Development and Application of a Fluorescent Postlabelling Assay for the Detection of N7-Alkylguanines

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by

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Abstract

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DNA reacts with many alkylating carcinogens to give N-alkylated bases as major products, which can be used as biomarkers of human exposure to carcinogens. However, quantitation of this DNA damage is found to be difficult, largely due to the instability of the modified bases and hazardous nature of some detection methods (^{32}P-postalabelling). This instability is utilised in an approach that uses a non-radioactive postlabelling technique to detect and quantify N7-alkylguanine bases. The technique is based on the reaction of phenylmalondialdehyde with N7-alkylguanines to give fluorescent pyrimidopurines, *i.e.* 7-phenyl-10-oxo-1-alkyl-9,10-dihydropyrimido-[1,2,a]-purines. The specificity of the assay is improved by the use of immunoaffinity purification of the adducts prior to fluorescent postlabelling. N^2-Carboxymethyl-N7-ethylguanine was coupled with methylated bovine serum albumin, and used to immunise mice, to successfully produce monoclonal antibodies specific for N7-ethylguanine. The monoclonal antibodies were subsequently used to manufacture immunoaffinity columns, which were incorporated into the fluorescent postlabelling assay. A method for the preparation of suitably functionalised N7-alkylguanine derivatives for use in preparing monoclonal antibodies is also described which requires fewer steps and uses more readily available starting materials than previously described methods.

The sensitivity and application of the approach is exemplified by the quantitation of N7-methylguanine and N7-ethylguanine in DNA. Calf thymus DNA treated *in vitro* with synthesised 2-diazopropanoic acid (a possible precursor to an ethylating agent, formed from alanine in tobacco after undergoing nitrosation and decarboxylation on burning), dimethylsulphate, diethylsulphate and exposed to tobacco smoke, was analysed by HPLC fluorescence. The assay is shown to be very sensitive with a limit of detection being approximately 0.8 pmol of adduct for a given sample of DNA. This has enabled the detection of one N7-methylguanine adduct/10^6 nucleotides from 1 mg of DNA. Unfortunately, the assay was unsuccessful in detecting significant levels of N7-ethylguanine from DNA exposed to tobacco smoke and 2-diazopropanoic acid.

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Abbreviations

7-AlkGua	N7-alkylguanine
7-EtGua	N7-ethylguanine
7-MeGua	N7-methylguanine
7-HOEtGua	N7-hydroxyethylguanine
7-HOPrGua	N7-hydroxypropylguanine
7,9-diHOPrGua	N7,9-dihydroxypropylguanine
7-CEtGua	N7-carboxyethylguanine
7-CMeGua	N7-carboxymethylguanine
7-EtAde	N7-ethyladenine
AP	apurinic or apyrimidinic
Phmal	phenylmalondialdehyde
Phmal-7-AlkGua	phenylmalondialdehyde derivative of N7-alkylguanine
Phmal-7-MeGua	phenylmalondialdehyde derivative of N7-methlguanine
Phmal-7-EtGua	phenylmalondialdehyde derivative of N7-ethylguanine
PAH	polyaromatic hydrocarbons
NNAL	4-(methylnitrosoamino)-1-(3-pyridyl)-butanal
NNK	4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
CT DNA	calf thymus deoxyribonucleic acid
mBSA	methylated bovine serum albumin
Ov	ovalbumin
ATP	adenosine triphosphate
Ig	immunoglobulin
CS	cell supernatants
PEG	polyethylene glycol
PBS	phosphate buffered solution
EDC	1-ethyl-3-dimethylaminopropylcarbodiimide
HPLC	high performance liquid chromatography
IA	immunoaffinity

ECD	electrochemical detection
UV	ultraviolet
IR	infrared
TLC	thin layer chromatography
ELISA	enzyme-linked immunosorbent assay
NMR	nuclear magnetic resonance
MS (FAB)	mass spectrometry (fast atom bombardment)
EI	electron impact
MW	molecular weight
m/z	mass to charge ratio
ppm	parts per million
ODS	octadecyl silyl
BDS	base-deactivated silica

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CHAPTER 6

Introduction

Chapter 1. Introduction

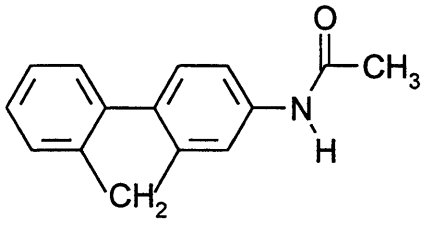
Neoplasms and malignant tumours account for one fifth of the deaths annually in the United Kingdom (Pitot and Dragan, 1991). A neoplasm ('new growth') consists of a mass of cells which have undergone a series of fundamental, heritable and irreversible changes in their physiology and structure. Cancer research indicates that the transformation of a normal cell to a neoplastic cell is probably caused by an alteration to the nuclear DNA, causing a permanent alteration in the coding sequence of the bases on the DNA backbone, which leads to abnormal proteins with altered or disabled function inherited by the daughter cells after division. This is known as the somatic mutation theory, and there is much experimental evidence in its support. Carcinogenesis may be actively induced by a variety of different agents, and these have been classified into four distinct categories; chemical, physical, biological and genetic (Table 1.1). The subject of this chapter will be a discussion of the various types of DNA damage that are associated with causing cancer and methods employed to detect and quantify the extent of damage.

Table 1.1. General classification of carcinogenic agents (Pitot and Dragan, 1991).

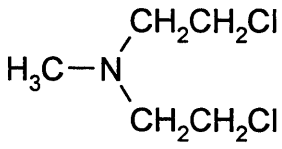
Class of Carcinogenic Agent	Examples
Chemical	Polycyclic aromatic hydrocarbons, aromatic amines and halides, diet, hormones, polymer surfaces and alkylating agents.
Physical	Ionising (X and γ ray, particle radiation) and ultraviolet radiation.
Biological	Papilloma, herpes, retro and hepadna viruses.
Genetic	Transgenesis by enhancer-promoter-oncogene constructs; selective breeding.

