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DEVELOPMENT OF A ^{32}P -POSTLABELING ASSAY FOR O^6 -METHYLGUANINE

By

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ABSTRACT

Monitoring of the promutagenic DNA adduct, O⁶-methylguanine, in the exfoliated cells (e.g. oral mucosal) of tissues from individuals exposed to tobacco-specific N-nitrosamines may aid in the evaluation of tissue-specific risk of carcinogenesis. People with high levels of this adduct could be identified and the appropriate intervention taken. Current techniques for detection of O⁶-methylguanine are unsuitable for the measurement of low adduct levels in small tissue samples.

This thesis describes the development of a ³²P-postlabeling method for detection of O⁶-methylguanine in microgram amounts of DNA. In the first part of the project I synthesized O⁶-methyldeoxyguanosine 3'-monophosphate (O⁶mdG3'p), needed as a chromatography marker. This was achieved using a two step approach; preparation of O⁶-methyldeoxyguanosine (O⁶mdG) followed by chemical phosphorylation with KH₂PO₄ in formamide. The identity of the compound was confirmed by U.V. spectroscopy and enzymatic analysis. This is the first reported synthesis of the modified nucleotide. Using the synthetic marker, high performance liquid chromatography (HPLC) procedures were then developed for isolation of O⁶mdG3'p from digested DNA prior to postlabeling.

The basic method for analysis of O⁶-methylguanine by ³²P-postlabeling comprises five steps, a) digestion of DNA containing O⁶-methylguanine to deoxynucleoside 3'-monophosphates using micrococcal nuclease and spleen phosphodiesterase, b) isolation of O⁶mdG3'p from normal nucleotides using reverse-phase HPLC, c) ³²P-labeling of O⁶mdG3'p to give O⁶-methyldeoxyguanosine 3',5',³²P-bisphosphate (O⁶mdG3'5'p) using ³²P-ATP and polynucleotide kinase, d) 2-dimensional polyethyleneimine cellulose thin layer chromatography (PEI-TLC) to resolve O⁶mdG3'5'p from other radioactive

materials and e) autoradiography and scintillation counting to quantitate O^6 mdG3'5'p.

Several variations of the basic technique were then investigated with the goal of improving the sensitivity. One modification, encompassing a second HPLC purification of O^6 mdG3'5'p showed the greatest sensitivity: 0.5 micromole O^6 -methylguanine per mole normal nucleotide ($0.5 \mu\text{mole/mole}$). Using this method, O^6 -methylguanine was detected in the DNA of mammalian tissue-culture cells treated with an agent, N-methyl-N-nitrosourea (MNU) known through independent techniques to form this adduct.

The development of ^{32}P -postlabeling methods for detection of other small DNA adducts is feasible using the approach described here for O^6 -methylguanine. The performance of the ^{32}P -postlabeling method for O^6 -methylguanine equals that of the best available methods when microgram quantities of DNA are assayed. This new method will complement existing techniques.

CONTENTS

	Page
ABSTRACT	ii
CONTENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
ACKNOWLEDGEMENTS	xi
GENERAL INTRODUCTION	1
1. Environment and Human Cancer	1
2. An Overview of Chemical Carcinogenesis	1
3. DNA Adducts and Chemical Carcinogenesis	2
4. Detection of DNA Adducts in Humans-Molecular Cancer	
Epidemiology	5
5. ³² P-Postlabeling Assays to Measure DNA Damage	6
6. The Significance of O ⁶ -methylguanine in Carcinogenesis	11
6.1 Formation of O ⁶ -methylguanine	11
6.2 Historical Perspectives	12
6.3 Repair of O ⁶ -methylguanine	12
6.4 Correlation of O ⁶ -methylguanine Persistence with	
Carcinogenesis by N-Nitrosamines	14
6.5 The Molecular Mechanism of Mutagenesis by O ⁶ -	
methylguanine	15
6.5.1 In Vitro	15
6.5.2 In Vivo	16
6.6 Initiation of Carcinogenesis through Activation of	
Cellular Proto-oncogenes by O ⁶ -methylguanine Induced	
Mutation	17

7. Ultra-sensitive Methodology for Detection of O ⁶ -methylguanine DNA Adducts	18
8. Current Problem	19
9. Objectives	20
MATERIALS	21
METHOD DEVELOPMENT	24
1. Synthesis of O ⁶ -methyldeoxyguanosine 3'-monophosphate	24
Introduction	24
Methods and Results	26
1.1 Preparation of O ⁶ -methyldeoxyguanosine	26
1.2 Preparation of O ⁶ -methyldeoxyguanosine 3'- monophosphate	32
2. O ⁶ -methylguanine Quantitation by ³² P-Postlabeling Analysis	40
Introduction	40
Methods and Results	43
2.1 Method #1: Single HPLC Purification-Diphosphate Level	43
2.2 Method #2: Dual HPLC Purification-Diphosphate Level	53
2.3 Method #3: Single HPLC Purification-Monophosphate Level	54
2.4 Method #4: Dual HPLC Purification-Monophosphate Level	55
3. Performance of the ³² P-Postlabeling Assays for O ⁶ - methylguanine	58
Introduction	58
Methods and Results	60
3.1 Reproducibility of the Assay for Synthetic O ⁶ mdG3'p	60
3.2 Reproducibility of DNA Digestion	60
3.3 Recovery of O ⁶ -methyldeoxyguanosine 3'-monophosphate	63
3.4 Sensitivity	64

DETECTION AND QUANTITATION OF O ⁶ -METHYLGUANINE IN THE DNA OF TISSUE-CULTURE CELLS TREATED WITH N-METHYL-N-NITROSOUREA	68
Introduction	68
1. Detection and Quantitation of O ⁶ -methylguanine in the DNA of CHO Cells treated with N-methyl-N-nitrosoourea	69
Methods	69
Results	70
2. Detection and Quantitation of O ⁶ -methylguanine in the DNA of C3H10T1/2 Mouse Embryo Fibroblast Cells treated with N-methyl-N- nitrosoourea	74
Methods	74
Results	75
DISCUSSION AND CONCLUSIONS	83
1. Method Development	83
2. Comparison of Methods	84
3. Factors Limiting the Performance of the ³² P-Postlabeling Assays	86
3.1 Reproducibility	86
3.2 Recovery	87
3.3 Sensitivity	88
4. Detection and Quantitation of O ⁶ -methylguanine in Mammalian Tissue-Culture Cells Treated with N-Methyl-N-nitrosoourea	90
5. Recommendations to Enable Application of the Method to Human Studies	90
REFERENCES	93

LIST OF TABLES

	Page
Table 1 Reproducibility of the ^{32}P -postlabeling assay for synthetic O^6 -methyldeoxyguanosine 3'-monophosphate	61
Table 2 Reproducibility of DNA digestion	62
Table 3 Sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine-method #1	65
Table 4 Performance characteristics of the ^{32}P -postlabeling assays for O^6 -methylguanine	85

LIST OF FIGURES

	Page
Figure 1 Schematic representation of the multistage process of carcinogenesis	3
Figure 2 Basic procedure for detecting DNA-carcinogen adducts using ^{32}P -postlabeling	7
Figure 3 Chemical structure of $\text{O}^6\text{mdG3}'\text{p}$	25
Figure 4 HPLC elution profile of isomeric methyldeoxyguanosines resulting from the methylation of deoxyguanosine with ethereal diazomethane	27
Figure 5 HPLC chromatogram of once purified O^6mdG	29
Figure 6 HPLC analysis of twice purified O^6mdG	30
Figure 7 U.V. spectrum of O^6mdG in ddH_2O	31
Figure 8 HPLC analysis of O^6mdG /formamide/ KH_2PO_4 phosphorylation mixture before (A) and after (B) 1.5 hours heating at 100°C	33
Figure 9 Putative O^6mdG monophosphates in crude phosphorylation mixture, separated by semi-preparative HPLC.	34
Figure 10 HPLC analysis of isomeric $\text{O}^6\text{mdG3}'$ and 5'-monophosphates isolated from the reaction mixture	36
Figure 11 HPLC elution profiles of purified $\text{O}^6\text{mdG5}'\text{p}$ (A) and $\text{O}^6\text{mdG3}'\text{p}$ (B)	37
Figure 12 U.V. spectrum of $\text{O}^6\text{mdG3}'\text{p}$ in ddH_2O	38
Figure 13 Characterization of $\text{O}^6\text{mdG3}'\text{p}$ using 5'-nucleotidase of <i>C. adamanteus</i> venom	39
Figure 14 Conversion of $\text{O}^6\text{mdG3}'\text{p}$ to O^6mdG by nuclease P1	41
Figure 15 1-Dimensional PEI-TLC autoradiogram of putative ^{32}P -labeled $\text{O}^6\text{mdG3}'5'\text{p}$ derived from labeling of $\text{O}^6\text{mdG3}'\text{p}$ with gamma ^{32}P -ATP and polynucleotide kinase	42
Figure 16 Summary of the ^{32}P -postlabeling methods for O^6 -methylguanine	44
Figure 17 Isolation of $\text{O}^6\text{mdG3}'\text{p}$ from DNA digested with micrococcal nuclease and spleen phosphodiesterase using reverse-phase HPLC.	47
Figure 18 Representative autoradiograms for postlabeling analysis of O^6 -methylguanine in $1\ \mu\text{g}$ DNA using method #1 (single HPLC purification-diphosphate level)	51

Figure 19	Representative autoradiograms for ^{32}P -postlabeling analysis of O^6 -methylguanine in 1 μg DNA using method #3 (single HPLC purification-monophosphate level)	56
Figure 20	Purification of ^{32}P -labeled $\text{O}^6\text{mdG5}'\text{p}$ using reverse-phase HPLC (method #4)	57
Figure 21	Sample autoradiograms for ^{32}P -postlabeling analysis of O^6 -methylguanine in 1 μg DNA using method #4 (dual HPLC purification-monophosphate level)	59
Figure 22	Sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine-method #1 (single HPLC purification-diphosphate level)	66
Figure 23	Sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine-method #2 (dual HPLC purification-diphosphate level)	67
Figure 24	Detection and quantitation of O^6 -methylguanine in CHO cells treated with MNU	72
Figure 25	Dose-response of O^6 -methylguanine in the DNA of CHO cells treated with MNU	73
Figure 26	Detection and quantitation of O^6 -methylguanine in the DNA of C3H10T1/2 cells treated with MNU (method #1)	77
Figure 27	Autoradiograms of DNA from C3H10T1/2 cells exposed to various doses of MNU (method #2)	79
Figure 28	Dose-response of O^6 -methylguanine in the DNA of C3H10T1/2 mouse-embryo fibroblast cells treated with MNU	81
Figure 29	Survival of C3H10T1/2 mouse-embryo fibroblast cells exposed to MNU	82

LIST OF ABBREVIATIONS

O ⁶ mdG	O ⁶ -methyldeoxyguanosine
O ⁶ mdG3'p	O ⁶ -methyldeoxyguanosine 3'-monophosphate
O ⁶ mdG5'p	O ⁶ -methyldeoxyguanosine 5'-monophosphate
O ⁶ mdG3'5'p	O ⁶ -methyldeoxyguanosine 3',5' ³² P-bisphosphate
dA3'p	deoxyadenosine 3'-monophosphate
dA3'5'p	deoxyadenosine 3'5' ³² P-bisphosphate
MNU	N-methyl-N-nitrosourea
AT	O ⁶ -alkylguanine-DNA-alkyltransferase
HPLC	high performance liquid chromatography
PEI-TLC	polyethylene-imine thin layer chromatography
μmole	micromole (10 ⁻⁶ mole)
μg	microgram (10 ⁻⁶ gram)
μl	microliter (10 ⁻⁶ liter)

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GENERAL INTRODUCTION

1. Environment and Human Cancer

The majority of human cancers are induced by exogenous agents in the environment. This conclusion arises primarily from epidemiological data (descriptive and analytical) indicating temporal trends in incidence and changing risks in migrant populations (Doll et al. 1966, Waterhouse et al. 1982, Kolonel et al. 1980). Genetic and congenital factors are believed to be responsible for no more than 2% of the total cancer burden (Higginson and Muir 1979, Knudson 1977). Exposure to chemicals through diet, use of tobacco, and occupation may account for up to 85% of all cancer mortality in North America (Doll and Peto 1981).

2. An Overview of Chemical Carcinogenesis

Most of our knowledge concerning the mechanisms of chemical carcinogenesis is necessarily derived from studies on cancer induction or its modulation in experimental animal systems. Many identified human carcinogens cause tumors in animals (Searle 1984). Several carcinogens active in animals were initially discovered because of industrial exposure and carcinogenesis in humans; benzo(a)pyrene and β -naphthylamine for example (Doll and Peto 1981). There is also evidence that at the cellular level, humans by and large do not differ qualitatively from experimental animals in response to carcinogens.

Although tumors were chemically induced in animals prior to 1940 (Yamagiwa and Ichakawa 1915, Sasaki and Yoshida 1935), it was not until 1961 that the covalent binding of a carcinogen, dibenz(a,h)anthracene, to the DNA of mouse skin was reported (Heidelberger and Davenport 1961). A multitude of ensuing work showed that most animal carcinogens bind to the bases of DNA to form covalent DNA-carcinogen addition products or adducts (Hemminki 1983).

Moreover, the organotropy of the carcinogen could often be predicted by the extent of DNA binding in the target tissue (Kriek et al. 1984). These studies were primarily conducted using radioactive carcinogens, followed by isolation of DNA and identification of the radioactive adducted bases by chromatography (Baird 1979). Subsequent work indicated that the DNA binding of a chemical may be a more accurate predictor of carcinogenicity than mutagenicity.

Studies following the metabolism of carcinogens showed that they either directly formed highly reactive electrophilic intermediates or are enzymatically converted to these species by detoxification processes. The electrophiles or pre-carcinogens are thought to react with nucleophilic bases of DNA, forming adducts (Miller and Miller 1981). Recently it has been shown that the DNA adducts formed in rodents by major classes of carcinogens are also induced in human cells by the same agents (Autrup and Harris 1983).

3. DNA Adducts and Chemical Carcinogenesis

A simplified schematic diagram of the current model for chemical carcinogenesis is represented in Figure 1 (Harris 1985). The multistage process is divided into several steps necessary for tumor induction but which may be separated both temporally, and mechanistically (Bertram et al. 1987). The concepts of tumor initiation, promotion, conversion and progression have arisen from studies in experimental animal carcinogenesis. This model may be used as an intellectual framework to consider the role that DNA adducts may play in multi-stage carcinogenesis.

The earliest event in chemical carcinogenesis, tumor initiation, is comprised of several steps, a) exposure to the carcinogen, b) transport of the carcinogen to the target cell, c) activation to its ultimate electrophilic metabolite if the agent is a pre-carcinogen and d) DNA damage leading to an

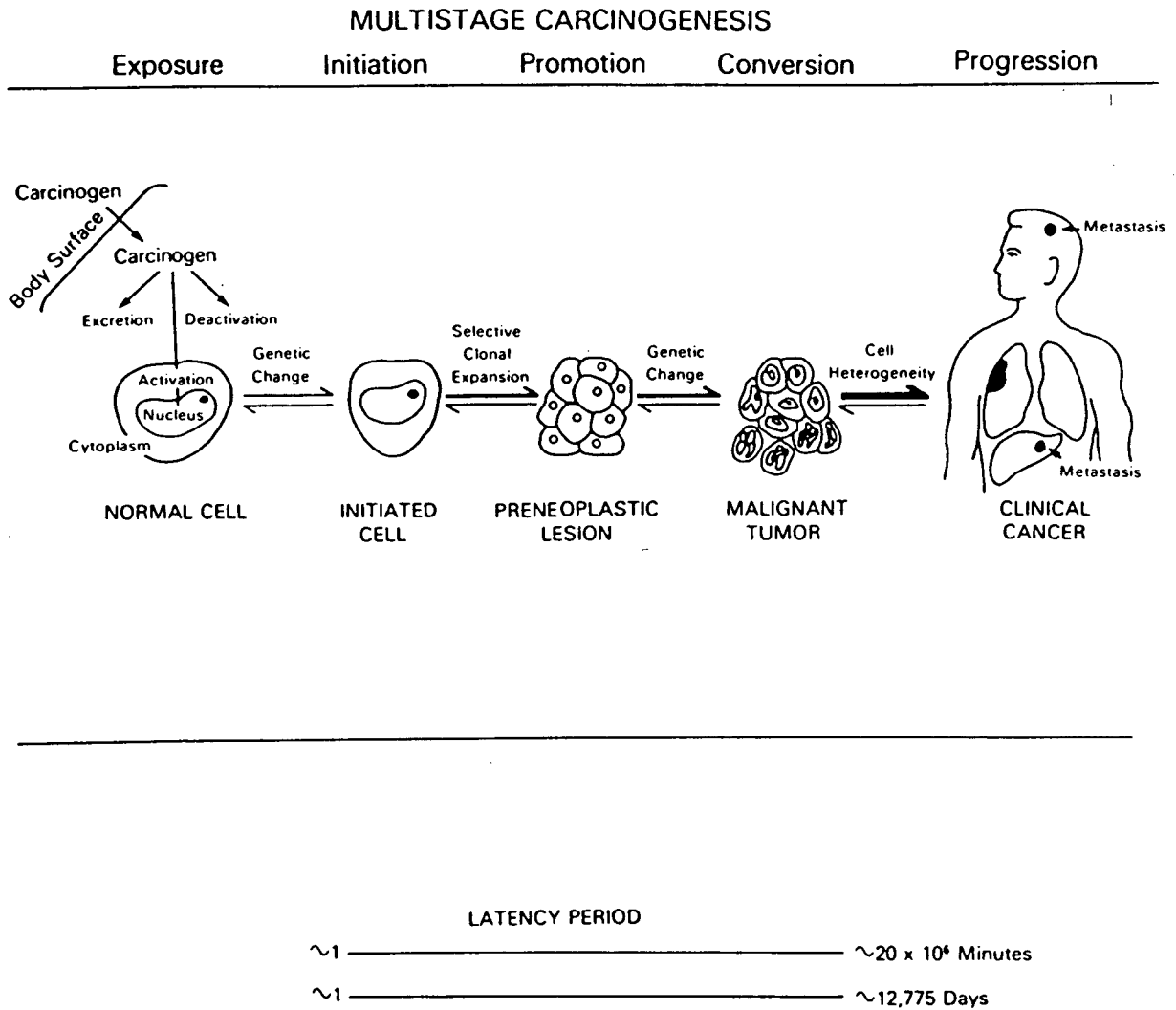


Figure 1 Schematic representation of the multistage process of carcinogenesis.

inherited change and the preneoplastic "initiated" cell. Metabolic activation and DNA damage probably occurs within hours of exposure. Current evidence suggests that DNA replication must occur before repair of the DNA adduct to create a stable biological lesion. Thus, replication before repair or error-prone repair can fix the lesion in DNA as a mutation. The requirement for replication may in part explain the high frequency of neoplasms in proliferating tissues (Cayama et al. 1978).

Cancer is thought to be the result of complex interactions between multiple environmental factors and both acquired and inherited host factors (Harris 1983). As such, DNA adducts should only be considered as necessary for the initiation of chemical carcinogenesis but not sufficient for tumor induction. Crucial are determinants of tumor promotion, progression, conversion and factors which can enhance or inhibit these processes.

The suggestion that persistent DNA adducts lead to initiation of carcinogenesis is corroborated by two lines of evidence. First, people who suffer from DNA repair deficiency syndromes are prone to the development of cancer (Knudson 1977). Examples are xeroderma pigmentosum, Fanconi's anemia and ataxia telangiectasia. Cultured cells from these individuals are unable to remove DNA modifications and are hypersensitive to the induction of mutation after chemical treatment. Second, qualitatively similar adducts are formed in animal species both sensitive and resistant to the tumorigenic effects of a chemical, but often a greater total amount of adducts are formed in target organs of sensitive species (reviewed by Wogan and Gorelick 1985).

4. Detection of DNA Adducts in Humans- Molecular Cancer Epidemiology

Data from animal studies and human cells in vitro indicate that formation and persistence of DNA adducts should be a necessary event in the initiation of chemical carcinogenesis in humans.

Detection of DNA adducts in humans could provide information on the mechanistic link between exposure to cancer causing chemicals and subsequent risk for tumor development (Maugh 1984). DNA adducts may be a better indication of risk for cancer than quantitation of exposure because they are markers of direct genetic damage. Molecular cancer epidemiology entails the establishment of a causal relationship between the formation and persistence of a particular adduct(s) and a particular tumor (Perera 1987). Identification of an exposure-related DNA adduct in tissues at risk could aid in the tracing of the chemical responsible.

Detection of an environmentally related DNA adduct indicates that exposure to genotoxic chemicals has occurred, and that the person is at risk. The value of a DNA adduct as an accurate early indicator of neoplasia can be established only through risk verification by following populations with different levels of adducts for many years. Once a causative lesion has been identified, detection in exposed individuals may serve to identify increased risk and allow opportunity for early intervention.

The detection of low levels of DNA damage in humans has been possible recently due to the development of several ultra-sensitive methodologies. The most important are ^{32}P -postlabeling (reviewed by Watson 1987), immunoassays (reviewed by Strickland and Boyle 1984), and synchronous fluorescence spectrophotometry (Vahakangas et al. 1985). Each method has particular advantages and optimal applications. Ideally the use of two or more

techniques in a validation scheme would serve to offset problems with specificity and accuracy associated with use of a single method.

Availability of these techniques has initiated an explosion in research concerning the measurement of DNA adducts in humans at risk for cancer due to occupation or lifestyle. Exposure related adducts have been detected in peripheral blood lymphocytes of foundry workers and in the placentae and lungs of smokers, for example (Harris et al. 1985, Everson et al. 1986). One interesting observation is the detection of DNA damage in so-called unexposed populations illustrating perhaps the unavoidability of exposure to genotoxic agents in the environment (Harris et al. 1985, Everson et al. 1986).

5. ³²P-Postlabeling Assays to Measure DNA Damage

To address the need for a sensitive and generally applicable test to detect directly the presence of chemically altered bases in DNA, the postlabeling method was developed by Randerath and co-workers in 1981 (Randerath et al. 1981). This test evolved from methods used to analyse modified constituents in RNA and normal bases in DNA (Randerath et al. 1972, Reddy et al. 1981).

The basic procedure encompasses the following steps: a) isolation of DNA from cells exposed to carcinogens, b) DNA digestion to a mixture of normal and adducted deoxynucleoside 3'-monophosphates using micrococcal endonuclease and spleen phosphodiesterase, c) transfer of ³²P-label from ³²P-ATP to the 5' position of digestion products mediated by T4 polynucleotide kinase and d) mapping of the ³²P-labeled adduct nucleotides by multi-directional chromatography on PEI-cellulose thin layers, followed by autoradiography. A schematic diagram illustrating the method is shown in Figure 2. The method is quantitative because the extent of DNA adduction can be determined by

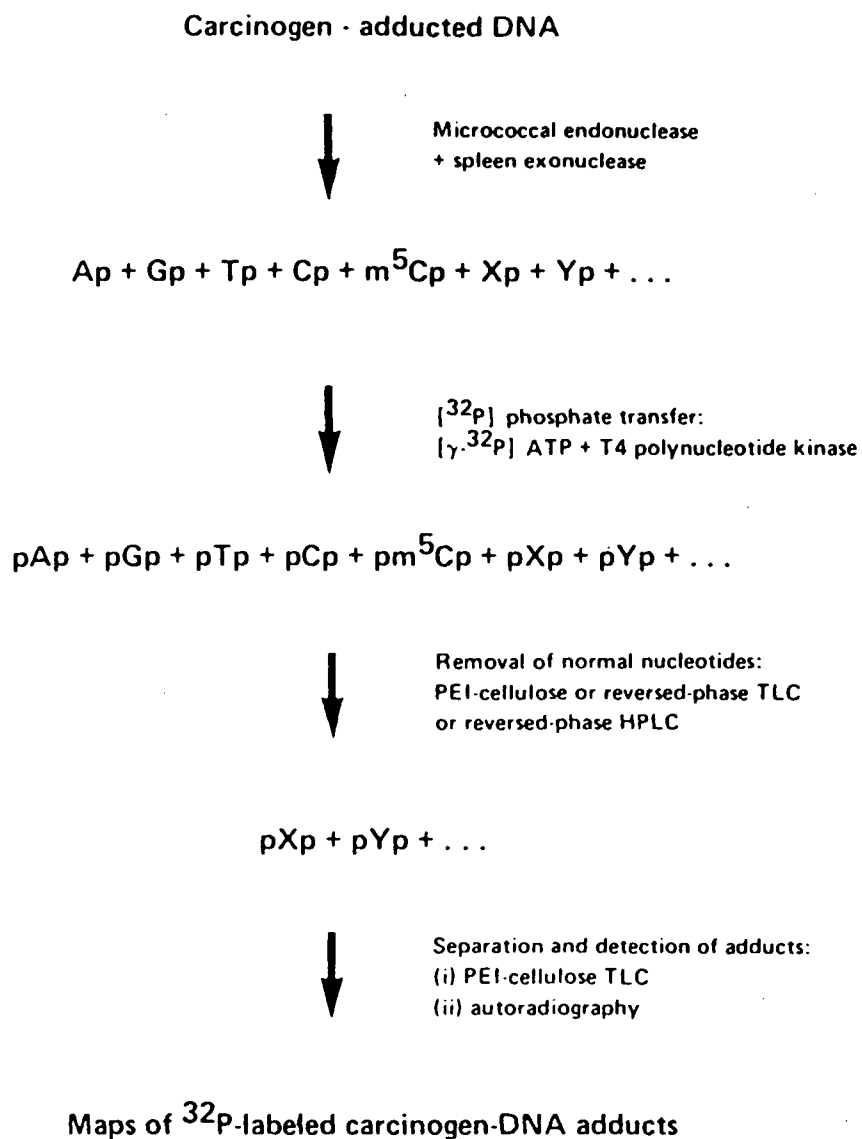


Figure 2 Basic procedure for detecting DNA-carcinogen adducts using ³²P-postlabeling.

scintillation counting of ^{32}P -labeled nucleotides on the chromatograms (Randerath et al. 1981).

