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GOODROW, TAMRA LISS

AN HPLC METHOD FOR MEASUREMENT OF THE PERSISTENCE OF METHYLATED GUANINES IN HEPATOTOXICANT-EXPOSED RATS

THE UNIVERSITY OF ARIZONA M.S. 1985

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AN HPLC METHOD FOR MEASUREMENT OF THE PERSISTENCE OF METHYLATED GUANINES IN HEPATOTOXICANT-EXPOSED RATS

by

Tamra Liss Goodrow

A Thesis Submitted to the Faculty of the COMMITTEE ON TOXICOLOGY In Partial Fulfillment of the Requirements For the Degree of MASTER OF SCIENCE In the Graduate College THE UNIVERSITY OF ARIZONA

1985
STATEMENT BY AUTHOR

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[Signature] 12-27-64

I. G. SIPES
Professor of Pharmacology and Toxicology
To my family,
whose support and faith in me has enabled me

to undertake and complete this endeavor.
ACKNOWLEDGMENTS

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ABSTRACT

The effect of pretreatment with trichloroethylene (TCE) in drinking water upon the persistence of 0-6 methylguanine adducts in rat liver DNA was examined. Rats were given TCE ad libitum in their drinking water at 5 mg/ml for one or two weeks and then given a single dose (15 mg/kg) of the carcinogen, dimethylnitrosamine (DMN). The 0-6 methylguanine present in hepatic DNA that formed as a result of the bioactivation of DMN was assayed using a high performance liquid chromatography (HPLC) technique. This assay was established through modification of published methods. The established procedure was sensitive to 0.125 ng for 0-6 methylguanine and 3.0 ng for N-7 methylguanine and it allowed baseline resolution of N-7 methylguanine from adenine. The TCE was found to have no effect upon the persistence of 0-6 methylguanine in hepatic DNA, when the DNA was isolated 14 hours after dosing with DMN.
INTRODUCTION

Human Exposure to Dimethylnitrosamine

Human exposure to exogenous dimethylnitrosamine (DMN) occurs via many sources. The highest level of human exposures to exogenous nitrosamines occurs as a result of occupational exposure. The levels of exposure as well as the numbers of workers affected are greatest in the rubber and tire industries. Exposure to DMN and other nitrosamines is linked to their formation from reaction of vulcanisation accelerators based on amines and nitrosating agents that are used to retard vulcanization (Speigelhalder and Preussman, 1983). The levels of DMN in the air in rubber and tire factories have been measured and the mean concentrations varied between 1-10 ug/m$^3$ but have been reported to be as high as 140 ug/m$^3$ in certain working areas (Fine, 1982a and Speigelhalder and Preussman, 1983). In addition, measurable concentrations of up to 1.5 ug/m$^3$ have been found in tire sales rooms, mounting halls, and stock rooms. Other occupational exposures have been reported in the leather tanning industry, amine factories, and rocket fuel factories (Fine, 1982a). Non-occupational sources of human exposure to DMN include food, alcoholic beverages, and tobacco smoke (Lijinsky and Epstein, 1970, Hoffman et al., 1982). Measurable concentrations of DMN have been found most consistently in three groups of food products: cured meats and fish, alcoholic beverages, and cheeses. DMN was found in 100% of 395 samples of cured
meat and sausages, albeit that the concentrations in 68% of these were less than 0.5 ppb. (Preussman et al., 1982) The same group has found concentrations as high as 25 ppb in some cured meats and analysis by other groups has confirmed the results described above (Fiddler, 1975, Fine, 1982a). High concentrations of DMN in cured fish have also been shown repeatedly. This is thought to result from the use of NaNO$_2$ and/or NaNO$_3$ in the relatively high amine-containing fish. Studies done in the United Kingdom, Hong Kong, and Japan showed concentrations of 1-9, 10-1000, and 0.5 to 5 ppb DMN respectively in fish (Fiddler, 1975, Fine, 1982). It has also been shown that free dimethylamine and trimethylamine can be formed during storage of fish. Both of these amines are known to yield DMN with NO$_3^-$ even under mildly acidic conditions. Also, factors such as temperature, time, and cooking method have been shown to be important in the formation of DMN in nitrate and nitrite-treated meats and fish (Fiddler, 1975).

Significant concentrations of DMN have been found in cheeses by many investigators. Preussman et al. (1982) found that 23% of some 209 samples of cheese analyzed contained DMN in ranges of 0.5 to 4.9 ppb. Another study showed slightly higher levels of 1 to 15 ppb in 21 different varieties of cheeses tested. It is thought that the presence of DMN in some cheeses could be the result of addition of nitrate to some cheeses to prevent the growth of clostridia (Fine, 1982a). Alcoholic beverages, and beers in particular are the third major source of food-related exposures to DMN. In early studies, 66% of 200 beer samples were found to contain greater than 0.5 ppb DMN.
This was traced to the malt kilning (drying) process. Due to the relatively high beer consumption of the adult population, measures were taken to change the existing kilning method. Subsequent studies in 1981 showed that only 24% of 450 samples tested contained DMN in concentrations greater than 0.5 ppb (Preussman et al., 1982). Other recent studies have shown that DMN concentration in 180 samples of domestic beers ranged from undetectable levels (0.2-0.4 ppb) to 13 ppb with an average of 1 ppb (Fine, 1982a).

**Exposure to DMN via Endogenous Formation**

Due to the significant level of human exposure to nitrites, nitrates, and secondary amines, the possibility of in situ intragastric formation has been examined. Four approaches have been taken. The first approach concerns the chemistry of nitrosamine formation. In vitro nitrosation of secondary amines by nitrite was shown in both dilute acid solutions and in the gastric juices of various species, including man (Mirvish, 1975). The proposed reaction is as follows: $2\text{HNO}_2 \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O}$, then $\text{R}_2\text{NH} + \text{N}_2\text{O}_3 \rightarrow \text{R}_2\text{N} \text{NO} + \text{HNO}_2$. It has also been shown to be pH dependent (Lijinsky and Epstein, 1970, Mirvish, 1975).

The second approach taken prescribed the use of in vivo tumor induction studies. In the majority of these studies done both in rats and mice, nitrite and a secondary amine were given orally and the number of tumors induced were counted. Tumors characteristic to the nitrosamine compound were found to be present after dosing with
N-methylbenzene and morpholine but not with the more basic amines: diethylamine and dimethylamine. In another model system examining induction of lung adenomas in mice, Mirvish (1975) reported that adenomas were produced following long term administration of nitrite in drinking water and dosing with morpholine, piperazine, n-methyl-aniline, and some methylureas, but not dimethylamine. In contrast to the results shown in some of these long term studies, the third approach to the problem showed some evidence that DMN might be formed.

Experiments done by Asahina et al (1971) showed liver necrosis in rats following gavage of large doses of dimethylamine and NaNO₂. They attributed this to intragastric DMN formation. Similarly, administration of aminopyrine and nitrite by gavage produced liver necrosis that was also attributed to DMN formation (Lijinsky and Greenblatt, 1972). Formation of DMN from reaction of aminopyrine and nitrite has been shown in vitro (Mirvish, 1975). In another experiment done by Friedman et al. (1973), formation of N-7 methylguanine and inhibition of RNA and protein synthesis were observed in the liver following gavage of dimethylamine and nitrite in mice.

The last approach used to examine the possible in situ formation of nitrosamines consisted of analysis for their presence in human body fluids. Nine studies done in the late 1970's and early 1980's showed the presence of volatile nitrosamines, including DMN and diethylnitrosamine (DEN) in the urine or blood of healthy human volunteers. These findings however have been disputed due to
criticism of the analytical methods used (Fine, 1982b). Since that time, new techniques have demonstrated the presence of a non-volatile (non-carcinogenic) nitrosamine, nitrosoproline in urine samples of humans fed proline and nitrate (Fine, 1982b). In another study, Simenhoﬀ et al. (1982) demonstrated the presence of DMN in 120 out of 161 blood samples from a widely varying hospital population. The amounts measured in this study were well above the analytical limits of detection. It must be noted, however, that one cannot rule out some extent of exogenous exposure that might be contributing to the DMN found. The overall results of these various approaches certainly suggest that intragastric formation is probable, however more work is required to provide conclusive evidence of this.

Additional studies examining possible in situ formation of DMN in the bladder have strongly suggested that this also occurs, particularly in those individuals with bladder infections. Measurement of significant concentrations of DMN in the urine of individuals with Proteus mirabilis infections and nitrosamine formation following experimental bladder infections in rats have supported this (Hill et al., 1973).

The demonstrated high potential for exogenous exposure to DMN as well as the possibility of endogenous formation of this compound justifies further study of its toxic and carcinogenic properties.

Carcinogenicity and Bioactivation of DMN

In vivo carcinogenicity of DMN was first demonstrated in rats by Magee and Barnes (1956). Subsequent studies by other investigators
confirmed carcinogenicity in all of the animal species tested and DMN was demonstrated to be one of the most powerful carcinogens known. A definite pattern emerged in additional studies. Large interspecies variation and organ specificity in tumor induction was demonstrated. The incidence of tumors was highest in the kidney, followed by the liver and the lung. These organs constitute the major sites of biotransformation. Other studies done at this time demonstrated that alkylation of proteins and nucleic acids occurred predominantly in these tissues (Magee and Hultin 1962, Magee and Farber 1962, Lawley et al, 1968, and Swan and Magee, 1968). This precipitated speculation on a bioactivating pathway that would include formation of an alkylating species.

The bioactivation pathway currently most widely accepted is shown in Figure 1. The postulated first and only enzymatic step is mediated by a cytochrome P-450-associated hydroxylase. The intermediate formed from the -hydroxylation, methylhydroxymethyl-nitrosamine, has been shown to be sufficiently stable to be able to diffuse away from the cytosolic enzymes and into the nucleus for subsequent rearrangement and interaction with nuclear nucleic acids (Swann 1982). Following hydroxylation, formaldehyde is stochiometrically released and a series of rearrangements occur resulting in the diazonium hydroxide. The release of N₂ gives the putative alkylating agent: an electrophilic carbonium ion. The last step is consistent with studies showing that methylation of the 0-6 position of guanine proceeds solely through Sn1 nucleophilic
Figure 1. Proposed pathway of DMN bioactivation to a methylating species.
substitution. The energy required for formation of the carbonium ion for this reaction is produced via the release of molecular N₂ (Swann, 1982). Isolation of intermediary metabolites has not been achieved. However, structure-activity relationships, analogy with nitrosoalkylureas, and experiments with nitrosohydroxyalkylamines have been consistent with the view that the alkylating agent is a methyldiazenium hydroxide (Swann, 1982). In vitro studies using human liver slices have verified human capacity for activation of DMN to an alkylating species (Montesano and Magee, 1970).

