of large amounts of plant DNA. As plant DNA is not commercially available, initial efforts focused on isolating DNA from pea sprouts. Pea seedlings were grown as described by Bendich and Bolton (1967). Sprouts represent an ideal tissue from which to isolate DNA because of the absence of woody tissue that could physically sequester DNA. Sprout tissue contains chloroplastic DNA even though chlorophyll is not present. Therefore, DNA isolated from sprouts contains a mixture of nuclear, mitochondrial, and chloroplastic DNA. Pea seeds were prepared for growth by soaking 20 min in a 1.0 % (v/v) bleach solution to eliminate fungal contamination. After rinsing and soaking the seeds in water for 1 h, they were distributed on a moist vermiculite bed and maintained in the dark. Distilled water was added to the sprouts as necessary to keep the preparation moist. Seedling harvest occurred on the seventh day of growth and involved removal of the cotyledon followed by thorough rinsing of the remaining sprout. Immediately following this preparation, the sprouts were extracted for DNA.

Initially, the DNA extraction method described by Rogers and Bendich (1988) was considered. This method made use of cetyltrimethylammonium bromide (CTAB) surfactant to disrupt cellular membranes. However, on further examination, it was felt that this procedure was unnecessarily complex for our objective of isolating bulk quantities of DNA. Therefore, the more straightforward DNA isolation scheme of Bendich and Bolton (1967) was used for large-scale DNA isolation. This procedure is summarized in Figure 2.1.

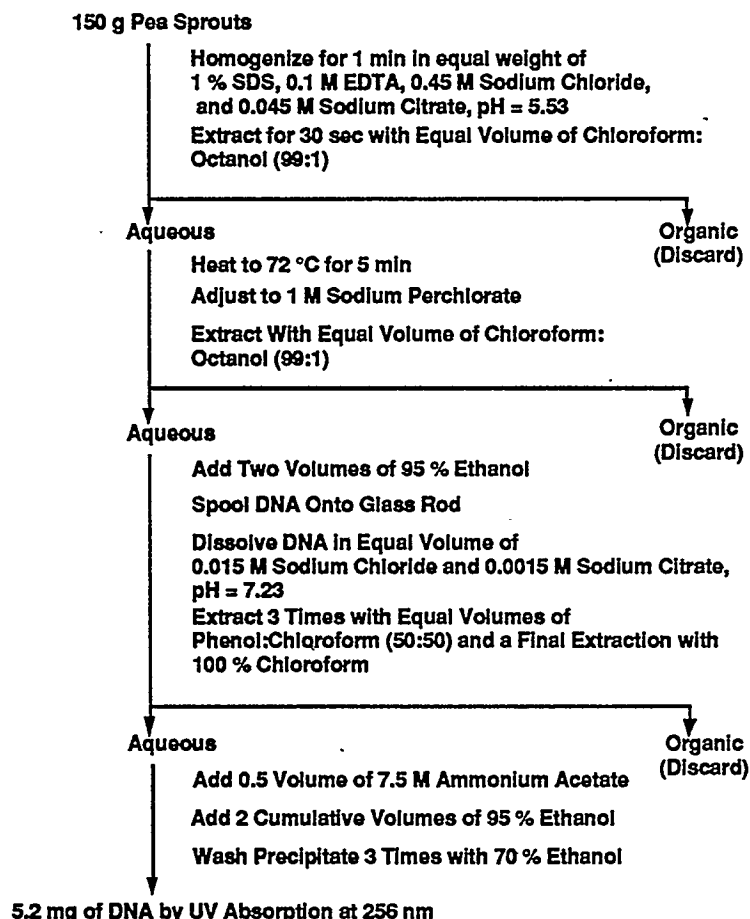


FIGURE 2.1. Flowchart Illustrating the Isolation of DNA from Plant Tissue.

2.3 IN VITRO DNA ADDUCTION

Our experimental rationale was to isolate sufficient quantities of plant DNA to perform an *in vitro* TNT adduction. Calf thymus DNA was later substituted to provide a simpler nucleotide composition that would maximize chromatographic visualization of adducts. Adduction procedures for TNT closely mimicked conditions defined by Beland and co-workers for their pioneering work with 1-nitropyrene (Howard et al. 1983). The bioreactive intermediates produced from TNT by the hypoxanthine/xanthine oxidase system are illustrated in Figure 2.2. Briefly, DNA, TNT, xanthine oxidase, and hypoxanthine are incubated under anaerobic conditions. In this system, hypoxanthine is successively oxidized to uric acid as the enzyme xanthine oxidase is reduced. The reduced form of the enzyme cycles back to the oxidized form by reducing TNT. As TNT undergoes stepwise nitroreduction, some of the bioreactive intermediates can be expected to adduct DNA.

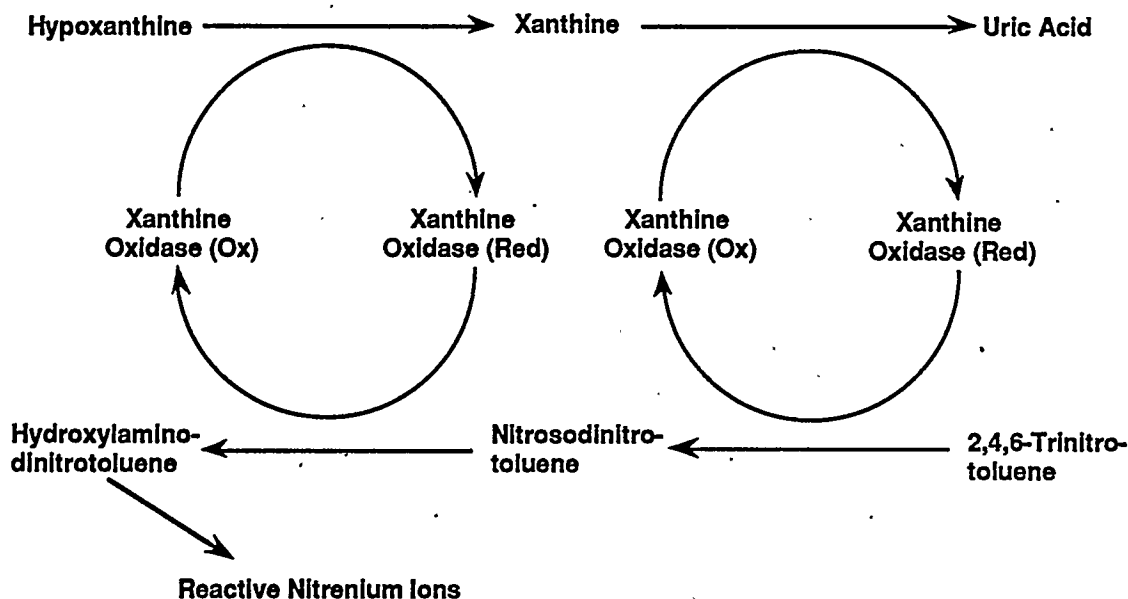


FIGURE 2.2. Hypoxanthine/Xanthine Oxidase System for Producing Nitroreduction Bioreactive Intermediates.