In recent years, the original method has been modified to accomodate the detection of a diverse array of aromatic and non-aromatic DNA adducts (Gupta et al. 1982, Reddy et al. 1984). The chemical class of adducts detected depends on the chromatographic conditions employed in the final PEI-TLC step. Resolution of bulky aromatic adduct deoxynucleoside 3',5', ^{32}P -bisphosphates requires the use of solvents containing high concentrations of urea. Smaller, less hydrophobic adducts (e.g. alkyl adducts) migrate with the solvent front under these conditions and are not retained on the chromatogram.

Bulky, aromatic adducts are detected with the greatest sensitivity using the ^{32}P -postlabeling method. This is because these compounds can be effectively isolated as a class from the normal nucleotides using chromatography. This is accomplished by elution of labeled normal nucleotides off the chromatograms onto a paper wick using urea solvents, leaving the aromatic adducts at the origin (Gupta et al. 1982).

The extent of DNA damage in humans exposed to environmental carcinogens is expected to be less than 1 altered base in 10^7 to 10^8 normal bases (Everson et al. 1986, Randerath et al. 1986). To broaden the application of the postlabeling assay to human studies, the sensitivity of the method has been improved. This has been achieved for detection of aromatic adducts through the efficient removal of normal nucleotides prior to postlabeling. Pre-labeling isolation of adducts allows the analysis of more DNA using less ^{32}P -ATP, resulting in lower background counts. Extraction of aromatic adducts from the DNA digest using n-butanol followed by postlabeling after solvent removal is one example (Gupta 1985). In another modification of the procedure, DNA digests are injected onto a reverse-phase HPLC column and the normal nucleotides removed by elution with ammonium formate solvent. The

aromatic adducts (retained on the column) are then recovered by elution with methanol (Dunn and Stich 1986, Dunn et al. 1987, Dunn and San 1988). The resistance of nucleotides adducted with aromatic or bulky non-aromatic groups to the 3'-dephosphorylating action of nuclease P1 is the basis for yet another recent advance (Reddy and Randerath 1986).

In another approach, a 10-100 fold increase in sensitivity was noted when DNA digests were labeled with a limiting amount of carrier-free (high specific activity) ATP. Adducted nucleotides were labeled preferentially over normal nucleotides under these conditions (Randerath et al. 1985).

Evidence for the identity of an adduct detected by the postlabeling assay may be obtained by co-chromatography with an authentic marker compound. Postlabeling assays for the detection of specific small adducts have been developed. Techniques for the detection of 5-methylcytosine, bromodeoxyuridine, a formaldehyde induced adduct, and N⁷-(2-oxoethyl)guanine (the principal vinyl-chloride adduct), have recently been developed (Wilson et al. 1986, Bodell and Rasmussen 1984, Fennell et al. 1987, Watson et al. 1987).

5.1 Applications of the Postlabeling Technique

The ability of numerous chemicals of diverse structure to bind to DNA in vitro has been reported using the postlabeling assay (Randerath et al. 1981, Gupta et al. 1982). DNA adducts in bacteria, and in mammalian tissue-culture cells exposed to polycyclic aromatic hydrocarbons have been assayed (Arce et al. 1987, Dunn and San 1988). The assay has been used extensively to measure the DNA adducts formed in various tissues of animals exposed to known carcinogens (Lu et al. 1986). The analysis of DNA adducts formed in tissues of animals exposed to mixtures containing unknown carcinogens (e.g. cigarette smoke condensate, diesel exhaust) has been reported (Randerath et al. 1986, Wong et al. 1986). Postlabeling analysis was used to detect age related

adducts in non-exposed laboratory rats (Randerath et al. 1986). The tissues of bottom dwelling fish from polluted waters show adducts when analysed (Dunn et al. 1987).

Currently, the method is being extensively applied to the detection and quantitation of adducts in humans voluntarily or involuntarily exposed to DNA damaging agents. Exposure related aromatic/bulky adducts have been detected in the placentae, oral mucosa, bronchus, white blood cells, bronchial tissue and lungs of smokers (Everson et al. 1986, Everson et al. 1987, Everson et al. 1987, Chacko and Gupta 1987, Randerath et al. 1987). Adducts have also been detected in the peripheral blood lymphocytes of foundry workers subjected to a known exposure to polycyclic aromatic hydrocarbons (Phillips et al. 1987).

5.2 Advantages of ^{32}P -Postlabeling Methods for the Detection of DNA Adducts.

Several features of the method make it suitable for the monitoring of human exposure to genotoxic chemicals and as a tool for studies of chemical carcinogenesis (reviewed by Watson 1987). Using this technique, the DNA damaging ability of any chemical or mixture in vivo or in vitro can be measured. The range of chemicals which can be tested is not limited by their availability in radiolabeled form. The detection of DNA binding using this assay does not require prior knowledge of the chemical identity of the adducts or carcinogens. Only 1-10 μg of DNA is required for an analysis. This makes possible the detection of DNA adducts in small biopsy specimens or exfoliated cells (e.g. oral mucosal) from exposed individuals without pooling the samples. The sensitivity of detection for aromatic/bulky adducts in a 10 μg DNA sample is about 1 adduct in 10^{10} nucleotides or 1-10 adducts per diploid mammalian genome (Reddy and Randerath 1986). For most adducts the method gives accurate quantitation and gives satisfactory reproducibility and recovery (Fennell et al. 1986, Gupta et al. 1982). The method is versatile in

that it allows for the simultaneous analysis of many different DNA adducts in a single DNA sample.

Studies employing dozens of test compounds have shown that each particular chemical or mixture produces a unique fingerprint pattern of DNA adducts (Reddy et al. 1984). In principle the identity of chemical exposure can be inferred by comparison of fingerprints between the test DNA sample and those produced by exposure of DNA to known chemicals. The creation of a databank composed of postlabeling fingerprints for all suspect chemicals has been proposed (Watson 1987).

The assay is more specific for known adducts than immunoassays because antibodies exhibit cross-reactivity with adducts of similar structure (Strickland and Boyle 1984). The study of adduct binding to defined DNA sequences and genes is encouraged due to the minimal amount of DNA required for analysis (Gupta 1984, Gupta et al. 1985). Finally, the method is potentially useful for investigating the repair and persistence of DNA adducts. It should also be applicable to the investigation of the effects of chemopreventative agents and/or metabolic inhibitors on adduct formation/persistence.

6. The Significance of O⁶-methylguanine in Carcinogenesis

6.1 Formation of O⁶-methylguanine

O⁶-methylguanine is formed by the reaction of an electrophilic diazonium ion with the O⁶ oxygen of guanine in DNA in vitro or in vivo. Methylating agents (e.g. some N-nitrosamines and N-nitrosamides) react with DNA giving this adduct plus more than 10 other methyl adducts (Singer 1985). O⁶-methylguanine is a minor product of methylation and usually accounts for between 0.1% and 7% of all methyl adducts.

6.2 Historical Perspectives

Loveless was the first to propose that alkylation at the O⁶ position of guanine is the critical event in carcinogenesis and mutagenesis by alkylating agents (Loveless 1969). He correlated the ability of chemicals to effect O⁶-methylation of deoxyguanosine with their mutagenic activity in T-even bacteriophages. The causative mechanism, according to Loveless was mispairing of O⁶-methylguanine with thymine due to deprotonation of guanine nitrogen N¹, leading to G/C → A/T transitions on replication.

6.3 Repair of O⁶-methylguanine

The biological importance of alkylation at the O⁶ position of guanine is emphasized by the fact that most cells possess a special repair pathway solely for this damage (Yarosh 1985). Olsson and Lindahl were the first to deduce the nature of this process in *E. coli* (Olsson and Lindahl 1980). They observed methyl group transfer from the O⁶ position of guanine in DNA to a cysteine group in an acceptor protein, generating a S-methyl-L-cysteine residue and restoring the guanine base. Later, this activity was attributed to a 37,000 MWt. protein deemed to be O⁶-alkylguanine-DNA-alkyltransferase (AT) (Demple et al. 1982). This protein is able to demethylate larger alkyl groups, albeit much less efficiently (Morimoto et al. 1985).

Normally, bacteria contain about 13-60 molecules of AT per cell (Mitra et al. 1982). However, on exposure to low levels of O⁶-methylguanine producing chemicals, the transferase gene is derepressed and upwards of 3000 molecules per cell are produced. This so-called adaptive response in bacteria, resulting in elevated levels of AT, confers resistance to the mutagenic and toxic effects of methylating agents (Samson and Cairns 1977).

Mammalian (including human) cells have an equivalent enzyme (Renard and Verly 1980). There has been some suggestion of a similar though less profound adaptive response to low levels of chemicals in animal cells (Montesano et al. 1979). In normal human tissues and cells the AT activity varies up to 1,000 fold between tissues and individuals (Grafstrom et al. 1984). In addition, the AT activity of human tissues is usually several orders of magnitude greater than that of the equivalent rodent tissues (Hall et al. 1985). Interestingly, the activity of the enzyme is highest in tissues (liver, digestive tract) which might be first exposed to dietary alkylating agents (Kyrtopoulos et al. 1984).

Several human tumor cell lines and cells from individuals with genetic DNA repair deficiency diseases lack the ability to repair O⁶-methylguanine (Day et al. 1980). Consequently, they are hypersensitive to the mutagenic, toxic and clastogenic effects of methylating agents (Domoradziki et al. 1984). Mammalian cells lacking AT activity have been transfected with the cloned AT gene (*ada*) of *E. coli*, resulting in the restoration of repair activity (Samson et al. 1986, Jelinek et al. 1988). The activity of AT can be diminished by the administration of the free base, O⁶-methylguanine, to cells (Dolan et al. 1985).

The repair of O⁶-methylguanine in most cells is biphasic, characterized by a rapid early phase followed by slow late repair (Shiloh and Becker 1981). This has been explained by the greater rate of repair of O⁶-methylguanine at high adduct levels (Scicchitano and Pegg 1982). AT has been referred to as a suicidal enzyme because once it has reacted with O⁶-methylguanine only RNA and protein synthesis can restore its level (Yarosh 1985). The enzyme is not regenerated after methyl group acceptance and therefore is a dead-end complex. Thus, the system is saturable. The loss of O⁶-methylguanine from DNA is coupled with stoichiometric formation of S-methyl-cysteine and therefore is

the exclusive mechanism for repair. The transfer and accepting of the methyl group are performed by the same protein.

6.4 Correlation of O⁶-methylguanine Persistence with Carcinogenesis by N-Nitrosamines

Numerous investigators have attempted to define a connection between the frequency of tumorigenesis in a target tissue and the persistence or lack of repair of O⁶-methylguanine. In early experiments, after a single administration of methylnitrosourea (MNU) to rats, O⁶-methylguanine was observed to be removed far less rapidly from the DNA of the brain (the principal target organ) than the liver which is not susceptible to MNU (Kleihues and Margison 1974). In a similar single dose trial the long-term fate of MNU derived O⁶-methylguanine was monitored. In brain, unlike lung, kidney or liver, this modified base tended to persist for much of the animal's lifetime (Kleihues and Bucheler 1977).

Experimental tumorigenesis by repeated chronic exposure to a low dose of carcinogen is probably more relevant to the biology of cancer in humans than single-dose protocols. In a landmark study, Margison and Kleihues (1975) measured the levels of O⁶-methylguanine in the DNA of rat tissues during weekly injections of MNU; a regime which selectively induces nervous system tumors. During 5 weekly injections of MNU, O⁶-methylguanine accumulated in the brain to an extent greatly exceeding that of the kidney, spleen, intestine and liver. The latter organ had less than 1% the O⁶-methylguanine compared to the brain. Also, between the first and fifth injections the ratio of O⁶-methylguanine to 7-methylguanine (the major methyl adduct) in cerebral DNA increased from 0.20 to 0.68.

O⁶-methylguanine has been connected to transformation in tissue-culture systems. Syrian hamster embryo cells treated with methyl methanesulphonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and MNU were assayed for transformation and induction/repair of O⁶-methylguanine (Doniger et al. 1985). On a molar basis, MNNG was approximately 100 and 500 fold more effective in transformation than MNU and MMS respectively. The formation of O⁶-methylguanine was the same for the 3 carcinogens at concentrations that induced equivalent transformation frequencies. For MMS, however the induction of 7-methylguanine was 30-fold higher.

6.5 The Molecular Mechanism of Mutagenesis by O⁶-methylguanine

Research in the past ten years has provided several lines of evidence that strongly suggest O⁶-methylguanine is responsible for the vast majority of gene mutations produced in mammalian cells by methylating agents (Pegg and Singer 1984).

6.5.1 In Vitro

Oxygen-alkylation of guanine results in the deprotonation of N¹ and the modified base can no longer recognize cytosine (Parthasarathy and Fridey 1986). Biochemical studies have shown that thymidine or uridine gets incorporated preferentially opposite this adduct in co-polymers for *E. coli* polymerase I (Abbott and Saffhill 1979) or RNA polymerase (Gerchman and Ludlum 1973). Also, O⁶-methyldeoxyguanosine 5'-triphosphate is preferentially incorporated against thymine on a template of calf-thymus DNA (Hall and Saffhill 1983) or pBR322 DNA (Toorchen and Topal 1983). Therefore, alkylated guanine acts as an adenine as far as base pairing is concerned and recognizes thymine.

6.5.2 In Vivo

6.5.2.1 Site-directed Mutagenesis

Recently, the advent of molecular techniques has enabled the genetic consequences of O⁶-methylguanine specifically incorporated in a known position in the genome to be assessed. In one case, DNA analysis of 60 mutant viral genomes revealed that O⁶-methylguanine induced exclusively G → A transition mutations (Loechler et al. 1984).

E. coli carrying the xanthine guanine phosphoribosyltransferase (gpt) gene on a pSV2gpt plasmid were exposed in vivo to MNU (Richardson et al. 1987). Using this forward mutation system and di-deoxy chain terminating DNA sequence methods, the type and frequency of specific DNA base changes (mutations) were compared to the level of O⁶-methylguanine in the bacterial genome. It was found that 100% of MNU induced mutations were G/C → A/T transitions and 82% of the changes occurred at the middle guanine of the sequence 5'-GG(A/T)-3'.

In vivo, O⁶-methylguanine directed to a specific location in ϕ X174 phage replicating form DNA has a mutagenic efficiency of 75% due exclusively to thymidine misincorporations (Bhanot and Ray 1986).

6.5.2.2 Studies Comparing the Level of O⁶-methylguanine Adduction and the Frequency of Specific Locus Mutation in Mammalian Cells

Newbold and co-workers reported the frequency of induction of 8-azaguanine resistance and ouabain resistance in Chinese hamster V79 cells treated with MMS, dimethylsulphate, and MNU (Newbold et al. 1980). They found that the mutagenicity reflects the carcinogenicity of these compounds and that differences in mutagenicity are paralleled by differences in levels of O⁶-methylguanine. In these repair defective cells, they observed a high

correlation between the concentration of O⁶-methylguanine and the frequency of mutation.

Suspensions of repair defective Chinese hamster ovary cells were exposed to MMS or MNU and assayed for mutation and a variety of DNA adducts including O⁶-methylguanine (Beranek et al. 1983). Only O⁶-methylguanine correlated strongly ($r=0.879$, probability less than 0.001) with the frequency of mutation at the hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) locus.

6.6 Initiation of Carcinogenesis through Activation of Cellular Proto-oncogenes by O⁶-methylguanine Induced Mutation

There is growing consensus that cellular proto-oncogenes are the genetic targets for initiation of carcinogenesis by chemicals (Barbacid 1986, Bishop 1987). For example, it is now believed that activation of H-ras proto-oncogenes by point mutations is a result of DNA damage produced by a carcinogen rather than as a consequence of the transformation process (Bohr et al. 1987).

The role of MNU induced O⁶-methylguanine mutation of the H-ras-1 oncogene in a rat mammary carcinogenesis model has been reported recently (Zarbl et al. 1985). A high percentage of rats administered a single dose of MNU develop mammary carcinomas. It was found that 83% of the chemically induced tumors contained an activated H-ras-1 locus as determined by two independent methods (NIH 3T3 transformation and restriction fragment length polymorphisms). All of the activated oncogenes had a G/C → A/T transition at the middle guanine of codon 12 (5'-GGA-3'). This observation supports the author's contention that mutations are caused by MNU directed methylation of the O⁶ position of the second guanine in codon 12. This is exactly what would be predicted if O⁶-methylguanine were responsible for mutation and neoplastic transformation.

In the rat mammary tumor model, cancer is achieved by a single dose of MNU. Due to the highly labile nature of MNU, adduct formation must occur within minutes of its administration. The results imply that malignant activation of the Ha-ras-1 locus by MNU induced G → A mutation is concomitant with initiation of carcinogenesis.

Human ras oncogenes have been found to be produced by a modification of a G → A transition or by a G → T transversion occurring at the same position (codon 12) as the transforming H-ras-1 gene in mammary carcinomas induced by MNU.

7. Ultra-sensitive Methodology for the Detection of O⁶-methylguanine DNA Adducts

The information presented above supports the current hypothesis that O⁶-methylguanine is the critical molecular lesion whose formation is necessary but probably not sufficient for the initiation of carcinogenesis by some N-nitrosamines (Pegg 1984).

Methods for the detection of O⁶-methylguanine in DNA include: a) HPLC followed by scintillation counting after administration of labeled carcinogens, b) HPLC coupled with fluorescence detection and c) radioimmunoassays. Only the latter two are applicable to human studies.

A typical sensitivity for the HPLC/fluorescence assay is 0.6 μmole/mole using 0.26 mg DNA (Herron and Shank 1979, Swenberg and Bedell 1982). The sensitivity of this assay is limited by the fluorescence at similar wavelengths of other compounds which may co-elute with O⁶-methylguanine on HPLC. Of the available methods, only immunoassays based on monoclonal antibodies appear sufficiently sensitive to detect O⁶-methylguanine in exposed humans (Wild et al. 1983, Wild et al. 1986, Castonguay et al. 1985). High sensitivities are achieved through HPLC recovery of O⁶-methyldeoxyguanosine

from several mg digested DNA before antibody binding. With these techniques the ability to detect low levels of adduct is limited only by the amount of DNA available for hydrolysis. The highest sensitivity for a radioimmunoassay reported in the literature is 0.008 $\mu\text{mole/mole}$ for HPLC analysis of 1 mg digested DNA (Umbenhauer et al. 1985). Recently a very high affinity murine monoclonal antibody giving 50% inhibition with 1 fmole O^6 -methyldeoxyguanosine has been reported (Parsa et al. 1987). If this amount of adduct arose from 1 mg DNA the sensitivity would be 0.0003 $\mu\text{mole/mole}$.

8. Current Problem

Exposure of humans to N-nitrosamines is associated with up to 30% of all cancer deaths in North America and is a significant contributor to disease in many countries (Bartch and Montesano 1984). The principal mode of exposure is through voluntary lifestyle practices, especially tobacco smoking and chewing (Hoffman and Hecht 1985, World Health Organization 1985). Other sources are the food, work and living environments (Singer et al. 1986, IARC 1978, Moloney et al. 1985). The bulk of carcinogenic activity in tobacco smoke and chewing tobacco extracts is due to a class of compounds known as tobacco specific N-nitrosamines (Hoffman and Hecht 1985).

Many of these agents are capable of forming O^6 -methylguanine in the cellular DNA of exposed individuals. The measurement of O^6 -methylguanine in exfoliated cells from susceptible tissues may be significant due to the mutagenic potential of this adduct and the strong correlation of DNA oxygen alkylation with the carcinogenic potency of N-nitrosamines.

To achieve high sensitivities, immunoassays for O^6 -methylguanine require milligram quantities of DNA and are therefore not applicable to the analysis of small samples of exfoliated cells from individuals. Cross-reactivity of even monoclonal antibodies with other adducts reduces the selectivity of this

method. There is a requirement for an alternative method able to detect low levels of O⁶-methylguanine in microgram quantities of DNA.

9. Objectives

The goals of this project were:

1. To develop a ³²P-postlabeling assay for the detection and quantitation of O⁶-methylguanine adducts in microgram quantities of DNA. Method development proceeded chronologically in two stages:
 - a) Synthesis and characterization of O⁶-methyldeoxyguanosine 3'-monophosphate (O⁶mdG3'p).
 - b) Development of chromatographic procedures for the isolation of O⁶mdG3'p from digested DNA and quantitation of O⁶-methyldeoxyguanosine 3',5',³²P-bisphosphate (O⁶mdG3'5'p) after postlabeling.
2. To evaluate the performance of the method and ascertain the feasibility of its application to molecular epidemiology studies in humans exposed to N-nitrosamines.
3. To detect and quantitate O⁶-methylguanine in the DNA of mammalian cells treated with a carcinogen (MNU) known to form this adduct, using the new methodology.

MATERIALS

1. Chemicals and Enzymes

Deoxyguanosine was purchased from Boehringer Mannheim, Dorval, Quebec. Diazomethane in ether was prepared by distillation of an alkaline solution of N-methyl-N-nitrosotoluenesulfonamide (Diazald, Sigma Chemical Company). The distillation apparatus was from the Aldrich Chemical Company. Anhydrous KH_2PO_4 (ACS) was purchased from Fisher Scientific Company. Redistilled formamide was from Bethesda Research Laboratories. Lyophilized snake venom, nuclease P1, and reagents for the preparation of ^{32}P -ATP were from Boehringer Mannheim, Dorval, Quebec or from the Sigma Chemical Company, St. Louis, MO. Polynucleotide kinase, cloned, was purchased from U.S. Biochemicals, Cleveland, OH. Proteinase K (Protease Type XI), ribonuclease-A (Type 1-A from bovine pancreas), micrococcal nuclease, spleen phosphodiesterase, and potato apyrase were purchased from the Sigma Chemical Company, St. Louis, MO. 2-Ethoxyethanol was from BDH Chemicals, Toronto, Ontario. Undenatured ethanol (100%) for DNA precipitations was distilled twice in glass. Phenol, for DNA extractions, was glass-distilled, equilibrated with Tris-Cl buffer pH 8.0, and stored frozen at -20°C before use. Isobutyric acid was from BDH Chemicals Ltd., Poole, England. MNU was from the Sigma Chemical Company, St. Louis, MO. and contained 25% by weight of a 3% acetic acid solution as a preservative. All other chemicals were reagent grade or better.

2. Equipment

U.V. spectra were obtained on a Lambda 3 UV/VIS spectrophotometer (Perkin-Elmer Corporation). The 'multi-tube' vortexer (Model 2600) was from Scientific Manufacturing Industries. Four ml polypropylene tubes were purchased from Elkay Products, Inc., of Shrewsbury, MA. Heat for the

phosphorylation was provided by a 'Reacti-Therm' heating module (Pierce Chemical Company, Rockford, Ill). Kodak 'X-Omat' XAR-5 X-ray film (8 X 10 in) was exposed to chromatograms in Kodak 'X-Omatic' cassettes (8 X 10 in) equipped with two Dupont Cronex 'lightening-plus' intensifying screens. Chromatograms were marked with phosphorescent ink using an 'Ultemit' autoradiography marker purchased from NEN Research Products.

3. Chromatography

HPLC columns for the chromatography of deoxynucleoside 3'-monophosphates, deoxynucleoside 5'-monophosphates, and deoxynucleoside 3',5'-bisphosphates were custom packed by Alltech, Deerfield, Il. They were 2.1 mm inside diameter X 100 mm and contained 5 micron ODS-3 reverse phase packing manufactured by Whatman, Clifton, NJ. Column eluant was monitored at 260 nm using a Beckman 165 UV absorption detector coupled to a Spectra-Physics SP4270 computing integrator. A Perkin-Elmer 2/2 HPLC pumping system was used to produce high-pressure solvent.

1 M ammonium formate pH 3.5 (for preparation of HPLC solvent) was made from glass distilled formic acid and purified ammonium hydroxide. One mole formic acid in 800 ml ddH₂O was titrated with ammonium hydroxide to pH 3.3, then the solution cooled to room temperature. The titration to pH 3.5 was then completed and the volume made up to one liter. Reagent grade ammonium hydroxide and formic acid were used to prepare 2.25 M ammonium formate pH 3.5 for TLC. Ammonium formate solutions for buffering of DNA digests and HPLC were made using glass distilled formic acid and ammonium hydroxide. HPLC fractions were dried down using a centrifugal vacuum evaporator (Model SVC 100H, Savant Corp., Hicksville, N.Y.).

PEI cellulose thin layer chromatography sheets (20 X 20 cm) were from Mackery Nagel, Germany and purchased through Brinkmann Instruments, Rexdale,

Ont. Cellulose coated TLC plates (20 X 20 cm, 0.1 mm layer thickness) were from E. Merck, Darmstadt, W. Germany and purchased through BDH, Toronto, Ontario, Canada.

4. Tissue-culture

The CHO cell line (wild type) was obtained from Dr. Lorne Whorton, University of Toronto, Canada. These cells are a subclone of the line originally described by Kao and Puck (Gen. 55; 513-518, 1967). They were cultured in Eagle's Minimum Essential Medium (MEM) containing Hank's salts supplemented with 7.5% NaHCO_3 , 10% fetal calf serum and antibiotics (kanamycin, fungizone, penicillin, and streptomycin). MNU was dissolved in 'wash' MEM which consisted of the above described 'growth' medium without fetal calf serum, kanamycin, and fungizone. The tissue-culture flasks (175 cm^2 for CHO experiments and 80 cm^2 for C3H10T1/2 experiments) were purchased from Nunclon (Nunc, Intermed). The cells were trypsinized using 0.1% trypsin in PBS.