Biotransformation of DMN to non-toxic species probably involves many pathways. The amount of DMN shown to be bound to tissue molecules is extremely small compared to the quantity of DMN administered (Margison and O'Connor, 1979). Some alternate pathways that have been proposed are denitrosation (Appel et al., 1980) and reduction (Grilli and Prodi, 1975). The extent of DMN participation in the bioactivation pathway described above in relation to other biotransforming pathways is still being debated.

**Interaction of DMN with Macromolecules**

Alkylation of proteins, RNA, and DNA has been demonstrated following treatment with DMN in vitro and in vivo (Brookes and Lawley, 1961, Magee and Farber, 1962, Magee and Hultin, 1962, Craddock and Magee, 1963). Although alkylation of proteins may contribute in part to the biological effects seen, protein alkylation is minor compared to nucleic acid alkylation (Magee and Farber, 1962, Magee and Hultin, 1962) and has thus received relatively little attention. Due
to this fact and to the more generally accepted theory that carcinogenesis initiation is linked to heritable changes in the genetic material (Miller and Miller, 1981), the remaining discussion will focus on nucleic acid alkylation with emphasis on alkylation of DNA.

**Distribution of Nucleic Acid Alkylation**

Alkylation within the animal is determined by route of administration, transport, stability of the compound, and distribution of activating enzymes (Pegg, 1983). After intraperitoneal injection, methylation of liver > kidney > lung > small intestine > testes (Swann and Magee, 1968) has been shown. The distribution appears to be most strongly influenced by distribution of metabolizing enzymes since organ metabolizing capacity follows roughly the same order. Intraorgan distribution according to cell type has also been suggested but not firmly established (Pegg, 1977a).

Subcellular distribution patterns show RNA to be methylated more extensively than DNA (Swann and Magee, 1968) with methylation of DNA, mRNA < rRNA < nuclear RNA (O'Connor et al, 1972, Pegg, 1983). Mitochondrial DNA is alkylated 2 to 4.5 times more than nuclear DNA and alkylation of bases in the dNTP pool may also theoretically occur (Pegg, 1983). A non random heterogenous distribution of alkylation at the molecular level has also been demonstrated (Pegg, 1983). This heterogenous distribution of alkylation in the nucleotide chain as well as in the individual bases is governed by a number of factors.

Protein complexation with DNA is one determinant of alkylation
location in DNA (Pegg, 1977a). Binding of proteins to DNA can protect potential sites on the DNA from alkylation. DNA conformation, secondary structures, and H bonding also affect the pattern of methylation in the polynucleotides and in the bases themselves (Pegg, 1977a, O'Connor et al., 1972). Atoms in the bases involved in H-bonding are postulated to be protected from alkylation. This is evident in cases such as with the N-1 and N-3 positions in adenine and guanine respectively. N-1 alkyladenine is the preferential minor product in RNA, however in DNA, binding to this atom is not detected (Lawley, 1966) or found in very small quantities (Pegg, 1983). Some positions that are involved in H bonding can be alkylated. These are the oxygen atoms in the 6 position of guanine, the 2 and 4 positions of uracil and thymine, and the 2 position of cytosine. The free electron pair on these oxygens allows alkylation to take place (Singer, 1983). See Figure 2. Relative nucleophilicity of the atoms also contributes to their reactivity towards the different alkylating agents and thus to the relative extent and distribution of methylation in the bases. This brings up the importance of the alkylating agent itself in determination of the relative abundance of each methylated product.

Alkylation may be mediated by at least two mechanisms. In the first type of reaction, the S_n1 reaction, alkylation occurs via a kinetically unimolecular reaction that is dependant upon the slow formation of a highly electrophilic carbonium ion. An increased S_n1 character, (i.e. ease of formation of the carbonium ion) is reflected
Figure 2. Base pairing and participation of atoms in hydrogen bonding. Solid pairs of dots represent e- pairs. From Singer (1983).
by a low Swain Scott factor, s. In the second type of reaction, the Sn2 reaction, the reagents form a transitory complex with the nucleophile and the kinetics are bimolecular. The Swain-Scott factors are high for these alkylating agents and they tend to react more exclusively with atoms of high nucleophilicity (Lawley, 1966, Pegg, 1977a). Accordingly, the alkylating agents of the Sn1 type react to a much greater extent with the less nucleophilic oxygen atoms and yield a greater abundance of these products while those of the Sn2 type react predominantly with the major nucleophilic center, the N-7 of guanine and give relatively little, if any alkylated oxygens (Lawley, 1966). The importance of these relative chemical reactivities becomes evident when one notes that the N-nitroso compounds, agents of the Sn1 type, are the most potent carcinogenic alkylating agents. Furthermore, the extent of Sn1 character, as reflected by a decreasing s factor can be correlated with increasing carcinogenicity (Singer, 1975, 1983) and increasing tendency to alkylate oxygens. This suggests that O-alkylation may be linked to the carcinogenic process.

Relative Abundance of Methylated Bases

The methylated bases and their relative abundance after in vivo administration of DMN is illustrated in Table 1. The base structures and where they are alkylated is shown in Figure 3. The percentages of minor products formed should be considered as approximations since not many measurements have been made and those that have been reported were found to vary from laboratory to laboratory.
Table 1. Relative abundance of methylated bases in DNA following *in vivo* administration of di-methylnitrosamine.

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<tr>
<th>Atom</th>
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<tr>
<td>N-1 methyl</td>
<td>adenine</td>
<td>0.7</td>
</tr>
<tr>
<td>N-3 methyl</td>
<td>adenine</td>
<td>8.0</td>
</tr>
<tr>
<td>N-7 methyl</td>
<td>adenine</td>
<td>1.5</td>
</tr>
<tr>
<td>N-3 methyl</td>
<td>thymine</td>
<td>0.3</td>
</tr>
<tr>
<td>O-2 methyl</td>
<td>thymine</td>
<td>0.1</td>
</tr>
<tr>
<td>O-4 methyl</td>
<td>thymine</td>
<td>0.7</td>
</tr>
<tr>
<td>N-3 methyl</td>
<td>cytosine</td>
<td>0.5</td>
</tr>
<tr>
<td>O-2 methyl</td>
<td>cytosine</td>
<td>0.1</td>
</tr>
<tr>
<td>N-3 methyl</td>
<td>guanine</td>
<td>0.8</td>
</tr>
<tr>
<td>N-7 methyl</td>
<td>guanine</td>
<td>68.0</td>
</tr>
<tr>
<td>O-6 methyl</td>
<td>guanine</td>
<td>7.5</td>
</tr>
<tr>
<td>alkylphosphates</td>
<td></td>
<td>12.0</td>
</tr>
</tbody>
</table>

from Pegg (1983).
Figure 3. Sites of DNA alkylation by DMN at physiological pH.
**Fate and Importance of DNA adducts**

The majority of the methylated adducts formed do not cause direct mispairing or miscoding in *in vitro* testing and are generally considered to be non-promutagenic lesions. However, a large number of these, including N-7 methylguanine, are released rapidly via spontaneous depurination and/or enzymic excision and may result in formation of apurinic sites. These sites may be repaired or persist. Their presence may result in some inhibition of DNA synthesis and they have been shown to be capable of producing substantial increases in reversion frequencies in assays using bacteriophages (Kunkel et al., 1981, Schaaper et al., 1982). Conversely, Drinkwater et al. (1980) demonstrated very poor correlations between the production of apurinic sites *in vitro* and production of missense mutations in bacterial assays. The role of depurinations in nitrosamine-induced mutagenesis may be thus considered somewhat ambiguous although possible (Singer, 1975, Schaaper et al., 1982). More attention has been directed towards those alkylated bases shown to be pro-mutagenic.

**Promutagenic DNA Adducts**

*In vitro* transcription studies employing synthetic copolymers made of alkylated and normal nucleotides and RNA polymerases have demonstrated misincorporation of nucleotides to be induced by N-1 methyladenosine, O-6 methylguanosine, O-2 and N-3 methylcytosine, N-3 and O-4 methyluridines, and possibly O-2 methyluridine (Gerchman and Ludlum, 1973, Singer and Kroger, 1979, Pegg, 1983). Thymidine is assumed to miscode by direct analogy with uridine. Studies using
deoxyribonucleotide templates have confirmed these results for N-3 methylcytosine (Ludlum, 1971) and O-6 methylguanine (Mehta and Ludlum, 1978). Miscoding is possible because methylation may permit the ability to form a stable base pair with both complementary and non-complementary bases (see Figures 4 and 5). Table 2 shows the nucleotides that are misincorporated for each methylated base. It should be noted that the use of RNA polymerases can only predict the possibility of miscoding in DNA in vivo. They cannot be considered analogous to DNA polymerases due to their lack of proofreading and editing functions. Nevertheless, they do show that misincorporation is possible and as a consequence of this, GC to AT and AT to GC transitions can be induced.

Until recently, most research efforts have been directed towards study of O-6 alkylguanine as the potentially pro-carcinogenic lesion. The other pro-mutagenic lesions were largely discounted because the results of 2 studies done by O'Connor et al. (1972, 1973) appear to rule them out. In both studies, O'Connor et al. compared molar proportions of methylated bases formed by the hepatocarcinogen DMN and the non-hepatocarcinogen, methylmethanesulphonate, (MMS). They found that O-6 alkylated guanines were not detectable when rats were treated with the latter compound however they were present in significant quantities in DMN-treated rats. Conversely, molar proportions of the minor products: N-3 methylcytosine, N-3 methylguanine, and N-1 methyladenine were approximately equal or even higher in the MMS-treated rats. These studies led to a general
Figure 4. Base pairing of O-6 methylguanine.
Figure 5. Base pairing of O-4 methylthymine.
Table 2. Transcription and subsequent misincorporation of polynucleotides due to the presence of alkylated bases.

<table>
<thead>
<tr>
<th>alkylated nucleoside</th>
<th>anomalous bases misincorporated (RNA template)</th>
<th>anomalous bases misincorporated (DNA template)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-1 methyladenosine</td>
<td>GMP,AMP,CMP,UMP</td>
<td>-</td>
</tr>
<tr>
<td>N-3 methylcytosine</td>
<td>GMP,AMP,CMP,UMP</td>
<td>UMP,AMP</td>
</tr>
<tr>
<td>N-3 methyluridine</td>
<td>GMP,AMP,CMP,UMP</td>
<td>-</td>
</tr>
<tr>
<td>(thymine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4 methyluridine</td>
<td>GMP,CMP</td>
<td>-</td>
</tr>
<tr>
<td>(thymine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6 methylguanosine</td>
<td>AMP,UMP</td>
<td>GMP,UMP</td>
</tr>
</tbody>
</table>

acceptance of their relative insignificance in liver carcinogenesis. Subsequent work thus dealt with 0-6 methylguanine almost exclusively until the 0-alkylated thymines were isolated, identified, and subsequently shown to be potentially pro-mutagenic lesions. In order to discuss the possible roles of the two lesions in hepatocarcinogenesis, the evidence implicating them as well as some background on their removal is needed.