Adduction reactions were performed in a 25-mL round-bottomed flask that was fitted with a magnetic stir bar and continuously purged with nitrogen. To prepare the adduction solution, 5 mg of hypoxanthine was dissolved with the aid of sonication in 10 mL of 50-mM potassium phosphate (pH = 5.8) adduction buffer to yield a 3.7-mM hypoxanthine solution. Next, 20 mg of calf thymus DNA was added to this solution and stirred until completely dissolved. Meanwhile, 250 μ L of a 2.12-mg/mL solution of TNT (0.5 mg) in methanol was added to the round-bottomed flask. Methanol solvent was then removed by immersing the flask in a 37 ± 0.5 °C water bath and purging with nitrogen. After the methanol was completely removed, hypoxanthine/DNA solution was added to the TNT-containing flask and the solution was degassed for 20 min. The adduction reaction was then initiated by adding of 55.6 μ L xanthine oxidase (final concentration of 0.10 unit/mL). DNA adduction was allowed to proceed for a 2-h period at which time the adducted DNA was purified by repetitive extraction with chloroform:isoamyl alcohol:phenol (24:1:25 v/v) and ethanol precipitations as described above. Purified DNA was then subjected to enzymatic hydrolysis and HPLC analysis again, as described in detail below. Reactions were always performed in parallel with blank reactions for comparison. A blank consisted of a solution that was identical in all aspects to the sample except the analyte was omitted. The blank solution was carried through the entire procedure including the 2-h "reaction" period, DNA isolation, hydrolysis, and HPLC analysis.

2.4 DNA HYDROLYSIS AND SEPHADEX LH-20 CHROMATOGRAPHY

In vitro studies of B[a]P involve reaction of B[a]P diol epoxide with calf thymus DNA followed by the enzymatic hydrolysis of DNA to either the individual nucleosides or 5'-mononucleotides. The enzymatic hydrolysis mixture is either analyzed directly (Jackim and Norwood 1990) or the adducted nucleotide/nucleoside fraction is chromatographically purified on Sephadex LH-20 (Jennette et al. 1977). During this purification, enzymes and normal bases are eluted from the column with buffer, whereas the adducted base fraction is eluted with a stronger mobile phase containing methanol. Generally, DNA is enzymatically digested to the nucleoside level before purification on LH-20. However, from an analytical perspective the isolation of nucleotides is more desirable. The nucleotide phosphate group facilitates analysis by capillary electroseparation techniques and enhances subsequent detection by on-line electrospray or fast atom bombardment mass spectrometry. Although capillary electroseparation techniques may be applied directly to the DNA hydrolysis mixture, isolating the adducts from normal bases and enzymes is clearly advantageous. Towards such an objective, Harvey et al. (1992) evaluated Sephadex LH-20 chromatography for purification of B[a]P-adducted nucleotides. Harvey et al. (1992) found that the B[a]P-adducted nucleotides were reasonably retained on Sephadex LH-20, although less than the corresponding adducted nucleosides. B[a]P-adducted nucleotides were eluted only after moderate proportions (40%, v/v) of methanol were added to the buffered mobile phase. In the traditional isolation procedure, pure methanol is used to elute the B[a]P-adducted nucleoside from LH-20. The lower affinity of the adducted nucleotide towards Sephadex LH-20 is easily rationalized because the phosphate group imparts a higher polarity to the molecule.

Adducted DNA will be subject to enzymatic hydrolysis to produce 5'-mononucleotides. To accomplish this task, adducted DNA, along with an appropriate control, will be incubated with deoxyribonuclease I and snake venom phosphodiesterase. Deoxyribonuclease I serves to cleave the DNA to oligonucleotides, whereas the phosphodiesterase successively cleaves off deoxynucleotide-5'-monophosphates from the oligonucleotide strands. Analysis of the hydrolysis mixture proceeds either directly on the crude hydrolysate or after fractionation on Sephadex LH-20. Should adducts be observed, they will be isolated and chemically characterized. As studies progress, advanced analytical techniques will be implemented to allow for high resolution separation with trace detection capabilities.

Initial hydrolysis studies were performed with 100 μ L of DNA solution (containing 62 μ g DNA). This solution was mixed with 50 μ L of tris(hydroxymethyl)aminomethane (TRIS) buffer [150 mM TRIS, 1 mM $MgCl_2$, 150 mM NaCl, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH of 7.4], 50 μ L deoxyribonuclease I solution (25 mg/mL containing 62.5 units/ μ L in 10 mM TRIS and 10 mM $CaCl_2$ adjusted to pH = 8.5) and 40 μ L of snake venom phosphodiesterase (0.5 units/mL in 10 mM TRIS and

10 mM CaCl₂ adjusted to pH = 8.5) to give a final volume of 240 µL. Deoxyribonuclease I (E.C. 3.1.21.1) type II and phosphodiesterase I (E.C. 3.1.4.1) type VII were obtained from Sigma (St. Louis, MO) and were purified from bovine pancreas and *Crotalus atrox* venom, respectively. The enzymatic digest mixture was incubated at 38°C for 2 h and analyzed by HPLC after centrifugation at 16,000 x g for 20 min. In instances where material for sephadex LH-20 chromatography was being prepared, the enzymatic hydrolysis reaction was scaled up by a factor of 10.

Sephadex LH-20 columns were packed with material that had been first equilibrated with 20 mM ammonium bicarbonate buffer (pH = 8.4). This resin was then packed in 18.0-cm x 0.8-cm interior diameter (ID) column blanks (Isolab, Akron, OH) as a methanol slurry. After achieving a 12.0-cm packing bed, the columns were thoroughly equilibrated with aqueous bicarbonate buffer. At this time, DNA hydrolysate sample aliquots were applied to the columns and successive 2.0-mL fractions collected. Absorbance at 254 and 280 nm were determined for each individual fraction. Fractions 1 to 4 were eluted with aqueous 20 mM ammonium bicarbonate buffer (pH = 8.4). Subsequent fractions were eluted with increasing proportions of methanol. Fractions 5 to 8, 9 to 12, and 13 to 16 were eluted with 20, 40, and 80% (v/v) methanol, respectively. Finally, fractions 17 through 20 were eluted with pure methanol.