Mouse-embryo fibroblast cells (C3H10T1/2 Clone 8) were obtained from the American Type Culture Collection, Rockville, Maryland. These cells are a subclone of a line of C3H mouse embryo cells initiated by C. Reznikoff, D. Brankow, and C. Heidelberger in 1972 (Cancer Res. 33: 3231-3238, 1973). They were cultured and exposed to MNU in Eagle's Basal Medium (BME) supplemented with 7.5% NaHCO_3 , 10% fetal calf serum, L-glutamine, and antibiotics (penicillin, streptomycin, kanamycin, and fungizone). For the colony forming assay the cells were grown in 60 mm dishes (with grid) obtained from Nunclon.

METHOD DEVELOPMENT

1. Synthesis of O⁶-methyldeoxyguanosine 3'-monophosphateIntroduction

DNA containing O⁶-methylguanine residues should give O⁶mdG3'p when digested with micrococcal nuclease and spleen phosphodiesterase. The structure of this compound is shown in Figure 3. Identification and quantitation of O⁶-methylguanine by ³²P-postlabeling required the availability of synthetic O⁶mdG3'p as a chromatography marker. No published synthesis was available for this compound.

Initially, two routes were investigated to prepare O⁶mdG3'p. Direct methylation of deoxyguanosine 3'-monophosphate with diazomethane was unsuccessful, presumably because phosphate methylation predominates. Diazomethane methylation of DNA followed by enzymatic digestion to deoxynucleoside 3'-monophosphates failed, likely for the same reason. Phosphorylation of O⁶-methyldeoxyguanosine, for which there are many reported syntheses (Farmer et al. 1973, Bernadou et al. 1983), using POCl₃ in triethylphosphate gave largely O⁶-methylguanine through probable acidic decomposition.

Amide catalysed condensation of deoxynucleosides with orthophosphate salts at elevated temperatures gives isomeric mixtures of deoxynucleoside monophosphates (Philipp and Seliger 1977, Schoffstall 1976, Schoffstall and Kokko 1978, Schoffstall et al. 1982, Schoffstall and Laing 1984). A preliminary experiment showed that reaction of excess KH₂PO₄ with deoxyguanosine for 9 hours in formamide at 100°C gives a major product that co-chromatographs with deoxyguanosine 3'-monophosphate.

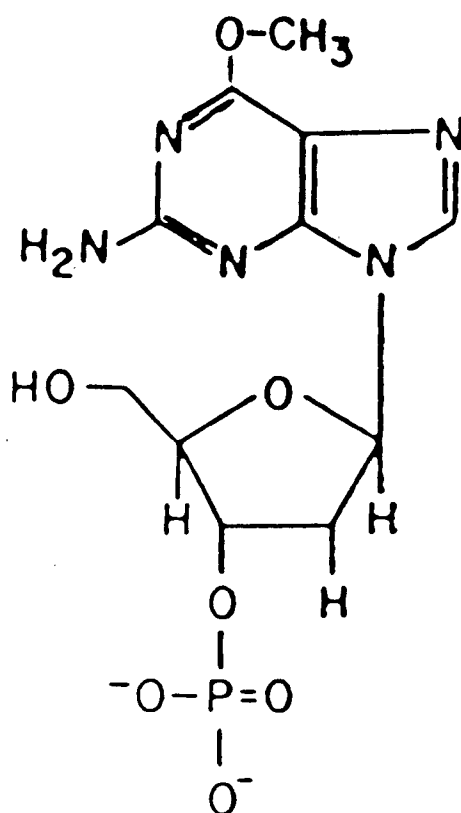


Figure 3 Chemical structure of 0⁶mdG3'p.

Therefore, it was decided to attempt the synthesis of $O^6\text{mdG}3'\text{p}$ in a two stage fashion; first, preparation of $O^6\text{mdG}$ by diazomethane methylation of deoxyguanosine (Farmer et al. 1973) then phosphorylation of the compound using KH_2PO_4 in formamide.

Methods and Results

1.1 Preparation of O^6 -methyldeoxyguanosine

Methylation of deoxyguanosine

To a suspension of 95 mg deoxyguanosine (.356 mmole) in 15 ml methanol was added 34 ml freshly prepared ethereal diazomethane (7.12 mmole). The resulting yellow suspension was loosely stoppered then stirred at room temperature. After two hours most of the deoxyguanosine had reacted, some diazomethane remained and an insoluble white precipitate appeared. The mixture was then reduced to dryness by rotary evaporation at 30°C . The residue left behind was dissolved in 1.5 ml 0.1 M ammonium formate pH 3.5 containing 20% methanol to give a yellowish solution. A small amount of insoluble white material was removed by centrifugation.

In addition to $O^6\text{mdG}$, N^1 and N^7 -methyldeoxyguanosine are major products of the methylation of deoxyguanosine with diazomethane (Farmer et al. 1973). Of these components, $O^6\text{mdG}$ should elute last from a reverse phase HPLC column (Abbott et al. 1980). When a small sample of the crude reaction mixture was fractionated on a semi-prep column, four major peaks were seen (Fig 4). The last peak (15 min) was tentatively identified by U.V. spectrophotometry as $O^6\text{mdG}$ (see below).

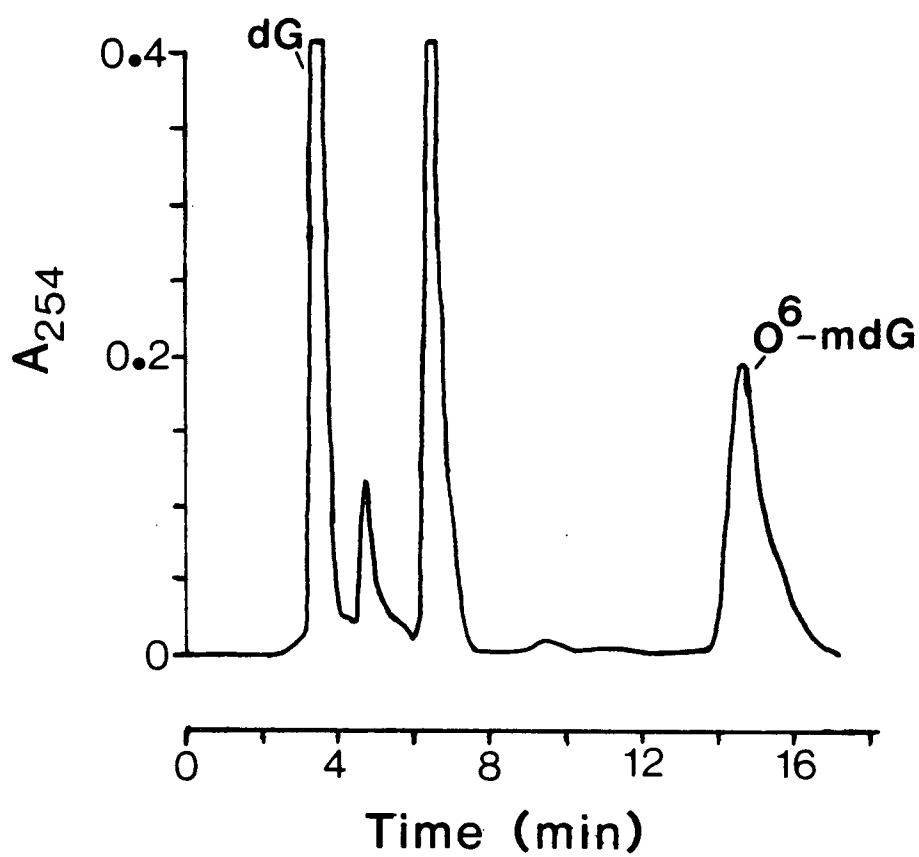


Figure 4 HPLC elution profile of isomeric methyldeoxyguanosines resulting from the methylation of deoxyguanosine with ethereal diazomethane.

Isolation and Purification of O⁶-methyldeoxyguanosine

O⁶mdG was isolated and purified on a Whatman semi-preparative HPLC column (0.9 cm X 25 cm) containing 10 micron ODS-3 reversed phase packing. Ammonium formate (0.1 M, pH 3.5) containing 20% methanol was pumped through the column at 5 ml/min using a Perkin Elmer pumping system. Eluant was monitored at 254 nm using an ISCO (Instrumentation Specialities Company) Type 6 optical unit coupled to an ISCO UA5 monitor.

O⁶mdG was recovered from 1500 μ l crude reaction mixture by injecting 200 μ l aliquots onto the column and collecting the last peak as it eluted. This fraction (approximately 160 ml) was reduced to dryness at room temperature using a centrifugal spin drier. The whitish product remaining was dissolved in 2 ml ddH₂O then a small portion analysed by HPLC as a check for purity. Several minor impurities were present including one that eluted just after O⁶mdG.

To remove trace contaminants from O⁶mdG, a second HPLC purification was performed. In 200 μ l aliquots, the impure product was injected onto the column and the majority of the O⁶mdG peak collected. An impurity that co-eluted with the peak tail was excluded (Fig 5). The fraction (approximately 108 ml) was dried down at room temperature using the centrifugal spin-drier, then the purified O⁶mdG dissolved in 2 ml ddH₂O and analysed by HPLC (Fig 6).

Identification of O⁶-methyldeoxyguanosine

A U.V. spectrum of the product (Fig 7) was in close agreement to the literature values for O⁶mdG (Farmer et al. 1973). Observed absorbance maxima (nm): 246, 278.5; minima: 224, 259. Literature values for maxima: 247, 278; minima: 260. The yield of O⁶mdG after HPLC recovery was 27.3 mg (27.3% of theoretical).

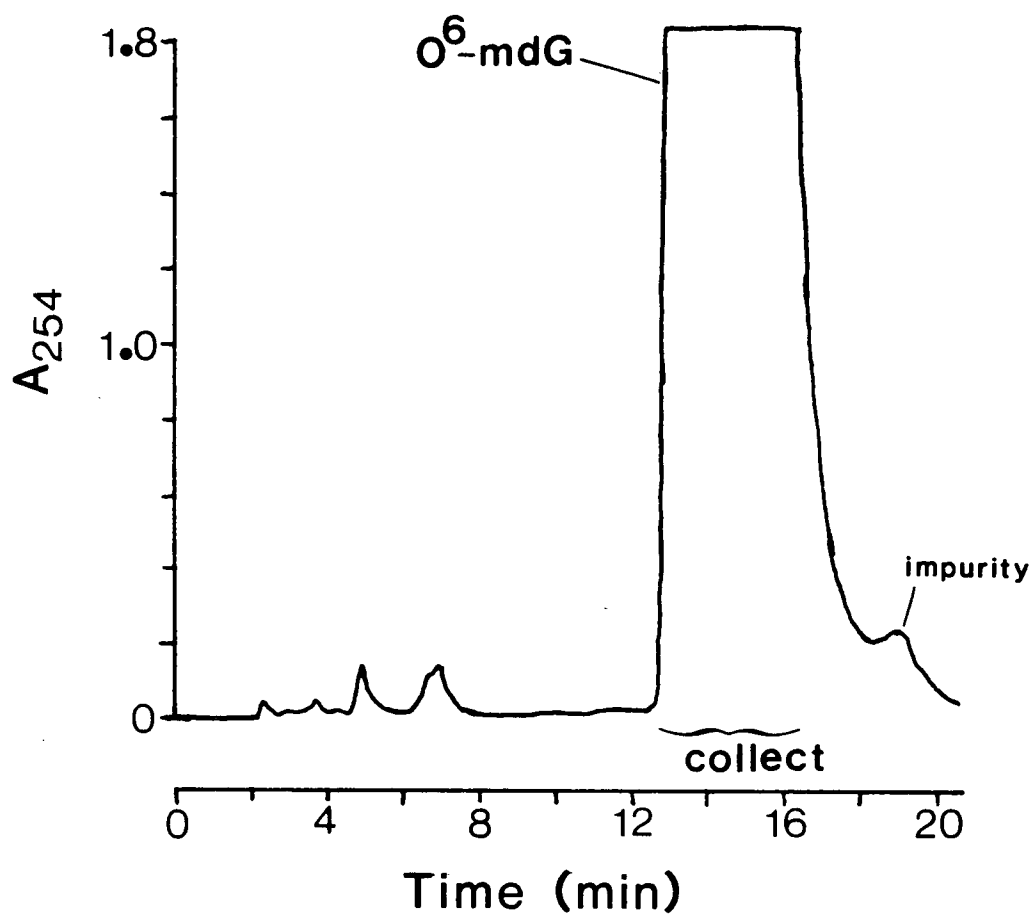


Figure 5 HPLC chromatogram of once purified O^6 mdG. The product was purified a second time by collecting the front portion of the peak to exclude a slower running impurity.

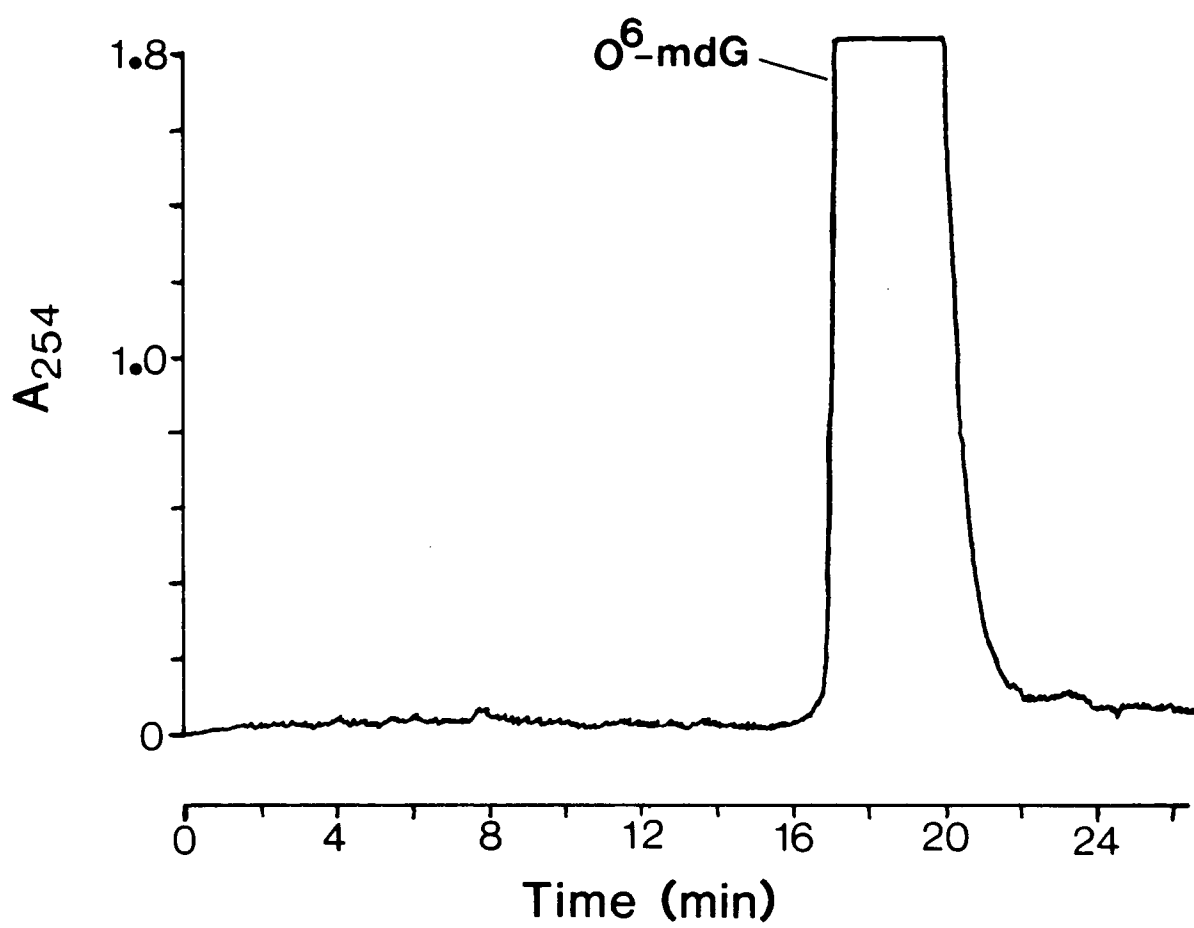


Figure 6 HPLC analysis of twice purified O^6 mdG.

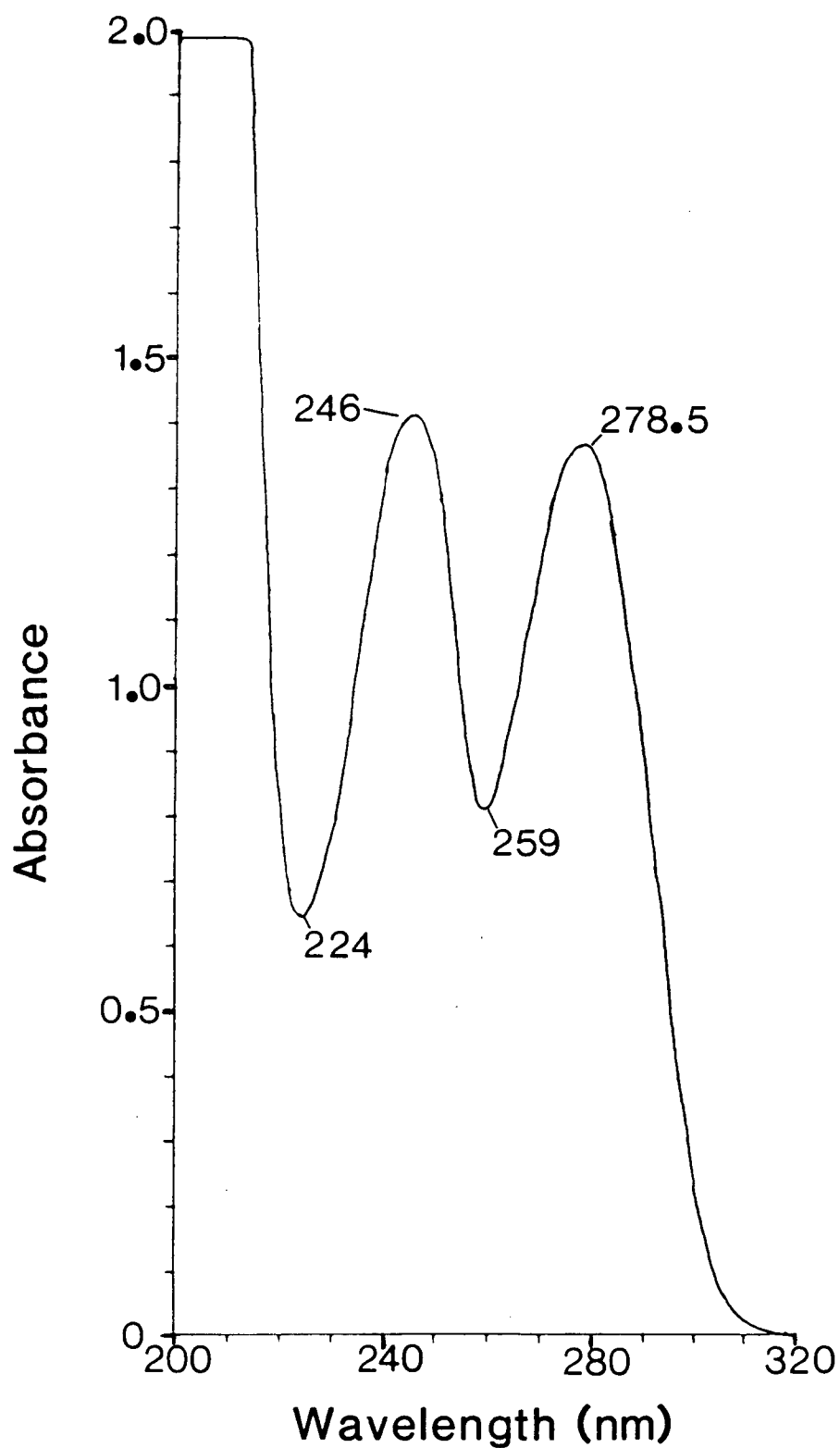


Figure 7 U.V. spectrum of 0⁶mdG in ddH₂O. Observed absorbance maxima (nm): 246, 278.5. Minima: 224, 259. Literature values (Farmer et al. 1973) for maxima (nm): 247, 278. Minima: 260.

1.2 Preparation of O⁶-methyldeoxyguanosine 3'-monophosphate

Reaction of O⁶-methyldeoxyguanosine with Potassium Phosphate in Formamide

To 13.8 mg purified O⁶mdG (.049 mmole) in a 4 ml polypropylene tube, was added 134.8 mg anhydrous KH₂PO₄ (1 mmole) and 2.4 ml formamide (60.4 mmole). On thorough mixing, some KH₂PO₄ and all of the O⁶mdG dissolved in the formamide. The tube was then stoppered and the mixture heated to 100°C in a heating block.

To monitor the phosphorylation reaction, the reaction mixture was resolved on an analytical reverse-phase HPLC column (Brownlee labs). A guard column (2.1 mm X 30 mm) was connected in series with the main column (2.1 mm X 100 mm) and eluted at 0.5 ml/min with 0.1 M ammonium formate pH 3.5 containing 10% methanol. On this column the mono- and diphosphates of O⁶mdG would be expected to elute much earlier than the parent alkylated nucleoside (Dunn, B. personal communication). After 1.5 hours heating, 1 µl of the reaction mixture was analysed by HPLC. Compared to a chromatogram of the mixture before heating (Fig 8A), a broad peak appeared that ran faster than O⁶mdG (Fig 8B) and increased in area with time. The peak reached its maximum size after nine hours.

Isolation and Purification of Putative O⁶-methyldeoxyguanosine 3'- and 5'-monophosphates

When the phosphorylation mixture was fractionated on a semi-preparative reverse-phase column the new peak was resolved into two separate peaks which were tentatively designated as the monophosphates of O⁶mdG (Fig 9). The phosphorylated products were recovered together from the crude reaction mixture by injecting 100 µl aliquots onto the semi-preparative HPLC and collecting the appropriate fraction. Solvent was removed in the spin-drier

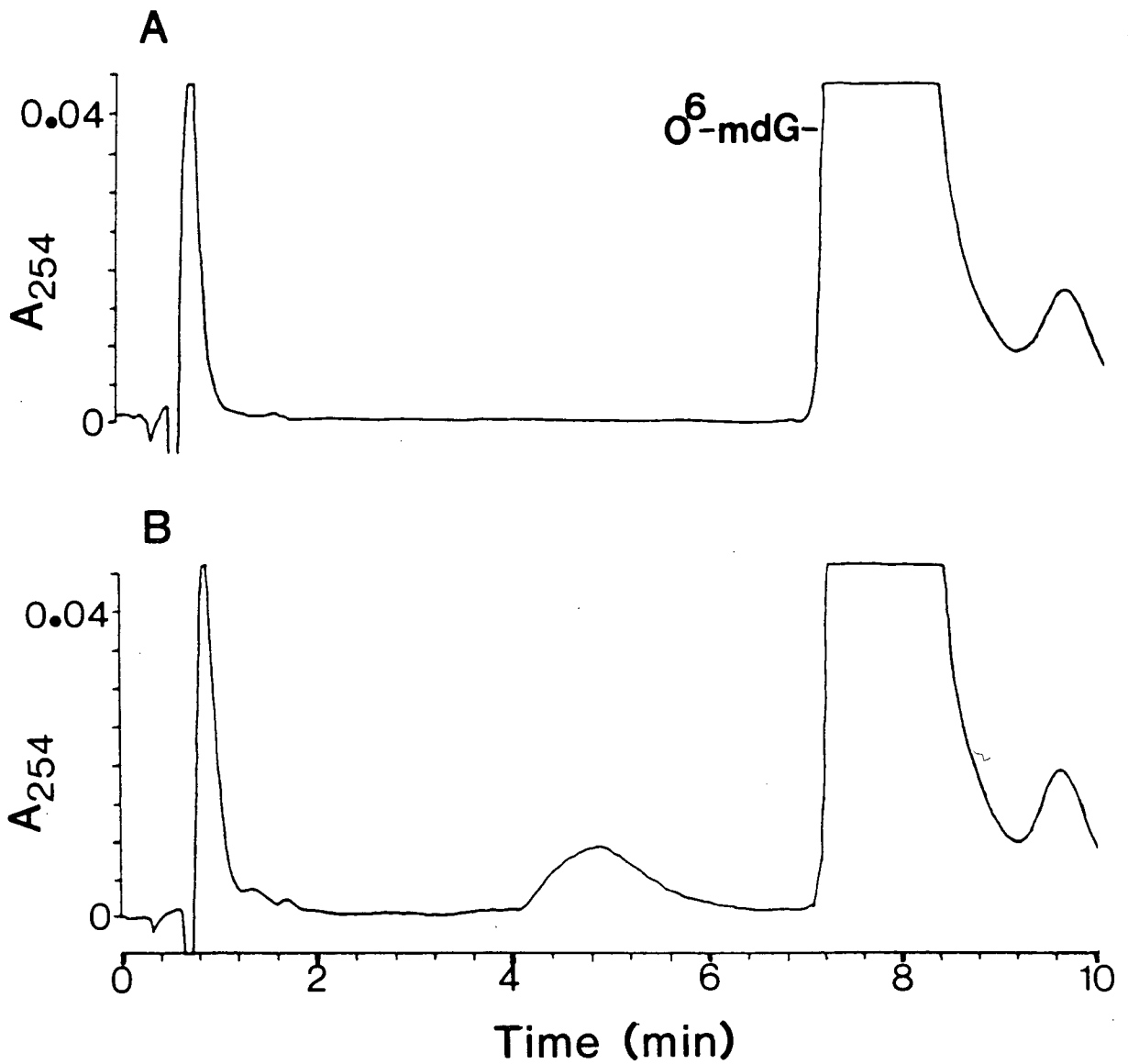


Figure 8 HPLC analysis of $O^6\text{mdG}/\text{formamide}/\text{KH}_2\text{PO}_4$ phosphorylation mixture before (A) and after (B) 1.5 hours heating at 100°C .