**0-6 Methylguanine**

Some of the first evidence implicating 0-6 methylguanine as a possible pro-carcinogenic lesion concerned correlations made between the ability of alkylating agents to produce tumors and their alkylation at the 0-6 position of guanine. The studies described above by O'Connor et al. showed that the hepatocarcinogen DMN alkylated the 0-6 position of guanine while the non-hepatocarcinogen MMS didn't. Other studies showed that diethylnitrosamine (DEN), an alkylating agent with a higher efficiency for O-alkylation than DMN, was a more potent carcinogen than DMN. A correlation between an organ's susceptibility to tumorigenesis and accumulation or persistence of 0-6 methylguanine has also been shown.

The most well known studies correlating accumulation of 0-6 methylguanine to carcinogenesis were done by Margison and Kleihues (1975) and Kleihues and Magee (1973). Injections of N-methyl-N-nitrosourea were given in a dosing regimen selectively known to induce tumors of the nervous system. After termination, analysis of 0-6 methylguanine content in a number of tissues showed that the
amount of this lesion in the brain was significantly greater than that found in the kidney and much greater than that found in the liver. Increases observed in 0-6/N-7 methylguanine ratios over the 5 week dosing period in the former study led to the observation that 0-6 methylguanine accumulated in the brain and to a lesser extent in the kidney but not in the liver.

That the kidney is less efficient than the liver at removal of 0-6 methylguanine has been shown by many investigators (Pegg, 1977b). This might explain why in adult rats, a single dose of DMN is sufficient for induction of kidney tumors but multiple daily doses are required for induction of liver tumors (Pegg, 1977b). Indeed, experiments using mammalian cell extracts show that the rate of demethylation is highest in the liver followed by kidney, small intestine, colon, lung, and brain (Pegg, 1978, Snow et al, 1983). Lewis and Swenberg (1980) further showed that demethylating activity differed in the various hepatic cell populations; it was 28 times greater in hepatocytes than in non-parenchymal cells (NPC). Since the relative removal capabilities determine the persistence of 0-6 methylguanine, factors affecting this may have significant importance in tumor induction.

Various treatments given prior to dosing with an alkylating agent may influence 0-6 methylguanine removal. Unlabeled N-methyl nitroso urea (NMU) given in vivo in a single dose less than 40 hours prior to treatment with labeled NMU has shown some "inhibition" of removal of the lesion. However, if NMU or methyl methane sulfonate
(NMS) are given at low doses repeatedly before treatment with a labeled alkylating agent, no effect on removal of the lesion is seen (Margison, 1981). In vitro studies with rat liver extracts have shown that transfer of methyl groups from 0-6 methylguanine to a protein is stimulated by pretreatment of rats with thioacetamide, carbon tetrachloride, dimethylhydrazine, DEN, and DMN but not by NMU or streptozotocin (Pegg and Perry, 1981). A partial hepatectomy also caused a maximal 6-fold increase in removal activity (Pegg et al., 1981). Removal activity might be caused by the increase in cell division brought about through liver regeneration that was induced by these agents since most of them are hepatotoxic (Pegg et al., 1981a). Subsequent studies have shown removal to be due to an enzyme or "carrier protein" that is currently called either 0-6 Methyltransferase or 0-6 Transmethylase.

0-6 Methyltransferase

0-6 Methyltransferase is a protein containing a cysteine residue to which a methyl or ethyl group is stoichiometrically transferred. It is not a glycosylase and the demethylated guanine is left intact. Removal of the ethyl group from 0-6 ethylguanine is slower than removal of the methyl group. Removal of methyl groups from other bases was not demonstrated (Pegg, 1983, Pegg et al., 1983). Since this protein was demonstrated to become inactivated after incubation with methylated DNA, it is thought that regeneration of the cysteine group acting as the alkyl group acceptor does not occur (Pegg et al., 1983). This might explain why pretreatment with a substantial
dose of an alkylating agent less than 40 hours prior to dosing with a labeled alkylating agent would "inhibit" or decrease the removal of the O-alkylated products. The slower removal of O-6 ethyl groups could also be one reason why DEN is a more potent carcinogen than DMN. However, recent evidence casts doubt upon the role of O-6 alkylguanine in nitrosamine-induced carcinogenesis and favors the role of O-4 alkylthymine.

Some evidence suggesting that O-6 alkylguanine may not be the only critical lesion is linked to an important exception to the correlation between the accumulation or persistence of O-6 alkylguanine in target tissues and their susceptibility to tumorigenesis. A study done by Nicoll et al. (1977) showed that the liver did not markedly accumulate O-6 methylguanine when rats were treated with DMN in a regimen that was known to induce liver tumors in 100% of the rats. The O-6 methylguanine concentration was only 85% higher after 10 doses than after one dose. Margison et al. (1977) also found that levels of O-6 methylguanine were undetectable during a 24 week long study using a dosing regimen causing liver carcinogenesis. They found however that accumulation of the lesion occurred in the non-target organs: kidney and lung. One explanation is that the types of neoplasms seen may be the result of differential accumulation among the cells in the liver. As mentioned previously, Lewis and Swenberg (1980) demonstrated differential repair of O-6 methylguanine between hepatocytes and NPCs with the latter group of cells accumulating a 28-fold greater concentration of O-6 methylguanine. Since NPC DNA
constitutes only 10-20% of liver DNA (Lewis and Swenberg, 1980), marked accumulation in these cells during chronic treatment might not be observed in studies where "whole" liver DNA is assayed. Indeed, in a study treating animals chronically with a 1,2 dimethylhydrazine dosing regimen causing angiosarcomas in 90% of the animals, accumulation of O-6 methylguanine in NPC was significant (Bedell et al., 1982). In contrast, the one day O-6 methylguanine level in hepatocytes was up to 28 times that observed at later exposure times, suggesting induction of removal processes. The NPCs demonstrated doubled O-6 methylguanine concentrations during the first 8 days and consistently high O-6/N-7 methylguanine ratios throughout the 28 day duration of the experiment. These ratios were from 2-10 times greater than those seen in hepatocytes throughout the exposure period. The high incidence of angiosarcomas may thus be due to accumulation of O-6 methylguanine in the NPCs, but O-6 methylguanine accumulation does not appear to be implicated in the 40% incidence of hepatocarcinomas that are also induced by this dosing regimen.

Additional evidence suggesting that the other pro-mutagenic lesions are responsible for nitrosamine-induced hepatocarcinogenesis comes from a study employing a 28 day DEN dosing regimen that selectively causes nearly 100% incidence of hepatocellular carcinomas. The DNA from hepatocytes and NPCs both showed O-6 ethylguanine concentrations generally below the limits of detection over the whole 28 day period. Both groups of cells also exhibited similar levels of de novo DNA synthesis (Swenberg, 1984a). This suggested further that
other pro-mutagenic lesions might be responsible for hepatocellular initiation.

0-4 alkylguanine was strongly implicated in subsequent studies performed by Swenberg et al. (1984b). A DEN dosing schedule similar to that described above was used and hepatocyte and NPC DNA was isolated and analyzed for ethylated products using high affinity antibodies. 0-6 ethylguanine did not accumulate in hepatocytes and decreased in concentration from 28-77 days. 0-4 ethylthymine was however found to accumulate in hepatocytes over the first 28 days and to remain approximately stable for the duration of the 77 day exposure period. Interestingly, the NPCs were found to contain approximately half as much 0-4 ethylthymine as the hepatocytes. These data indicated that hepatocarcinoma induction might be correlated with the presence and persistence of 0-4 ethylthymine. An additional factor implicating 0-4 methylthymine is that many of the correlations that implicate 0-6 alkylguanine as a pro-carcinogenic lesion also apply to 0-4 alkylthymine (Pegg, 1983, Singer, 1979, 1981, Scherrer et al., 1980).

0-6 Alkylguanine and 0-4 Alkylthymine

From the various lines of evidence given above, it is clear that the roles of these pro-mutagenic lesions in hepatocarcinogenesis have not been completely elucidated. This allows for more study of the lesions themselves and factors influencing their removal. A primary objective therefore of this work was to develop a working method for measurement of some of these adducts, in particular,
0-6 methylguanine and N-7 methylguanine, to use as a tool for further research.

**HPLC Analysis of Methylated DNA**

High performance liquid chromatographic methods using cation exchange are well suited for separation of methylated and unmodified purine bases. Although the bases are both relatively weak acids and weak bases, they are easily protonated at a relatively low pH, giving them a net positive charge. In cation exchange, separation occurs primarily as a result of reversible adsorption mediated by electrostatic binding of the cationic solutes to the anionic sites on the column. Anion exchange and reversed-phase columns may also be used in separation of DNA bases however these would be incompatible with the use of fluorimetric detection, since they would necessitate dissolution of the bases in a relatively high pH solution. It has been shown that the fluorescence of these compounds is greatly decreased in solutions of pH 5 and above (Swenberg and Bedell, 1982).

Use of fluorimetric detection provides a very sensitive and excellent means of detection of the methylated and unmodified bases. Fluorescence occurs during the deexcitation of a molecule that has attained a high energy state due to absorption of a photon. However, not all molecules that absorb photons will fluoresce. Fluorescence is generally a characteristic of those molecules possessing the highly delocalized electron systems that are typical of aromatic systems. Adenine and guanine possess systems such as these. It is interesting to note that the fluorescent intensity of 0-6 methylguanine is much
greater than that of guanine or adenine (Swenberg and Bedell, 1982). This strong fluorescence of O-6 methylguanine is particularly useful since this base may be present in amounts of as little as one millionth those of the other unmodified bases. N-7 methylguanine has only moderate fluorescent intensity but it is much greater than that of adenine. This may be exploited since 7-methylguanine and adenine peaks are difficult to separate. The smaller peak width of adenine permits better apparent resolution between the peaks.

Fluorescence detection has the additional advantage of alleviating the need for use of costly radioactively-labeled carcinogens, many of which are not commercially available. A combined HPLC technique with fluorescence detection permits less stringent sample size requirements and may thus avoid the need for pooling of tissues and large numbers of animals. It also allows for both resolution and quantitation of the bases in a single run. This method may be employed in studies using any methylating agent and may also be used for further examination of the importance of O-6 methylguanine in carcinogenesis and of factors affecting its repair. In the work described herein, it is used to determine if the hepatotoxin, trichloroethylene (TCE) affects repair of O-6 methylguanine.