1.1. Identification of Chemical Carcinogens

Much of the evidence that human cancer is caused by chemical substances comes from epidemiological studies that first began in the 18th century. Observations made by the physician John Hill, showed that a high incidence of nasal cancers occurred as a consequence of using tobacco snuff and in 1775, the surgeon Sir Percival Pott described the occurrence of cancer in the scrotum of a number of young male patients who had previously been employed as chimney sweeps in their childhood (Miller, 1978). Pott suggested that the soot, to which the men had been exposed in their youth, was the causative agent of their condition. This was one of the first suggestions of a chemical causing cancer in humans. Similar observations were made some time later by Butlin (1892). Various other observations of increased cancer incidences in certain occupational groups (urinary bladder cancer correlated with aniline dye industry) were made in the 19th century. The polycyclic aromatic hydrocarbon (PAH), benzo[*a*]pyrene was identified as the first pure chemical carcinogen in 1933, and was isolated from coal tar. Various experiments by different groups of scientists had shown that coal tars and their extracts induced skin cancer in mice (Miller, 1978). Extensive studies followed which provided a large literature on the chemical features that were required for the carcinogencity of PAHs. It was starting in the late 1940s that the aromatic amines (2-acetylaminofluorene), inorganic chemicals (zinc beryllium silicate and beryllium oxide), nitrogen mustards and the wide range of alkylating agents were identified as having carcinogenic activity. As can be seen from Figure 1.1., the chemical carcinogens can be structurally very diverse. The possible metabolic transformation to ultimate carcinogens was shown by Miller and Miller in 1947. They found that a metabolite of N,N-dimethyl-4-aminoazobenzene covalently bound to the hepatic proteins of rats fed this dye. Further studies by different groups of researchers, lead to the generalisation that chemical carcinogens needed to be activated to the ultimate carcinogens (electrophilic species), and that this was usually carried out by a category of enzymes collectively referred to as the mixed-function oxidases (Cooper *et al.*, 1995). The known exceptions are the direct-acting alkylating or acylating agents.



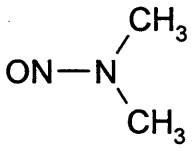
2-Acetylaminofluorene



Nitrogen Mustard

BeO

Beryllium Oxide



Dimethylnitrosamine

Figure 1.1. The structures of some of the earliest identified chemical carcinogens.

By the 1960s, Brookes and Lawley (1960; 1961) had shown that DNA was the target for chemical carcinogens with their studies on mustard gases and alkylating agents. The covalently bound products were referred to as DNA adducts. The detection and quantitation of these DNA adducts has become important as they are thought to be relevant to the mechanism of chemical carcinogenesis as well as providing valuable information for the evaluation of human exposure to chemical carcinogens in molecular epidemiology studies. Many animals studies have suggested that DNA adducts play a key role in the initiation of carcinogenesis.

Chemical carcinogens fall into many different structural groups. These include, the polycyclic aromatic hydrocarbons, aromatic amines, N-nitroso compounds, azodyes, alkylating agents and a number of inorganic compounds. This is just one way of categorising carcinogens, but they could equally be classified by the method in which they are encountered or derived by humans; occupational, diet, medicine, tobacco smoke and environmental (Figure 1.2.).

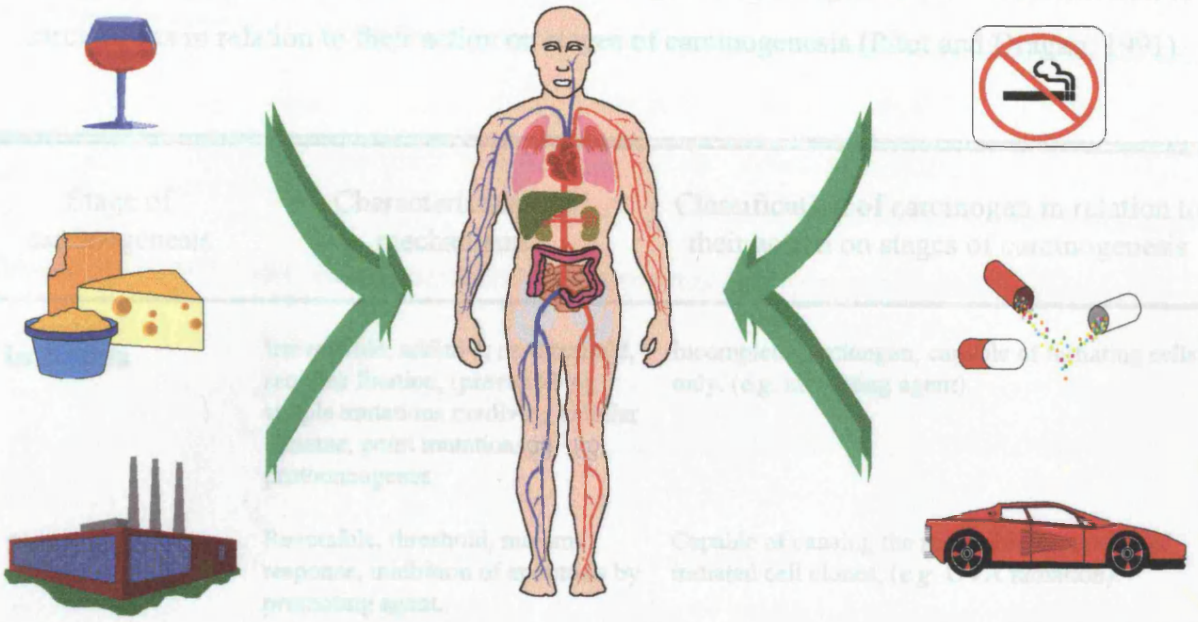


Figure 1.2. Ubiquitous exposure of chemical carcinogens to humans *via* (*from top right clockwise*) tobacco smoke, chemotherapeutic medication, environment, occupation and diet.

1.1.2. Multistage Nature of Carcinogenesis

The development of a fully malignant tumour involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, *etc.*). Carcinogenesis is thought to occur *via* multiple stages (Table 1.2.) and may occupy the life span of an individual (Pitot and Dragan, 1991; Weinstein, 1981). Many model systems have been used to study the process of tumour development in animals. By studying epidermal carcinogenesis in the mouse (Foulds, 1954), two distinct stages were identified, termed **initiation** and **promotion**. Further work, experimenting with mammary adenocarcinomas in the mouse modified the concept of the stage of promotion to include all events after initiation of the neoplastic process. The term **progression** was used to describe all post-initiation events in neoplastic development. The transitions between these three successive stages can be enhanced or inhibited by different types of agents, suggesting that the individual stages may involve different mechanisms at the cellular and genetic levels.

Table 1.2. Characteristics and mechanisms of stages of carcinogenesis, and classification of carcinogens in relation to their action on stages of carcinogenesis (Pitot and Dragan, 1991).