2.5 ANALYTICAL SEPARATIONS

Capillary electroseparations were performed with a system assembled in-house as previously described (Harvey et al. 1992). Fused silica capillaries [1.0 m x 50 µm inside diameter (360 µm outside diameter)], obtained from Polymicro Technologies (Phoenix, AZ), were preconditioned by treatment with 0.1 M sodium hydroxide followed by a water rinse before equilibration with separation buffer. Detection windows were placed approximately 25 cm from the ground reservoir on these columns. Buffer reservoirs were contained within a Lexan^{TM1} box fitted with a safety interlock system to protect the operator from high-voltage contact. High-voltage and ground connections were provided through platinum electrodes immersed in the buffer reservoirs. Voltage was delivered to the capillary injection reservoir by a Spellman CZE 1000R power supply (Plainview, NY). A Keithly 175A autoranging multimeter (Cleveland, OH) was placed in series between the detector reservoir and ground to measure separation current. Introduction of sample to the separation capillary was accomplished hydrodynamically. An Isco^{®2} CV⁴ capillary electrophoresis absorbance detector (Lincoln, NE) was utilized at 245 nm for analyte detection. Chromatographic traces were recorded on a Hewlett-Packard^{®3} model 3393A integrator (Avondale, PA).

¹ Lexan is a trademark of General Electric Company, Schenectady, NY.

² Isco is a registered trademark of Isco Inc., Lincoln, NE.

³ Hewlett-Packard is a registered trademark of Hewlett-Packard Company, Palo Alto, CA.

HPLC analyses were performed on a Beckman Ultrasphere C-18 reversed-phase column (24 cm x 4.6 mm, $d_p = 5 \mu\text{m}$). Linear aqueous buffer-methanol gradients were delivered to the column at a flow rate of 1.0 mL/min by a Waters model 600E system controller and pump. Samples were introduced to the column by use of a Waters WISP model 710 automatic injector. As the column was developed, the eluting components were detected by a Waters 490E detector operated 254 nm.

3.0 RESULTS AND DISCUSSION

3.1 GENERAL CONSIDERATIONS

We found that the isolated pea DNA produced an exceedingly complex nucleotide chromatographic profile for some of these developmental efforts. While some of the preliminary analyses were conducted with the pea DNA, we felt that the numerous trace-level modified bases might obscure the presence of TNT-adducted nucleotides. Therefore, we opted to perform many of the tests in this developmental study with purified calf thymus DNA obtained from Sigma (St. Louis, MO) in the anticipation of returning to plant DNA in the future.

Several different analysis techniques were evaluated for profiling deoxynucleotide-5'-monophosphates. CZE, MECC, and conventional HPLC separations of deoxynucleotide-5'-monophosphate standards were performed to determine the most suitable analytical approach. Because our initial success depends upon careful comparisons between controls and adducted DNA, the separation technique needed to be, above all else, highly reproducible. Of the above techniques, HPLC is sufficiently advanced that high levels of reproducibility were expected on a routine basis. In contrast, during future extensions of this program where reproducibility may not be of foremost concern, the ultra-trace analytical techniques based on the recent capillary separation approaches can be expected to outperform traditional HPLC methods because of higher resolution, enhanced mass detection abilities, as well as unique and novel detector arrangements (Wallingford and Ewing 1989).

3.2 CZE EVALUATION

Initially, a preliminary evaluation of CZE techniques for separating 5'-monophosphate nucleotides was performed. Efforts focused on reproducing a CZE separation reported by Jackim and Norwood (1990). A standard solution containing 0.2 to 0.4 mg/mL each of the four normal deoxynucleotide-5'-monophosphates and 5-methyl-2'-deoxycytidine-5'-monophosphate was prepared in distilled water. The methylated cytidine phosphate was included in the standard because this modified base is commonly found in plants. CZE separations with a 40 mM sodium carbonate CZE buffer (pH = 9.6) resulted in four broad peaks that lacked adequate resolution. An electropherogram of the standard nucleotide mixture is presented in the top of Figure 3.1. Although this separation used the same buffer composition that Jackim and Norwood employed in their studies, in our hands this system did not provide adequate separation of test components. Because CZE separations are highly dependent on the fused silica composition, it is presumed that the difference observed is caused by slight differences in fused silica surface chemistry. In an effort to improve this separation, a fused silica column that contained a uniform and

defined surface coating was investigated. Coated CZE columns provide decreased electroosmotic flow and minimal analyte interactions with the capillary wall. A separation obtained with a CElect H-50 column (Supelco, Bellefonte, PA) is shown in the bottom of Figure 3.1. Although the nucleotide separation is improved over that obtained on bare silica, separations on the coated column were time consuming and failed to provide baseline separation of the standard components. Furthermore, although the column coating is reportedly stable towards hydrolysis within a defined pH range, we experienced migration times with substantial variation particularly when the column was maintained in separation buffer overnight. These observations argue against the prolonged stability of the column coating at relatively high pH values. Based on this experience, we concluded that CZE on bare or coated fused silica columns did not meet the reproducibility criterion necessary for our studies.

3.3 MECC EVALUATION

Micellar electrokinetic systems were next investigated for their potential to provide reproducible separations of nucleotide standards. Micellar separation systems are reported to offer rapid high-resolution separations of nucleotides (Liu et al. 1989). The buffer used for our studies was 40 mM sodium carbonate containing 50 mM CTAB at pH = 9.6. As expected, the high concentration of cationic surfactant caused a reversal in electroosmotic flow. It was therefore necessary to reverse the electrode polarity to maintain electroosmotic flow towards the detector. As illustrated in the lower portion of Figure 3.2, the micellar electrokinetic separation resolves all five nucleotides within 10 min; however, a phantom peak that elutes after the other nucleotides (8.02 min) was also observed. At present, we have no explanation for the appearance of this phantom peak other than it seems to be associated with the concentration of 2-deoxyguanosine-5'-monophosphate. An electropherogram of hydrolyzed pea DNA produced under the same conditions is shown in the top of Figure 3.2. The five nucleobases in the pea DNA hydrolysate are clearly shown by comparison of this electropherogram to the nucleotide standard. This separation technique remains potentially useful; however, the mechanism underlying the appearance of the artifact peak must first be understood. Although micellar approaches appear encouraging, the artifact inconsistency prompted us to continue to search for a separation system that will allow reproducible profiling of enzymatic DNA hydrolysates.