**Trichloroethylene**

Trichloroethylene is a highly volatile solvent that has been used for a variety of purposes during the last 75 years. It has been used as an anesthetic, as a component of grain fumigant mixtures, in the dry cleaning industry, and as a solvent for decaffeination of
coffee. Its principle use however, has been in industry where it is an effective degreasing agent (Waters et al., 1977). Although its use has been restricted in the United States, widespread human exposure to the compound has occurred both in the workplace and in the environment in general. Exposure to a significant population has recently taken place due to TCE contaminated municipal water wells in various parts of the nation. The high potential for human exposure has recently spurred much interest in the mechanisms and forms of toxicity of TCE.

**Toxicity of TCE**

TCE has been demonstrated to be toxic to a variety of organs in a number of species. Cardiotoxicity and toxicity to the central nervous system has been shown in man (Waters et al., 1977). TCE is carcinogenic in liver of B6C3Fl mice of both sexes but has little effect on Osborne-Mendel rats (Weisburger, 1977). Renal toxicity has been demonstrated in some studies and has been absent in others (Waters et al., 1977). Hepatotoxicity also occurs particularly when TCE is administered to phenobarbital-induced rats. Allemand et al. (1978) showed increased SGPT levels in both pretreated and non-pretreated rats and either centrilobular necrosis or cellular clarification in all of the phenobarbital-pretreated rats. TCE also resulted in a loss of cytochrome P-450 in pretreated rats but not in non-pretreated rats. Substantial liver enlargement has also been demonstrated in rats and other rodents due to TCE treatment Kjellstrand et al., 1981). Since the major site of metabolism, the liver, appears to be one of the major target organs, a bioactivated
form of TCE is thought to mediate its toxicity. This hypothesis is supported by the proposed biotransformation pathway of TCE.

**Biotransformation of TCE**

Following dermal, oral, or respiratory exposure to TCE, this compound is biotransformed via hydroxylation or epoxidation in P-450, mixed function oxidase-dependent reaction (Miller and Guengerich, 1982). The proposed biotransformation pathway is shown in Figure 6. The breakdown products, trichloroacetic acid, trichloroethanol, and trichloroethanol-glucuronide are excreted by the kidney. The epoxidation pathway is important because not only are epoxides expected to be highly reactive with cellular nucleophiles, but the observed binding of TCE to proteins and DNA appears to be dependent upon the presence of an epoxide. Van Duuren and Banerjee (1976), showed that binding of TCE to proteins and nucleic acids was greatly enhanced in rat hepatic microsomes by addition of a potent epoxide hydrazine inhibitor. In spite of this evidence, the possibility of another electrophilic species cannot be excluded. Van Duuren and Banerjee (1976) also showed that biotransformation of TCE was required for macromolecular binding. Covalent binding to protein in their in vitro experiments was reduced by benzoflavone and blocked completely by SKF-525A. It was also enhanced by administration of phenobarbital (Allemand et al., 1978, Van Duuren and Banerjee, 1976, Cunningham et al., 1981). Further studies showing greatly enhanced hepatotoxicity in phenobarbital-pretreated rats suggests that the toxicity is due to binding of TCE to tissue macromolecules.
Figure 6. Proposed pathway for bioactivation of TCE.
In vitro binding of TCE to DNA and proteins has been shown in numerous studies in rat hepatic microsomes (Banerjee and Van Duuren, 1978, Allemand et al., 1978, DiRenzo et al., 1982) and in rat hepatocytes (Cunningham et al., 1981). In vivo studies have also shown irreversible binding of TCE to proteins and DNA (Allemand et al., 1978, Pessayre et al., 1979). In both types of studies, binding to proteins is higher than to DNA or RNA. Protein binding is also substantially higher in mice that are sensitive to TCE-induced hepatocarcinogenesis than in rats (Banerjee and Van Duuren, 1978). Binding has also been shown to be 40 fold higher in liver than in muscle proteins (Allemand et al., 1978).

Due to this well demonstrated binding of electrophilic TCE species to liver proteins as well as the widespread potential for human exposure to both TCE and DMN, a study examining the possible effects of TCE treatment in vivo on the persistence of 0-6 methylguanine was performed. Considerable binding and subsequent inactivation of such a repair enzyme could possibly be one mechanism whereby TCE might "promote" carcinogenesis. Conversely, due to its hepatotoxic effects, TCE might possibly cause an induction of the enzyme similar to that caused by other hepatotoxic agents.
MATERIALS AND METHODS

Chemicals

Spermine.4HCl, spermidine.3HCl, reagent grade Trizma base (tris base), ethylene glycol-bis (B amino-ethylether)N,N'-tetraacetic acid (EGTA), phenylmethysulfonyl fluoride (PMSF), 2 mercaptoethanol, sodium lauryl sulfate (SDS), bovine albumin, fraction V(BSA), bovine pancreas type XII-A ribonuclease (ribonuclease A), ribonuclease T1, grade V in (NH4)2SO4, highly polymerized calf thymus DNA, type 1, ammonium salt of formic acid, sodium salt of N-lauroylsarcosine, guanine, and adenine were purchased from Sigma Chemical Co., St. Louis, MO. N-7 methylguanine was purchased from Vega Biochemicals, P.O. Box 11648, Tucson, AZ. O-6 methylguanine was synthesized by Dr S. Mufti by the method of Balsiger and Montgomery (1960) and its structure confirmed by mass spectroscopy. Enzyme grade ultrapure sucrose was purchased from Bethesda Research Labs, Inc., Gaithersburg, MD. Ethylenediaminetetraacetic acid (EDTA), 3,5, diaminobenzoic acid, dihydrochloride, 99% (DABA), tetraethylammoniumhydroxide (TEAH), trichloroethylene (TCE), 99% gold label, and HPLC grade carbon tetrachloride were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Glycerin, isoamyl alcohol, HPLC grade ammonium phosphate and HPLC grade 85% phosphoric acid were purchased from Fisher Scientific Co., Fairlawn, NJ. Emulphor was purchased from GAF Corporation. Dibasic potassium phosphate and trisodium citrate were
purchased from Mallinckrodt, Inc., Paris, KY and St. Louis, MO. respectively. Potassium phosphate and sodium perchlorate were purchased from MCB Chemists, Norwood, OH. Proteinase K was purchased from Beckman Instruments, Inc., Bioproducts Operations, Palo Alto, CA. Dehydrated 100% ethanol was purchased from U.S. Industrial Chemicals Co., New York, NY. Burdick and Jackson HPLC grade methanol was purchased from American Scientific and Chemical, 922 E. So. Pacific Dr. Phoenix, AZ.

**Animals**

Female Sprague-Dawley rats weighing 175-225 grams and obtained from the University of Arizona Division of Animal Resources colony were used for all experiments. The rats were transferred from the colony into a room with an independent air supply and a constant temperature of 22 C. Humidity was 45-55% and there was a 12 hour light and 12 hour dark cycle. The rats were kept in wire- bottomed cages to prevent enzyme induction possible from volatile components of the bedding material. They were given Wayne rat chow and deionized tap water ad libitum. At least 5 days were allowed to elapse between transfer of the rats from the colony and the beginning of any experiment. The rats were checked for apparent good health prior to and during experiments.

Cervical dislocation following partial asphyxiation by CO₂ was the method chosen for termination of rats in all experiments.
Nuclei Isolation

The method of Burgoyne et al (1970) as modified by Hewish and Burgoyne (1973), Lindell (1975), and Woll et al (1981) was used to isolate nuclei from rat liver. Following termination of each rat, the liver was rapidly excised and two grams homogenized immediately in 6 ml of a cold (4 C) buffer (250mM sucrose, 4mM EDTA, 2mM EGTA, 120mMKCl, 30mM NaCl, 0.3mM Spermine·4HCl, 1mM Spermidine·3HCl, 30mM Tris base, 14.3mM mercaptoethanol, 1mM PMSF, pH 7.4). The subsequent procedures were performed at 4 C in a "cold" room to lower activities of degradative enzymes. Four ml of each homogenate was pipetted into a 5/8 x 4 inch polyallomer tube. Eight ml of a "dense sucrose" buffer (2.1M sucrose, 2mM EDTA, 1mM EGTA, 60 mM KCl, 15mMNaCl, 0.15mM spermine·4HCl, 0.5mM spermidine·3HCl, 15mM tris base, 14.3mM mercaptoethanol, 1mM PMSF, pH 7.4) was added to each tube and the tubes mixed by inversion. Each tube was underlaid with an additional 4-5 ml of the "dense sucrose" buffer and the tubes were centrifuged at 25,000 rpm, 4 C for 40 minutes. The supernatant was carefully discarded and the nuclear pellet retained.

DNA Purification

DNA was purified from the nuclear pellet or from frozen liver sections in some later experiments. The nuclear pellet was resuspended in 8-9 ml of a lysis buffer (150 mM NaCl, 50 mM EDTA, 20 mM Tris Base, pH 7.6, 4% sodium lauroyl sarcosine) by gentle vortexing. A 5 mg/ml stock solution of Proteinase K was added to give a final concentration of 100 ug/ml. ZnCl₂ was added to make the
lysing buffer 1 mM in ZnCl₂. This prevented activity of 0-6 methyltransferase during the 37 C incubation period by inhibiting the enzyme (Pegg et al., 1983). The tubes were incubated overnight in a 37 C waterbath.

The contents of each tube were extracted with an equal volume of a 1:1 solution of phenol (redistilled at 160 C and collected over water) and chloroform-isoamyl alcohol (24:1). Phenol inactivates some nucleases and serves as a solvent for proteins (Kirby, 1964) and chloroform causes surface denaturation of proteins. Isoamyl alcohol reduces foaming and maintains stability of the aqueous and organic layers. The tubes were shaken gently for 20 minutes then centrifuged at 6000 rpm for 15 minutes at room temperature. The top aqueous layers were removed to clean, labeled tubes and extracted again as above. Subsequent extractions were performed as above with chloroform-isoamyl alcohol (24:1) alone until there was little or no visible protein at the aqueous-organic interface. This usually required from 2 to 4 more extractions. Sodium acetate was added to the apparent protein-free aqueous DNA extracts to give a 0.3 M concentration and 2.5 volumes of ice cold 70% ethanol were added to precipitate the DNA. Seventy percent ethanol was used to remove any solutes that were trapped in the DNA precipitate. The solution containing the DNA was mixed gently by swirling and refrigerated for at least 2 hours at -20 C.