Stage of carcinogenesis	Characteristics and mechanisms	Classification of carcinogen in relation to their action on stages of carcinogenesis
Initiation	Irreversible, additive, no threshold, requires fixation, (preventable), simple mutations involving cellular genome, point mutations in protooncogenes.	Incomplete carcinogen, capable of initiating cells only, (*e.g.* alkylating agent).
Promotion	Reversible, threshold, maximal response, inhibition of apoptosis by promoting agent.	Capable of causing the reversible expansion of initiated cell clones, (*e.g.* UVA radiation).
Progression	Irreversible, somatic aneuploidy, progressive karyotopic instability, complex genetic alterations, irreversible changes in gene expression, selection of neoplastic cells for optimal growth.	Capable of converting an initiated cell or a cell in the stage of promotion to a potentially malignant cell, (*e.g.* hepatitis B virus).

1.1.2.1. Initiation

Agents that initiate carcinogenesis do so by damaging cellular DNA. This is well illustrated by the studies on the chemical carcinogen, benzo[*a*]pyrene (Miller, 1978). The efficiency of initiation is related to the cellular DNA repair processes and persistence of DNA damage. Inhibition of metabolism of chemicals to their ultimate carcinogen forms can occur, and so blocking the stage of initiation (Wattenberg, 1978). The absence of threshold limits for initiating agents is evident from the studies on mutations that result from these agents, from the activation of proto-oncogenes to cellular oncogenes and from the deactivation of tumour suppressor genes.

1.1.2.1.1. Point Mutations

These mutations occur in DNA through base substitutions (Hoffmann and Fuchs, 1997; Venitt and Parry, 1984), leading to a codon change which specifies the insertion of a wrong amino acid into a polypeptide (*missense mutation*), or by the addition or deletion of bases which change the reading frame of the DNA because of the difference in codon (*frameshift mutation*). Alkylation occurring at the O^6-position of guanine is thought to be an important in induction of point mutations, the lesion results in the formation of GC to AT base pair transitions. When DNA is transcribed to mRNA, uracil is inserted in the place of guanine. The lesion at the O^4-position of thymidine results in the formation of AT to GC transitions.

The formation of apurinic or apyrimidinic (AP) sites resulting from the depurination of N3- or N7-alkylpurines and O^2-alkylpyrimidines also results in frameshift mutations following DNA repair (Loeb and Preston, 1986). It was the study on the implications of AP sites as intermediates in chemical mutagenesis with different carcinogenic agents on the *lac*I gene of *E. coli*, that lead to the induction of primarily GC to AT transition mutations. With examination of nearly 80 different mutable sites on the *lac*I gene, it was shown that even though the same type of transition mutations were observed, each carcinogen exhibited a unique mutational spectrum, *i.e.* each carcinogen has its own mutational 'hotspots' (clusters of mutations in spectrum) and 'cold spots' throughout the nucleotide sequence. Therefore, by determining the sequence distribution of DNA adducts in certain genes, it may be possible to correlate them with mutational 'hot spots' in specific cancers. Hence, this may allow the causal link of carcinogenic agents to that particular cancer (Dennisenko *et al.*, 1996).

Chromosomal (resulting from breakage and reunion of chromosomal material during cell cycle) and genomic (changes in the number of chromosomes in the genome, *i.e.* polyploidy) mutations are two other types of mutation which also result from the induction of the initiation step in carcinogenesis.

1.1.2.1.2. Oncogene Activation and Tumour Suppressor Genes.

Growth promoting proto-oncogenes are thought to regulate the proliferation of normal cells, which are counter-balanced by growth-inhibiting tumour suppressor genes. The H-*ras* gene is

a cellular proto-oncogene located on the inner side of the cell membrane. It shows evidence of tyrosine kinase activity, but its precise role is unknown. The twelfth codon (GCC) codes for the amino acid glycine, but in some tumour cells, a mutation is found in codon 12, giving the triplet GTC which codes for valine. This mutated gene sequence has transforming activity, and therefore, the proto-oncogene becomes activated by a mutation to an oncogene, resulting in the uncontrolled proliferation of cells (Weinberg, 1991). In contrast, tumour suppressor genes function as physiological barriers against clonal expansion or genomic mutability as well as hindering the metastasis of cells driven to uncontrolled proliferation by oncogenes. Therefore, these genes are vulnerable sites for DNA damage, and loss of tumour suppressor function can occur *via* damage to the genome through mutation or chromosomal rearrangement. The *p53* tumour suppressor gene is the most common example as it is mutated in about half of human cancers. The spectrum of *p53* mutations induced in human cancer can help identify particular carcinogens (UV radiation correlates with transition mutations at dipyrimidine sites; dietary aflatoxin B_1 exposure is correlated with GC to TA transitions that lead to serine substitution at residue 249 of p53 in hepatocellular carcinoma; exposure to tobacco smoke is correlated with GC to TA transitions in lung carcinomas), and the frequency and type of *p53* mutations can act as a molecular dosimeter of carcinogen exposure. These characteristics of *p53* mutations can then be combined to provide information about the molecular epidemiology of human cancer risk (Harris, 1995). The uncontrolled proliferation of cells due to oncogene activation and the tumour suppressor gene deactivation seem to produce the same end result, but they are both quite different, and both are required for progression of most tumours to full malignancy (Brown, 1995).

1.1.2.2. Promotion

The promotion stage of carcinogenesis is reversible in nature. This is evident in several model systems, where focal lesions are shown to regress when administration of promoting agents is stopped, but then reappear when promoting agent is re-administered (Pitot and Dragan, 1991). Promoting agents increase the chance of full malignancy, as they increase the proliferation rate of normal cells, but they do not interact with DNA directly (Miller and Miller, 1981). Apoptosis (programmed cell death) is thought to play a part in the regression of cells after withdrawal of a promoting agent, which show to inhibit apoptosis in preneoplastic lesions (Schulte-Hermann *et al.*, 1995).