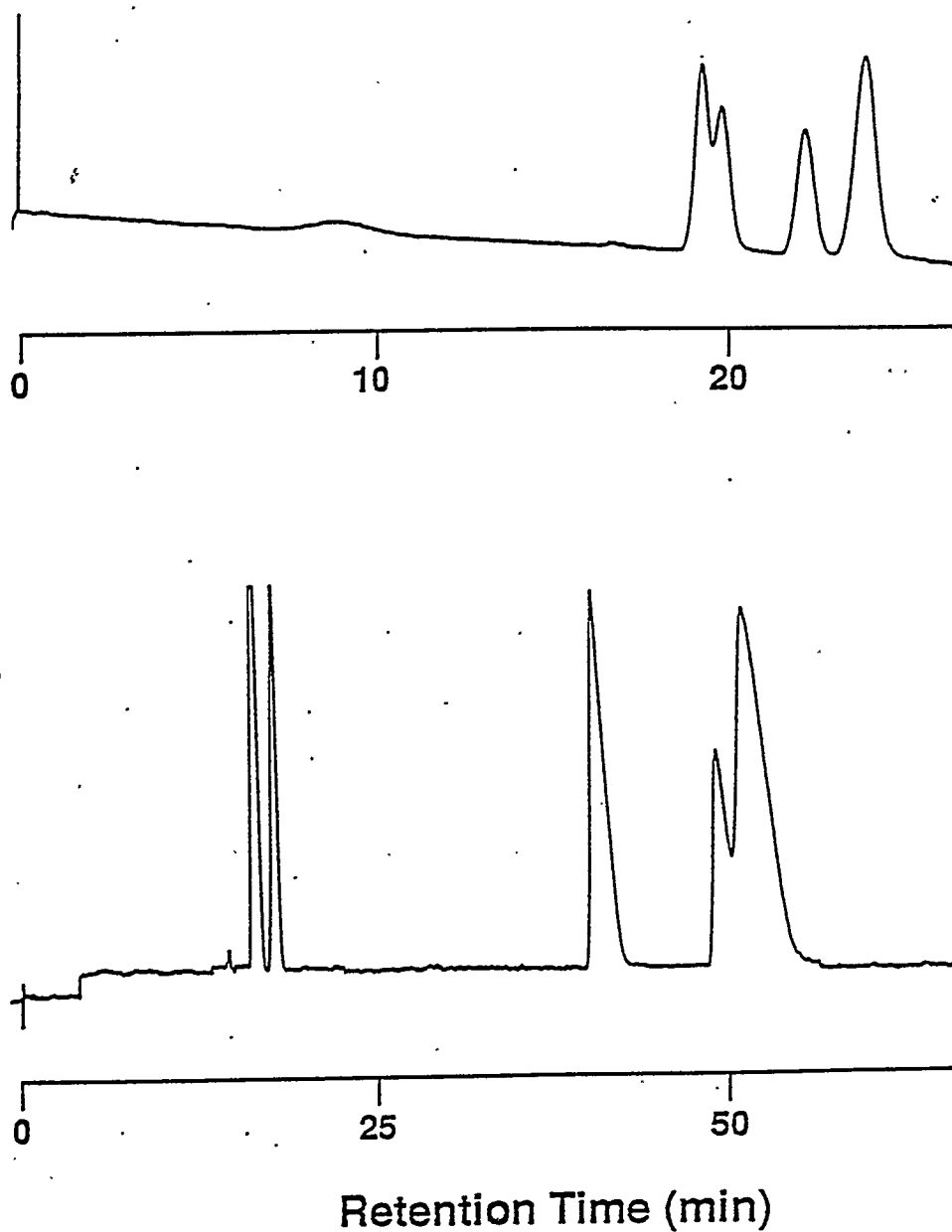


FIGURE 3.1. Electropherograms of Standard Nucleotides by Capillary Zone Electrophoresis on Bare (Top) and Coated (Bottom) Fused Silica Columns.