Following refrigeration, the solutions were centrifuged at 15,000 rpm, 0 C, for 15 minutes. The supernatant was decanted and the
tubes drained. The pellet was dried under a stream of N₂ and then resuspended by gentle shaking in 2.5 - 3.5 ml of a low salt buffer (10 mM Tris, pH 7.6, 10 mM NaCl, and 1 mM EDTA) to maintain the ionic strength of the dissolved DNA. Following resuspension, 5 M NaCl was added to give a concentration of 150 mM in NaCl. Ribonuclease T and pancreatic ribonuclease (dissolved at 2 mg/ml in 0.15 M NaCl, pH 5.0, immersed in a boiling water bath for 30 minutes to eliminate deoxyribonuclease activity, and aliquoted and stored at –20 C.) were added to give final concentrations of 50 units/ml and 50 µg/ml respectively. The DNA solutions were incubated at 37 C for 2-3 hours. Ten percent stock SDS was added to give a final concentration of 2% and Proteinase K was added to 50 µg/ml. SDS aids in the dissociation and denaturation of proteins due to unfolding of the polypeptide chain (Marko and Butler, 1951, Gross-Bellard, Oudet, and Chambon, 1973) and has been shown to increase the rate at which they are digested by the Proteinase K (Gross-Bellard et al., 1973, Butterworth, 1976). The DNA solutions were incubated for at least 3 hours at 37 C, extracted as above, made 0.3 M in sodium acetate, and then precipitated with ice cold:95% ethanol and stored at 4 C until further use.

**Hydrolysis of Purified DNA**

DNA purified and stored in ethanol at 4 C was centrifuged at 15,000 rpm, 0 C, for 15 minutes. The supernatant was decanted and the tubes inverted on absorbent paper for drainage. The ethanol remaining on the sides of the tubes was dried under a steady flow of N₂. Eight tenths to one ml of filtered 0.1 N HCl was added to each tube and the
tubes shaken gently in a Dubonoff shaking incubator in a 37 C water bath for 20 hours. The hydrolysates were filtered with Whatman nitrocellulose filters (0.65 μm) and stored in labeled, capped plastic scintillation vials at 4 C. Injection and analysis of the DNA samples was usually performed immediately following hydrolysis and filtration of the DNA samples.

Assay for DNA Content

Hydrolysates diluted for HPLC analysis were assayed for DNA concentration using the fluorimetric procedure of Kissane and Robins (1958). A standard solution of known DNA concentration was made by dilution of calf thymus DNA with 0.1 N HCl. This was heated and gently shaken for 20 hours to cause hydrolysis and depurination of the DNA as in the sample DNA hydrolysates. The stock solution was diluted to give known concentrations for standard curves. Fifty microliters of sample or standard DNA hydrolysates or 0.1 N HCl (used for a blank to zero the spectrofluorometer) were placed in Falcon 2054 tubes. For formation of the fluorescence derivatives, 125 ul of diaminobenzoic acid (30% w/v; DABA) was added to each tube and the tubes heated in a 70-80 C water bath for 35 minutes. After cooling, 1.0 ml of filtered 1 N HCl was added to each tube. Fluorescent intensity was read on an Aminco-Bowman spectrofluorometer with an excitation wavelength of 420 nm and an emission wavelength of 520 nm. DNA sample concentrations were determined using linear regressions of the standard curves.
**Fluorescence Spectra**

Fluorescence spectra for the mobile phase containing 0.06 M ammonium formate, pH 4.1, 8% methanol, 0-6 methylguanine (5 ng/ml), N-7 methylguanine (150 ng/ml) and adenine (250 ng/ml) were determined using the Perkin Elmer 650-10S fluorescence spectrofluorometer. The spectrofluorometer settings were: response=slow, sensitivity range=0.1, fine sensitivity=approximately 3/4 scale, and PM gain=normal. Excitation and emission scans were done from 220 nm to at least 450 nm. The emission wavelength was set at 360 nm, with a 9 nm slit width when excitation scans were being performed. The excitation wavelength was 290 nm, with a 9 nm slit width when emission scans were being done. The slit width of the parameter being scanned was 3 nm. The scan speed was 30 nm/min.

**HPLC Analysis of DNA Samples**

**Standard Solutions**

Stock solutions of 0-6 methylguanine, N-7 methylguanine, guanine, and adenine were made in 0.01 N HCl. The solutions were aliquoted into plastic scintillation vials and stored at -20 C. Working aliquots were diluted serially with 0.01 N HCl. Standard working concentrations for each base were as follows: 0-6 methylguanine: 100, 50, 40, 30, 20, 10, and 5 ng/ml, guanine: 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 ug/ml, and N-7 methylguanine: 600, 400, 200, and 100 ng/ml. The latter standard solutions were prepared in both 0.01 N HCl and in control DNA. A concentrated solution of
N-7 methylguanine was added to DNA isolated from untreated rats to give the above concentrations. Working standards were stored at 4 C until use.

Method Development Experiments

Ammonium phosphate and ammonium formate buffers of varying concentrations and pHs were tested during method development. The effect of adding methanol the mobile phase was also studied. The effects of various flow rates on separation and quantitation were noted. The following spectrofluorometric detector settings were optimized: excitation and emission wavelengths and slit widths, sensitivity ranges and response. These and other method development experiments are described further in the Results section.

Mobile Phase Preparation

Stock 0.1 ammonium formate buffer was made using the ammonium salt of formic acid and deionized, (0.2 um) filtered water. It was brought to a pH of 4.1 with the addition of concentrated HCl. The working buffer was made by addition of 80 ml HPLC grade methanol to 600 ml of 0.1 M ammonium formate, pH 4.1 and completion of the volume to 1 liter with deionized, filtered water. The 0.06 M ammonium formate, pH 4.1, 8% methanol buffer was filtered with a Whatman 0.65 um nitrocellulose filter and stored in ground glass stoppered bottles for no longer than 2 days before using. At least three liters of the buffer were made (and mixed to provide homogeneity) prior to running
any set of purine standards and DNA samples. The buffer was degassed by bubbling helium through it for 20 minutes just prior to use.

Apparatus Set-up

The equipment consisted of the following: an Altex solvent delivery pump, a Rheodyne loop injector fitted with a 50 ul injection loop, a Whatman Partisil-10 SCX strong cation exchange column (25 x 0.45 cm) employed with a Whatman column survival kit, a Perkin-Elmer 650-10S fluorescence spectrofluorometer equipped with an 18 ul flow cell, and a Spectra physics SP4100 computing integrator. Settings on the spectrofluorometer were: response switch= slow, zero suppression= off, sensitivity range= 1, fine sensitivity range= approximately 3/4 scale, PM gain= low, and excitation and emission wavelengths of 290 and 360 nm respectively. Slit widths were 12 nm for both. The column was equilibrated with 0.06 M ammonium formate, pH 4.1, 8% methanol at a flow rate of 1 ml/min for at least 45 minutes and then 2 ml/min for at least 20 minutes. The xenon lamp in the detector was allowed to warm up for at least 30 minutes.

Dilution of DNA Sample Solutions

In order to inject an approximately uniform amount of DNA onto the column, the following steps were taken: 20 ul of two different N-7 methylguanine spiked DNA standard solutions were injected. The adenine peak areas were averaged. Subsequently, 20 ul injections of each DNA sample solution were made. Adenine peak areas of the samples were compared to the average adenine peak area of the N-7 methylguanine standard solutions and the dilution factor calculated to give the
approximately same adenine concentration. The samples were then diluted with filtered 0.1 N HCl.

**Sample and Standard Runs and Analyses**

After a stable baseline had been achieved and the peak threshold evaluated by the spectrofluorometer, duplicate or triplicate injections of each of the purine standard solutions and single or duplicate injections of sample DNA solutions were made with a glass Hamilton syringe. Approximately 25 to 40 ug of DNA was usually injected. Peak areas were either calculated by hand, using height x width at one half height, (for 0-6 methylguanine and sometimes N-7 methylguanine) or by electronic integration. Concentrations of the purines in the DNA samples were determined from linear regressions of the standard curves. Adenine:guanine peak area ratios were determined and compared to the experimental means to detect possible incomplete hydrolysis of the samples.

**Animal Experiments**

**Dimethylnitrosamine Dose-Response Studies**

Female Sprague-Dawley rats weighing between 175 and 200 grams were dosed intraperitoneally (i.p.) between 9:15 and 10:15 a.m. with either dimethylnitrosamine (DMN) in 0.9% NaCl or 0.9% NaCl alone. Doses of DMN were 2.5, 5.0, 10.0, 20.0, and 40.0 mg/kg and volumes injected ranged from 0.4 to 0.8 ml. Rats were terminated 4 hours after dosing by cervical dislocation and their livers excised immediately. Nuclei were isolated and DNA purified from the liver
sections as described in *Nuclei Isolation* and *DNA purification* sections. The DNA samples were hydrolyzed and analyzed by HPLC.

**Determination of possible 0-6 methyltransferase Activity during Isolation and Purification Procedures**

Rats were treated with DMN, 30 mg/kg at 10:00 a.m. and terminated 4 hours later by cervical dislocation. Two, 2 gram liver sections were taken from each rat. One was isolated in a nuclei isolation buffers containing 1 mM ZnCl\(_2\). The other section was isolated and lysed in buffers containing no ZnCl\(_2\). As mentioned previously, ZnCl\(_2\) is a potent inhibitor of 0-6 methyltransferase. The concentrations of 0-6 methylguanine in DNA purified from nuclei isolated using both methods were analyzed and compared to determine whether 0-6 methyltransferase activity persists during the nuclei isolation procedure.

**TCE Drinking Water Studies**

To prepare a solution of drinking water that contained 5 mg TCE/ml of water, 3.45 ml of TCE was mixed with 10 ml Emulphor and then the volume was completed to 1 liter with deionized tap water. A solution of one percent Emulphor drinking water was made for controls by dissolving 10 ml Emulphor in 1 liter of deionized tap water. The drinking water was placed in foil covered and labeled water bottles with corks that had been soaked in 1% Emulphor water. The water bottles were filled nearly to the top to minimize loss of TCE due to equilibration of the volatile compound with the headspace. The water was replaced with freshly made TCE water or 1% Emulphor water every 2
or 3 days. TCE or Emulphor water was given ad libitum to the rats over a one or two week period.

At the end of the 1 or 2 week drinking water exposure period, the rats were dosed (between 9:15 and 10:30 a.m.) i.p. with DMN, 15 mg/kg in 0.9% NaCl or with 0.9% NaCl alone. There were two kinds of controls. One group received 1% Emulphor water or TCE water, but no DMN. This group allowed for comparison of concentrations of 0-6 methylguanine found in DMN treated rats vs those found in rats not treated with the methylating agent. The other group received DMN but no TCE in their water. This allowed for examination of the effects that TCE had on the concentration of 0-6 methylguanine after treatment with the methylating agent. Four hours after dosing with DMN, rats were terminated by cervical dislocation, the livers were excised, and nuclei isolation, DNA purification and HPLC analysis were performed as above.
RESULTS

HPLC Assay for Methylated Purines

Figures 7 and 8 show representative chromatograms obtained using the method developed in this research project. The mobile phase consisted of a 0.06 M ammonium formate buffer, pH 4.1, 8% methanol and the stationary phase consisted of a Partisil 10-SCX strong cation exchange column. Figure 7 depicts the elution profile of liver DNA isolated from an untreated rat. Figure 8 depicts the elution profile of liver DNA isolated from a rat treated with DMN, 15 mg/kg.