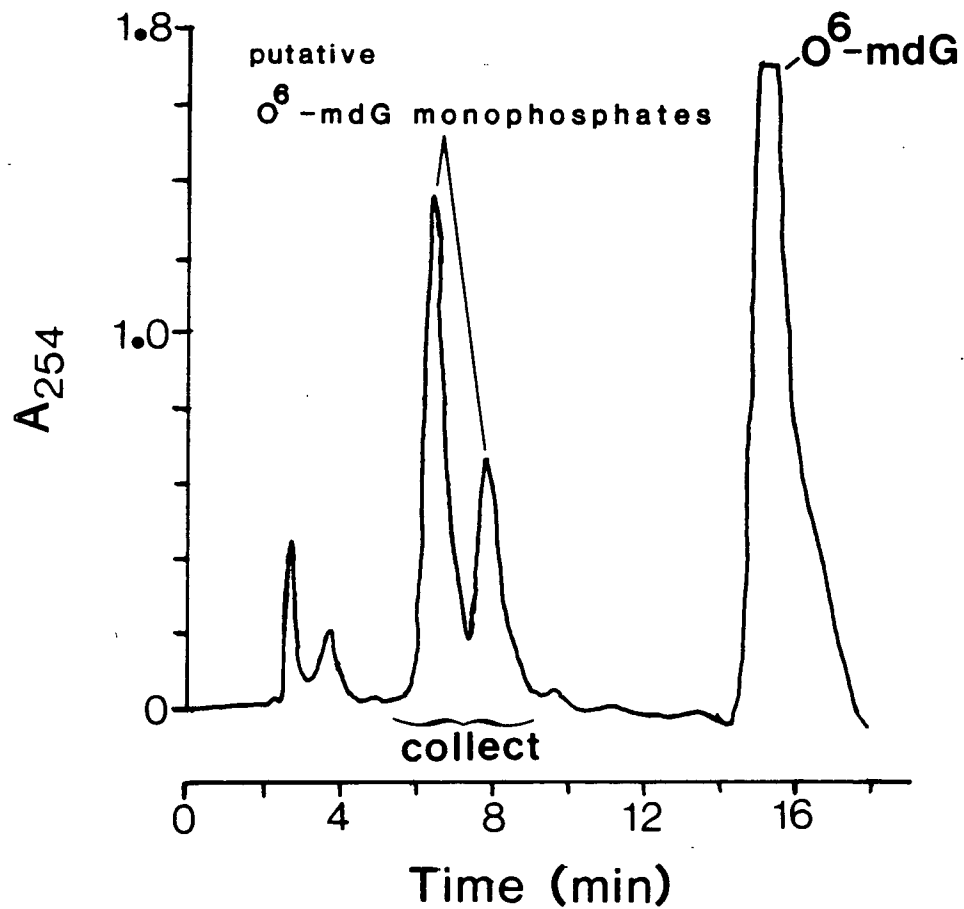


Figure 9 Putative O^6 mdG monophosphates in crude phosphorylation mixture, separated by semi-preparative HPLC. Both peaks were collected from 100 μ l injections of the reaction mixture.

then the residue dissolved in 1.5 ml ddH₂O. Figure 10 shows an HPLC chromatogram of the purified products. For reverse-phase HPLC of nucleotides, the 5' isomers generally run faster than the corresponding 3' isomers (Ramos and Schoffstall 1983). Therefore, for purposes of discussion, the first peak (Figure 10) was tentatively designated as the 3'-monophosphate of O⁶mdG.

The isomeric monophosphates were individually isolated from the mixture by injection of 100 µl aliquots onto the semi-preparative HPLC followed by separate collection of each peak. Both fractions were dried down then each of the purified products dissolved in 1.5 ml ddH₂O. The isolated monophosphates were isomerically pure as determined by HPLC (Fig 11). Based on U.V. absorption, the yields of the putative 3'- and 5'- monophosphates after HPLC isolation were 3.04 µmole (6.2%) and 3.86 µmole (7.9%) respectively.

Identification of O⁶-methyldeoxyguanosine 3'-monophosphate

Identity of the putative O⁶mdG3'p was confirmed by measurement of its U.V. spectrum and analysis of reaction products after exposure to enzymes with known specific activities.

The U.V. spectrum of putative O⁶mdG3'p (Fig 12) and O⁶mdG5'p (not shown) are similar to that of O⁶mdG (Fig 7).

Deoxynucleoside 5'-monophosphates are hydrolysed to inorganic phosphate and deoxynucleoside by the 5'-nucleotidase of *C. adamanteus* venom (Sulkowski et al. 1963, Etaix and Orgel 1978). Deoxynucleoside 3'-monophosphates are resistant to this activity. HPLC analysis revealed that putative O⁶mdG3'p was only slightly decomposed to O⁶mdG by treatment with lyophilized venom (Fig 13). Partial hydrolysis was probably caused by non-specific phosphatases present as impurities in the crude venom. O⁶mdG5'p was completely converted to O⁶mdG by this treatment (data not shown).

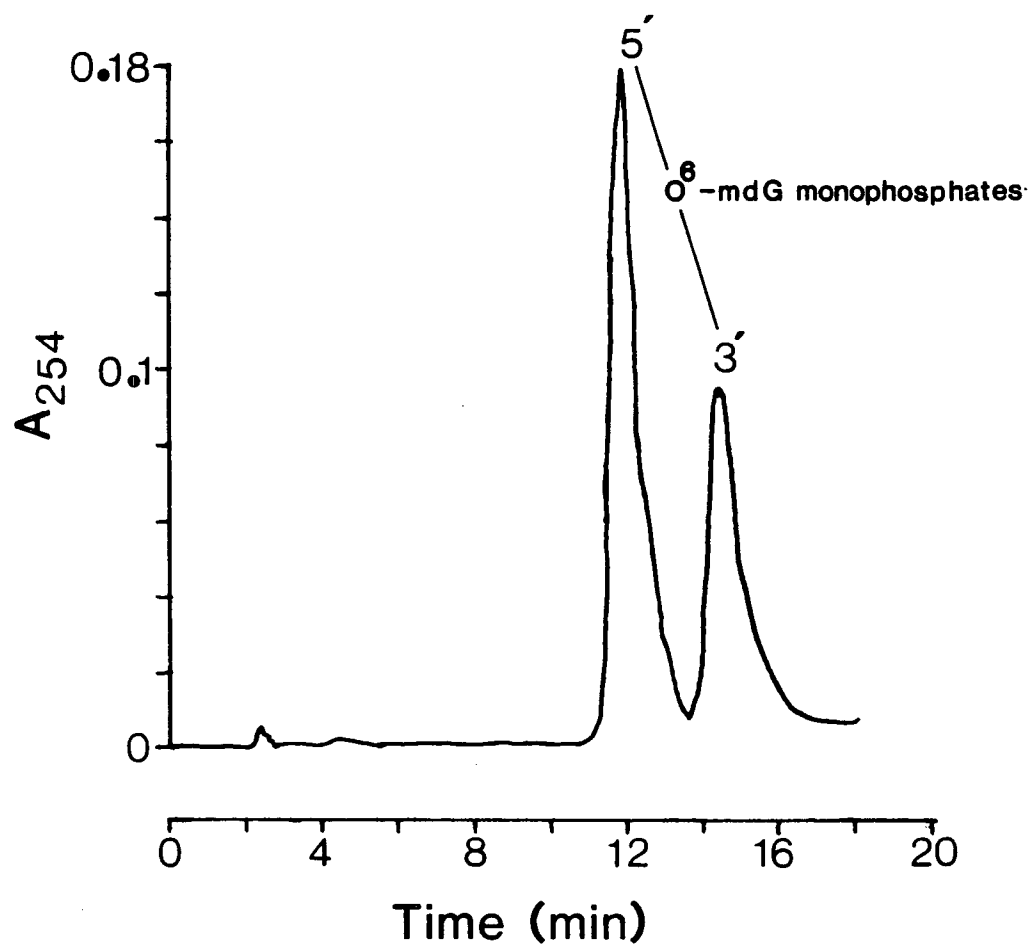


Figure 10 HPLC analysis of isomeric O^6 mdG $3'$ and $5'$ -monophosphates isolated from the reaction mixture.

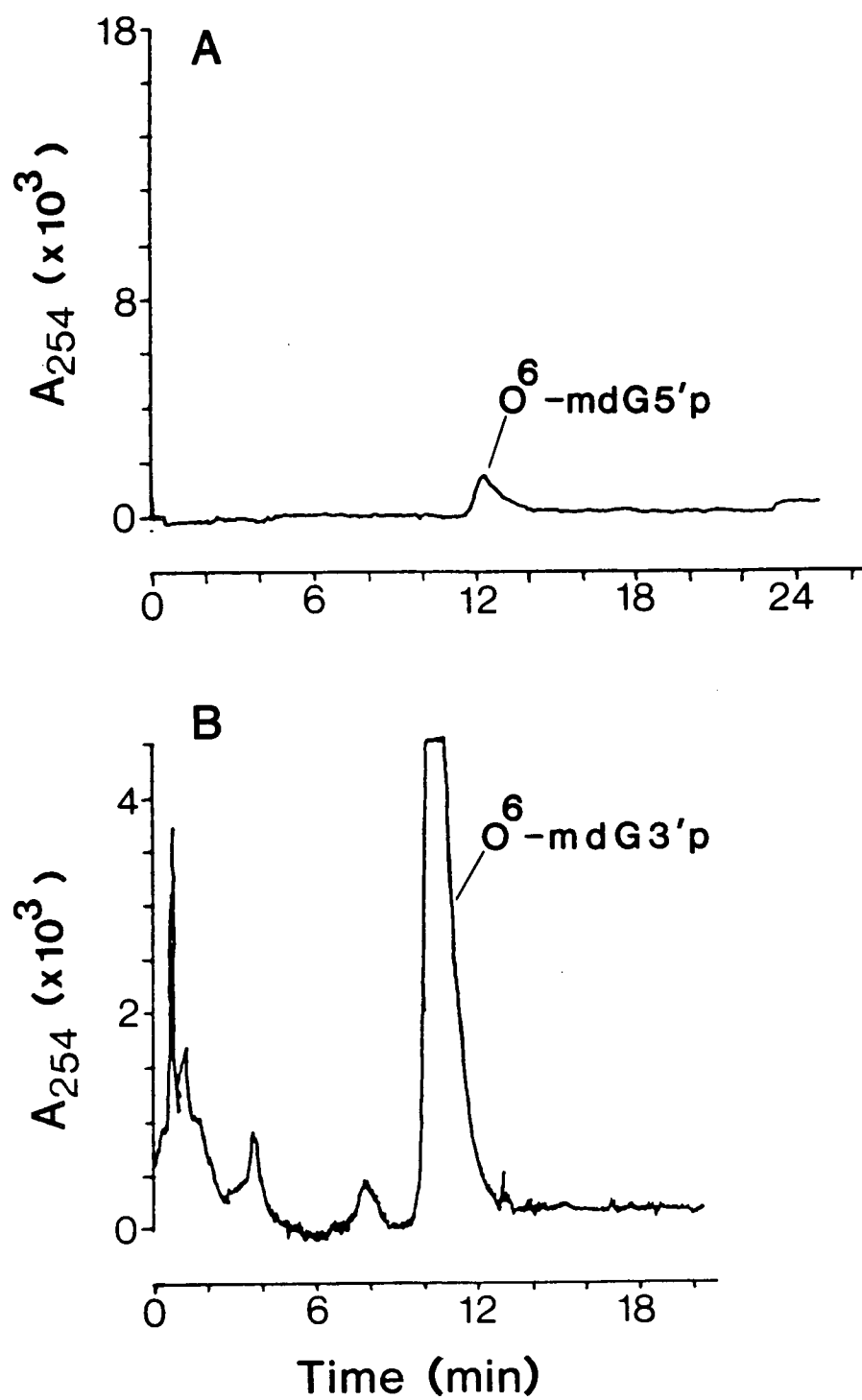


Figure 11 HPLC elution profiles of purified $O^6\text{mdG5'p}$ (A) and $O^6\text{mdG3'p}$ (B).

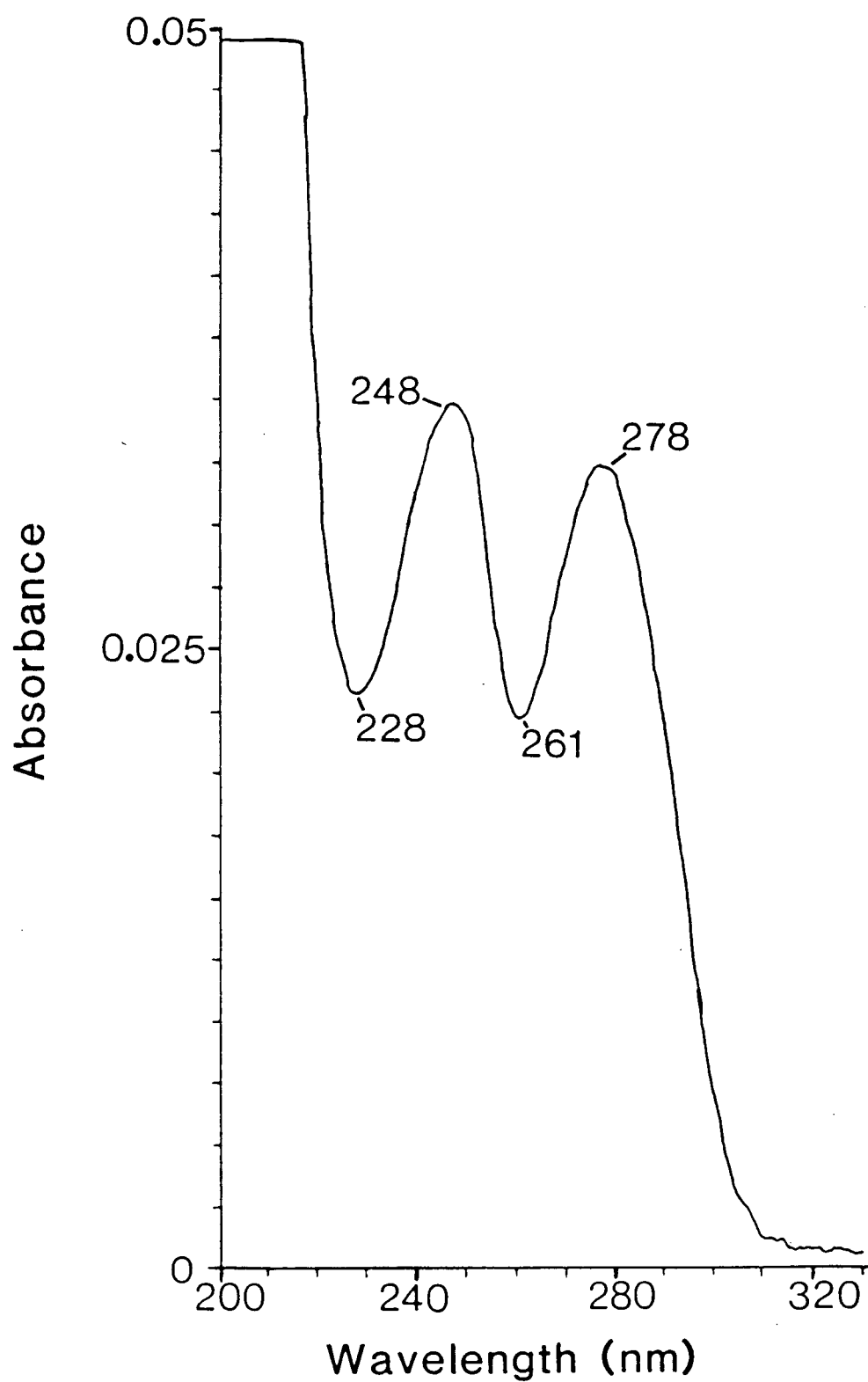


Figure 12 U.V. spectrum of $O^6mdG3'p$ in ddH_2O . Observed absorbance maxima (nm): 248, 278. Minima: 228, 261.

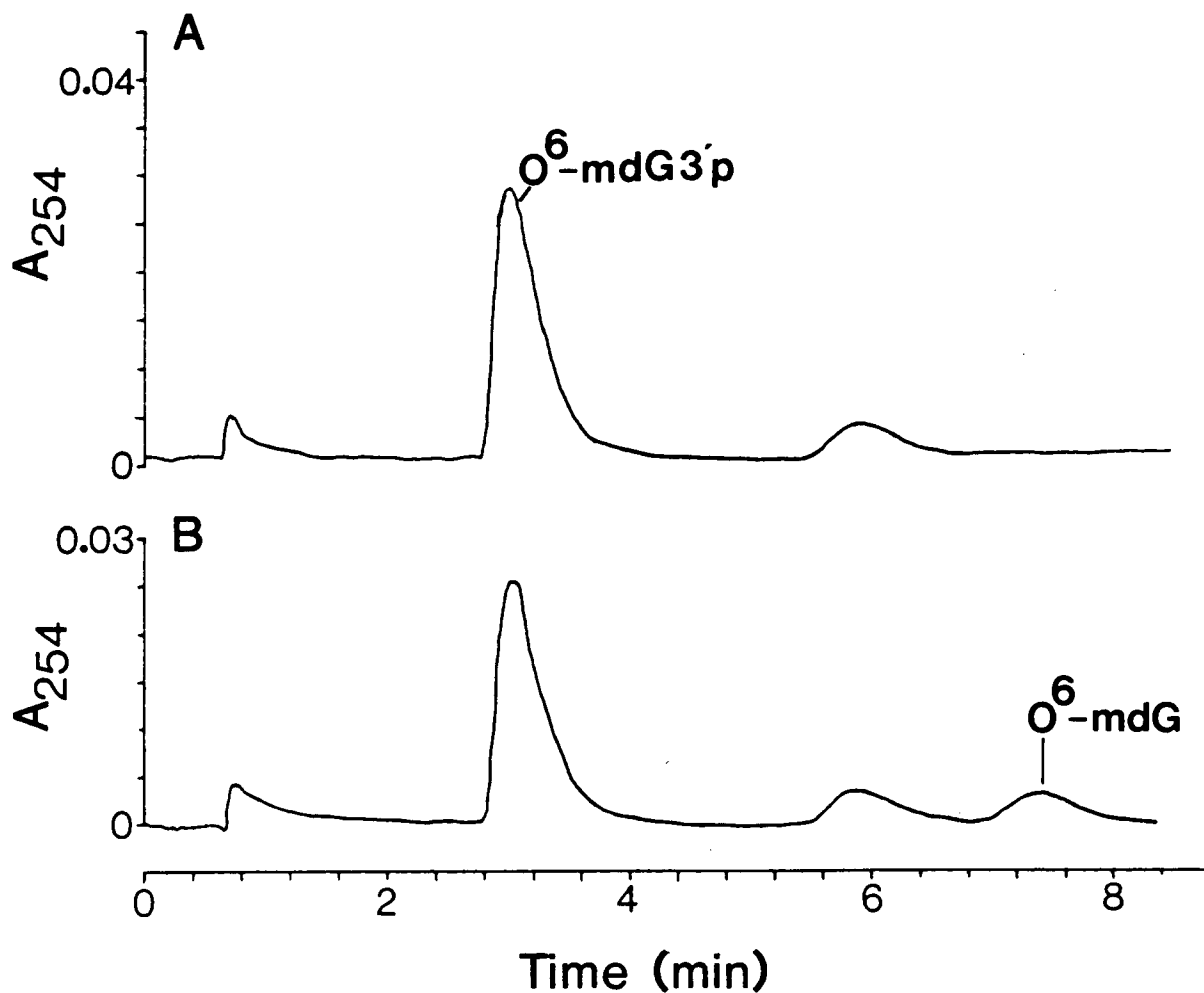


Figure 13 Characterization of O⁶mdG3'p using 5'-nucleotidase of *C. adamanteus* venom. Shown, are HPLC chromatograms of O⁶mdG3'p before (A) and after (B) exposure to venom for 5 minutes.

Deoxynucleoside 3'-monophosphates are hydrolysed to inorganic phosphate and deoxynucleoside by the 3'-nucleotidase activity of nuclease P1 (Reddy et al. 1984, Reddy and Randerath 1986). Deoxynucleoside 5'-monophosphates are resistant to this enzyme. Putative O^6 mdG3'p was converted to O^6 mdG by incubation with nuclease P1 at 37°C for five hours (Fig 14). O^6 mdG5'p was unchanged after the same treatment (data not shown).

Polynucleotide kinase catalyses the transfer of inorganic phosphate from the gamma position of 32 P-ATP to the 5' hydroxyl of deoxynucleoside 3'-monophosphates (Randerath et al. 1981). Deoxynucleosides and deoxynucleoside 5'-monophosphates are not substrates for this enzyme and therefore are not labeled. Incubation of putative O^6 mdG3'p with polynucleotide kinase and gamma 32 P-ATP gave rise to a labeled product that migrated as a distinct spot in a 1-dimensional PEI-TLC system (Fig 15). The labeled compound is O^6 mdG3'5'p. O^6 mdG5'p was not labeled under these conditions.

2. O^6 -Methylguanine Quantitation by 32 P-Postlabeling Analysis

Introduction

Using synthetic O^6 mdG3'p as a chromatography marker, HPLC and TLC (thin layer chromatography) procedures were developed for the detection and quantitation of O^6 -methylguanine in DNA by 32 P-postlabeling analysis. HPLC was used to isolate O^6 mdG3'p from DNA which had been digested to deoxynucleoside 3'-monophosphates with micrococcal nuclease and spleen phosphodiesterase. It was also used to purify 32 P-labeled O^6 mdG3'5'p from residual labeled normal nucleotides and other radioactive material (method #2). To measure O^6 -methylguanine in DNA it was necessary to resolve or separate O^6 mdG3'5'p from other radioactive compounds before scintillation counting; this was accomplished by PEI-TLC. To investigate enhancement of the

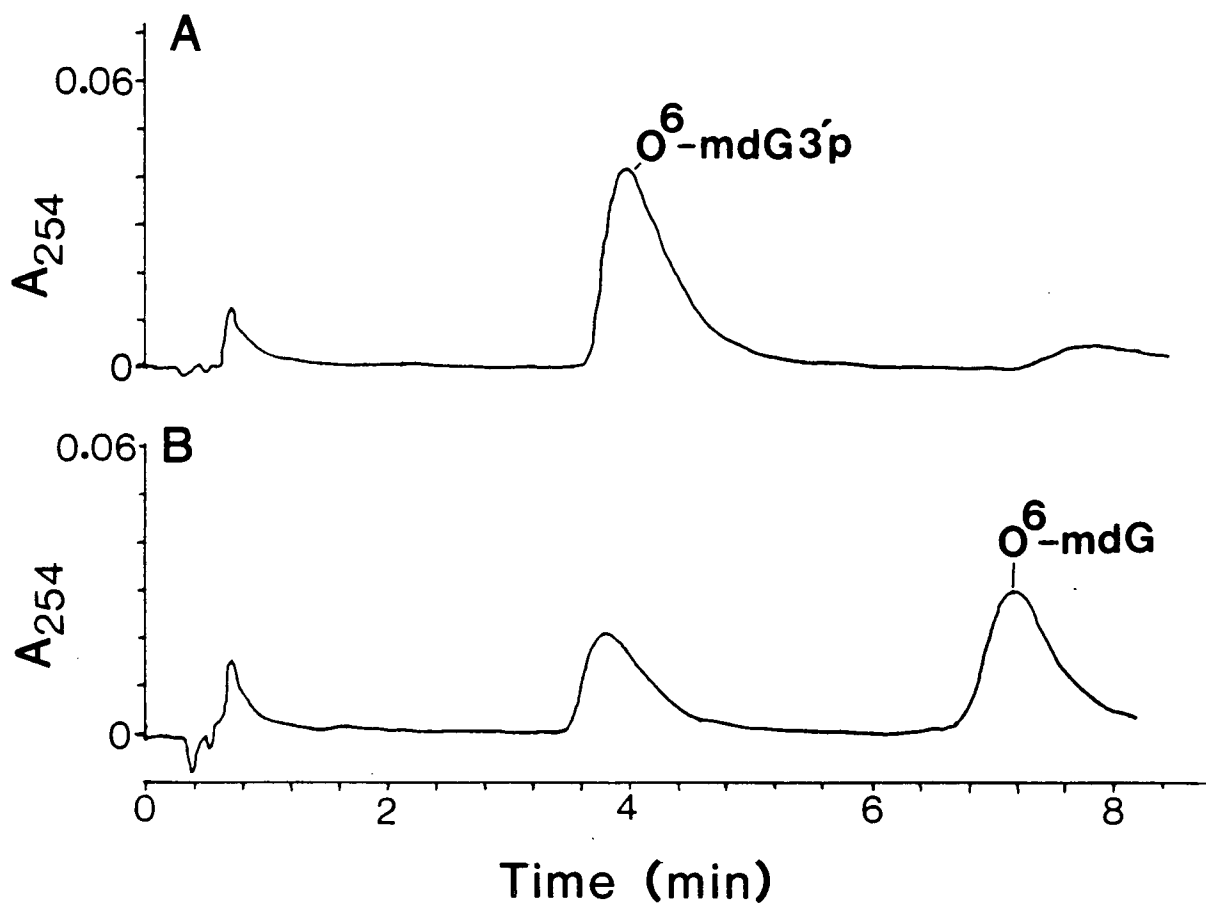


Figure 14 Conversion of O⁶mdG3'p to O⁶mdG by nuclease P1. HPLC chromatograms show O⁶mdG3'p before (A) and after (B) exposure to nuclease P1 for 5 hours at 37°C.