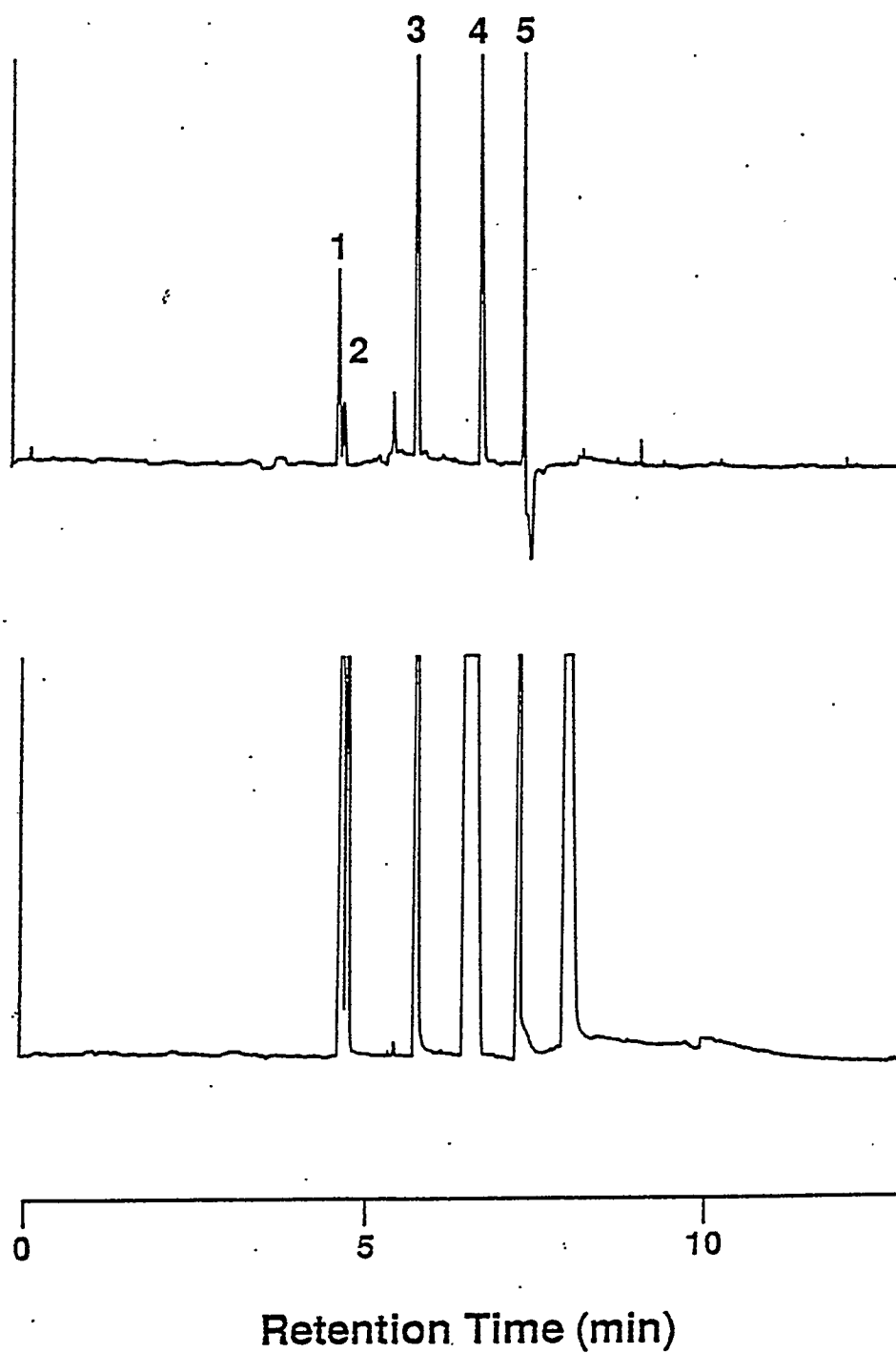


FIGURE 3.2. Electropherograms of Nucleotides Obtained from Enzymatic Hydrolysis of Pea DNA (Top) and Standard Nucleotides (Bottom) by Micellar Electrokinetic Capillary Chromatography. Identity of the Deoxynucleotide-5'-Monophosphates are 1) Cytidine, 2) 5-Methylcytidine, 3) Thymidine, 4) Guanosine, and 5) Adenosine.

3.4 HPLC RESULTS

HPLC nucleotide separations used a 30-mM potassium phosphate buffer (pH = 5.6)/methanol gradient. The HPLC mobile phase was ramped from 100% phosphate buffer to 40% methanol in a 20-min period. The final mobile phase composition was maintained for 20 min. Detection was by UV absorbance at 254 nm. The chromatogram shown in the bottom of Figure 3.3 illustrates a representative separation of the principal deoxynucleotide-5'-monophosphate standards. The order of elution of the deoxynucleotide-5'-monophosphates from the octadecyl silica column was cytidine, thymidine, guanosine, and adenosine. A common pyrimidine nucleotide found in DNA is 5-methylcytidine. In a separate chromatographic experiment, a standard of deoxy-5-methylcytidine-5'-monophosphate was analyzed and found to elute with a retention time of 8.78 min (between deoxynucleotide-5'-monophosphates of cytidine and thymidine). Separations by HPLC proved more reproducible, reliable, and rugged compared to the capillary electroseparations. Therefore, HPLC was implemented for subsequent DNA adduction studies.

The reversed phase HPLC separation described above was used to separate deoxynucleotide-5'-monophosphates that resulted from enzymatic hydrolysis of plant DNA. The HPLC chromatogram of the enzymatic hydrolysis mixture is shown in the top of Figure 3.3. The presence of four principal deoxynucleotide-5'-monophosphate bases are clearly indicated in this chromatogram. Additionally, the peak eluting with a retention time of 8.78 min indicates the presence of deoxy-5-methylcytidine-5'-monophosphate. Other peaks in this profile are caused by minor bases, although hydrolysis enzymes and impurities introduced through the extraction and hydrolysis procedure may also be represented. The nucleotide chromatographic profile of pea DNA was unexpectedly complex. Numerous trace-level modified bases could easily obscure TNT-adducted nucleotides during comparisons between controls and TNT-adducted DNA. Calf thymus DNA, on the other hand, contains a very low occurrence of modified bases. For this reason, commercially available calf thymus-DNA was substituted for pea DNA to maximize the probability of visualizing TNT adducts in the nucleotide profiles.