Assay Development

Three important questions had to be addressed when a mobile phase was considered for use in this assay: 1) Could it give a good separation of N-7 methylguanine from either adenine or guanine peaks? 2) Did the mobile phase ensure a low limit of detection for O-6 methylguanine? and 3) Did the mobile phase diminish the fluorescence of the bases? Essentially, one of two different salts were used in any of the mobile phase buffers tested. The first one tested was ammonium phosphate. The conditions that were employed were those used in the first published method for detection of methylated bases with HPLC and fluorimetric detection (Herron and Shank, 1979). When 0.05 M ammonium phosphate, pH 2.0 was used as in Herron and Shank's method, the limit for detection of O-6 methylguanine was low but there was no resolution of the N-7 methylguanine peak from the guanine peak in a
Figure 7. Elution profile of a DNA hydrolysate from a control rat, mobile phase: 0.06 M ammonium formate, pH 4.1, 8% methanol. Peaks 1 and 2 represent guanine and adenine respectively.
Figure 8. Elution profile of DNA hydrolysate from a rat treated with DMN, 15 mg/kg, mobile phase: 0.06 M ammonium formate, pH 4.1, 8% methanol. Peaks 1, 2, 3, and 4 represent guanine, N-7 methylguanine, adenine, and O-6 methylguanine respectively.
solution containing a relatively large amount of the compound. A sample chromatogram showing the elution profile of liver DNA obtained from a rat treated with 40 mg/kg DMN is shown in Figure 9. Subsequent studies performed in which the pH and molarity of ammonium phosphate were altered showed that pH 2 was optimal and molarities of between 0.035 and 0.05 could be used, but resolution of the N-7 methylguanine peaks from guanine peaks was not greatly improved.

The second mobile phase tested was one using the 0.02 M ammonium formate, pH 4.0 buffer that had been employed by Pegg et al., (1983a). The sample chromatogram in Figure 10 shows that the order of elution of the bases changes with use of this mobile phase. The N-7 methylguanine elutes just before the adenine rather than eluting after guanine and forms a shoulder on this peak as it does when using the ammonium phosphate buffer. Various optimization experiments were performed and showed that resolution of the adenine and N-7 methylguanine peaks was dependent upon the pH and molarity of the ammonium formate buffer. Lowering the pH was detrimental to the separation of the two while raising it slightly increased the amount of separation. However, raising the pH too much apppeared to decrease the peak sizes of the bases. Therefore, a pH of 4.0 was used. Increasing and decreasing ammonium formate molarity both caused resolution between the two bases to be decreased. Thus the ammonium formate buffer system originally used remained 0.02 M in ammonium formate and pH 4.0. A comparison of Figures 9 and 10 show that resolution of the
Figure 9. Elution profile of DNA hydrolysate from a rat treated with DMN, 40 mg/kg, mobile phase: 0.05 M ammonium phosphate, pH 2.0. Peaks 1 and 3 represent guanine and O-6 methylguanine. 2 indicates where N-7 methylguanine peak should elute.
Figure 10. Elution profile of DNA from a rat treated with DMN, 20mg/kg, mobile phase: 0.02 M ammonium formate, pH 4.0. Peaks 1, 2, 3, and 4 represent guanine, N-7 methylguanine, adenine, and O-6 methylguanine respectively.
N-7 methylguanine peak was better when the ammonium formate mobile phase was used than when the ammonium phosphate buffer was used.

In an attempt to enhance the separation of N-7 methylguanine and adenine further and to sharpen the O-6 methylguanine peak, a small amount of methanol was added to the ammonium formate buffer. A small methanol gradient had been reported to give earlier elution and a sharper peak for O-6 methylguanine when using an ammonium phosphate mobile phase (Swenberg and Bedell, 1982). It had also been included in an ammonium formate mobile phase used for separation of radiolabeled methylated bases (Beranek et al., 1980). It was demonstrated that not only did the addition of a small amount of methanol sharpen the O-6 methylguanine peak, but it also gave enhanced resolution of the N-7 methylguanine and adenine peaks. Figure 11 shows a chromatogram obtained when using a 0.02 M ammonium formate, pH 4.0, 2% methanol mobile phase. After the ability to separate the N-7 methylguanaine from the adenine had been verified, additional studies done using standards confirmed that the limits of detection of N-7 methylguanaine and O-6 methylguanaine were similar or better than those found using other methods. These are shown in Table 3.

Although the HPLC method described above appeared adequate for analysis of N-7 and O-6 methylguanaine, when a new Partisil 10 SCX column was obtained, testing was performed to determine whether it might enable a more optimal separation. After new mobile phase concentrations of ammonium formate and methanol had been determined for this column, it was shown that a better separation could indeed be
Figure 11. Elution profile of DNA from rat treated with DMN, 20 mg/kg, mobile phase: 0.02 M ammonium formate, pH 4.0, 2% methanol. Peaks 1, 2, 3, and 4 represent guanine, N-7 methylguanine, adenine, and O-6 methylguanine respectively.
Table 3. 0-6 and N-7 methylguanine limits of detection

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Approximate Limits of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6 MeGuanine</td>
</tr>
<tr>
<td>0.035 M ammon. phosphate, pH 2.0</td>
<td>0.1 ng.</td>
</tr>
<tr>
<td>0.05 M ammon. phosphate, pH 2.0</td>
<td>0.1 ng.</td>
</tr>
<tr>
<td>0.02 M ammon. formate, pH 4.0</td>
<td>0.1 ng.</td>
</tr>
<tr>
<td>0.02 M ammon. formate, pH 4.0,</td>
<td>0.1 ng.</td>
</tr>
<tr>
<td>2-2.5% methanol, old column</td>
<td></td>
</tr>
<tr>
<td>0.06 M ammon. formate, pH 4.1,</td>
<td>0.125 ng.</td>
</tr>
<tr>
<td>8% methanol, new column</td>
<td></td>
</tr>
</tbody>
</table>
achieved. A chromatogram using the mobile phase: 0.06 M ammonium formate, pH 4.1, 8% methanol is represented in Figure 12. The increased resolution is illustrated by comparison of Figures 11 and 12 which both represent elution profiles from the identical DNA solution. The volume injected was 10 ul less than that injected in Figure 11. It is also important to note that another peak was resolved using the latter system. In a later experiment performed in order to determine whether this peak was due to protein, a filtered hydrolysate of BSA and proteinase K was assayed. The protein peak had a retention time identical to that of the "unknown" peak eluting after guanine and just before N-7 methylguanine in some of the samples. The identical retention times of the protein and the unknown peaks suggested that the peak was indeed due to protein contamination of the DNA samples.

**Fluorimetric Spectra**

Fluorimetric spectra were determined for 0.06 M ammonium formate, pH 4.1, 8% methanol, N-7 methylguanine, O-6 methylguanine, and adenine in order to choose the most optimal excitation and emission wavelengths for the system. The fluorescence spectra are shown in Appendix A. The optimal wavelengths would ideally be chosen to allow for maximal fluorescence of the methylated bases and minimal fluorescence of the adenine since a decreased detector response to adenine would aid in resolution of N-7 methylguanine and adenine peaks. Unfortunately, the adenine excitation and emission maxima were found to be very similar to those of the N-7 and O-6 methylguanine as
Figure 12. Elution profile of DNA from a rat treated with DMN, 20 mg/kg, mobile phase: 0.06 M ammonium formate, pH 4.1, 8% methanol. Peaks 1,2,3,4, and 5 represent guanine, protein, N-7 methylguanine, adenine, and O-6 methylguanine respectively. A new SCX column was used.
shown in Table 4. The excitation and emission wavelengths chosen were 290 and 360 nm respectively.

**Limits of Detection**

The limits of detection obtained for O-6 methylguanine and N-7 methylguanine when using the 0.06 M ammonium formate, pH 4.1, 8% methanol mobile phase are listed in Table 3.

**Standard Curves**

Standard curves were initially run during method development in order to verify linear detector response and later to allow quantitation of each of the bases. Representative standard curves for each of the bases are shown in Figures 13-16. Note that the linearity of the O-6 and N-7 methylguanine curves was present even in the ranges of their limits of detection. DNA from control rats was spiked with known amounts of N-7 methylguanine and these solutions were used for the N-7 methylguanine standard curves.

In spite of the apparent linear detector response for O-6 and N-7 methylguanine throughout a range encompassing most of the DNA sample solutions, a uniformity in the amount of DNA injected for each analysis was attempted. As mentioned in the Methods section, this was achieved by dilution of all hydrolyzed DNA solutions to give approximately the same adenine peak area upon injection. DNA content was measured in each of 6 different hydrolyzed DNA solutions that had already been diluted for injection and analysis. The concentrations of DNA in 50 ul of each of these samples are shown in Table 5. Since
Table 4. Fluorescence spectra maxima.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation Max.</th>
<th>Emission Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mobile phase:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.06M ammonium formate, pH 4.1, 8% methanol</td>
<td>325 nm.</td>
<td>320.5 nm.</td>
</tr>
<tr>
<td>0-6 methylguanine in mobile phase</td>
<td>290-293 nm.</td>
<td>352-366 nm.</td>
</tr>
<tr>
<td>N-7 methylguanine in mobile phase</td>
<td>281-292 nm.</td>
<td>334-355 nm.</td>
</tr>
<tr>
<td>adenine in mobile phase</td>
<td>278-290 nm.</td>
<td>335-360 nm.</td>
</tr>
</tbody>
</table>
Figure 13. Guanine standard curve.
Figure 14. Adenine standard curve.
Figure 15. N-7 methylguanine standard curve.
Figure 16. O-6 methylguanine standard curve.
Table 5. Concentrations of DNA in hydrolyzed DNA samples.

<table>
<thead>
<tr>
<th>DNA Sample</th>
<th>ug DNA/50 ul aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.5</td>
</tr>
<tr>
<td>2</td>
<td>26.5</td>
</tr>
<tr>
<td>3</td>
<td>33.0</td>
</tr>
<tr>
<td>4</td>
<td>33.0</td>
</tr>
<tr>
<td>5</td>
<td>20.0</td>
</tr>
<tr>
<td>6</td>
<td>36.5</td>
</tr>
</tbody>
</table>
the injection volumes were always 50 ul, these values represent the amount of DNA injected for each analysis. The mean concentration of DNA injected was 29.3 ug. The values in the table were determined by linear regression of a standard curve made with known amounts of DNA that had been hydrolyzed in the same manner as the sample DNA solutions. The standard curve that was used is depicted in Figure 17.