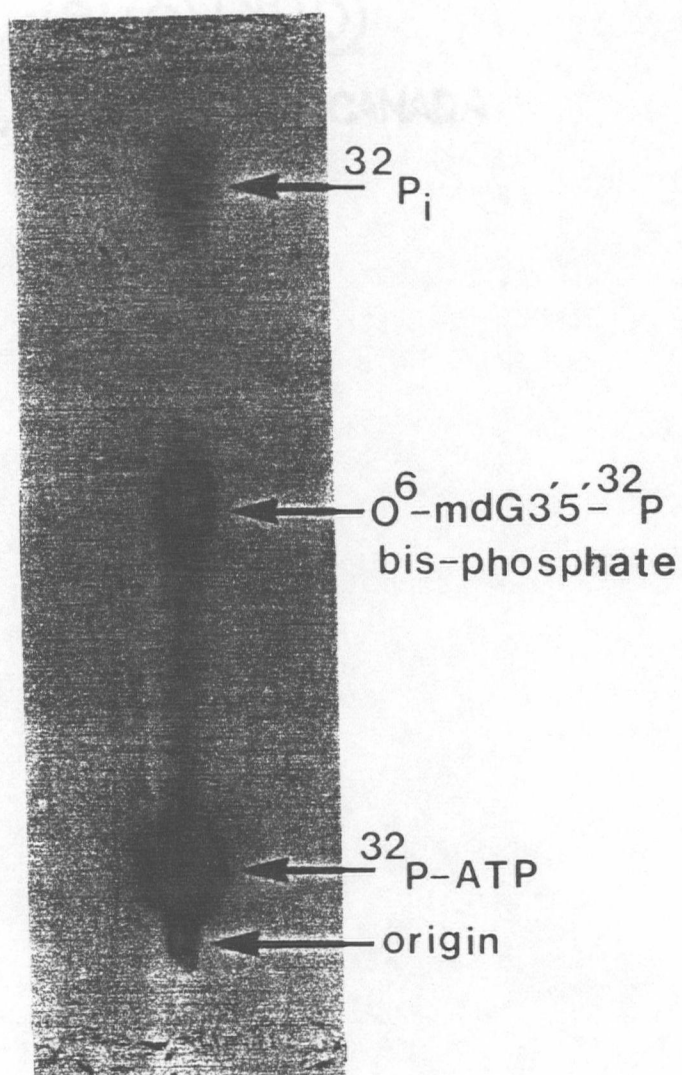


Figure 15 1-Dimensional PEI-TLC autoradiogram of putative ^{32}P -labeled $\text{O}^6\text{mdG3'5'p}$ derived from labeling of $\text{O}^6\text{mdG3'p}$ with gamma ^{32}P -ATP and polynucleotide kinase. Direction of solvent (2.25 M ammonium formate pH 3.5) migration is bottom to top.

assays' performance, ^{32}P -postlabeling methodology for analysis of O^6 -methylguanine at both the 3',5'-bisphosphate and 5'-monophosphate levels was developed (methods #3 and #4). The four methods are summarized in Figure 16 .

Methods and Results

2.1 Method #1: Single HPLC Purification-Diphosphate Level

DNA Extraction

DNA was isolated from tissue-culture cells using a modification of a previously described procedure (Maniatis et al. 1982). 20-100 mg tissue-culture cells were digested for 3 hours at 37°C with 500 μg proteinase K in 0.5 ml SET buffer (100 mM NaCl, 20 mM EDTA, 50 mM Tris-Cl pH 8.0) containing 0.5% sodium dodecyl sulfate (SDS). Samples were then successively extracted with an equal volume of phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1). The aqueous layer was mixed with the organic layer using a 'multi-tube' vortexer, and interface material discarded. DNA was precipitated with 2 volumes 2-ethoxyethanol and washed with 1 ml cold 70% ethanol. To digest RNA, the DNA was redissolved in 0.5 ml SET containing 50 μg heat treated pancreatic ribonuclease, and incubated for 1 hour at 37°C . To remove ribonuclease, 50 μg proteinase K in 10 μl SET buffer was added to the tubes which were incubated at 37°C a further hour. The phenol, phenol/chloroform/isoamyl alcohol, and chloroform/isoamyl alcohol extractions were repeated then the DNA recovered by 2-ethoxyethanol precipitation.

To remove residual RNA, the DNA was dissolved in 0.25 ml SET buffer and precipitated again with 2 volumes 2-ethoxyethanol. This was repeated. Finally, the purified DNA was dissolved in 1 ml ddH_2O and quantitated by its

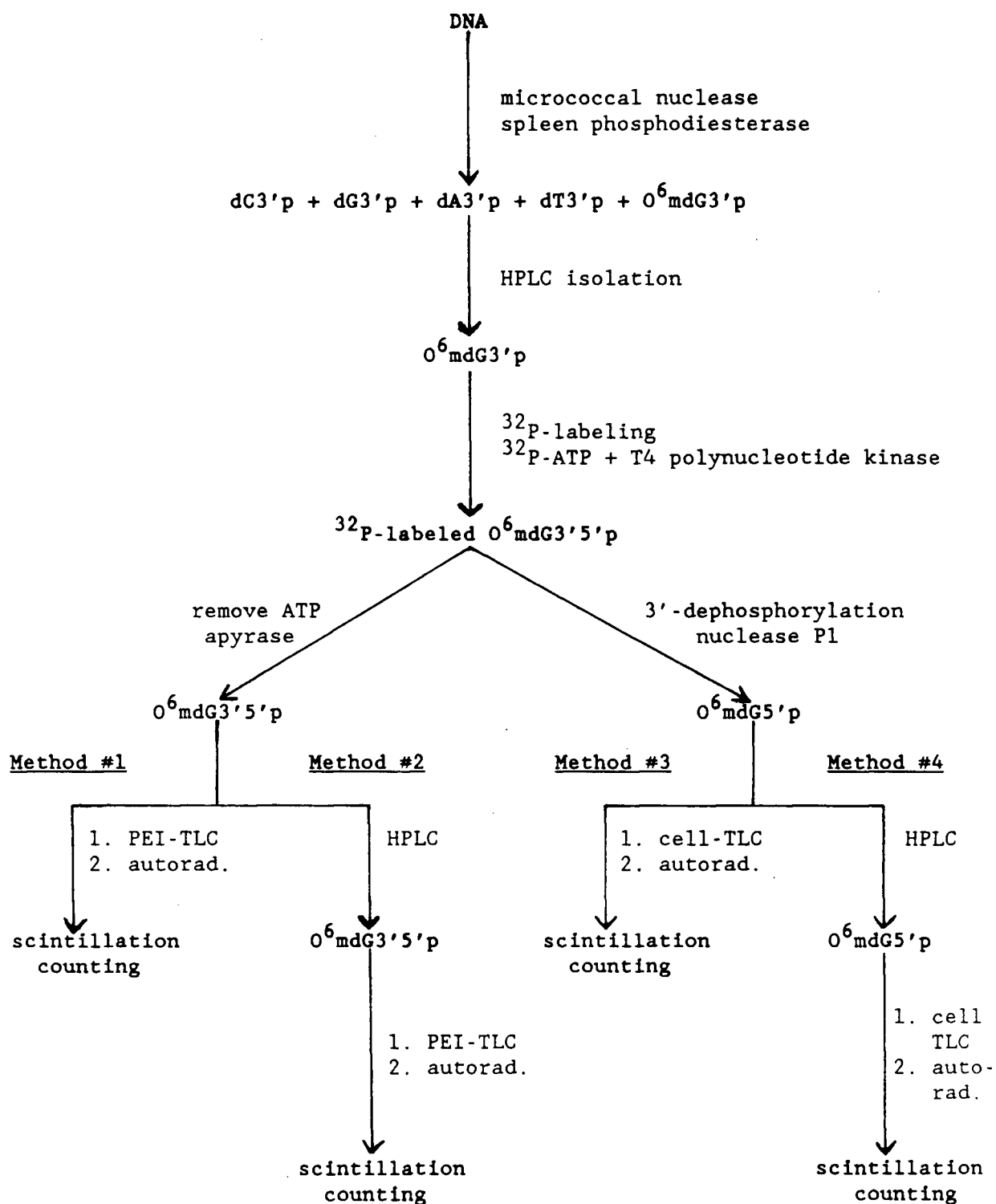


Fig 16 Summary of the ³²P-postlabeling methods for O⁶-methylguanine

U.V. absorption at 260 nm, using the relationship of 1 absorbance unit = 50 $\mu\text{g/ml}$ (Sueoka and Cheng 1967).

RNA associated background radioactivity interferes with labeled $^6\text{O}^6\text{mdG3}'5'\text{p}$ making quantitation problematical (see page 80 and Figure 26). Prior to postlabeling analysis the RNA content of the DNA was determined using a newly developed HPLC method (Dunn and San 1988). Preparations were further purified by ethoxyethanol precipitation if the contamination was significant (greater than 5%).

Enzymatic Hydrolysis of DNA

Aliquots of DNA solutions each containing 1.5 μg DNA (about 4.5 nmol DNA nucleotides) were reduced to dryness in 1.5 ml polypropylene tubes using a centrifugal vacuum evaporator. DNA was dissolved in 15 μl 10 mM CaCl_2 , 20 mM succinate buffer pH 6.0 containing 3 μg (0.36 units) micrococcal nuclease and 3 μg (0.006 units) spleen phosphodiesterase then digested for 3 hours at 37°C (Randerath et al. 1981). This treatment hydrolyses the DNA to deoxynucleoside 3'-monophosphates.

Isolation of $^6\text{O}^6$ -methyldeoxyguanosine 3'-monophosphate by reverse-phase HPLC

Reverse-phase HPLC was used to isolate femtomole (10^{-15} mole) quantities of $^6\text{O}^6\text{mdG3}'\text{p}$ from enzymatically digested DNA. The column was eluted with 95% 1 M ammonium formate pH 3.5 + 5% methanol at a flow rate of 0.4 ml/min.

Prior to HPLC analysis of DNA samples, 3 aliquots of 10 μl 0.0905 mM deoxyadenosine 3'-monophosphate standard (0.905 nmole) were injected onto the column. This allowed determination of the total amount of DNA nucleotides associated with each injection.

In a typical experiment, twelve DNA samples were analysed. DNA digests (15 μ l) were mixed with 15 μ l 2 M ammonium formate pH 3.5 and briefly centrifuged. For each sample, an injection 'chase' of 10 μ l 95% 1 M ammonium formate + 5% methanol was first drawn into a HPLC syringe (50 μ l capacity) followed by 20 μ l of the dilute DNA digest (about 3 nmoles DNA nucleotides) to give a total injection volume of 30 μ l. After the sample was injected onto the column, the appropriate eluant fraction containing O^6 mdG3'p was collected in a 1.5 ml polypropylene tube (Figure 17).

Experiments with synthetic marker O^6 mdG3'p showed that O^6 mdG3'p typically eluted from the column in a volume of 300 μ l, 4.75-5.5 minutes after injection. To guard against peak drift caused by variable chromatographic conditions, the actual collection fraction was increased to 500 μ l (4.5-5.75 minutes). Solvent and buffer were removed by vacuum evaporation in a centrifugal spin-drier at room temperature for 12 hours.

After sample DNA injections, a positive control consisting of approximately 1×10^{-14} moles synthetic O^6 mdG3'p was chromatographed and analysed along side the others. This, and a sample composed of the same amount of O^6 mdG3'p directly labeled without HPLC allowed calculation of the adduct recovery and subsequently, the adduct levels.

32 P-Postlabeling

Dried residues from the evaporated HPLC fractions were dissolved in 5 μ l ddH₂O. They were then 32 P-labeled by adding 3 units of polynucleotide kinase, 1 μ l of kinase buffer (0.2 M Bicine-Cl pH 9.5, 0.1 M MgCl₂, 0.1 M dithiothreitol and 10 mM spermidine), 20 uCi gamma 32 P-ATP and water to give a total volume of 10 μ l. All samples were incubated for 3 hours at 37°C (Dunn et al. 1987). Excess 32 P-ATP was converted to inorganic phosphate and ADP by treatment of the labeled digest with 0.05 units apyrase (in 5 μ l 10 mM bicine-

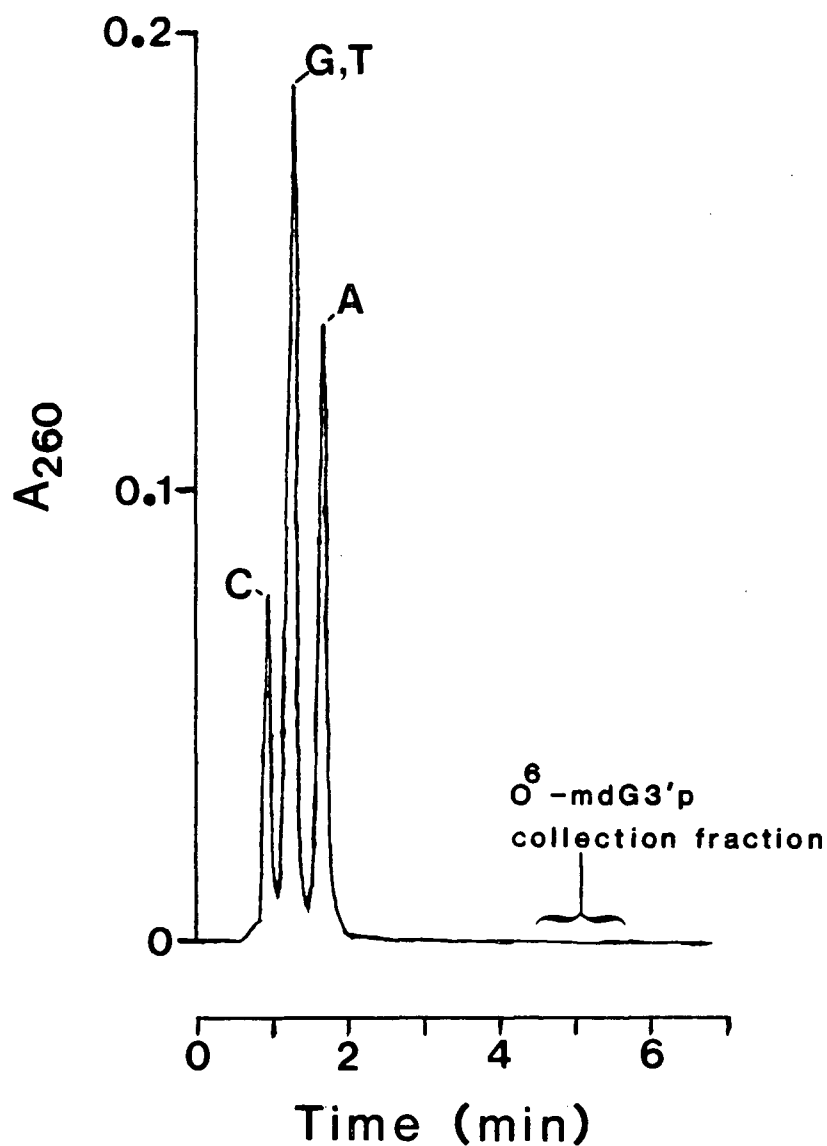


Figure 17 Isolation of O⁶mdG3'p from DNA digested with micrococcal nuclease and spleen phosphodiesterase using reverse-phase HPLC. The HPLC chromatogram is from the analysis of 1 μ g methylated DNA (about 3 nmole DNA nucleotides) containing 1 μ mole/mole O⁶-methylguanine. C,G,T, and A are the 3'-monophosphates of deoxycytidine, deoxyguanosine, thymidine, and deoxyadenosine respectively.

Cl pH 9.5 buffer) for 1/2 hour at room temperature (Gupta et al. 1982). Samples were diluted to 100 μ l with 85 μ l ddH₂O and either immediately chromatographed on PEI-cellulose or stored overnight at -20°C.

Preparation of Gamma 32 P-ATP and Determination of its Specific Activity

32 P-ATP was synthesized on a weekly basis, usually two days before it was to be used for labeling. Using a previously described procedure, a cocktail of glycolytic pathway enzymes catalysed the reaction between ADP and inorganic 32 P_i to give the desired product (Gupta et al. 1982). The preparation was managed so that the concentration of 32 P-ATP was 100 uCi/ μ l on the day of labeling. Completeness of the reaction was checked prior to labeling by one-dimensional TLC of the mixture on PEI-cellulose. For preparation of the kinase reaction mixture the 32 P-ATP was diluted with ddH₂O.

For each batch of 32 P-ATP made the specific activity was determined by labeling four 5 μ l samples of 10^{-7} M deoxyadenosine 3'-phosphate (5×10^{-13} moles) (Reddy and Randerath 1986). The samples were chromatographed on PEI-cellulose using 0.3 M ammonium sulfate buffered with 10 mM Na phosphate pH 7.5. The spots containing 32 P-labeled deoxyadenosine 3',5'-bisphosphate were cut out and their radioactivity measured by scintillation counting (Dunn and San 1988). Assuming 100% labeling, the specific activity of 32 P-ATP was calculated and it typically had a value of 3000 Ci/mmole. This is less than the theoretical figure of 9000 Ci/mmole and may be due to the presence of non-radioactive inorganic phosphate in the mixture, derived from one or more of the reagents used.

Quantitation of ^{32}P -ATP Consumed during the Kinase Reaction

To accurately measure O^6 -methylguanine in DNA by postlabeling, all of the $\text{O}^6\text{mdG3}'\text{p}$ present in the HPLC fraction must be labeled; this requires a molar excess of ^{32}P -ATP. In some assays, the amount of excess ^{32}P -ATP left after labeling was monitored by analysing a small portion of the reaction mixture before apyrase treatment (Dunn and San 1988). This was accomplished by dipping the tip of a wooden toothpick into the reaction mixture then rinsing the toothpick in 20 μl water. A small aliquot of the dilute mixture (5 μl) was then chromatographed on PEI-cellulose in one dimension using 0.3 M ammonium sulphate. Unused ^{32}P -ATP was estimated by comparing the radioactivity in the ^{32}P -ATP spot to the radioactivity in the normal nucleotide spots.

Two-Dimensional PEI-Cellulose Thin Layer Chromatography

$\text{O}^6\text{mdG3}'5'\text{p}$ was resolved from residual radioactive normal nucleotides and other material by two-dimensional thin layer chromatography (TLC) on PEI-cellulose.

Commercially manufactured sheets were washed and marked before use. To remove impurities from the sheets they were individually rinsed for 2 minutes in methanol, then twice in deionized water and finally in 0.225 M ammonium formate pH 3.5. Then, using a hair-dryer, the sheets were blown dry for 10 minutes. To facilitate orientation of the chromatograms with the autoradiogram, the sheets were marked with phosphorescent ink. The chromatographic origin (2 cm in from the lower left-hand corner) and directions of solvent development were indicated on the sheets using a soft (#6B) pencil.

The two-dimensional solvent system for chromatography of normal nucleotides used by Gupta and co-workers was modified for optimal resolution of O^6 mdG3'5'p from other spots (Gupta et al. 1982). 10 μ l of the diluted radioactive sample (10% of the labeled HPLC fraction) was slowly spotted at the marked origin. After blow drying the spot, the chromatograms were developed to the top edge in 2.25 M ammonium formate pH 3.5 (D1) ; this took about 1.5 hours. Chromatography was performed in closed tanks with a solvent depth of about 1 cm. After D1, ammonium formate was removed from the chromatograms using a current of warm air from a hair dryer. Chromatograms were then soaked vertically for 5 minutes in deionized water then for 5 minutes in 1 mM NaH_2PO_4 pH 7.5. Orientation during soaking was the same as for D2. After blow drying for 10 minutes the chromatograms were developed to the top edge in 0.3 M ammonium sulphate + 0.01 M NaH_2PO_4 pH 7.5. This run (D2 at right angles to D1) also required about 1.5 hours. Finally, the sheets were blown dry for the next stage, autoradiography.

Autoradiography and Measurement of Radioactivity in the O^6 -methyldeoxyguanosine 3',5'-bisphosphate spot

Chromatograms were autoradiographed with X-ray film in cassettes equipped with 2 intensifying screens for 2 hours at room temperature (Dunn and Stich 1986). Film was then developed for 5 minutes in Kodak D-19, immersed in a stop bath for 1 minute then fixed until clear (5-10 minutes). After rinsing films in running tap water for 10 minutes, they were dried using an automatic roller drier. Representative autoradiograms are shown in Figure 18. Chromatographic conditions in the HPLC step were such that O^6 mdG3'p eluted at about three times the retention time of dA3'p, which was the last normal nucleotide. This resulted in traces of all four normal nucleotides being

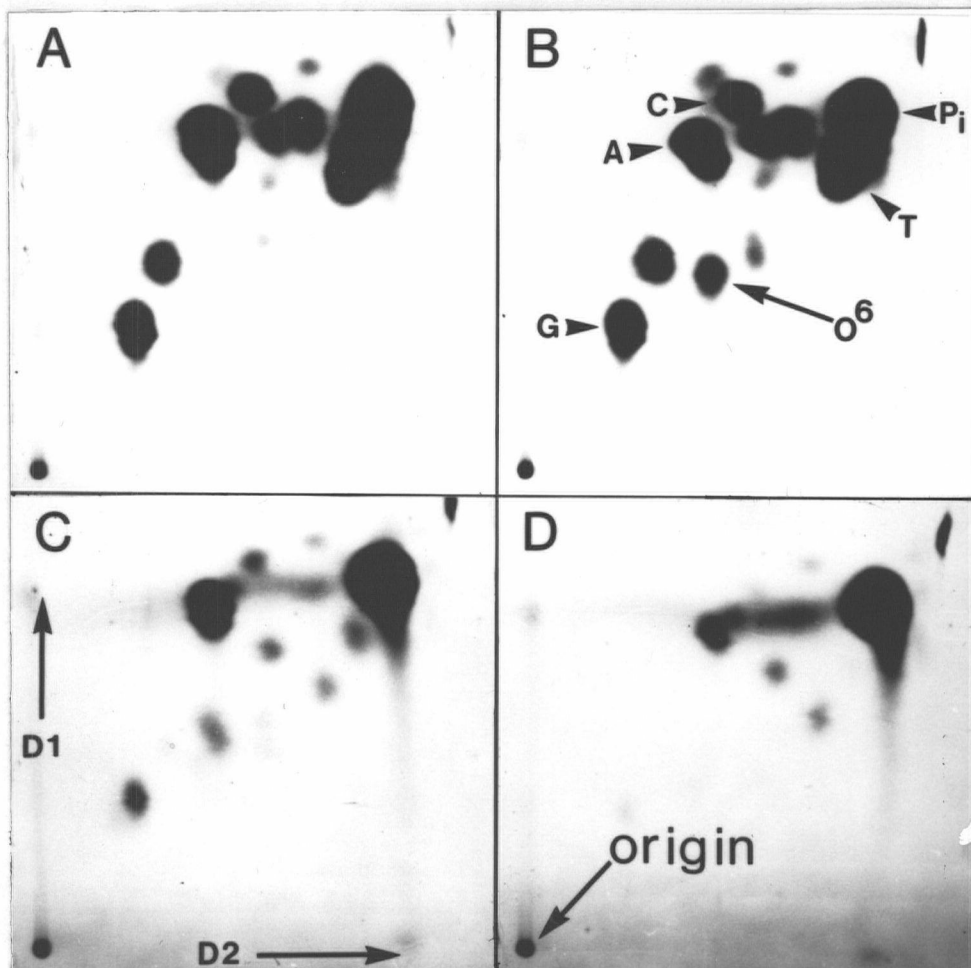


Figure 18 Representative autoradiograms for postlabeling analysis of O^6 -methylguanine in $1 \mu\text{g}$ DNA using method #1 (single HPLC purification-diphosphate level).

- A. DNA from untreated CHO cells.
 - B. DNA containing $55 \mu\text{mole/mole}$ O^6 -methylguanine from CHO cells treated with MNU.
 - C. Positive control consisting of 4×10^{-14} moles synthetic $O^6\text{mdG3'p}$ injected onto the HPLC.
 - D. Blank sample consisting of $500 \mu\text{l}$ HPLC solvent collected from column before DNA injections.
- G, A, C, T and O^6 are the ^{32}P -labeled 3'5' biphosphates of deoxyguanosine, deoxyadenosine, deoxycytidine, thymidine and $O^6\text{mdG}$ respectively.

present in the O^6 mdG3'p fraction, however these are well resolved from O^6 mdG3'5'p by 2-dimensional PEI-TLC (Fig 18).

With the aid of a light box, the outline of the O^6 mdG3'5'p spot on the film was traced onto the face of the TLC chromatograms using a soft pencil. The circled section of chromatogram was then punched out using a 13 mm diameter 'art' punch (Dunn and San 1988).

The level of radioactivity in the O^6 mdG3'5'p spot was determined by liquid scintillation counting. The radioactive chip was combined with 5 ml toluene based scintillation fluid in a plastic screw top scintillation vial. Samples were then counted for 10 minutes at a counting efficiency of essentially 100%.

Quantitation of O^6 -methylguanine

The amount (moles) of deoxyadenosine 3'-monophosphate in a digested DNA sample that was injected onto the HPLC was determined by comparison of its peak area with that of a known amount of standard analysed during the same chromatographic run. This number was divided by 0.29 (the prevalence of adenosine in DNA from vertebrates) to give the total moles of DNA nucleotides associated with each kinase reaction (Sober 1968).

The moles of O^6 mdG3'5'p was calculated by dividing the specific activity of 32 P-ATP into the O^6 mdG3'5'p counts. To correct for HPLC associated losses, this value was divided by the recovery (determined from the positive control). Finally, this number was multiplied by ten to give the total moles of O^6 mdG3'5'p since only 10% of the kinase reaction was analysed by TLC. For all analyses, the specific activity of 32 P-ATP was determined on the same day as the O^6 mdG3'5'p spots were counted.

Division of moles of O^6 mdG3'5'p by total moles DNA nucleotides for a sample gave the level of O^6 -methylguanine in the DNA. This was conveniently expressed as micromoles adduct per mole normal nucleotides (μ mole/mole).

2.2 Method #2: Dual HPLC Purification-Diphosphate Level

For further purification of O^6 mdG3'5'p by reverse-phase HPLC, 15 μ l HPLC carrier was added to the 15 μ l apyrase treated kinase reaction (see page 46) and the mixture centrifuged briefly. HPLC carrier was 2 M ammonium formate pH 3.5 containing 2 mM of the 3',5'-bisphosphates of deoxycytidine, thymidine and deoxyadenosine, and 10 mM potassium dihydrogen phosphate. Preceded by 10 μ l of an HPLC solvent 'chase', all of the radioactive solution was drawn up into an HPLC syringe to give an injection volume of 40 μ l per sample. This was then injected onto a reverse-phase HPLC column and eluted with 1 M ammonium formate pH 3.5 containing 1% methanol at a flow rate of 0.4 ml/min. The eluant fraction containing O^6 mdG3'5'p was collected in a 1.5 ml polypropylene tube and reduced to dryness for 12 hours at room temperature in a centrifugal spin-drier.