1.1.2.3. Progression

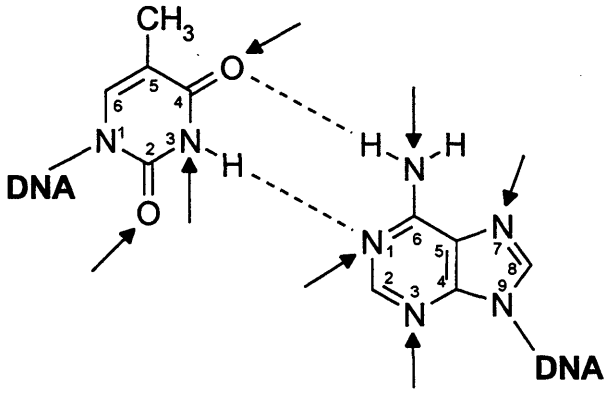
The development of irreversible, aneuploid malignant neoplasms and karyotypic instability distinguish the stage of progression from the two preceding stages. Alterations in the structure of the genome of the malignant cell during this stage are directly related to the increased growth rate, invasiveness, metastatic capability and biological changes in the malignant cell. Chemical agents that act only during progression (promotion to progression) have not been identified in any specific system, but agents such as benzoyl peroxide (free radical generator - a complete carcinogen, able to induce transformations from induction through to progression), do act as agents which induce progression in epidermal carcinogenesis (Pitot and Dragan, 1991).

1.2. Formation of DNA Adducts from Alkylating Agents

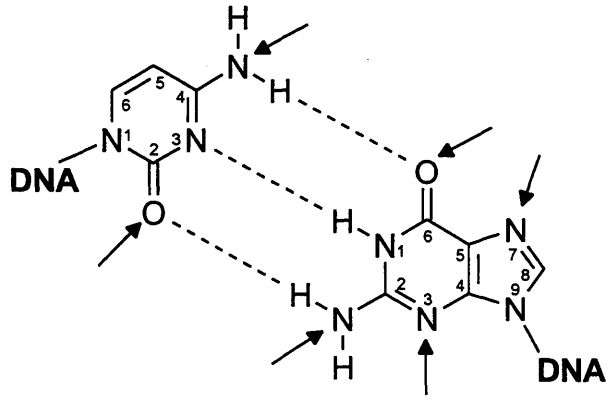
Figure 1.3. illustrates the sites of cellular DNA that readily undergo alkylation. The arrows represent the major nucleophilic sites for attack (Lawley and Brookes, 1963). The phosphate (DNA backbone) also undergoes alkylation, but is not represented in this figure. As well as those sites illustrated, covalent binding of chemicals can occur at other sites, *i.e.* C-8 position of guanine (aromatic amines) and the N-3 position of cytosine.

As can be seen in Table 1.3., the percentage of alkylation at different sites varies with the alkylating reagent used. Two specific class of simple methylating and ethylating agents are shown; the N-nitroso compounds (metabolically activated to alkylating species, *via* an α-hydroxy intermediate) and the alkylsulphates. Alkylation predominantly occurs on the exocyclic nitrogen and oxygen atoms, and ring nitrogen atoms of the purine and pyrimidine bases. But one consistent observation is that the N-7 position of guanine is alkylated to the greatest extent, no matter what alkylating species is used (except for ethylphosphates from reaction of diethylnitrosamine and ethylnitrosourea). There has been no correlation between N7-alkylguanine (7-AlkGua) incidence and tumour induction, even though the N7- position of guanine is known to be the most frequent site of alkylation (Magee *et al.*, 1976). The pattern of DNA alkylation and the stabilities of the individual alkylation products are assumed to be essential factors in determining the carcinogenic and mutagenic effects of alkylating agents (Singer and Essigman, 1991). Miscoding lesions in cellular DNA are presumed to be caused

by alkylation occurring at the O^6-position of guanine, O^4- and O^2-positions of thymine and the O^2-position of cytosine. Alkylphosphotriesters are thought to interfere with DNA-handling enzymes (Takeda *et al.*, 1983), whereas other lesions may lead to mutational or toxic events by producing apurinic sites. Ethylating agents are considered to be more mutagenic than their methyl counterparts (Den Engelse *et al.*, 1986; Jansen *et al.*, 1994) even though total ethylation is less than total methylation. This is probably due to the extents of alkylation at sites like the oxygen atoms of thymine, cytosine and phosphate groups. On these groups, extents of ethylation are higher than methylation. As the half lives of ethylated products are much greater than corresponding methylated products (high repair capacity), the ethyl adducts tend to persist, inducing mutations.



Thymine - Adenine



Cytosine - Guanine

Figure 1.3. Sites of alkylation in cellular DNA indicated by arrows.

Table 1.3. Relative proportions of methylated and ethylated bases present in DNA after exposure to carcinogenic alkylating agents *in vitro*. (Adapted from Pegg, 1984; Singer and Grunberger, 1983).

Alkylated Bases	Percentage of Total Alkylation by:					
	DMN, MNU, DMH	DEN, ENU	MMS	EMS	DMS	DES
N1-alkyladenine	0.7-1.3	0.2-0.3	1.2-3.8	1.7	1.9	2.0
N3-alkyladenine	8-9	4	10.4-11.0	4.9	18	10
N7-alkyladenine	1.5-1.7	0.3-0.4	1.8-1.9	1.1	1.9	1.5
N3-alkylguanine	0.8	0.6	0.6-0.7	0.9	1.1	0.9
N7-alkylguanine	**67-68**	**12**	**83**	**65**	**74**	**67**
O^6-alkylguanine	6.3-7.5	8	0.3	2	0.2	0.2
N3-alkylcytosine	0.5-0.6	0.2	<1.0	0.6	<2.0	0.7
O^2-alkylcytosine	0.1	3.5	nd	nd	nd	nd
N3-alkylthymine	0.3	0.8	nd			nd
O^2-alkylthymine	0.1	7.0-7.4	nd			nd
O^4-alkylthymine	0.1-0.7	1-4	nd			nd
alkylphosphate	12-17	53-57	0.8-1.0	13		16

DMN, dimethylnitrosamine; MNU, methylnitrosourea; DMH, dimethylhydrazine; DEN, diethylnitrosourea; ENU, ethylnitrosourea; MMS, methylmethane sulphate; EMS, ethylmethane suphate; DMS, dimethylsulphate; DES, diethylsulphate; nd, not detected.

1.2.1. Possible Mechanisms of Alkylation

There are various opinions on the mechanism of alkylation on DNA by simple alkylating agents and these will be discussed briefly. Reviews by Swenson (1983) and Beranek (1990) have discussed three of the chemical theories involved in methylation and ethylation of DNA. Firstly, the Ingold concept of nucleophilic substitution involves S_N1 (dependent on formation of electrophilic carbocation intermediate) and S_N2-type (bimolecular and dependent on steric

accessibility) reactions. In this concept, electrophilic reactivity is dependent on the stability of the carbocation and the extent of lability of the leaving group. The more reactive alkylating agents (alkyl nitrosoureas; tending to react with O atoms) proceed *via* an S_N1 mechanism, whilst the less reactive alkylating agents (alkyl sulphates; tending to react with N atoms) utilise an S_N2 mechanism (Lawley, 1974). The second theory on alkylation mechanism, covers the Swain-Scott equation for relative reaction rates. This theory explains the various tendencies for alkylating agents to attack the various sites on DNA due to their relative electrophilicity, given by electrophilicity constant, **s** (Swain and Scott, 1953). The value of **s** is determined experimentally based on the relative reactivity of the methyl cation formed from methyl bromide, with an **s** value of 1.0. Low **s** values correlate with alkylating agents reacting *via* S_N2 and **s** values approximating to 1.0 correlate with alkylating agents reacting *via* S_N1.