Validation of this method was achieved through comparison of the data obtained from HPLC analysis with those reported and obtained using radioactively labeled DMN. The in vivo experiments providing these data are described below.

Hydrolysis of DNA Samples

Complete hydrolysis and depurination of the purified DNA samples was checked by comparison of adenine:guanine peak area ratios. The mean adenine:guanine ratios in the samples was found to be 0.320 ± 3%. Hence if peak ratio values differed substantially from this value, it was assumed that incomplete hydrolysis had occurred and the samples were subjected to additional gentle shaking in a 37 C water bath and reassayed.

Animal Experiments

Prior to performing experiments designed to determine a dose-response relationship between the dose of DMN administered and the moles of N-7 and O-6 methylguanine formed, it was necessary to determine whether any O-6 methyltransferase activity might be taking place during the approximately 2 1/2 hour-long nuclei isolation
Figure 17. DNA standard curve using hydrolyzed calf thymus DNA.
procedure. This was done by isolation of one portion of liver from a
DMN-treated rat in nuclei isolation and DNA lysing buffers containing
ZnCl₂, a known potent in vitro inhibitor of 0-6 methyltransferase
activity (Pegg et al., 1983a). Nuclei from another liver portion from
the same rat was isolated in buffers not containing ZnCl₂. Table 6
shows the moles of 0-6 methylguanine/ moles of guanine x 10⁶ when
nuclei from 2 rats were isolated as described above. It is apparent
that even though 37% of the 0-6 methyltransferase activity has been
shown to reside in the nuclear fraction (Pegg, 1983a), there was no
significant activity of 0-6 methyltransferase during the isolation
procedure. This activity was decreased or absent either due to the 4 C
temperature at which the procedure was carried out or due to the
presence of EDTA and KCl in the buffers. EDTA and KCl have been shown
to have the ability to inhibit 65-75% of the 0-6 methyltransferase
activity in vitro, when present in concentrations of 1 mM and 100 mM
respectively. The EDTA and KCl concentrations in the 2 nuclei
isolation buffers (homogenization and dense sucrose) used were 2 and 4
mM EDTA and 60 and 120 mM KCl. After observation of the results from
this experiment, it was felt that addition of ZnCl₂ to nuclei
isolation buffers and the DNA lysing buffers was not necessary.

DMN Dose Response Studies

A dose response curve for DMN injected i.p. in rats is
depicted in Figure 18. Experiments to give this dose-response curve
were performed both in order to validate the method that had been
developed and to determine a suitable dose for use in subsequent TCE
Table 6. Effect of 0-6 methyltransferase inhibitor: ZnCl₂ on concentration of 0-6 methylguanine in hydrolyzed DNA samples.

<table>
<thead>
<tr>
<th>Rat</th>
<th>moles 0-6 methylguanine/moles guanine x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Buffers</td>
</tr>
<tr>
<td>1</td>
<td>625.83</td>
</tr>
<tr>
<td>2</td>
<td>488.25</td>
</tr>
</tbody>
</table>
Figure 18. Dose response curve for DMN. Each point represents the mean value obtained from 2 to 4 rats. Bars represent standard deviations from mean values.
studies. This experiment provided a means of validation for the HPLC method because the same measurements of moles of 0-6 methylguanine /moles of guanine x 10^6 using radioactively labeled DMN and following nearly identical in vivo experimental protocols had been reported (Pegg and Hui, 1978). Table 7 shows a comparison of measurements of 0-6 methylguanine using either 14C-labelling or HPLC analysis with fluorimetric detection. Since 3 out of 4 of the measurements were within 92% agreement with the values obtained using 14C-DMN, this method was considered suitable for measurement of 0-6 methylguanine.

The determination of a suitable dose of DMN for use in the TCE drinking water studies was made after observation of the dose-response curve shown in Figure 18. The curve appears to level off when doses above 20 mg/kg are given. This probably represents saturation of enzymes metabolizing and bioactivating DMN to its methylating species. The dose of 15 mg/kg was chosen to avoid this part of the curve which would give 0-6 methylguanine values that might be greatly dependent upon a particular animal's maximal capacity for bioactivation of DMN to a methylating species. It was also chosen to allow for measurement of 0-6 methylguanine concentrations above and below that expected from the administered dose. The 0-6 methylguanine concentration might be different due to either interspecies variation or various other factors affecting removal of the methylated base.
Table 7. Amount of 0-6 methylguanine present in liver DNA as measured by HPLC analysis with fluorimetric detection or by C 14-DMN

<table>
<thead>
<tr>
<th>Dose of DMN</th>
<th>moles 0-6 MeGua/moles Gua x 10^6</th>
<th>% Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg body wt.</td>
<td>C¹⁴-DMN*</td>
<td>HPLC Analysis</td>
</tr>
<tr>
<td>2.5</td>
<td>57</td>
<td>37.6</td>
</tr>
<tr>
<td>5.0</td>
<td>155</td>
<td>147.4</td>
</tr>
<tr>
<td>10.0</td>
<td>322</td>
<td>350.3</td>
</tr>
<tr>
<td>20.0</td>
<td>652</td>
<td>691.3</td>
</tr>
</tbody>
</table>

a from Pegg and Hui (1978).
TCE Drinking Water Experiments

One of the objectives of this work was to determine whether short term administration of TCE in drinking water would affect the removal of 0-6 methylguanine in rats that were treated with DMN. Although time did not permit completion of this work, the results obtained and shown in Figure 19 suggest that TCE neither induced nor inhibited removal of 0-6 methylguanine when given in drinking water concentrations of 5 mg TCE/ml water during a one or two week period. Since TCE might be expected to affect cytochrome P-450 levels which are responsible for bioactivation of DMN to the active species, it was necessary not only to compare the absolute amounts of 0-6 methylguanine present but also to compare the 0-6/N-7 methylguanine ratios in each of the groups. As seen in table 8, the 0-6/N-7 methylguanine ratios did not differ significantly. As will be discussed presently in the Discussion section, additional time points would be needed for more conclusive evidence demonstrating TCE's lack of effect on the 0-6 methylguanine removal system.
Figure 19. Effect of TCE on persistence of 0-6 methylguanine. Solid bars represent pretreatment with TCE, 5 mg/ml 1% emulphor water. Hatched bars represent pretreatment with 1% emulphor water alone. Values shown represent the mean of 2-4 rats and the bars represent the standard deviations from these means.
Table 8. 0-6 methylguanine: N-7 methylguanine ratios following DMN administration and pretreatment with TCE or 1% Emulphor in the drinking water.

<table>
<thead>
<tr>
<th>duration of treatment</th>
<th>1% Emulphor</th>
<th>Treatment</th>
<th>5 Mg TCE/ml</th>
<th>1% Emulphor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>0.102 + 0.006</td>
<td>0.111 + 0.013</td>
<td>0.106 + 0.007</td>
<td>0.094 + 0.009</td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
An HPLC Assay for Methylated Purines

The primary goal of this work was to provide a working analytical procedure that would allow quantification and separation of methylated purines from unmodified purines. In particular, a method enabling analysis of O-6 and N-7 methylguanine was established due to the widespread interest in the possible role of the former adduct in nitrosamine-induced carcinogenesis. The working method that was established and described herein employs a Partisil 10 SCX strong cation exchange column with an isocratic mobile phase of 0.06 M ammonium formate, 8% methanol, pH 4.1 for separation of the bases. Detection and quantification are achieved through the use of fluorimetric detection.

A strong cation exchange column was chosen for use in the separation of the purines both because of its prevalent use in published methods for purine separation and because it permits the use of a large range of mobile phase pHs (Herron and Shank, 1979, Belinsky et al, 1982, Swenberg and Bedell, 1982). A weak cation exchange column has a much higher affinity for its counter ions and thus will not exchange them with cationic solute molecules unless a high pH is used. In addition, at a pH below 4.0, the capacity of such a column declines. However, mobile phases of low pH are critical for both separation and quantification of the bases. Since the purines are
weak bases, a relatively low pH is required for them to be in the cationic forms necessary for use with cation exchange methods (Zakaria and Brown, 1981). A low mobile phase pH is also required for maximal fluorescence and thus high sensitivity for the purine bases (Swenberg and Bedell, 1982). Protonated purines have been found to be more fluorescent (at room temperature) than non-protonated purines (Eisinger and Lamola, 1971).

As mentioned previously, fluorescence detection was chosen for quantification of bases due to its high sensitivity and additional properties that are distinctly advantageous for use in this system. Fluorescent intensity is highly dependent upon the molecular structure of a compound. Accordingly, the fluorescent intensities of O-6 and N-7 methylguanines, adenine, and guanine differ greatly. Advantage may be taken of the moderate fluorescence of N-7 methylguanine compared to the relatively weak fluorescence of adenine (Herron and Shank, 1979). The differences in fluorescent intensity aids in the resolution of these closely eluting peaks because the decreased fluorescence intensity of adenine narrows its peak width. Additionally, the very strong fluorescence of O-6 methylguanine relative to the other bases is especially advantageous in this situation where it is present in minute concentrations compared to those of the other bases in the sample DNA hydrolysates. Having considered the reasoning behind the analytical methods chosen, a review of the work on method development and the results will be discussed.
Separation

A better separation of the purines was demonstrated when the ammonium formate buffer was used than when the ammonium phosphate buffer was used. A pH of 4.1 was found to be the optimum pH for separation in the ammonium formate mobile phase. This pH is low enough for protonation of the weakly basic purines (Zakaria and Brown, 1981). When the cation exchange column is used, the more highly protonated the bases are, the longer they are retained on the column. Protonation in turn is dependent upon both the pH of the mobile phase, and the $pK_{ab}$ of each purine. The $pK_{ab}$ values are a reflection of the basicity of the purines. At a pH below their $pK_{ab}$ values, the NH$_2$ groups on the bases are protonated and positively charged. Above the $pK_{ab}$, they are present in a neutral form. The uncharged (neutral) bases are not retained significantly. The order of elution thus would be expected to correspond to the increasing $pK_{ab}$ values of the bases. This is illustrated in the sample chromatograms where it was demonstrated that guanine eluted first with the lowest $pK_{ab}$ of 3.2, followed by N-7 methylguanine and adenine with $pK_{ab}$ values of 3.5 and 4.15 respectively. Although the $pK_{ab}$ of 0-6 methylguanine was not available, observation of the elution profile would suggest that its $pK_{ab}$ would be greater than 4.15.