Experiments with 32 P-labeled synthetic O^6 mdG3'5'p under the same chromatographic conditions showed that the bisphosphate typically eluted from the column in a volume of 500 μ l, 3.75-5 minutes after injection. To compensate for experimental variability in elution positions, the actual collection fraction was increased to 700 μ l (3.5-5.25 minutes).

The dried radioactive residue was dissolved in 20 μ l 10 mM ammonium formate pH 3.5 and slowly spotted at the origin of a pre-treated PEI-cellulose sheet marked for 2-dimensional TLC. The spot was blown dry then chromatography, autoradiography (12 hours), and scintillation counting performed as described previously.

2.3 Method #3: Single HPLC Purification-Monophosphate Level

After ^{32}P -postlabeling of the HPLC fraction (see page 46), 7.5 μl of nuclease P1 in buffer (consisting of 1.5 μl 5 mg/ml nuclease P1 in water, 3.75 μl of 0.25 M Na acetate pH 5.0, 2.25 μl 0.3 mM ZnCl_2) was added to the solution. This treatment converts ^{32}P -labeled deoxynucleoside 3'5'-bisphosphates to the corresponding ^{32}P -labeled deoxynucleoside 5'-monophosphates. Incubation was at 37°C for one hour. The sample was then diluted to 50 μl by the addition of 21.5 μl ddH_2O and 11 μl 'TLC carrier' and stored overnight at -20°C . 'TLC carrier' was 2.5 mg each of ATP, and the 5'-monophosphates of deoxycytidine, thymidine, deoxyguanosine and deoxyadenosine plus 150 μl of 2.6×10^{-3} M unlabeled $\text{O}^6\text{mdG5'p}$ dissolved in 500 μl ddH_2O .

Compared to deoxynucleoside bisphosphates, 2-dimensional TLC of ^{32}P -labeled $\text{O}^6\text{mdG5'p}$ requires different solvents and thin layer chromatogram characteristics. The 2-D TLC system employed by Wilson and co-workers to quantitate 5-methylcytosine by ^{32}P -postlabeling at the 5'-monophosphate level was therefore adapted for this purpose (Wilson et al. 1986).

Cellulose plates were marked for 2-dimensional TLC using a soft pencil and for autoradiography using phosphorescent ink. The plate was spotted with 2 μl of the dilute radioactive sample at the origin (2 cm from the lower left-hand corner) and the spot allowed to air-dry. The chromatograms were developed to the top edge (D1) using isobutyric acid: H_2O : concentrated ammonium hydroxide (66:20:1). This run took about 10 hours. They were then dried overnight in a fumehood. Next day, D1 was repeated. On the third day, the chromatograms were turned 90° and run to the top edge (D2) in saturated ammonium sulphate: isopropanol: 1 M sodium acetate (80:2:18). This run required about 9 hours. Finally the plates were air dried overnight in a fumehood.

The chromatograms were autoradiographed for 7 hours at room temperature. The O^6 mdG5'p spot was scraped off the plate using a spatula and the radioactivity measured by scintillation counting. Typical autoradiograms are shown in Figure 19.

32 P-ATP migrated as a distinct spot in the two-dimensional TLC system used (see Figure 19). Measurement of radioactivity in this spot allowed verification of excess 32 P-ATP in the kinase reaction. Alternatively, using a toothpick, a small sample of the kinase reaction was analysed after nuclease P1 treatment, by 1-dimensional PEI-cellulose TLC.

2.4 Method #4: Dual HPLC Purification-Monophosphate Level

To the 17.5 μ l nuclease P1 treated kinase reaction (see page 54) was added 17.5 μ l 'HPLC carrier' and the mixture briefly centrifuged. 'HPLC carrier' was 1.5 M ammonium formate pH 3.5 containing 0.57 mM of the 5'-monophosphates of deoxycytidine, thymidine, deoxyguanosine, deoxyadenosine, and O^6 -methyldeoxyguanosine plus 0.57 mM ATP. Along with a 'chase' of 10 μ l HPLC solvent the entire mixture was injected onto the column and eluted with 1 M ammonium formate pH 3.5 plus 1% methanol at 0.4 ml/min. With the detector (254 nm) sensitivity set at 0.5 AUFS, the peak corresponding to cold O^6 mdG5'p was collected in a 1.5 ml polypropylene tube (Figure 20). Then, 1 μ l of diluted TLC carrier (1:10 in ddH₂O) was added to the fraction and buffer/solvent removed by evaporation in a centrifugal spin-drier overnight at room temperature.

A typical collection fraction for O^6 mdG5'p was 4 minutes to 6 minutes 30 seconds after injection (volume=1 ml). This corresponded to a peak center elution time of 4 minutes 30 seconds. The fraction was relatively large owing to the pronounced tailing of the peak.

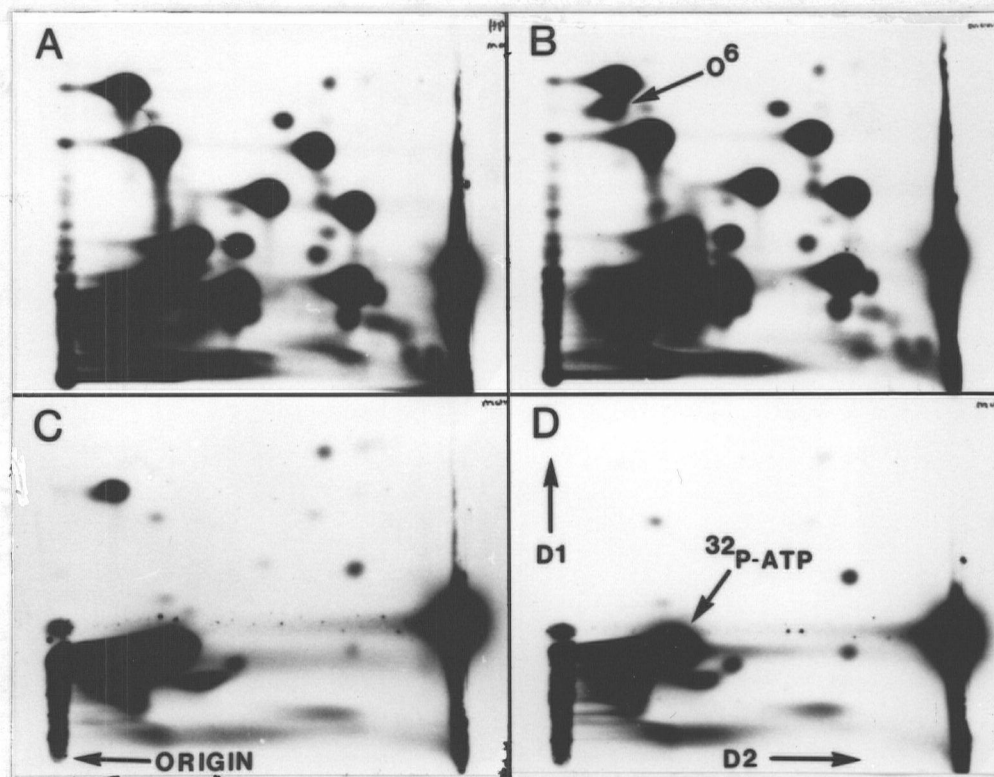


Figure 19 Representative autoradiograms for ^{32}P -postlabeling analysis of O^6 -methylguanine in $1\text{ }\mu\text{g}$ DNA using method #3 (single HPLC purification-monophosphates).

- A. DNA from untreated C3H10T1/2 mouse embryo fibroblast cells.
- B. $1\text{ }\mu\text{g}$ digested C3H10T1/2 DNA spiked with $27.5\text{ }\mu\text{mole/mole}$ synthetic $\text{O}^6\text{mdG3'p}$.
- C. Positive control consisting of 6.87×10^{-14} moles synthetic $\text{O}^6\text{mdG3'p}$ injected onto the HPLC.
- D. Blank sample consisting of $500\text{ }\mu\text{l}$ HPLC solvent collected from the column before DNA injections.

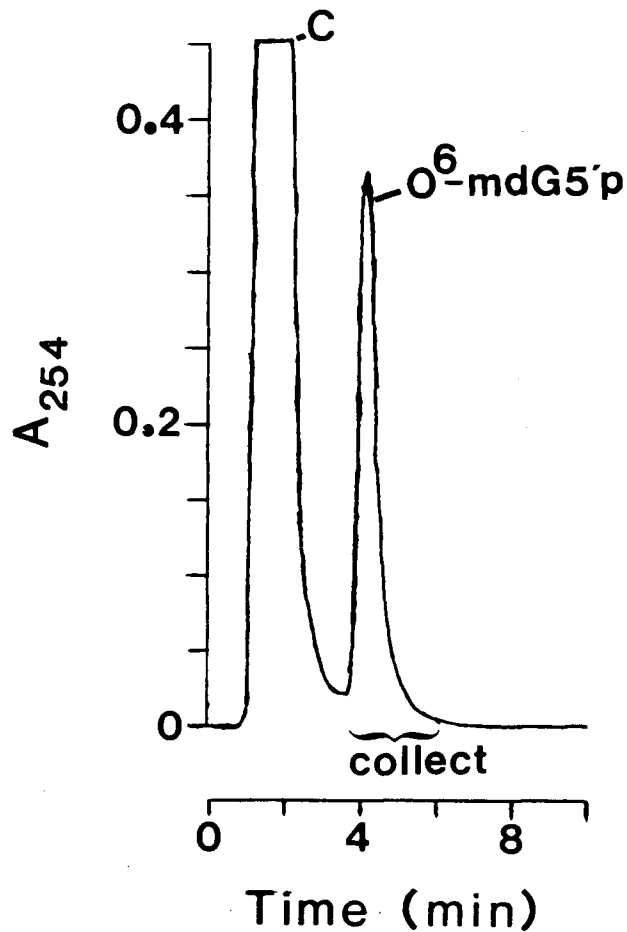


Figure 20 Purification of ^{32}P -labeled $O^6\text{mdG5'p}$ using reverse-phase HPLC (method #4). Cold $O^6\text{mdG5'p}$ added to the nuclease P1 treated kinase mixture serves as a U.V. marker for the recovery of ^{32}P -labeled $O^6\text{mdG5'p}$.

C. Peak due to unlabeled ATP and deoxynucleoside 5'-monophosphates in 'HPLC carrier'.

The radioactive residue was dissolved in 10 μ l 10 mM ammonium formate pH 3.5. This solution was then spotted, in 2 μ l aliquots with drying in between, onto the origin of a cellulose TLC plate marked for 2-dimensional TLC. Chromatography, autoradiography and scintillation counting were performed as described previously. Sample autoradiograms are shown in Figure 21. Confirmation of excess 32 P-ATP in the kinase reaction after nuclease P1 treatment was established by assaying a small aliquot of the radioactive material using a toothpick as described previously.

3. Performance of the 32 P-Postlabeling Assays for O⁶-methylguanine

Introduction

Three principal determinants of an assay's performance are its reproducibility, recovery and sensitivity (Garner 1985, Lohman et al. 1984).

A measure of the reproducibility is the standard deviation of adduct levels on replicate analysis of a single DNA sample. The recovery is the fraction of O⁶mdG3'p released on enzymatic digestion of DNA which is finally counted as labeled O⁶mdG3'5'p on the TLC chromatograms, after passage through various HPLC/TLC purifications before and after postlabeling. Sensitivity refers to the lowest level of adduct which can be reliably detected above background for a given amount of DNA analysed.

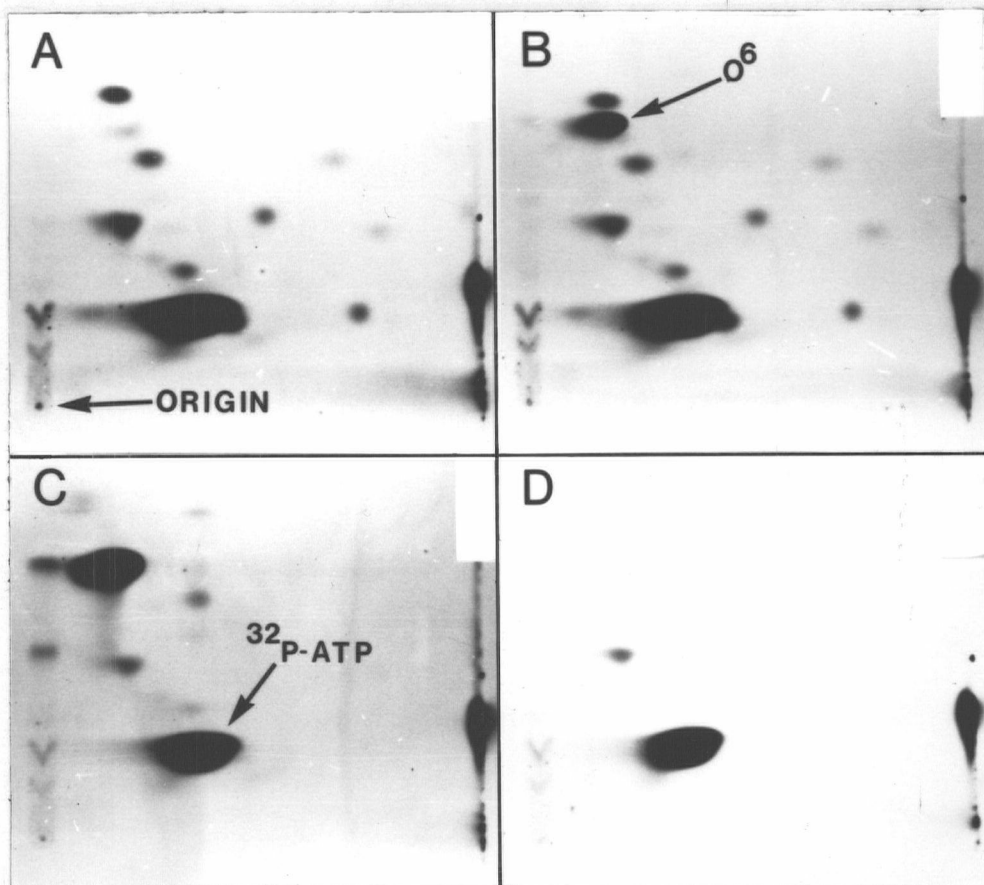


Figure 21 Sample autoradiograms for ^{32}P -postlabeling analysis of O^6 -methylguanine in $1\ \mu\text{g}$ DNA using method #4 (dual HPLC purification-monophosphate level).

- A. DNA from untreated C3H10T1/2 cells.
- B. $1\ \mu\text{g}$ digested DNA spiked with $17\ \mu\text{mole/mole}$ synthetic $\text{O}^6\text{mdG3'p}$.
- C. Sample consisting of directly labeled synthetic $\text{O}^6\text{mdG3'p}$ with no HPLC purification.
- D. Assay blank created by the labeling of $5\ \mu\text{l}$ ddH_2O .

Methods and Results

3.1 Reproducibility of the Assay for Synthetic O⁶mdG3'p

HPLC of replicate injections of standard followed by postlabeling of the fraction, TLC analysis and counting of the O⁶mdG3'5'p spots can give an estimate of the assay reproducibility within a single sample of digested DNA. This is one source of variation contributing to the overall assay reproducibility between separately digested aliquots from a single DNA sample.

First, a solution consisting of 15 μ l 2.83 X 10⁻⁹ M O⁶mdG3'p and 15 μ l 2 M ammonium formate pH 3.5 was prepared. Then, four 5 μ l aliquots of this solution were separately injected onto the HPLC and the appropriate fraction collected. O⁶mdG3'p was measured in the samples according to method #1. Values for the radioactivity in the adduct spots are presented in Table 1. From these data, the standard deviation for replicate analysis of O⁶mdG3'p from a single sample is \pm 6%.

3.2 Reproducibility of DNA Digestion

An estimate of the reproducibility of enzymatic release of O⁶mdG3'p from DNA may be obtained by determining the standard deviation of DNA nucleotide yields from multiple separately digested aliquots of a single DNA sample using HPLC. The values for replicate digestion of 1 μ g DNA are shown in Table 2. For 10 experiments involving separate DNA samples analysed at different times, the standard deviations of digestion range from \pm 0.9% to \pm 7.3%.

TABLE 1

REPRODUCIBILITY OF THE ^{32}P -POSTLABELING ASSAY FOR SYNTHETIC O^6 -METHYLDEOXYGUANOSINE 3'-MONOPHOSPHATE

<u>Sample</u>	<u>^{32}P-$\text{O}^6\text{mdG3'5'p}$ Radioactivity (cpm)</u>
1	3411.0
2	3542.8
3	3932.0
4	<u>3686.0</u>
Mean	3643.0 ± 223 (SD)

Note: Replicate injections (1-4) of standard $\text{O}^6\text{mdG3'p}$ on the reverse-phase HPLC column were analysed according to method #1. Values were corrected by subtracting a column blank value of 6770.4 cpm. The column blank consisted of 500 μl solvent collected from the column prior to actual samples and analysed in the same manner.

TABLE 2

REPRODUCIBILITY OF DNA DIGESTION

<u>Replicates (n)^a</u>	<u>Nucleotides (nmole)^b</u>	<u>Standard Deviation</u>
10	2.56	0.07
10	2.20	0.16
10	2.26	0.05
4	2.14	0.08
4	2.32	0.02
6	2.27	0.05
4	2.64	0.03
7	2.60	0.18
4	2.07	0.08
4	<u>2.32</u>	<u>0.03</u>
Mean = 2.34		Mean = 0.08

Note: The table shows the results of 10 independent trials where 1 μ g (determined by U.V.) aliquots of a single DNA sample was separately digested using MN/SPD. Nucleotides were quantitated by HPLC analysis and measurement of the dA3'p peak.

^a Denotes the number (n) of 1 μ g DNA aliquots digested for each trial.

^b The mean yield of DNA nucleotides from digestion of n aliquots of 1 μ g DNA.

3.3 Recovery of O⁶-methyldeoxyguanosine 3'-monophosphate

To determine the recovery of O⁶mdG3'p from digested DNA, DNA from unexposed cells was digested, then spiked with a known amount of synthetic marker, and analysed by postlabeling. The HPLC recovery of O⁶mdG3'p was determined by comparison of spot intensities between the purified DNA sample and one consisting of the same amount of O⁶mdG3'p directly labeled (no HPLC).

In one experiment, 1.5 µg C3H10T1/2 DNA was digested using micrococcal nuclease and spleen phosphodiesterase then spiked with 8.42×10^{-14} moles O⁶mdG3'p. 2/3 of this mixture was injected onto the HPLC and the adduct subsequently quantitated using method #1. At the same time, 5.61×10^{-14} moles O⁶mdG3'p (equal to the amount of standard in the spiked DNA sample) was analysed by direct labeling without HPLC purification. Comparison of spot intensities for duplicates of each type of sample gave a recovery of 81%. In a similar experiment 1.5 µg digested calf-thymus DNA was spiked with a lower level of O⁶mdG3'p (1.58×10^{-14} moles). The recovery in this case was 88%. In two experiments without DNA involving 1.5 and 1.7×10^{-14} moles O⁶mdG3'p, recoveries were 86% and 82% respectively.

The recovery of O⁶mdG3'5'p for methods #2-#4 was determined in the same manner. In experiments using method #2 without DNA the recovery was 39% and 37% when 1.13 and 1.69×10^{-14} moles O⁶mdG3'p was analysed.

For duplicates of 1 µg digested C3H10T1/2 DNA spiked with 5.61×10^{-14} moles marker O⁶mdG3'p and analysed using method #3, the recovery was 85%. Adding a second HPLC purification before the final chromatography (method #4) dropped the recovery to 51%.

For all experiments, adduct levels were corrected for recovery, using internal controls or 'historical' recovery values.

3.4 Sensitivity

The sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine may be estimated as twice the background measured for a control DNA sample. In this context, 'control' DNA is DNA isolated from cultured cells or tissues not exposed to methylating agents and therefore assumed to contain no 'exogenous' O^6 -methylguanine. In five separate experiments DNA isolated from C3H10T1/2 cells and CHO cells was analysed using method #1 under identical conditions and the estimated sensitivity, corrected for recovery, determined (Table 3). The average sensitivity is $6.5 \pm 1.5 \mu\text{mole/mole}$.

To confirm the sensitivity estimated from blank values of control DNA, samples of digested DNA were spiked with known amounts of synthetic marker $\text{O}^6\text{mdG3}'\text{p}$ and analysed. In a typical experiment, $1 \mu\text{g}$ samples of digested C3H10T1/2 DNA were spiked with synthetic $\text{O}^6\text{mdG3}'\text{p}$ to simulate levels of O^6 -methylguanine in DNA at 0.1, 0.7, 1.4, and $6.8 \mu\text{mole/mole}$. Visual examination of the resulting autoradiograms (Fig 22) show that $\text{O}^6\text{mdG3}'\text{p}$ in digested DNA to a level of $6.8 \mu\text{mol/mol}$ is clearly detectable above background. In contrast, the autoradiograms of DNA spiked with $1.4 \mu\text{mol/mole}$ $\text{O}^6\text{mdG3}'\text{p}$ or lower are indistinguishable from those of control (unspiked) DNA.

The estimated sensitivity of method #2 for analysis of C3H10T1/2 DNA was $0.5 \mu\text{mole/mole}$. The actual sensitivity was assessed using DNA samples spiked with $\text{O}^6\text{mdG3}'\text{p}$ as described above. Calf-thymus DNA spiked with $0.2 \mu\text{mole/mole}$ $\text{O}^6\text{mdG3}'\text{p}$ on analysis gave a $\text{O}^6\text{mdG3}'5'\text{p}$ spot and was visually distinguishable from the control sample (Fig 23).

The estimated sensitivity (twice background) for detection of O^6 -methylguanine in $1 \mu\text{g}$ C3H10T1/2 DNA analysed using methods #3 and #4 was 8.5 and $2 \mu\text{mole/mole}$ respectively.

TABLE 3

SENSITIVITY OF THE ^{32}P -POSTLABELING ASSAY FOR O^6 -METHYLGUANINE-METHOD #1

<u>DNA Source (cell type)</u>	<u>Sensitivity ($\mu\text{mole/mole}$)</u>
C3H	8.4
C3H	5.1 ^a
C3H	5.2 ^a
C3H	6.1
CHO	7.6

mean = $6.5 \pm 1.5(\text{SD})$

Note: In five separate experiments, independent isolates of 1 μg DNA from untreated CHO and C3H10T1/2 cultures were analysed for O^6 -methylguanine using method #1. The sensitivity was estimated as twice the molar equivalent of the radioactivity in the $\text{O}^6\text{mdG3'5'p}$ zone of the chromatogram. Values were corrected for an adduct recovery of 84%.

^a Mean of duplicate analyses.

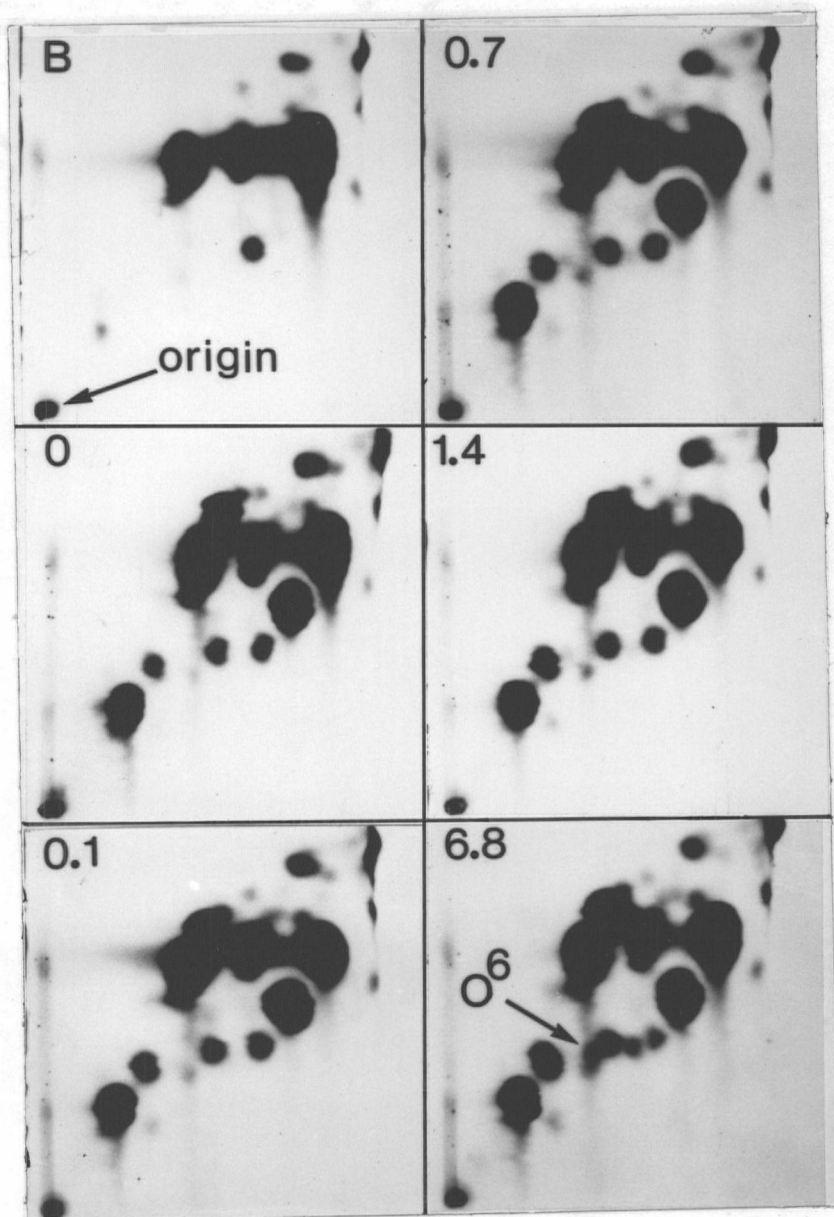


Figure 22 Sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine-method #1 (single HPLC purification-diphosphate level). Digested samples of $1\text{ }\mu\text{g}$ DNA were spiked with the indicated equivalent levels ($\mu\text{mole/mole}$) of synthetic $\text{O}^6\text{mdG3'p}$ and then assayed.