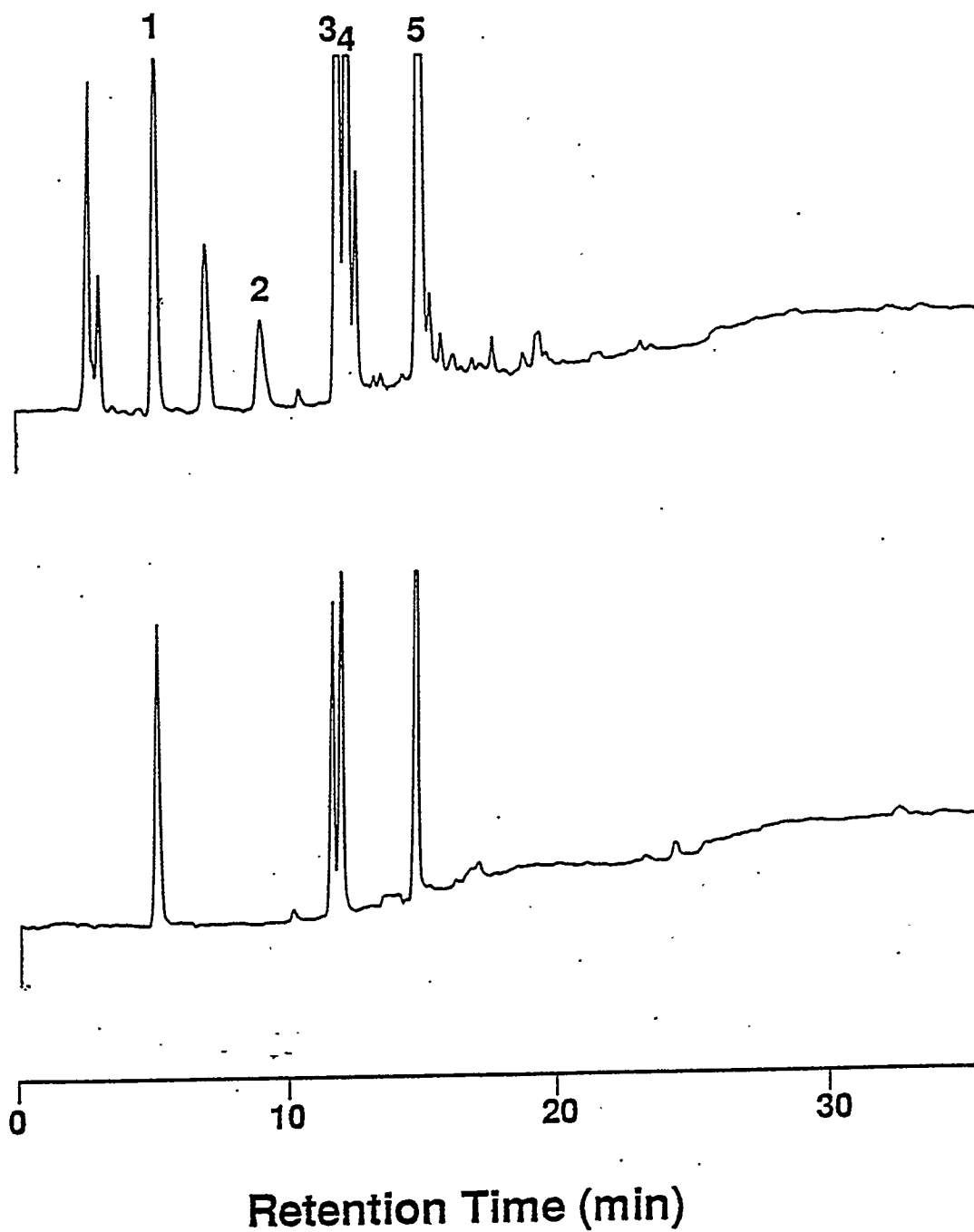


FIGURE 3.3. HPLC Chromatograms of Nucleotides Obtained from Enzymatic Hydrolysis of Pea DNA (Top) and Standard Nucleotides (Bottom). Identity of Deoxynucleotide-5'-Monophosphates are 1) Cytidine, 2) 5-Methylcytidine, 3) Thymidine, 4) Guanosine, and 5) Adenosine.

3.5 ADDUCTIONS: TNT AND 1-NITROPYRENE

A preliminary reaction was performed with 1-nitropyrene as described by Howard et al. (1983). The only deviation from the adduction procedure outlined in the methodology section was that 1-nitropyrene was added in a small volume of dimethylsulfoxide (DMSO). During the course of this reaction, the intense yellow-colored nonfluorescent nitropyrene was transformed to the highly fluorescent reduction product, 1-aminopyrene, thereby ensuring adequate conditions existed for substrate reduction. The indirect inference can be made that reactive intermediates were formed in the reaction sequence towards the 1-aminopyrene product.

Several TNT adduction reactions were performed as described above. During the course of the reaction, the TNT adduction solution turned distinctly yellow. This color indicated formation of amino homologs of TNT and served to verify that correct nitroreduction conditions existed during reaction. Color formation was not observed in the blank adduction solutions. After DNA purification and enzymatic hydrolysis, the nucleotides from TNT-adducted DNA were chromatographically profiled by HPLC. A comparison of the crude hydrolysis mixture to the blank is presented in Figure 3.4. The four principal nucleotides were prominent in both TNT-adducted and blank hydrolysates. Of particular interest were two peaks eluting within the 21.4- to 22.6-min retention window in the adducted TNT sample chromatogram. These peaks were not present in the blank solution and may represent TNT-adducted nucleotides. The two nitro-reduction isomers shown in Figure 1.1 could possibly be the identity of these two peaks.

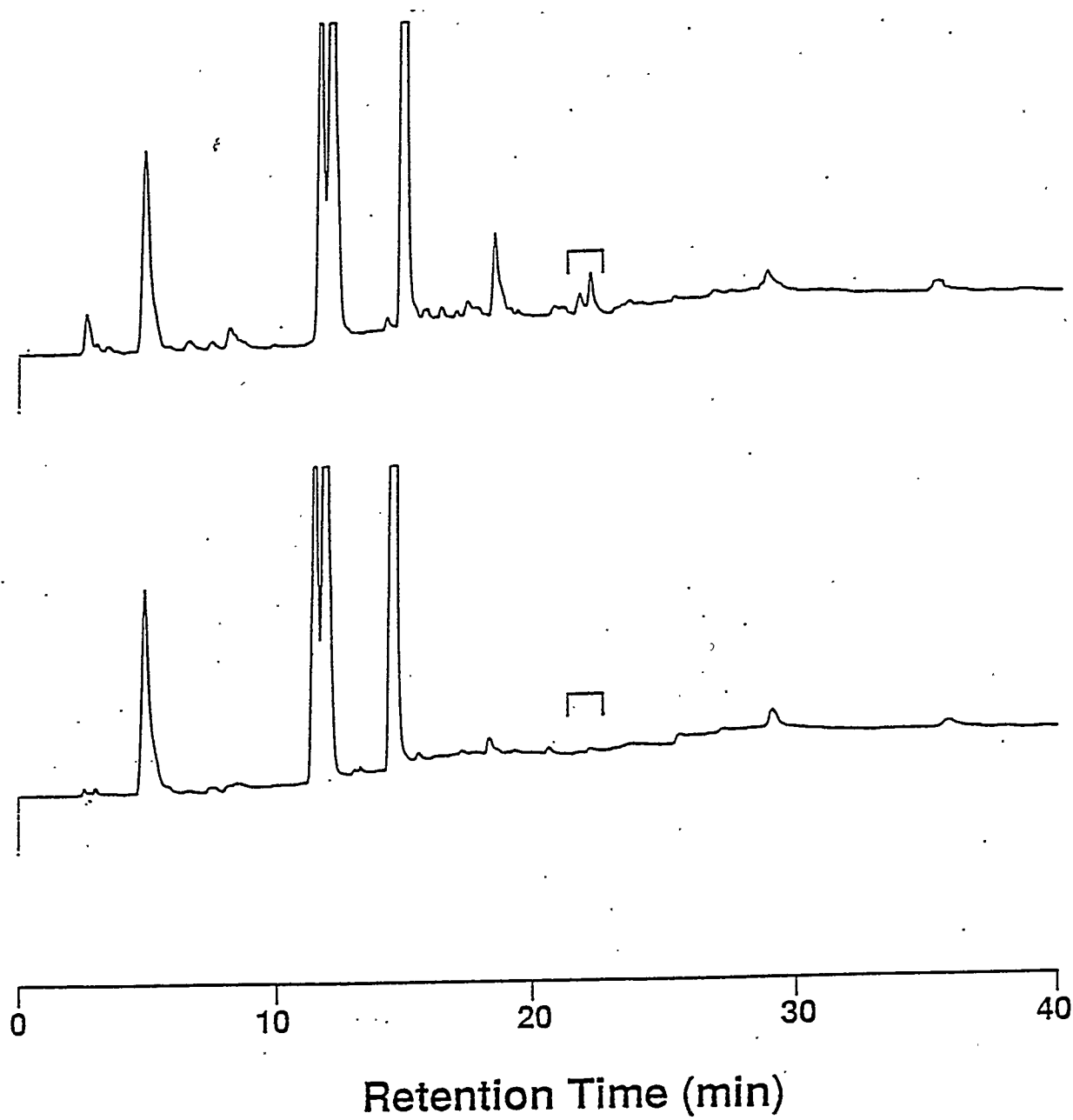


FIGURE 3.4. HPLC Profile of Hydrolysate from TNT-Adducted DNA (Top) Compared to Control DNA (Bottom).