Differential retention and subsequent separation of the purine bases is a result primarily of the ion exchange processes described above. However, other factors may play a role in the separation process. The size and shape of the bases as well as the capacity and
type of functional groups on the bonded phase affect the retention and separation of these compounds. Importantly, partitioning processes between the aqueous mobile phase and the bonded benzene sulfonic acid phase may also occur. This partitioning might provide an explanation for the decreases in retention times and peak widths of the adenine and N-7 methylguanine peaks that are seen following the addition of methanol to the ammonium formate mobile phase. The addition of methanol may decreases the polarity of the mobile phase slightly and may cause the purines to partition more readily from the nonpolar bonded phase benzene environment into the slightly non-polar mobile phase.

Limits of Detection

The limit of detection of a particular analyte may be theoretically defined as that concentration of the analyte required to give a signal (or peak height) twice that of the standard deviation of the background noise (Skoog and West, 1980). As mentioned previously, very low detection limits were possible for 0-6 methylguanine due to its strong fluorescent intensity. The limit of detection for N-7 methylguanine was higher due to its weaker fluorescence. However this did not constitute a major problem since there is always approximately ten-fold more N-7 methylguanine formed as 0-6 methylguanine, when DMN is the methylating agent (Pegg, 1977a). The limits of detection for 0-6 methylguanine and N-7 methylguanine were approximately 0.75 and 21.0 picomoles respectively. These detection limits were similar to those reported in the literature.
Limits of detection reported for 0-6 methylguanine range from 0.3-2.0 picomoles and those reported for N-7 methylguanine range from 10.0-42.0 picomoles (Herron and Shank, 1979, Belinsky et al., 1982, Swenberg and Bedell, 1982, Lindamood et al., 1984).

Linearity of Detection

The standard curves presented in Figures 7-10 illustrate the ranges of linearity of detection for the purine bases. The standard curve for guanine shows a linear detection range from 150-4000 ng. Other curves ran prior to the one illustrated demonstrated linearity of detector response for guanine when amounts of the base in the range of 8 ng to 4000 ng were plotted. The lower amounts were not used in the standard curves normally because these were always greatly exceeded in the samples. Linearity of detector response was shown for adenine in amounts between 37.5 and 750 ng. This range can probably be extended to higher amounts. However, since the amount of adenine was not measured routinely, standard curves including higher adenine amounts were not attempted. Linearity of detection was demonstrated for N-7 and 0-6 methylguanines from their approximate limits of detection, 3.75 and 0.125 ng to 30 and 2.5 ng respectively. Most of the samples analyzed contained N-7 and 0-6 methylguanine concentrations within this range. Some samples that were analyzed showed 0-6 methylguanine but not N-7 methylguanine because the latter was below its limit of detection. For these samples, it was not possible to calculate the 0-6:N-7 methylguanine ratios.
Reproducibility

The reproducibility of the HPLC assay was tested by comparing peak areas following repeated injections of the standard solutions of the bases. In general, the deviations from the mean (peak areas) after triplicate injections were less than 5%. However, periodically, a slightly larger deviation was seen. During the early stages of method development, problems were encountered with the reproducibility between repeated injections. However, these problems were solved when the injection method was changed to use of an injection loop for injection of constant volumes of samples or standards.

The reproducibility of the HPLC assay performed on different days was also examined. It was found that the day to day peak areas were reproducible when assays were done on consecutive days, using the same batch of mobile phase buffer. Since running the standards and samples took no longer than three consecutive days, it was not conclusively established whether peak area reproducibility would be present on subsequent days. However, reproducibility was not always evident when assays were performed more than two or three weeks apart using different batches of mobile phase buffer. In general, guanine peak areas were reproducible and the standard curves done previously were often used. However, occasionally the variation in 0-6 methylguanine peak areas measured on different dates was consistently larger than 5%, as shown in Table 9. Since 0-6 methylguanine was the main compound of interest, and because consistent variations in measurements of this adduct greater than five percent could greatly
Table 9. Mean peak areas obtained for 4 different concentrations of 0-6 methylguanine on three different dates.

<table>
<thead>
<tr>
<th>ng 0-6 methylguanine</th>
<th>mean peak area, 9-18-84</th>
<th>mean peak area, 10-1-84</th>
<th>mean peak area, 10-12-84</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>31.84</td>
<td>33.67</td>
<td>31.44</td>
</tr>
<tr>
<td>1.0</td>
<td>61.80</td>
<td>62.37</td>
<td>64.36</td>
</tr>
<tr>
<td>2.0</td>
<td>121.40</td>
<td>119.02</td>
<td>129.90</td>
</tr>
<tr>
<td>2.5</td>
<td>151.60</td>
<td>148.92</td>
<td>163.49</td>
</tr>
</tbody>
</table>
affect interpretations of the results, a conservative approach was taken and new standard curves were run each time samples were analyzed. Table 9 also illustrates how the reproducibility of these peak areas was greater at some times than other times. This may have been due to fluctuations in detector response.

The possible effect of TCE on removal of 0-6 methylguanine

As mentioned in the Introduction, the second objective of this research project was to determine whether a dosing regimen providing TCE in the drinking water had any effect upon the removal of 0-6 methylguanine in DNA. Interest in this question was in part due to the recent possible widespread human exposure to TCE in drinking water as well as to the probable simultaneous widespread exposure of man to methylating agents. It was additionally due to the fact that although highly debated, 0-6 methylguanine is still strongly implicated as a pro-carcinogenic lesion. Hence, any compound that might enhance or inhibit its removal would merit study.

TCE might affect removal of 0-6 methylguanine for the following reasons: 1) TCE has been shown to be hepatotoxic and to cause necrosis in rats whose drug metabolizing enzymes have been induced with phenobarbital (Allemand et al., 1978, Kjellstrand et al., 1981). The regenerative response following hepatic necrosis has been shown to induce the enzyme that results in the removal of 0-6 methylguanine from DNA when enzyme activity is measured in vitro (Pegg and Perry, 1981) 2) TCE has been shown to be activated to an electrophilic species (Waters et al., 1977, Miller and Guergerich,
1982) and to bind to proteins (Allemand et al., 1978, Van Duuren and Banerjee, 1976, Cunningham et al., 1981). Accordingly, it might be expected to bind to the nucleophilic sulphur of the methyl-transferring cysteine on 0-6 methyltransferase and thus inhibit the removal of the methyl group from 0-6 methylguanine.

The inhibition of removal of the pro-mutagenic 0-6 methylguanine lesion might be envisioned as one possible mechanism whereby the carcinogenicity of TCE seen in mice could be explained. If these mice are exposed to unknown methylating agents a decreased efficiency in methyl group removal would result in an increased persistence of 0-6 methylguanine. Cell division and transcription of the altered DNA therefore would be more likely to occur before the lesions were repaired. It has been demonstrated that the target tissues for nitrosamine-induced carcinogenesis are those in which the lesion persists for a long enough period of time that cell division is likely to occur (Pegg, 1983).

**Experimental Protocol and Results**

Although TCE has not been demonstrated to be carcinogenic in rats, this species was selected for these experiments primarily because most of the work pertaining to 0-6 methylguanine persistence has been done on these animals. This enabled validation of the HPLC method developed by comparison with data obtained in identical experiments using $^{14}$C-DMN. Nevertheless the use of rats for a study on the influence of TCE upon 0-6 methylguanine removal may still be justified despite the apparent lack of carcinogenicity in these
animals. Since mice have significantly lower rates of 0-6 methylguanine removal than rats (Lawley, 1976), compounds that inhibit this removal (by binding to 0-6 methyltransferase for example) may have more detrimental effects in the former species.

The results from experiments performed using the drinking water dosing regimen outlined in the Materials and Methods section showed that neither induction nor inhibition of the removal processes were apparent. The results from these studies may not however be representative of the effects of TCE on removal processes for two reasons. The first reason is that 0-6 methylguanine concentrations must be measured at additional, later time points. As mentioned above, the expected ratio of O-6/N-7 methylguanine formation is 0.11 (Pegg, 1977a). Since the methyl group of the 0-6 methylguanine is removed more rapidly than that of the N-7 methylguanine, this ratio should decrease with time. The ratios obtained in these experiments (see Table 8) show that the O-6/N-7 ratios were still very close to 0.11. This indicates that substantial removal of 0-6 methylguanine had not occurred. Even though removal processes have been shown to occur immediately after i.p. dosing with DMN, measurement of O-6 methylguanine concentrations at later time points, when a substantially greater amount of removal has occurred, would give a better indication of whether TCE had any effect on these processes.

The second reason that TCE-induced changes in O-6 methylguanine removal activity may not have been shown concerns the relative lack of hepatotoxicity of TCE. As mentioned above,
hepatotoxic agents causing a regenerative response in the liver have been shown in vitro to be capable of inducing 0-6 methylguanine removal processes. TCE has, however been shown to be only slightly hepatotoxic or non-hepatotoxic in uninduced rats (Waters et al., 1977, Allemand et al., 1981). The only indications of hepatotoxicity in these rats are increased liver-to-body weight ratios (Kjellstrand et al., 1981) or slightly increased SGPT levels (Allemand et al., 1981). In contrast, hepatonecrosis and toxicity have been shown morphologically and biochemically (highly elevated SGPT levels) in rats whose drug metabolizing enzymes have been induced with phenobarbital (Allemand et al., 1978). This would be expected since these enzymes are assumed to be responsible for the activation of TCE to its electrophilic species (Waters et al., 1977, Miller and Guerngerich, 1981) Since the animals used in these studies were not induced, hepatonecrosis due to TCE would not be expected and the regenerative processes postulated to cause induction of 0-6 methyltransferase would not be present. In addition, the doses used in the studies cited above were significantly greater (0.25-2 ml TCE/kg) than what could be obtained in a drinking water study. The doses were limited in the latter type of study both due to the solubility of the TCE in water and the palatability of water containing high concentrations of TCE.

In conclusion, it is felt by the author that TCE is not hepatotoxic enough to cause induction of 0-6 methylguanine removal
systems. The electrophilic species of TCE may however bind non-specifically to 0-6 methyltransferase, the enzyme responsible for removal of the 0-6 methyl group from guanine. It would be interesting to examine whether this might be occurring. This would best be examined by dosing a phenobarbital-induced rat with radiolabeled TCE followed by isolation of the 0-6 methyltransferase protein and measurement of binding of radioactive species.
APPENDIX

Fluorimetric Spectra
Figure A.1. Excitation spectra of 0.06 M ammonium formate, pH 4.1, 8% methanol.
Figure A.2. Emission spectra of 0.06 M ammonium formate, pH 4.1, 82% methanol.
Figure A.3. Excitation spectra of adenine.
Figure A.4. Emission spectra of adenine.
Figure A.5. Excitation spectra of N-7 methylguanine.
Figure A.6. Emission spectra of N-7 methylguanine.
Figure A.7. Excitation spectra of 0-6 methylguanine.
Figure A.8. Emission spectra of 0-6 methylguanine.
References