B. Column blank resulting from the labeling of $500\text{ }\mu\text{l}$ HPLC solvent collected from the column before injection of DNA samples.

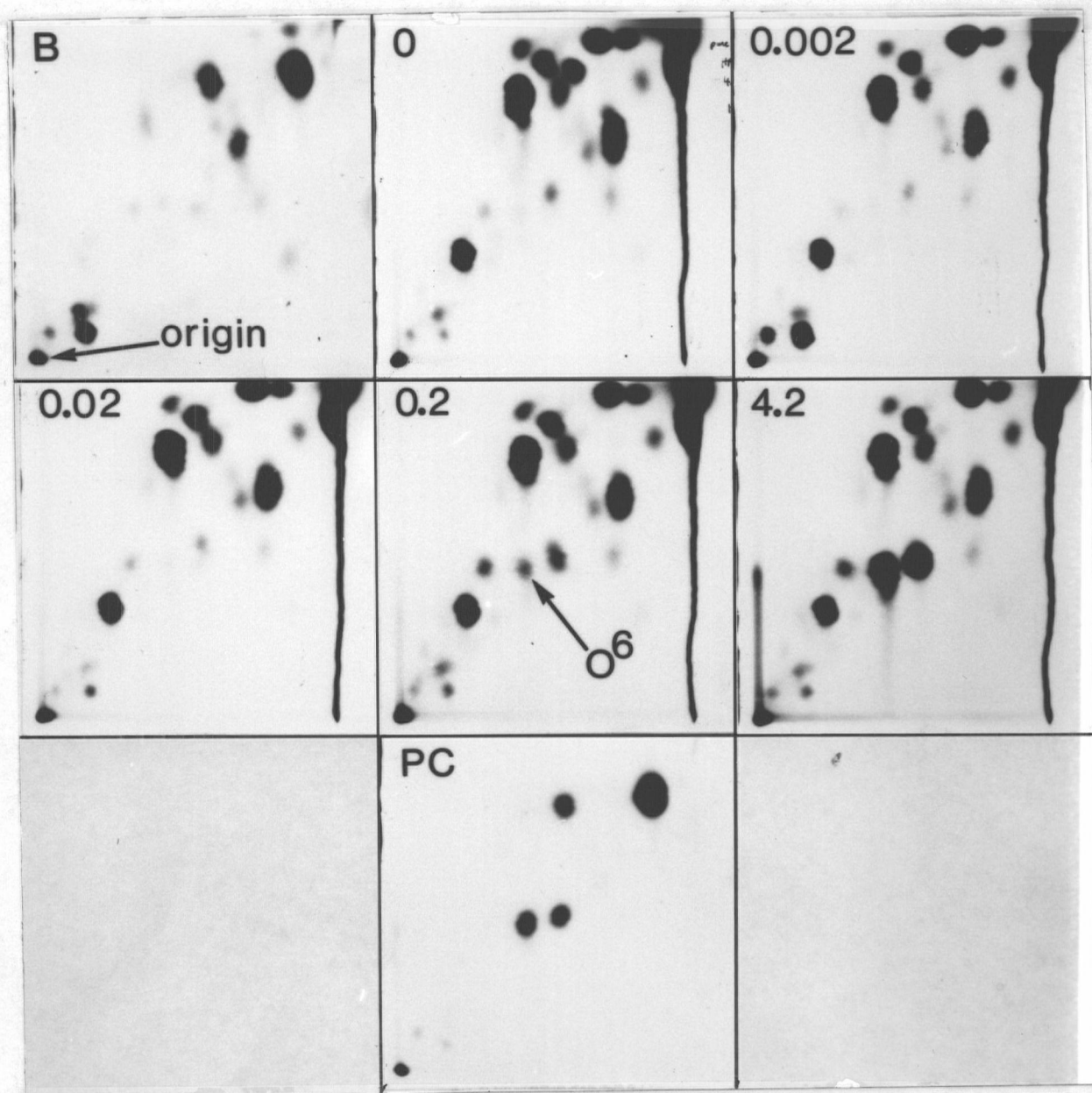


Figure 23 Sensitivity of the ^{32}P -postlabeling assay for O^6 -methylguanine-method #2 (dual HPLC purification-diphosphate level). Digested samples of 1 μg calf-thymus DNA were spiked with the indicated levels ($\mu\text{mole/mole}$) of $\text{O}^6\text{mdG3'p}$ then assayed.

B. Column blank.

PC. Positive control. 1×10^{-15} moles synthetic $\text{O}^6\text{mdG3'p}$ injected onto the column after DNA injections.

DETECTION AND QUANTITATION OF O⁶-METHYLGUANINE IN THE DNA OF
TISSUE-CULTURE CELLS TREATED WITH N-METHYL-N-NITROSOUREA

Introduction

N-Methyl-N-nitrosourea (MNU), a model direct-acting carcinogen (IARC 1978), induces the formation of O⁶-methylguanine adducts in DNA both in vitro and in vivo (Beranek et al. 1980). This has been established primarily through treatment of cells with radiolabeled MNU, followed by isolation of the DNA, acidic depurination and scintillation counting of labeled O⁶-methylguanine after HPLC isolation. The labeled purine has been identified by co-elution with authentic marker (Lutz 1979).

Treatment of Chinese Hamster Ovary (CHO) cells with MNU results in the formation of O⁶-methylguanine residues (Morris et al. 1983, Beranek et al. 1983). Most CHO cell lines are unable to excise/repair this adduct (Goth-Goldstein 1980). C3H10T1/2 cells, in contrast, possess alkyltransferase activity and therefore are capable of repair. Nevertheless, O⁶-methylguanine has been detected and quantitated in C3H10T1/2 cells exposed to MNU (Topal and Baker 1982).

As a trial of the newly developed ³²P-postlabeling assays for O⁶-methylguanine, DNA from both repair deficient CHO cells and repair proficient C3H10T1/2 cells treated with MNU was analysed for the presence of O⁶mdG3'5'p using methods #1 and #2.

1. Detection and Quantitation of O⁶-methylguanine in the DNA of CHO Cells
Treated with N-Methyl-N-nitrosourea

Methods

CHO cells were seeded into flasks containing 50 ml MEM supplemented with antibiotics and 10% fetal calf serum at a density of 0.5×10^6 cells per flask. The cells grew to confluence (2×10^6 cells per flask) after two days incubation at 37°C in a humidified atmosphere containing 5% CO₂.

10 flasks containing confluent CHO cell cultures were treated with MNU (0 to 19.3 mM). First, growth medium was poured off the cells, then a pre-weighed amount of MNU was quickly dissolved in 50 ml 'wash' MEM and the resulting solution immediately added to the flask, to cover the cells. For the control flask, 50 ml of 'wash' MEM (without MNU) was added to the cells. Exposure of the cells to MNU was for 3 hours at 37°C in a humidified incubator fed 5% CO₂ and 95% air.

After treatment, the medium containing MNU was removed from each flask and the monolayer rinsed for a few seconds with 10 ml of 0.1% trypsin. The rinse was discarded then the monolayer detached by trypsinization for 5 minutes at room temperature. The resulting cell suspension was then transferred to a 17 X 100 mm centrifuge tube. The cells were pelleted by centrifugation for 5 minutes at 2000 rpm. The supernatant was poured off then 10 ml phosphate buffered saline (PBS) added and the cells recentrifuged to remove the last traces of trypsin. Once the PBS rinse was removed the cells were dispersed in 1 ml fresh PBS, transferred to a 1.5 ml polypropylene tube and stored at -70°C.

DNA was extracted from CHO cells according to the method described on page 43.

The level of O⁶-methylguanine in the DNA of CHO cells treated with MNU was measured using method #1. Preceding the analysis of digested DNA samples, a 'column blank' consisting of 0.5 ml HPLC solvent through the column was collected. Digested CHO DNA from the control sample was injected onto the HPLC first followed by the treatment samples in order of exposure to increasing concentrations of MNU. After injection and collection of the control DNA, a positive control sample consisting of 2×10^{-14} moles O⁶mdG3'p was injected onto the column and the appropriate fraction collected.

Results

In the case of the two highest doses of MNU used (9.6 and 19.3 mM), the medium pH was 6 at the end of the exposure period, compared to 7.4 before treatment. The pH for the other doses remained unchanged compared to control. Also, most of the monolayer detached during treatment for the two highest doses and as a result very few cells were recovered. These samples were therefore, not analysed. The average (based on U.V.) yield of DNA extracted from one flask of CHO cells was 15 μ g.

Autoradiograms of the column blank, O⁶mdG3'p positive control and CHO DNA samples are shown in Figure 24. The films of DNA from MNU treated CHO cells show a spot that co-chromatographs with O⁶mdG3'5'p in 2-dimensional TLC by comparison with autoradiograms of the positive control. The spot intensity increases with higher doses of MNU. From the radioactivity of the adduct spots and using the specific activity of ³²P-ATP the level of O⁶-methylguanine in the CHO DNA was determined. The quantitative data are presented in Figure 25.

Figure 24 Detection and quantitation of O^6 -methylguanine in CHO cells treated with MNU. Autoradiograms show the results of exposing cells to the indicated concentrations (mM) of MNU and then analysing the DNA using method #1 (single HPLC purification-diphosphate level).

B. Column blank consisting of 500 μ l HPLC solvent from the column before DNA injections.

PC. Positive control - 2×10^{-14} moles synthetic O^6 mdG3'p injected onto the column after DNA injections.

Circled area- O^6 mdG3'5'p spot visible on original autoradiogram.

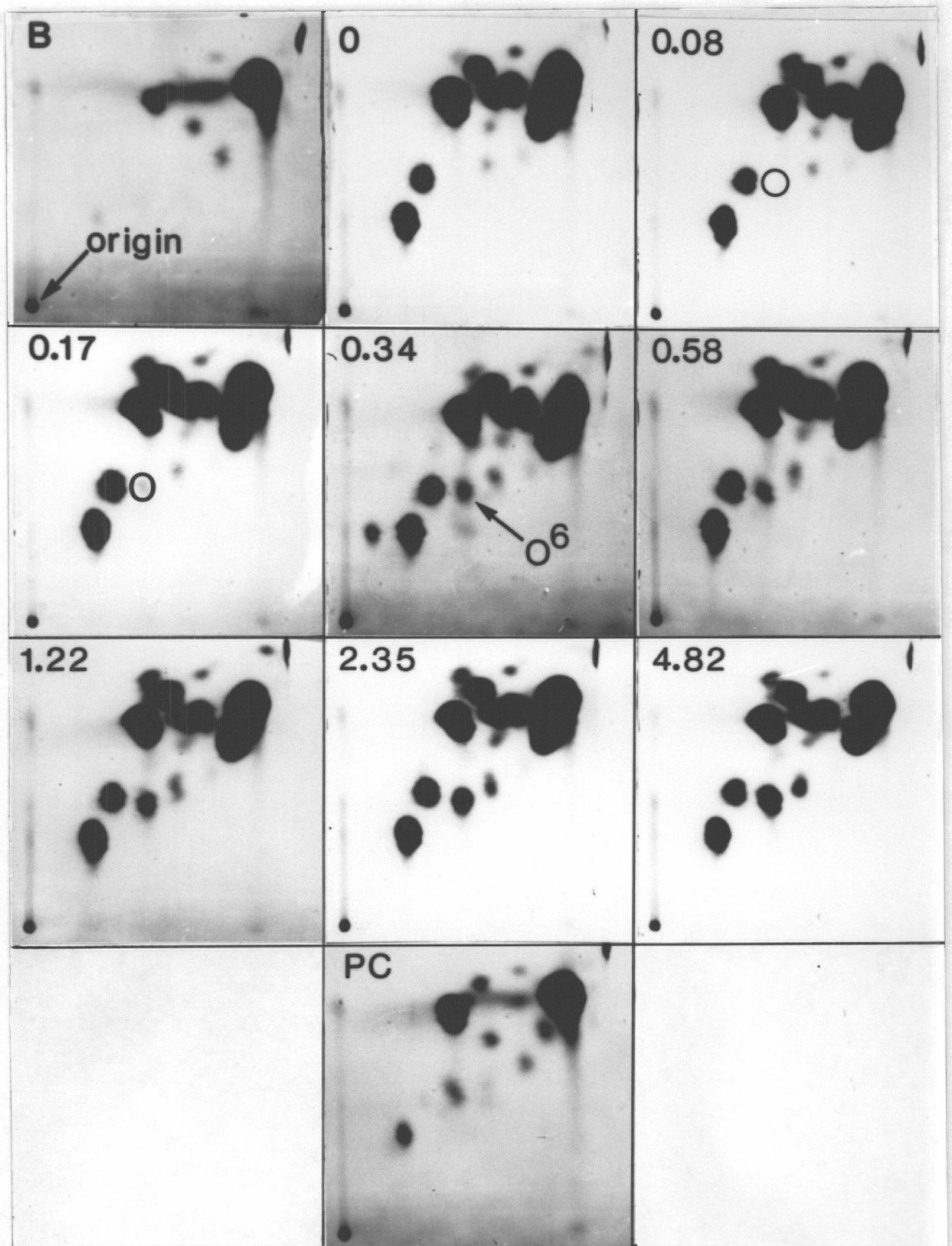


Figure 24

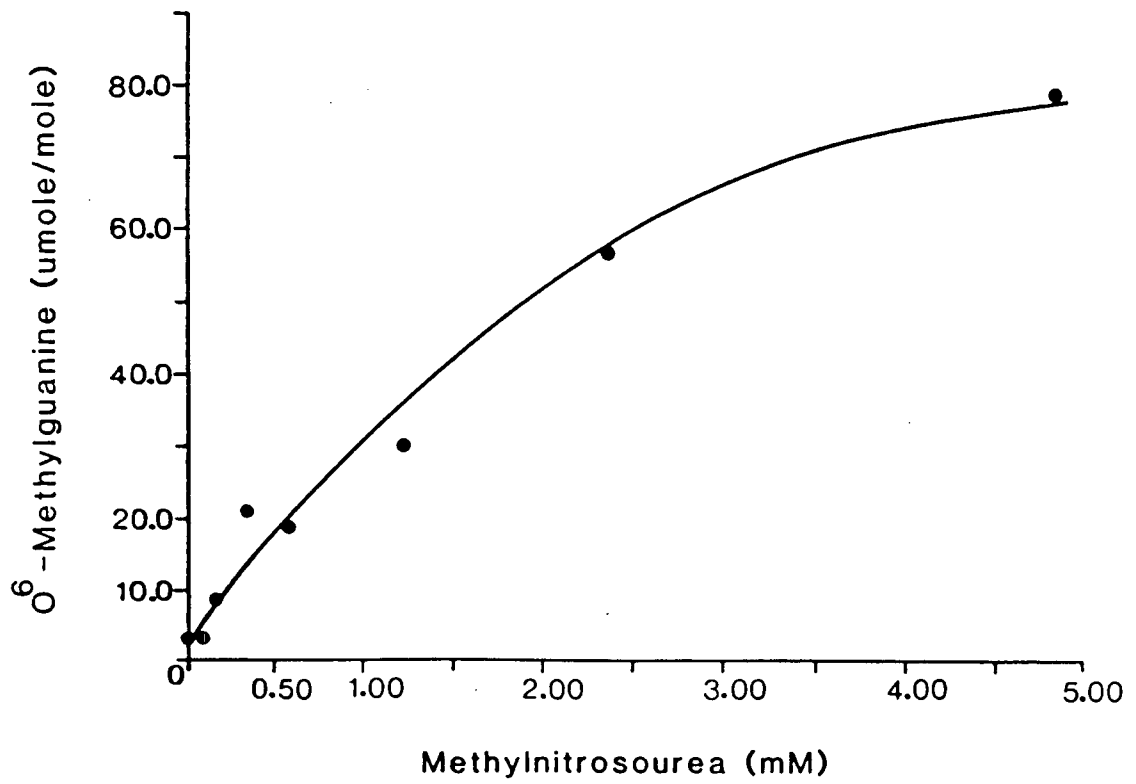


Figure 25 Dose-response of O^6 -methylguanine in the DNA of CHO cells treated with MNU.

2. Detection and Quantitation of O⁶-methylguanine in the DNA of C3H10T1/2 Mouse Embryo Fibroblast Cells treated with N-Methyl-N-nitrosourea

Methods

Mouse embryo fibroblast cells (passage 13) were seeded into 16 flasks (each containing 20 ml BME supplemented with 10% fetal calf serum and antibiotics) at a density of 0.5×10^6 cells per flask. After four days incubation at 37°C visual inspection revealed that the cells had grown to confluence.

Excluding control (no chemical) flasks, the freshly confluent C3H10T1/2 cells were exposed in duplicate to 7 different concentrations of MNU. For a given dose of MNU, medium was removed from the flasks then 20 ml of medium containing freshly dissolved MNU was added to each flask. Treatment was for 3 hours at 37°C in a humidified incubator fed 5% CO₂ and 95% air. Cells were harvested by trypsinization according to a modification of the procedure described previously for CHO cells.

DNA from C3H10T1/2 cells exposed to MNU was analysed for O⁶-methylguanine using method #1. In relation to cell lines which are incapable of repairing this adduct (e.g. CHO cells) the level of O⁶-methylguanine in C3H10T1/2 cells treated with an equivalent dose of MNU would be expected to be significantly lower. To increase the chances of detecting O⁶-methylguanine in C3H10T1/2 cells, 10% of the diluted labeled kinase reaction was further purified by reverse-phase HPLC, according to method #2. A column blank consisting of 700 µl HPLC solvent passed through the column was obtained before actual sample injections. Scintillation counting was done only for samples analysed using method #2.

The survival of C3H10T1/2 cells exposed to MNU was determined by a colony forming assay (Reznikoff et al. 1973). 200 cells were seeded into each of 24-60 mm counting dishes containing 5 ml BME + 10% FCS and antibiotics. This was done at the same time and with the same cells used for O⁶-methylguanine analysis (see above).

One day after seeding, the cells were exposed in triplicate (3 dishes/dose MNU) to the same concentrations of MNU used for the postlabeling analysis (see above). For each level of MNU, the medium was removed from 3 dishes then 5 ml medium containing freshly dissolved MNU was added to each dish. Incubation was for 3 hours at 37°C. After treatment the chemical was replaced with 5 ml fresh 'growth' medium and the cells allowed to grow for a further 8 days (a medium change was made on the fifth day).

The survival of C3H10T1/2 cells exposed to MNU in terms of their colony forming capacity was determined by comparing the number of colonies in dishes treated with MNU to those in the control dishes (without MNU). In preparation for colony counting, the dishes were rinsed twice in distilled water, fixed in 95% ethanol, air dried and the colonies were visualized by staining with 0.1% methylene blue. Colonies containing 20 or more cells were included in the analysis.

Results

The average yield of DNA isolated from two confluent flasks was 60.7 µg (based on U.V. absorption). Autoradiograms of C3H10T1/2 DNA analysed using method #1 are shown in Figure 26. A spot that co-chromatographs with labeled synthetic O⁶mdG3'5'p is present for MNU concentrations of 1.27 mM and higher. Maps of DNA, analysed using method #2 are shown in Figure 27. Adduct spots are present for all concentrations of MNU used. An MNU/O⁶-methylguanine dose-

Figure 26 Detection and quantitation of O^6 -methylguanine in the DNA of C3H10T1/2 cells treated with MNU. Shown, are autoradiograms resulting from the exposure of cells to the indicated dose (mM) of MNU followed by postlabeling analysis of DNA using method #1 (single HPLC purification-diphosphate level).

B. Column blank.

PC. Positive control consisting of 2×10^{-14} moles synthetic O^6 mdG3'p injected onto the column after DNA injections.

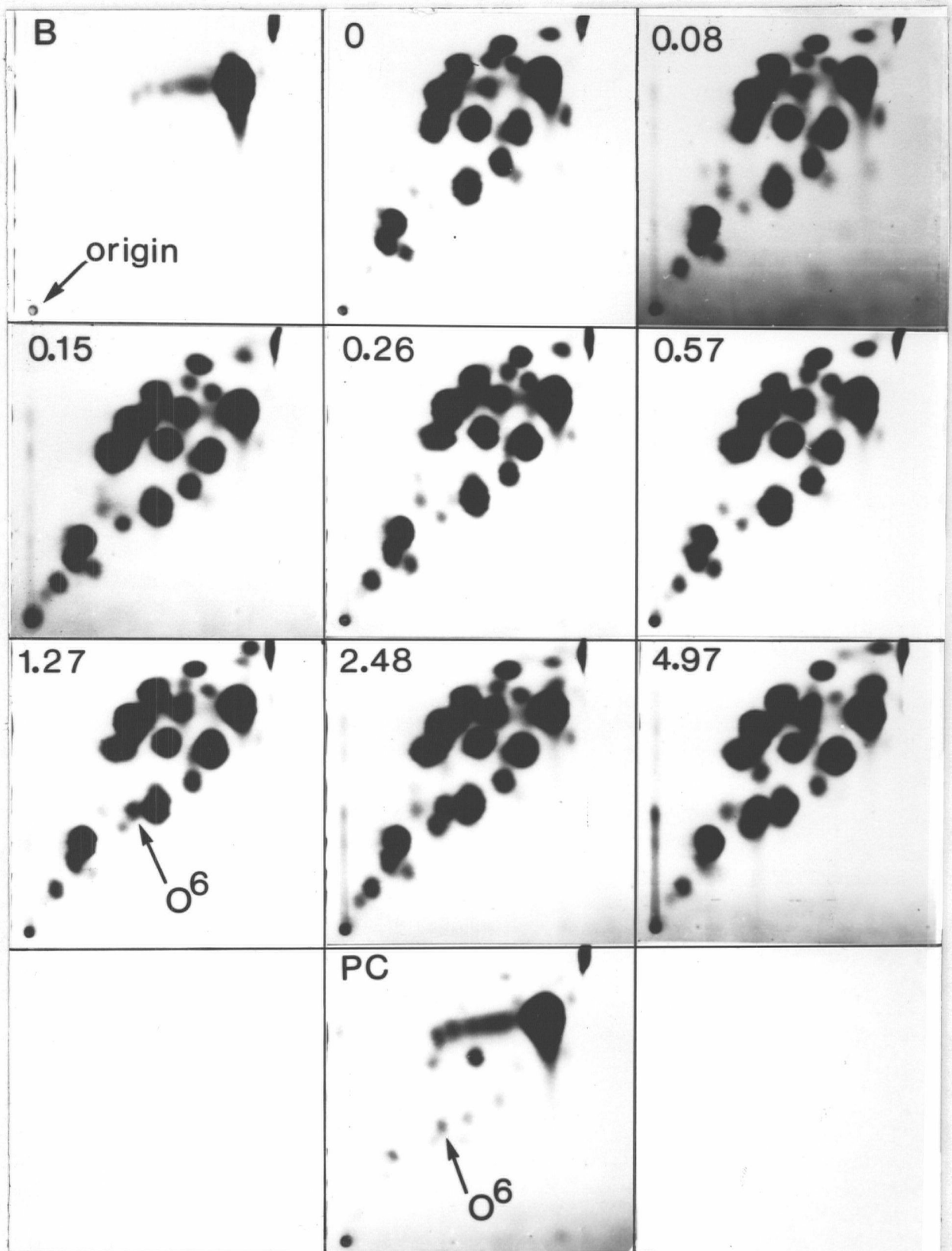


Figure 26

Figure 27 Autoradiograms of DNA from C3H10T1/2 cells exposed to various doses (mM) of MNU. DNA was analysed by ^{32}P -postlabeling using method #2 (dual HPLC purification-diphosphate level).

B. Column blank.

PC. Positive control= 2×10^{-14} moles synthetic $\text{O}^6\text{mdG3}'\text{p}$ injected onto the column after DNA injections.

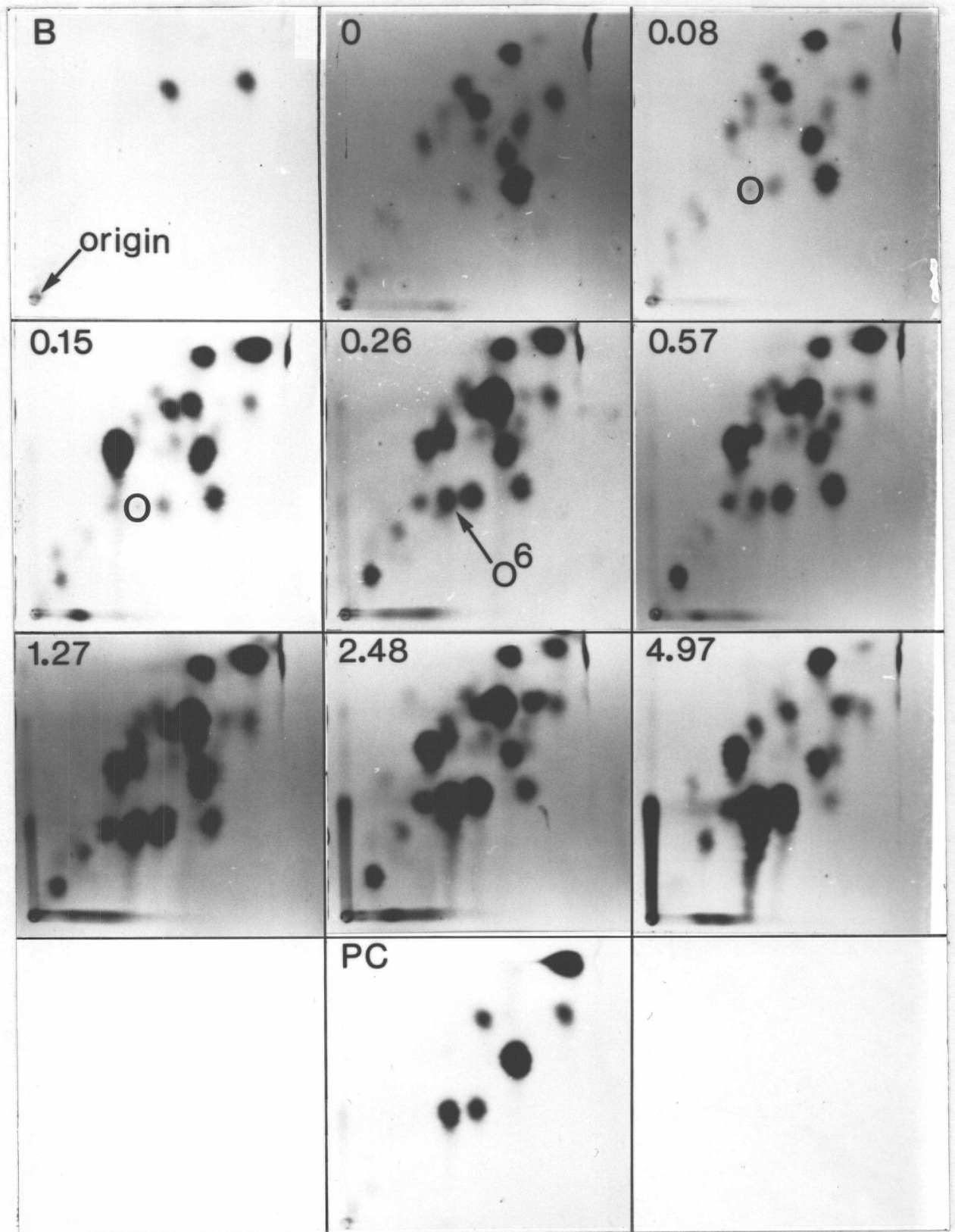


Figure 27

response curve illustrating adduct levels determined using method #2 is shown in Figure 28.