3.6 LH-20 CHROMATOGRAPHY

Figure 3.5 shows the results of separating nucleotides on sephadex LH-20. This figure compares sephadex LH-20 fraction absorbances at 254 nm for the adducted TNT (top) and control (bottom) hydrolysate samples. As is shown in Figure 3.5, fraction 14 from the adducted sample appears to have an enhanced absorbance relative to the corresponding control fraction. This fraction was scrutinized by HPLC analysis to determine if the suspected TNT adducts (which were observed in the whole hydrolysate) were responsible for the observed absorbance. Surprisingly, chromatograms of fraction 14 from the adducted and control samples were identical. There was no indication of any peaks within the 21.4- to 22.6-min retention time range. Samples were rerun with a modified mobile-phase gradient that would elute more strongly retained UV absorbing compounds. The modified gradient ran from 0 to 80% methanol with a final 20-min hold at 80% methanol. Again, the chromatograms between the control and adducted sample were identical. At present, we cannot account for the higher absorbance observed in the TNT-adducted sephadex LH-20 fraction 14.

The possibility exists that TNT adducts were not separated from the normal nucleotides on Sephadex LH-20. As sephadex LH-20 fraction 2 contained the majority of normal nucleotides, fraction 2 from the control and sample were concentrated and chromatographically compared. This comparison gave no indication of compounds other than the normal nucleotide bases. Next, broad fractions were pooled in an attempt to locate the suspected TNT-adducted nucleotides. Three pools were created: 1) fraction 1 plus 3 through 5; 2) fractions 6 through 13; and 3) fractions 14 through 20. These pooled fractions were concentrated by evaporation under a stream of nitrogen and analyzed by HPLC. Chromatographic profiles of the three pooled TNT-adducted fractions are presented in Figure 3.6. The bracket above the top chromatogram in this figure represents the retention window where suspected TNT adducts eluted in the crude hydrolysate. This experiment was not able to locate the suspect TNT adducts. At present, we have no explanation for the loss of the suspected TNT adducts upon Sephadex LH-20 chromatography.

At this time it was critical to perform several TNT adductions to repeat and verify formation of peaks appearing in the 21.4- to 22.6-min retention window. A total of three additional addition reactions were performed along with the appropriate blanks. The two characteristic peaks (tentative TNT adducts) were observed in one of the TNT addition hydrolysates, albeit in slightly lower concentration than the original reaction. Interestingly, suspect TNT-adducted nucleotides were not visible in the other two TNT addition reactions. The results for this experiment should not necessarily be viewed as ambiguous. Because adducts appear in very low concentrations, failure to visualize the suspected adducts in several of the addition reactions could simply reflect a lower conversion efficiency for these particular reactions.

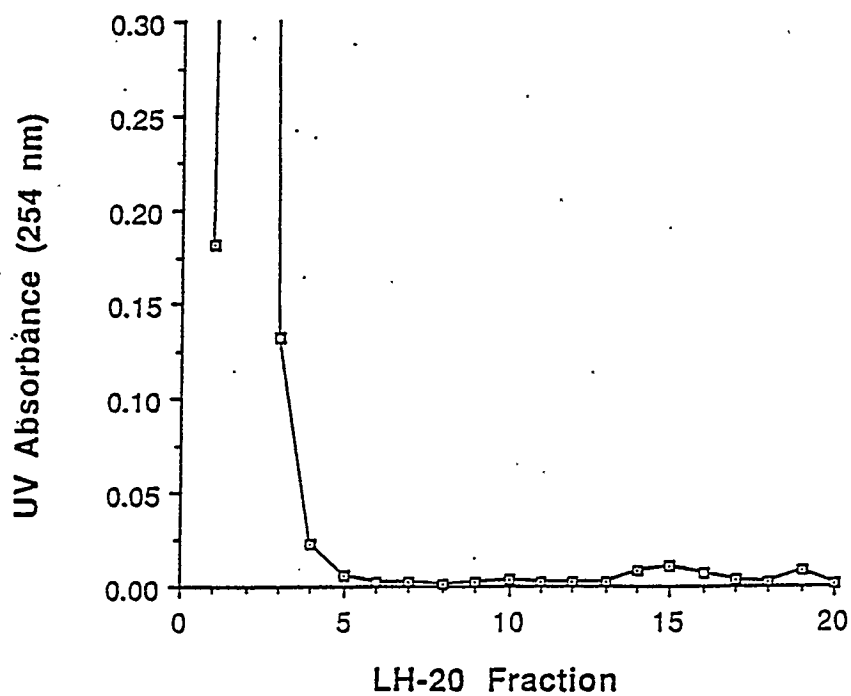
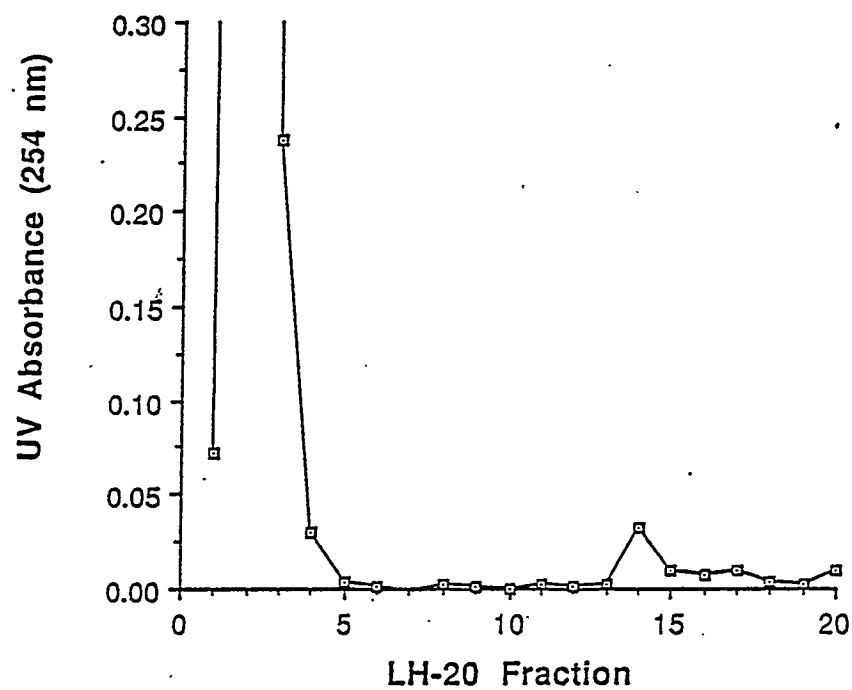


FIGURE 3.5. UV Absorbance at 254 nm for Sephadex LH-20 Fractions from Hydrolysates of TNT Adducted DNA (Top) and Control DNA (Bottom).