The more reactive alkylating agents are less discriminating towards nucleophiles (as they follow S_N1), therefore the less nucleophilic atoms in DNA (O atoms) become proportionately more reactive. The Swain-Scott principle is violated when nitrogen and oxygen nucleophiles are combined. It has been proposed that, instead of using the terms S_N1 and S_N2, the terms highly oxyphilic and low oxyphilic should be employed (Loechler, 1994). All these studies correlate the observations quite successfully, but none of them really explain the underlying physical interactions responsible. Saffhill *et al.*, (1985) have gone some way to explaining these interactions and they proposed an alternative mechanism to the Swain-Scott and Ingold concepts, for alkylation on DNA. Their proposition was that hard acids (electrophilic species) have a preference for hard bases (nucleophilic species), and that soft acids prefer soft bases. Alkylating agents are considered to be intermediate in their activities as hard or soft acids. Equally, the ring N and exocyclic O atoms of purine and pyrimidine bases in DNA are intermediate in their basicity. Therefore the N and O atoms will be preferentially alkylated in DNA. But O atoms are stronger bases than N atoms, and since electrophilic species formed by the N-nitroso compounds are stronger acids than alkanesulphonates, N-nitroso compounds will preferentially bind to O atoms than will the alkanesulphonates (Table 1.3.). The relative strength of an alkylating species, as an acid, is increased as the alkylating agent becomes more branched, and as shown in Table 1.3., ENU ethylates oxygen sites to greater extent than nitrogen sites in DNA. Propylation, butylation, *etc.*, have not been studied in depth as

methylation and ethylation, but are known to occur, with internal rearrangement of the alkyl groups also occurring, as a complication.

1.2.2. DNA Repair of Alkylated Bases

The persistence of alkylated DNA adducts results predominantly from the failure of DNA repair. Every type of organism so far tested has been found to posses efficient DNA repair mechanisms to ensure that particular alkylated oxygen and nitrogen atoms do not accumulate in the genome (Samson, 1992). The repair of damage of alkylated DNA in living cells occurs by two main processes for alkyl adducts (Singer and Hang, 1997); 1) direct reversal by removal of only a modified group and 2) base excision repair. For DNA adducts occurring *via* other processes such as acylation and free radical attack (bulky adducts or pyrimidines dimerised by action of UV radiation), nucleotide excision repair is another process which is utilised.

1.2.2.1. DNA Repair Alkyltransferases

The *E.coli* Ada protein, playing a central role in the **Ada**ptive response of *E.coli* to alkylating agents, was the first DNA repair methyltransferase to be discovered. The Ogt methyltransferase is also expressed by *E.coli* in the non-adapted state. The protein responsible for O^6methylguanine repair was shown to repair O-alkyl adducts *via* a suicide mechanism (Lindahl *et al.*, 1988). O^6-Methylguanine is repaired in DNA by the action of a methyltransferase protein which catalyses the transfer of the methyl group to a cysteine-residue within its own sequence to form S-methylcysteine (Lindahl, 1982). Essentially DNA repair methyltransferases are not enzymes because they are consumed in the reaction. There are two active sites on the protein, centered on Cys-321 (transfers methyl groups from either O^6-methylguanine or O^4-methylthymine) and Cys-69 (transfers methyl group from methyphosphotriester). Activation of the transcription of at least four genes whose products enable *E.coli* to recover from the toxic effects of alkylating agents also occurs. The capacity for rapid repair is limited to the number of molecules of the methyltransferase present. This number is both species and organ specific. Rat liver hepatocytes contain much more methyltransferase molecules (60,000/cell) than rat nonparenchymal cells (12,000/cell). In studies of dimethylnitrosamine exposure to rats, there was a relatively high incidence of

tumours resulting from alkylated nonparenchymal cells, which is consistent with O^6-methylguanine as the critical lesion since the capacity for repair of O^6-methylguanine is much less in these cells (Pegg, 1984).

1.2.2.2. DNA Glycosylases - Base Excision Repair

DNA glycosylases catalyse the hydrolytic cleavage of the N-glycosylic bond linking the damaged base to deoxyribose in DNA, producing an AP site. The undamaged DNA sequence is then restored by the consecutive action of AP endonuclease, exonuclease, DNA polymerase and DNA ligase enzymes (Friedberg, 1985). This is termed a short patch repair, as only the damaged base is excised. There are least 8 types of DNA glycosylase, each one specific for the removal of one or more damaged bases (Chetsanga *et al.*, 1981; Mattes *et al.*, 1996; Thomas *et al.*, 1982).

1.2.2.3. Nucleotide Excision Repair

Nucleotide excision repair is a long patch repair as it involves the excision of a base sequence of 20 nucleotides. The DNA sequence is restored by the action of DNA polymerase and DNA ligase enzymes. Patients with the autosomal recessive condition, *xeroderma pigmentosum*, are deficient in the excision step, so are therefore predisposed to DNA damage caused by sunlight and tend to develop skin tumours (McGee *et al.*, 1992).