The autoradiograms from analysis of C3H10T1/2 DNA (Figure 26) using method #1 show many additional background spots compared to autoradiograms of CHO DNA (Figure 24), including one that partially interferes with O^6 mdG3'5'p. Using an HPLC method, these spots were traced to a markedly higher RNA content for the C3H10T1/2 samples and are presumably residual ribonucleoside 3'5'-bisphosphates. Additional HPLC purification of labeled O^6 mdG3'5'p largely removed this putative RNA associated spot, reducing interference and improving the sensitivity of quantitation. RNA contamination of DNA samples can also be eliminated by repeated ethoxyethanol precipitations (data not shown).

Results of the assay for cell survival are shown in Figure 29. The plating efficiency of these cells was 25%. These data show that MNU is very toxic toward C3H10T1/2 cells. From Figure 28 and interpolation of the curve in Figure 29, the level of O^6 -methylguanine associated with 50% survival was 3.5 μ mole/mole.

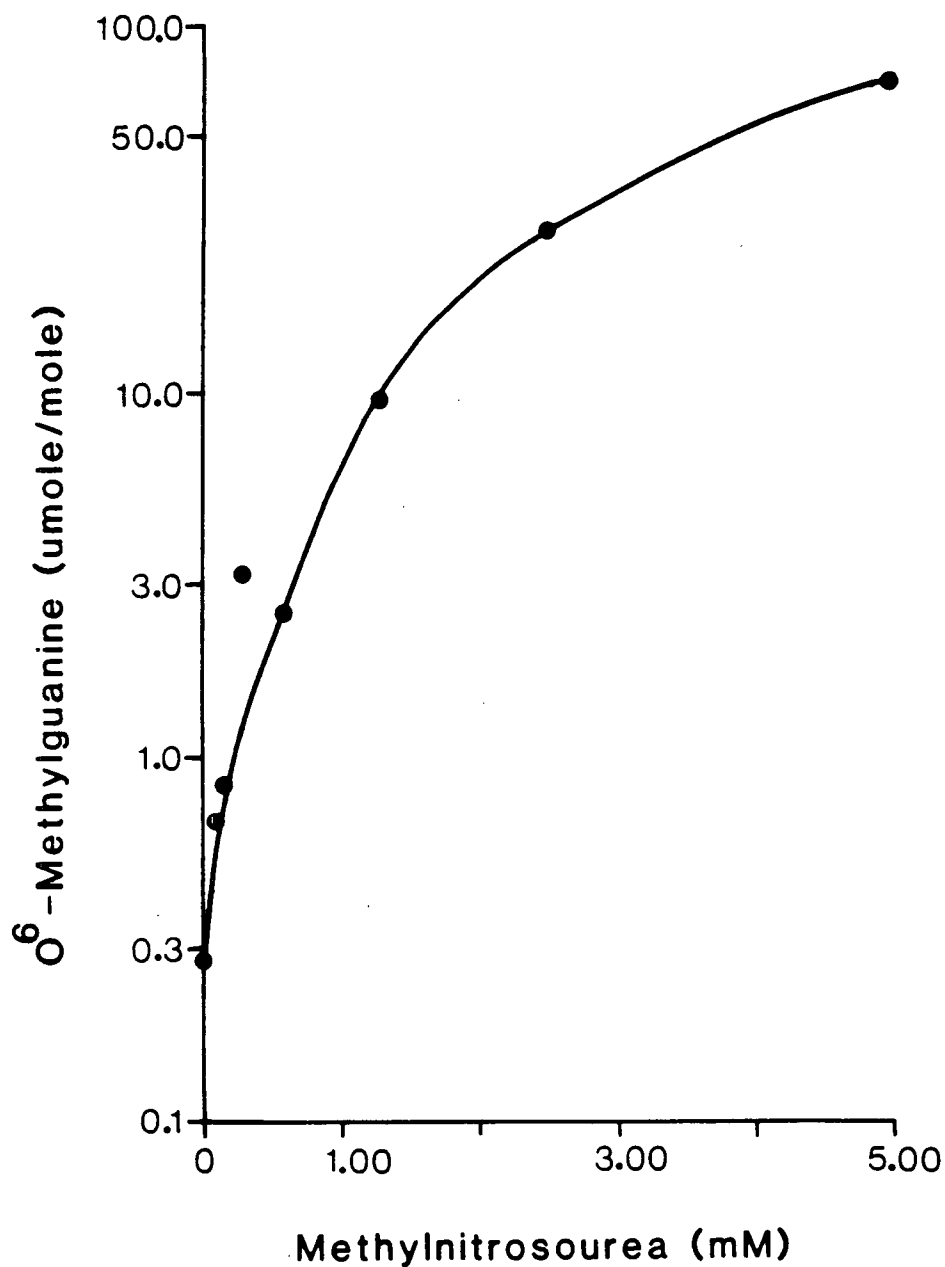


Figure 28 Dose-response of O⁶-methylguanine in the DNA of C3H10T1/2 mouse-embryo fibroblast cells treated with MNU. For each dose of MNU, DNA was analysed for O⁶-methylguanine by ³²P-postlabeling using method #2.

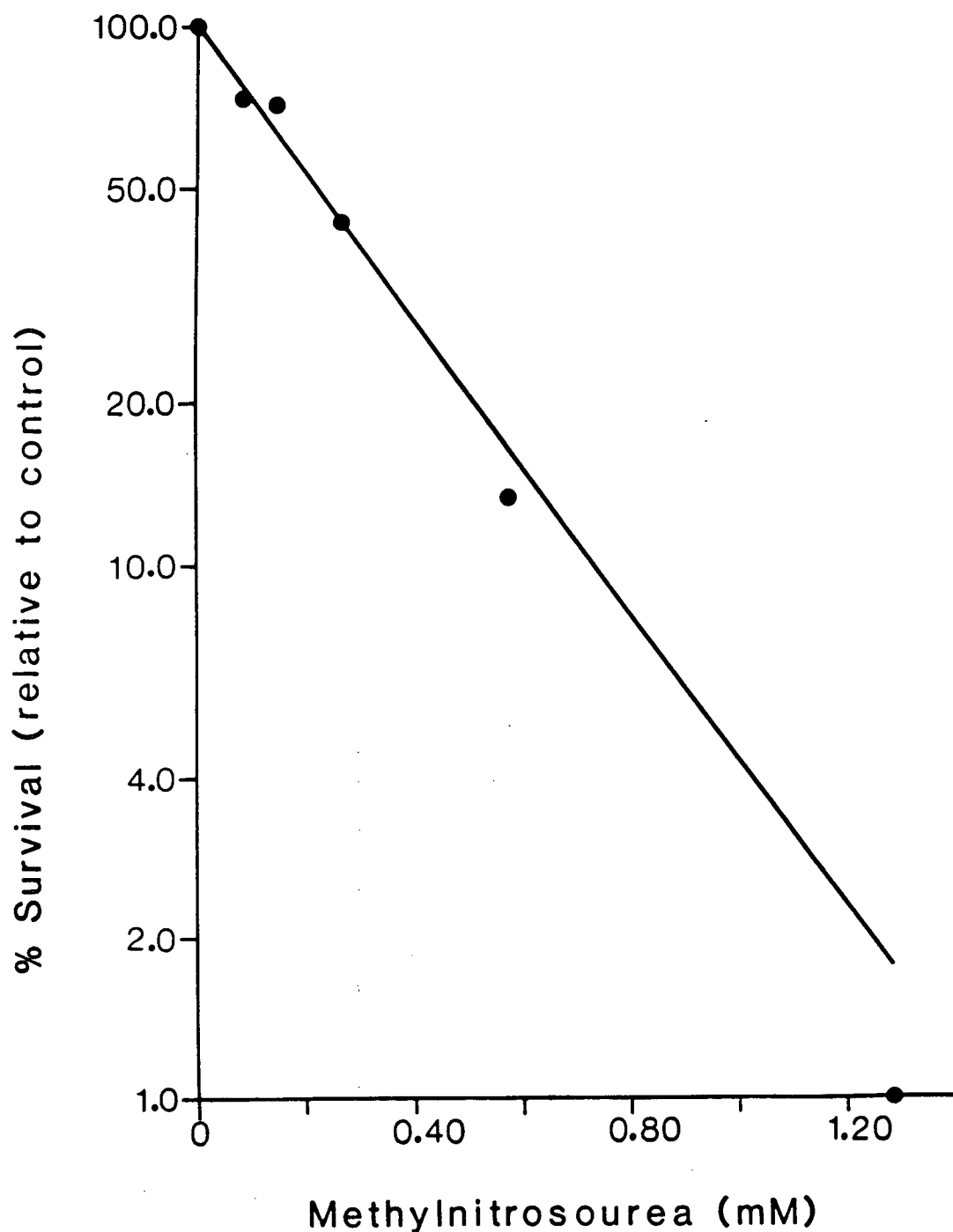


Figure 29 Survival of C3H10T1/2 mouse-embryo fibroblast cells exposed to MNU. 200 cells were seeded into dishes and the next day, exposed to MNU for 3 hours at 37°C. After treatment, the medium was replaced and the cells incubated at 37°C for a further 8 days. Each data point represents an average of three plates per dose.

DISCUSSION AND CONCLUSIONS

The primary goals of this project were to develop a sensitive ^{32}P -postlabeling assay for O^6 -methylguanine requiring only microgram quantities of DNA and to assess the feasibility of its application to adduct detection in humans.

1. Method Development

$\text{O}^6\text{mdG3}'\text{p}$, required as a chromatography marker for development of the method, was prepared using a novel two-stage approach. Similarity of the U.V. spectrum of $\text{O}^6\text{mdG3}'\text{p}$ to that of O^6mdG indicates retention of the O^6 -methylated guanine base. Conversion to O^6mdG by nuclease P1 and resistance to hydrolysis by 5'-nucleotidase is confirmation that the compound is a deoxynucleoside 3'-monophosphate. This is the first reported synthesis of the modified nucleotide. There are syntheses available for the deoxynucleoside form of many DNA adducts. The authentic standards necessary for the development of postlabeling assays for several other alkylated DNA adducts such as O^4 -methylthymine might therefore be synthesized by this route. The ability of $\text{O}^6\text{mdG3}'\text{p}$ to be ^{32}P -labeled by polynucleotide kinase and ^{32}P -ATP is further confirmation of the compound's identity and is the basis for a method to measure O^6 -methylguanine in DNA by postlabeling.

Reverse-phase HPLC was used to isolate $\text{O}^6\text{mdG3}'\text{p}$ from normal nucleotides before postlabeling. HPLC purification reduces the amount of ^{32}P -ATP required for quantitative labeling of $\text{O}^6\text{mdG3}'\text{p}$ and the associated level of radioactivity in the assay. $\text{O}^6\text{mdG3}'\text{p}$ is susceptible to enzymatic digestion by nuclease P1, therefore enrichment procedures based on selective dephosphorylation of normal nucleotides are not applicable (Reddy and Randerath 1986). HPLC enrichment resulted in an approximately 1000 fold

reduction in the amount of normal nucleotides relative to $O^6\text{mdG3}'\text{p}$ (data not shown). HPLC chromatography of DNA digests enabled the exact amount of digested normal nucleotides associated with a kinase reaction to be determined. This should improve the accuracy since assumptions based on U.V. quantitated DNA before digestion are eliminated. The recovery and analysis of several different adducts in one chromatographic run is possible if the corresponding markers are available.

After postlabeling of the HPLC fraction containing $O^6\text{mdG3}'\text{p}$, $O^6\text{mdG3}'5'\text{p}$ was resolved from residual labeled normal nucleotides using two-dimensional PEI-cellulose thin-layer chromatography; this is necessary for quantitation. This embodies the basic procedure (method #1) for analysis of O^6 -methylguanine by ^{32}P -postlabeling. The procedural steps of this method and three variations (methods #2-#4) investigated to improve the sensitivity are summarized in Figure 16.

2. Comparison of Methods

The performance characteristics of the four methods are given in Table 4. HPLC purification of $O^6\text{mdG3}'5'\text{p}$ (method #2) lowered the background enabling the detection of ten times less adduct compared to method #1 even though the recovery dropped significantly to 38%. The improved performance is at the expense of exposure to more radioactivity and a slightly longer analysis time. It has been reported that conversion of labeled normal nucleotides and adduct nucleotides, resulting from the analysis of DNA treated with methylating agents, to their respective 5'-monophosphates reduces the background in the adduct area of the TLC chromatograms (Reddy et al. 1984). This prompted the development of methods #3 and #4 based on treatment of the kinase reaction with nuclease P1. In contrast to the literature evidence, the results show that analysis at the 5'-monophosphate level did not improve the sensitivity.

TABLE 4

PERFORMANCE CHARACTERISTICS OF THE ^{32}P -POSTLABELING ASSAYS FOR O⁶-METHYLGUANINE

<u>Method</u>	<u>Time</u> ^a (days)	<u>Radiation</u> ^b	<u>Sensitivity</u> ^c ($\mu\text{mole/mole}$)	<u>Recovery</u> ^d (%)
1	5	low	6.5 ± 1.5	84
2	6	high	0.5	38
3	9	low	8.5	85
4	10	high	2.0	51

^a Time of analysis from tissue to result.

^b Approximate operator exposure.

^c Estimated sensitivity defined as twice the background radioactivity in the adduct zone for analysis of 1 μg DNA from untreated tissue-culture cells. The value for method #1 was extracted from Table 1. The rest of the values are from a single experiment and are the average of duplicate analyses.

^d HPLC analysis of 1 μg DNA spiked with standard O⁶mdG3'p compared to the same amount of standard O⁶mdG3'p labeled directly. The data for methods #1 and #2 are averages from two experiments.

Methods #3 and #4 are much longer, technically more difficult and expose the operator to more radiation compared to methods #1 and #2. In conclusion, this work shows that methods #1 and #2 are superior to methods #3 and #4 and that purification of O⁶mdG3'5'p by HPLC (method #2) allows detection of 0.5 micromole O⁶-methylguanine per mole of normal nucleotide in 1 microgram of DNA.

3. Factors Limiting the Performance of the ³²P-Postlabeling Assays

3.1 Reproducibility

The standard deviation for replicate analysis (method #1) of synthetic O⁶mdG3'p is $\pm 6\%$ (Table 1). This is evidence that the reproducibility of the assay is high for repeated samplings from a single DNA digest. The major 'within digest' variation probably arises from errors in cutting out the O⁶mdG3'5'p spot along with infringement of the adduct zone by background spots. The reproducibility of DNA digestion determined by separate injection and HPLC analysis of DNA aliquots taken from a single solution is higher within an experiment than between (Table 2). The higher between experiment variation in digestion could arise from errors in U.V. quantitation of DNA. Variable RNA contamination of samples could contribute because HPLC quantitation is specific for DNA nucleotides. These data suggest that the release of O⁶mdG3'p from DNA is reproducible and that multiple analysis of single DNA solutions are not necessary. The assay reproducibility as determined from multiple analysis of one DNA sample should be higher than the reproducibility of DNA digestion determined here. This is because the adduct levels (what the assay measures) are expressed as a ratio of moles of adduct to the digestion yield for each individual injection and this ratio should be independent of digestion. The reproducibility of the assay may be reduced at

low adduct levels because the background forms a larger percentage of the radioactivity measured in the O^6 mdG3'5'p zone.

3.2 Recovery

The recovery of O^6 mdG3'p after enzymatic release from DNA was about 85% for methods #1 and #3 (Table 4). Further HPLC purification of the labeled adduct (methods #2 and #4) reduced the recovery to below 50%.

The adduct recoveries determined here for O^6 mdG3'p are quite consistent with those found for other adducts measured using postlabeling methodology. Recoveries of labeled adducts were 40-50% and 70% for DNA modified in vitro with N-hydroxy-2-aminofluorene and a diol-epoxide of benzo[a]pyrene respectively (Gupta et al. 1982). Postlabeling analysis of DNA modified in vitro with 2-acetylaminofluorene, benzidine, and 4-dimethylaminoazobenzene gave adduct recoveries of 45-50% (Reddy et al. 1984). Finally, analysis by 32 P-postlabeling of defined mixtures of normal deoxynucleoside 3'-monophosphates and synthetic N^2 -(dehydro-estragol-1'-yl)-deoxyguanosine-3'-phosphate showed that recovery of the labeled adduct was about 60% that of the normal nucleotides (Fennell et al. 1986).

There are two mechanisms by which O^6 mdG3'p could be lost during the process of chromatography and labeling. These 'sinks' are either chemical/enzymatic or physical. Under the first mentioned category, decomposition of the adduct nucleotides in low pH (3.5) ammonium formate during HPLC and TLC may be important. O^6 mdG is about 50 times less stable to depurination than the unmethylated deoxynucleoside in acidic conditions (Farmer et al. 1973). Losses incurred when a second HPLC purification is performed may in part be due to simply a longer contact with low pH solvent. Drying down the adduct nucleotides may temporarily expose them to a combination of high strength ammonium formate/low pH and accelerate the

decomposition. Physical losses may occur through irreversible absorption. This can happen during HPLC itself or through incomplete uptake of the dried down fraction from the tube before labeling. Using a radiation detector, it was apparent that not all of the radioactivity was recovered from the tube after addition of the HPLC carrier preparatory to the second purification (data not shown).

3.3 Sensitivity

The sensitivity of the postlabeling technique for DNA adduct detection is enhanced when the target adducts are separable as a class from the normal nucleotides before postlabeling. Limits of detection for aromatic and/or bulky adducts are very low (0.0001 to 0.001 $\mu\text{mole/mole}$) because in these cases normal nucleotides are effectively eliminated by extraction, chromatographic isolation or enzymatic digestion prior to labeling. $\text{O}^6\text{mdG3}'\text{p}$ differs by only a methyl group from unmodified nucleotides and therefore is difficult to resolve from them. Development of a sensitive postlabeling method to detect this adduct represents a significant challenge to the chromatographer.

It is apparent from this work that the sensitivity of the method is not hampered by an inability to detect $\text{O}^6\text{mdG3}'\text{p}$ but rather by the background. This can be demonstrated by, for example assuming that an adduct level giving 100 dpm above background is measurable with some reliability. If the ^{32}P -ATP specific activity is 3000 Ci/mmol then this corresponds to 7.51×10^{-18} moles adduct. Assuming the adduct arose from analysis of 1 μg DNA or about 3 nmole DNA-p then the level of adduct in the DNA would be 0.005 $\mu\text{mole/mole}$ thereby defining the possible sensitivity in the absence of chromatographic background.

HPLC isolation of O^6 mdG3'p from enzymatically digested DNA does not remove all normal nucleotides and compounds which are labeled by 32 P-ATP and polynucleotide kinase. Some of this labeled residual material elutes with and very close to O^6 mdG3'5'p in the two dimensional TLC system. These impurities therefore, reduce the sensitivity and reproducibility of the assay. The major contributions to the background seen in control samples are a) non-specific radioactivity from 32 P-ATP and oligonucleotides, b) assay and RNA related background spots and c) tailing radioactivity from residual dA3'5'p. An additional purification stage before PEI-TLC reduced these impurities (method #2). This was accomplished by chromatography of the labeled kinase mixture on a reverse-phase column followed by collection of the O^6 mdG3'5'p fraction.

The theoretical sensitivity of the postlabeling assay when applied to human DNA will depend on the 'baseline' levels of O^6 -methylguanine. Sources of this adduct are ubiquitous N-nitrosamines in the environment and non-enzymatic methylation of DNA by the intracellular methyl group donor, S-adenosyl-L-methionine (Barrows and Shank 1981, Rydberg and Lindahl 1982). Baseline levels will also be influenced by the alkyltransferase activity of the particular tissue.

The sensitivity of the method (0.5 μ mole/mole) exceeds that of techniques based on HPLC fluorescence or scintillation counting of 14 C labeled purines after treatment with labeled chemicals (Herron and Shank 1979, Baird 1979). It performs about as well as immunoassays and radioimmunoassays which typically require larger (1000 μ g) samples of DNA (Wild et al. 1983, Castonguay et al. 1985). The 32 P-postlabeling assay for O^6 -methylguanine should be more selective than immunoassays for this adduct because of the numerous chromatographic isolations and purifications done before quantitation.

4. Detection and Quantitation of O⁶-methylguanine in Mammalian Tissue-Culture Cells Treated with N-Methyl-N-nitrosourea

³²P-Postlabeling analysis of DNA from repair proficient (C3H10T1/2) and repair deficient (CHO) mammalian tissue-culture cells treated with MNU gives a compound that co-chromatographs with synthetic marker O⁶mdG3'p or O⁶mdG3'5'p in four different systems (Figures 24, 26 and 27). This was not observed for untreated cultures. Under similar treatment conditions, MNU has been shown to induce the formation of O⁶-methylguanine in these cell lines using methods completely unrelated to postlabeling (Morris et al. 1983, Beranek et al. 1983, Topal and Baker 1982)).

In the trial with C3H10T1/2 cells, the lower background resulting from an additional HPLC purification enabled the detection of adduct at all dosage levels, even that allowing 70% survival (Fig 27). These data suggest a high negative correlation (coefficient= -0.992) between the survival of C3H10T1/2 cells and levels of O⁶-methylguanine for low doses of MNU. This result is consistent with other evidence which suggests that this adduct plays a central role in cell killing by N-nitrosamines (Doniger et al. 1985).

5. Recommendations to Enable Application of the Method to Human Studies

The level of O⁶-methylguanine adducts in the DNA of exfoliated cells from humans chronically exposed through habit or environment to N-nitrosamines is probably very low. There is both direct and indirect evidence that suggests this may be the case. First, the capacity of most human tissues studied for repair of this lesion is very high (Grafstrom et al. 1984, Hall et al. 1985). Secondly, some tissues from people exposed to putative N-nitrosamines have been analysed for O⁶-methylguanine using radioimmunoassays. In one report, this adduct was not detected in the placentae of 10 women who smoked during

pregnancy, using a radioimmunoassay with a sensitivity of 0.19 $\mu\text{mole/mole}$ (Everson et al. 1987). In another report, esophageal tissues from a population at an elevated risk for esophageal cancer due to possible N-nitrosamine exposure from the diet were analysed for this adduct (Umbenhauer et al. 1985). The radioimmunoassay used in this study detected a high value of 0.05 $\mu\text{mole/mole}$ O⁶-methylguanine in one individual and lower values in others when 1000 μg of DNA was employed.

To be useful for the detection of low O⁶-methylguanine levels (less than 0.05 $\mu\text{mole/mole}$) in small samples of DNA from exfoliated human cells, the sensitivity of the postlabeling method must be increased by a factor of ten; this should be the focus of future method development.

The required improvement in sensitivity could be achieved through analysis of more DNA (up to ten micrograms) in combination with reduction of radioactive background in the adduct zone. Lowering of background might be effected by increasing the efficiency of the chromatographic separations inherent in the method. This is preferred over including more HPLC purification steps which would cause further losses of adduct. Improving the resolution of O⁶mdG3'p from normal nucleotides in the initial HPLC step using different solvent systems perhaps in combination with other columns may lower the level of impurities in the adduct fraction. HPLC separations using a solvent of higher pH may also improve the recovery of O⁶mdG3'p.

The accuracy of the ³²P-postlabeling assay for O⁶-methylguanine should be directly validated. Simultaneous measurement /analysis of a DNA sample containing O⁶-methylguanine using the postlabeling assay and another method would serve this purpose. For example, O⁶-(¹⁴C)-methylguanine forms in the DNA of CHO cells treated with ¹⁴C-labeled MNU (Goth-Goldstein 1980). After DNA extraction, some of the material could be depurinated in acid and isolated from the hydrosylate by HPLC using authentic O⁶-methylguanine as a U.V.

marker. The labeled purine could then be quantitated by scintillation counting. Several other aliquots of the DNA could be analysed by postlabeling and the levels of adduct determined using the different methods directly compared.

More data on the reproducibility of the method should be obtained. Of relevance is the standard deviation of values from replicate analysis of aliquots of a DNA sample containing O⁶-methylguanine. The sensitivity of the method could be substantially improved if the level of background were reproducible. The adduct levels could then be derived by subtraction of background radiation in control samples from that of samples containing low levels of O⁶-methylguanine.

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