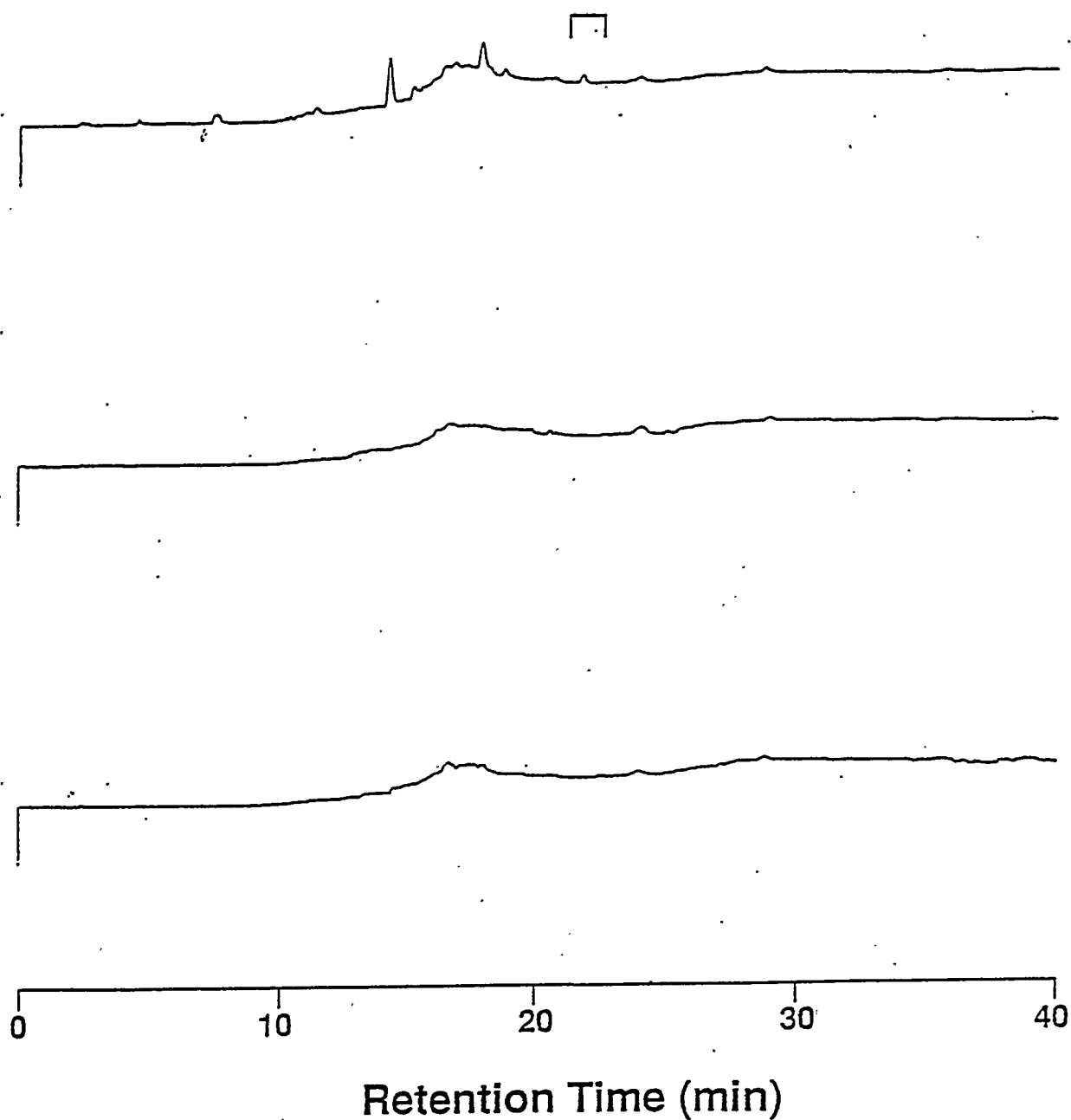


FIGURE 3.6. HPLC Fractions for Pooled Sephadex LH-20 Fractions from TNT-Adducted Hydrolysate. Chromatograms Represent Fractions 1 and 3 through 5 (Top), 6 through 13 (Center), and 14 through 20 (Bottom).

4.0 SUMMARY AND CONCLUSIONS

The present study performs preliminary ground work towards identifying adducts formed between DNA and TNT. Our research strategy has been to adduct calf thymus DNA with TNT *in vitro* using an established hypoxanthine/xanthine oxidase enzyme system to produce nitro-reduction reaction intermediates. Once adducted, DNA was purified and enzymatically cleaved to the 5'-monophosphate nucleotide level. Subsequent HPLC profiling of the nucleotides was used to identify suspected TNT adducts.

Chromatographic comparisons between control and TNT-adducted hydrolysates allowed the tentative identification of two TNT-adducted nucleotides. Chromatographic profiles of TNT-adducted DNA hydrolysates featured these compounds eluting in the 21.4- to 22.6-min retention window. The suspect adducts were not observed in all TNT adduct reactions performed, perhaps because of the formation at concentrations below the analytical detection limit. These studies can be added to the mounting evidence that specific TNT DNA adducts occur.

Further work is clearly needed to understand the variables leading towards reproducible high-yield TNT adduction reactions. To extend the studies performed here, unambiguously verifying formation of these specific TNT adducts is necessary. Studies with TNT have been considerably more difficult than DNA adduct studies of carcinogens that form fluorescent adducts. Because of the enhanced selectivity and sensitivity possible with fluorescence detection, adducts are far easier to identify and quantitate. A viable approach for extending the present studies would be to perform *in vitro* adductions with radiolabeled TNT. Adducted nucleotides could then be identified by radiochromatographic means.

Although TNT adducts have been visualized with postlabeling techniques, insufficient amounts of material are used to provide complete structural information. Similarly, capillary separation techniques can be expected to be ideal for trace analysis and detection; however, capacity is insufficient to provide material for structural studies.

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