1.3. Use of Carcinogen-DNA Adducts as Biomarkers for Cancer

In human biomonitoring there are two types of measurements that can be made to determine the extent of DNA damage. They are firstly, the measurement of biological responses, such as mutations, sister chromatid exchanges and chomosome aberrations, and then secondly, the measurement of levels of chemicals, and their metabolites and/or derivatives in body fluids and tissue (Wogan and Gorelick, 1985). DNA adducts are thought to represent events leading to mutation and/or malignant tumours, therefore measurement of DNA adducts would be a good indicator of exposure to carcinogenic agents (Shields and Harris, 1991). But does the presence of carcinogen-DNA adducts in humans indicate that the person is going to develop cancer? I believe that this question is answered quite adequately by Phillips, (1996):

> "*In theory, the presence of adducts in an individual indicates that the person is at risk of developing cancer, although the influence of modulating factors on the carcinogenic process will make it unlikely that the magnitude of the risk can be calculated from a single parameter such as the level of DNA adducts.*"

So essentially, it is important to measure the levels of DNA adducts in humans, but even then it cannot be confirmed that presence of adducts will lead to the formation of cancer, only that the individual is at risk. Most chemicals require metabolic activation to exert their carcinogenic effects, and the amount of levels of adduct detected results from the action of competing activation and detoxification pathways to produce an ultimate carcinogen, DNA repair capacity and cell turnover. An important determinant of cancer susceptibility is the interindividual variation in carcinogen metabolism (Harris, 1989). For instance, the issue of measuring adducts is complicated by the removal of adducts from DNA by chemical or enzymatic processes at different rates, even within the same cell. The kinetics and the extent of DNA adduct removal in human tissues is relatively unknown. If cells are highly efficient at DNA repair, adducts, although formed may go undetected. If cells replicate quickly, then only short-term exposures can be measured in the tissue. So in the production of adducts, dosimetry needs to be considered. There is the internal dose (amount of genotoxic compound absorbed into organism) and the biologically active dose (amount of chemical needed to induce a biological response, *i.e.* adduct). The amount of internal dose can be related to the

biological dose for risk estimation, but to account for differences in genetic susceptibility, and differences in absorption, metabolism and excretion, the biological dose is more relevant.

Measurement of DNA adducts is all relative depending on what body tissue or fluid is examined, and allows the determination of the biologically effective dose. DNA adducts measured *in situ*, within the cell, would give the most direct evidence of genotoxic exposure, whereas adducts measured in excreted body fluids would only represent total recent exposure. Even the measurement of DNA adducts in the lung can vary depending upon where the tissue segment is taken from. Studies by Blömeke *et al.*, (1996), have shown that levels of N7-methyl- and N7-ethyldeoxyguanosine 3'-monophosphate are not distributed throughout the human lungs with any specific pattern and that for most individuals a random lung sample would not be representative of other parts of the lungs. Therefore, some individuals might be misclassified due to highly variable N7-alkyldeoxyguanosine 3'-monophosphate levels. One extremely important consideration to be undertaken when measuring levels of adducts in DNA extracted from human tissue, is the removal of RNA. For example, N7-methylguanosine is a natural constituent of RNA at a level of 1 adduct/500 guanosines. Therefore a 1% contamination of DNA with RNA would result in an apparent level of 1 adduct/5 x 10^4 normal nucleotides (Bianchini and Wild, 1994).

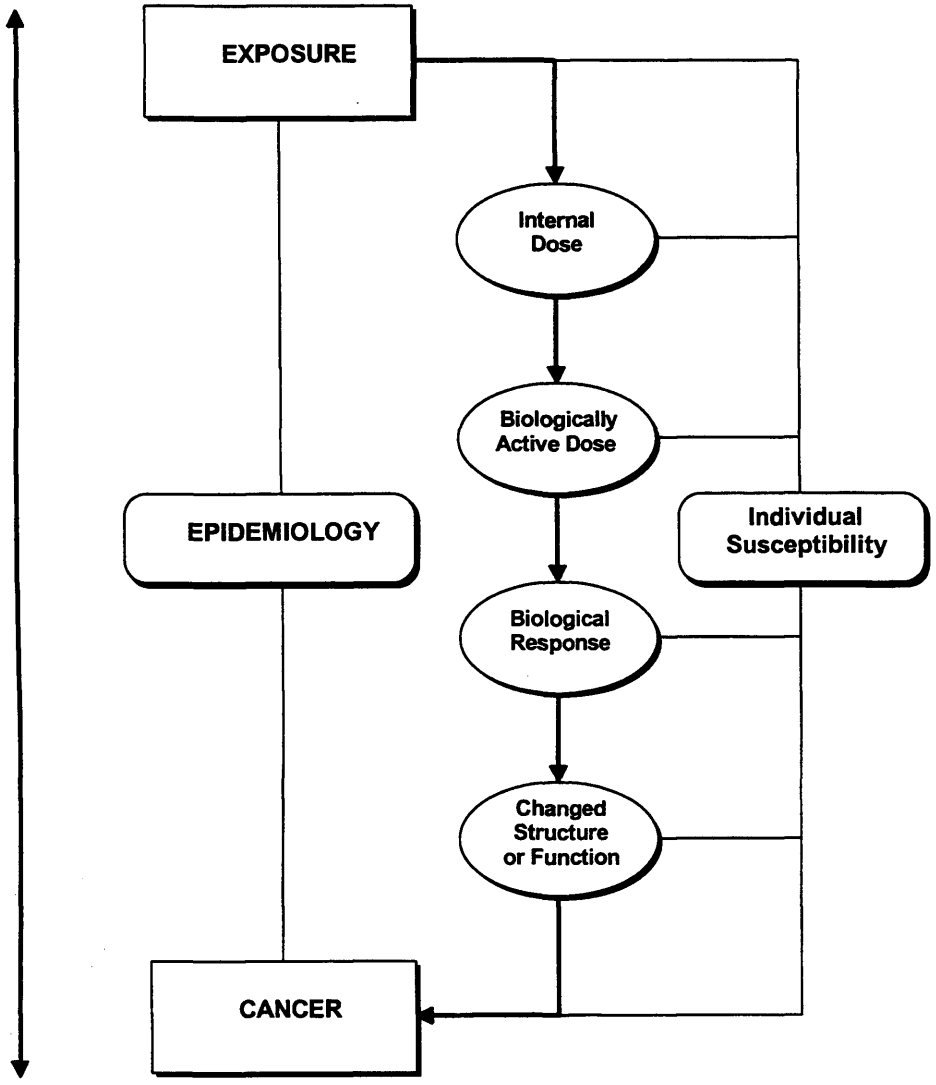
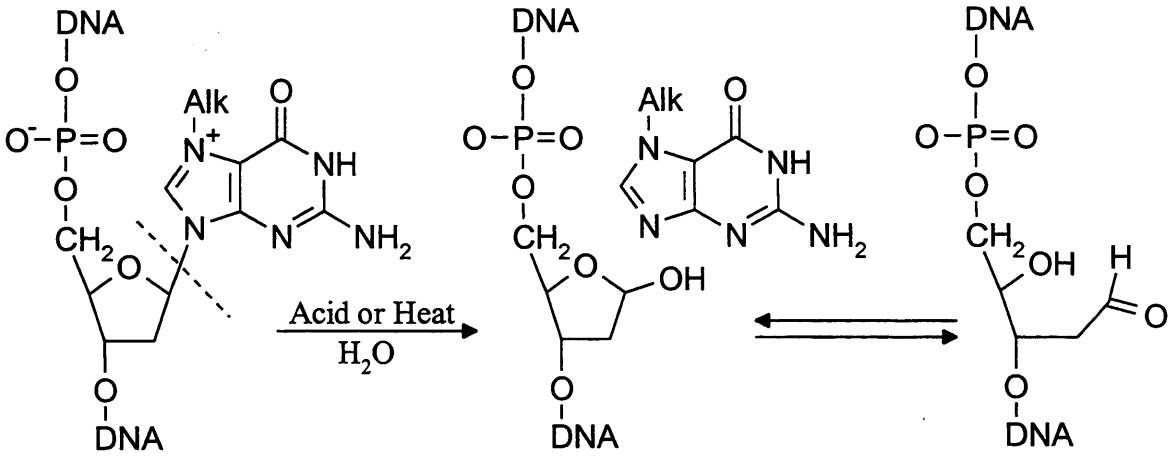


Figure 1.4. The biomonitoring of exposure to genotoxic compounds. (Decaprio, 1997; Farmer *et al.*, 1996; Henderson, 1995)

1.3.1. Alternative Biomarkers for DNA Damage

1.3.1.1. Detection of Apurinic/Apyrimidinic Sites in DNA

Abasic (apurinic/apyridiminic) sites are common lesions in DNA, produced from the spontaneous hydrolysis of the N-glycosylic bond under physiological conditions (Scheme 1), which is accelerated by the modifications of bases (Talpaert-Borlé and Liuzzi, 1983). Alkylation forms an unstable quaternary ion intermediate, which imparts a positive charge on the purine/pyrimidine ring systems, and N-glycosylic bond cleavage stabilises the charge. In general, alkylation increases the rate of depurination by at least six orders of magnitude. Depurination can also occur due to DNA glycosylases. Alkylated bases (7-AlkGua) can be induced to depurinate be heat treatment and the measurement of abasic sites can act as a surrogate biomarker for DNA adducts. Work in this field has included the reaction of the aldehyde group in the abasic site with [^{14}C]methoxyamine (Talpaert-Borlé and Liuzzi, 1983) and the specific tagging of abasic sites with biotin residues, which are then quantified by an enzyme-linked immunosorbent assay (Kubo *et al.*, 1992). Both methods are not comparable in sensitivity with methods for detecting DNA adducts, and also lead to erroneous results from quantitation of abasic sites produced due to factors other than the alkylation being correlated. Conversely, this makes them good methods for quantitation of total abasic sites in DNA.



Scheme 1. N7-Alkylated guanine bases can be easily depurinated by heat or mild acid hydrolysis to afford quantifiable abasic sites (or 7-AlkGua bases).

1.3.1.4. Protein Adducts

The use of protein adducts (haemoglobin and albumin) as surrogate biomarkers for DNA adducts is largely governed by their availability and long lifetime (Farmer, 1995; Skipper and Tannenbaum, 1990). From the quantitative viewpoint, protein adducts are considered to be better dosage indicators than DNA adducts. This is because, whilst most DNA adducts are efficiently repaired, protein adducts tend to persist for the lifetime of circulating erythrocytes, approximately 120 days for haemoglobin and 45 days for albumin in humans (Chang *et al.*, 1994). A wide range of genotoxic compounds are able to react with proteins. There are a variety of nucleophilic sites within proteins where covalent adduct formation may occur with electrophilic genotoxic agents (Farmer and Sweetman, 1995). The adduct modified amino acids of the proteins are isolated by hydrolysis with acid and subsequently derivatised for GC-MS analysis. Haemoglobin and albumin are considered to be non-target site macromolecules, but at low carcinogen doses, there is a reasonable correlation between protein adducts and DNA adducts. In conditions of unknown exposure, it is unlikely that protein adducts give an accurate prediction of DNA adducts, so therefore this precludes them from being true biomarkers *per se*, but they do provide useful information about exposure to carcinogens (Farmer, 1994).

1.3.1.3. Sister Chromatid Exchange

This is an indirect measure of genetic damage providing a useful marker of the biologically effective dose of a carcinogenic agent, where the exact mechanism is unknown. Promoting agents can be detected with this method, which involves taking slides of cells (blocked in mitosis) and examining the mitotic spread to determine whether two sister chromatids have exchanged material during mitosis (Sorsa *et al.*, 1982).

1.3.1.4. Chromosomal Aberrations

Chromosome aberrations are thought to arise from misrepair of lesions in peripheral blood lymphocytes and precursor cells in bone marrow and thymus (Aitio *et al.*, 1992). An assay is employed to assess the structural integrity and number of chromosomal aberrations in peripheral blood lymphocytes, and is carried out by arresting the cells in metaphase.

1.4. Analytical Methods for Measuring DNA Adducts

At present, there are three common types of analytical methods which are employed to detect and quantify DNA adducts (Chang *et al.*, 1994). These are ^{32}P-postlabelling (most sensitive and commonly used), immunological techniques (radioimmunoassay, enzyme-linked immunosorbent assay [ELISA] and immunoslot blot assay) and fluorometric methods. Other methods of detection, including mass spectrometry, will also be discussed briefly. Most important to the work in this thesis, are the detection methods that are currently available for detecting 7-AlkGua adducts. The N7-position of guanine is alkylated to the greatest extent in DNA, and it is a relatively long-lived lesion (but not considered a promutagenic lesion), therefore the detection and quantification of N7-alkyguanine adducts is potentially a good biomarker of recent exposure to genotoxic agents. Table 1.4. outlines some methods that are currently employed to detect and quantify 7-AlkGua lesions in DNA. Most of the methods are capable of detecting 1 adduct/10^6 normal nucleotides, but all the methods are fairly specific for that adduct. Therefore, it would be ideal if a method could be devised which could detect and quantify a series of 7-AlkGua adducts.

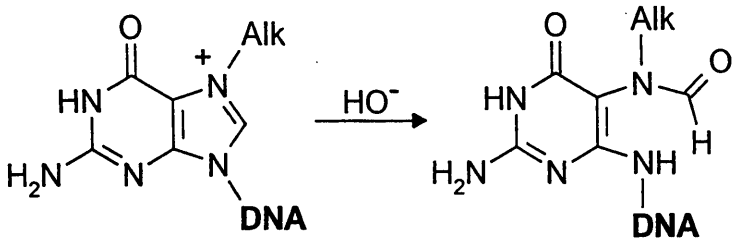
Table 1.4. Table showing various analytical techniques employed to detect and quantify N7-alkylated guanine compounds.

N7-AlkGua	Method of Detection	Levels Detected	Reference
7-MedG	HPLC/fluorescence Dansylation	0.3 adduct/10^6 nucleotides (100 μg)	Jain and Sharma, 1993
7-AlkGua, nucleosides + nucleotides	HPLC/fluorescence Phenylglyoxal	ns (ns)	Yonekura *et al.*, 1994
RO7-MeGua 7-Megua	ELISA HPLC-ECD	1.1 adducts/10^6 nucleotides 0.4 adducts/10^6 nucleotides (ns)	van Delft *et al.*, 1997
7-MedGp	^{32}P-postlabelling HPLC-ECD	3 adducts/10^6 nucleotides 6 adducts/10^6 nucleotides (100 μg)	Haque *et al.*, 1997
7-EtdGp	^{32}P-postlabelling	4 adducts/10^6 nucleotides (ns)	Blömeke *et al.*, 1996
7-EtdGp 7-MedGp	^{32}P-postlabelling	0.3 adducts/10^8 nucleotides (100 μg)	Kato *et al.*, 1993
7-MeGua	HPLC/UV	1 adduct/10^4 nucleotides (1 mg)	Lawrence *et al.*, 1981
7-EtGua	HPLC-ECD	6 adducts/10^6 nucleotides (100 μg)	Singh *et al.*, 1997
7-EtGua	IA-HPLC/fluorescence	4.8 adducts/10^6 nucleotides (1 mg)	Durand and Shuker, 1994
RO7-EtGua	ELISA	2.2 adducts/10^6 nucleotides (ns)	van Delft *et al.*, 1991
7-MeGua	GC-MS Phenylmalondialdehyde	ns (ns)	Sabbioni *et al.*, 1986

dGp-deoxyguanosine 3'-monophosphate, ECD-electrochemical detection, RO-ring opened, IA-immunoaffinity purification, parentheses-amount of DNA analysed, ns-not stated.

1.4.1. Some Physical and Chemical Properties of Alkylated Guanine Adducts

Alkylation at the N7- and N3-positions of guanine in DNA result in the formation of N7-alkyl- and N3-alkyldeoxyguanosine moieties, which can readily be hydrolysed in acid or neutral thermal conditions, to afford 7-AlkGua and N3-alkylguanine and leaving behind abasic sites (Jones and Robins, 1963). Alkaline treatment of 7-AlkGua results in the formation of a 5-alkylforamidopyrimidine derivative (Scheme 2), occurring *via* the imadazole ring opening reaction by nucleophilic attack of a hydroxyl ion at the C-8 position of the guanine adduct (Chetsanga and Makaroff, 1982; Chetsange *et al.*, 1982). The rate of ring opening is determined by the nature of the alkyl substituent. The more electron-withdrawing groups accelerate the reaction (Müller and Eisenbrand, 1985). The ring-opened N7-alkyguanine adducts in DNA are shown to stabilise the adduct against spontaneous depurination. The O^6-alkylguanine adducts are much more stable and do not easily depurinate. Mild acid conditions are required to depurinate O^6-alkylguanine.



N7-Alkyldeoxyguanosine ring-opened N7-Alkyldeoxyguanosine

Scheme 2. Alkaline treatment of 7-AlkGua affords the 5-alkylformamidopyrimidine derivative.

1.4.2. Mass Spectrometry for the Detection of DNA Adducts

Mass spectrometry is a valuable technique for the detection and structural characterisation of DNA adducts (Farmer and Sweetman, 1995). There is no mass spectral screening method for the determination for the total exposure to genotoxic agents (as in ^{32}P-postlabelling for DNA adducts). So therefore, this limits the extent to which mass spectrometry can be used as a tool

for studying biomarkers of exposure, as in the studies of exposure to complex mixtures (*i.e.* tobacco smoke). There are many mass spectrometric assays which are coupled to other analytical detection techniques, and these include, gas chromatography (GC), electron capture (EC), electrospray (ES), matrix-assisted laser desorption/time of flight (MALDI-TOF) and liquid chromatography (LC). The majority of DNA adducts lack the volatility and thermal stability required for detection by EC-MS, and therefore conversion to an electrophore is required (Giese, 1997). Gas chromatography-mass spectrometry has been used for the detection and quantification of alkylpurine adducts in human urine with a great deal of success (Prevost *et al.*, 1993; Shuker and Bartsch, 1994) and MALDI-TOF has been used for sequencing alkylated oligonucleotides. But none of the mass spectrometric assays provide the sensitivity of ^{32}P-postlabelling or immunslot-blot, partially because of the large amounts of sample required. A mass spectrometric technique coming to the fore is accelerator mass spectrometry (AMS), which allows the detection of 1 adduct/10^{11} normal nucleotides, but the DNA-damaging agent has to be radioactively labelled. The requirement of the assay is to convert a ^{14}C-labelled adduct into graphite or CO_2 prior to analysis (Farmer and Sweetman, 1995).

1.4.3. ^{32}P-Postlabelling for Detecting and Quantifying DNA Adducts.

Presently, the majority of detection and quantification of DNA damage is done by ^{32}P-postlabelling, a method pioneered by Randerath *et al.* (1981). The method conforms to most of the requirements for an assay to be applicable in human exposures, in that it sensitive (able to detect 1 adduct/$10^{9\text{-}10}$ normal nucleotides), requiring only small quantities of DNA (1-10 μg), is applicable to unknown adducts formed from complex mixtures (but identification is a problem) and is able to quantify the adducts. But the procedure does have its disadvantages; it is expensive (enzymes and [γ-^{32}P]ATP being costly), time consuming and hazardous (handling of radioactivity involved).

1.4.3.1. The General Procedure for ^{32}P-Postlabelling of DNA Adducts

The ^{32}P-postlabelling assay involves the introduction of P-labelled 5'-monophosphate groups into modified 3'-deoxynucleotides, obtained by the enzymatic digestion of adducted DNA

(Keith and Dirheimer, 1995; Watson, 1987; Gupta, 1985). The procedure requires at least 5 steps for good results (adapted from Beach and Gupta, 1992):

1) Enzymatic Hydrolysis. The initial step involves the hydrolysis of DNA to the 3'-nucleoside monophosphate level using a mixture of calf spleen phosphodiesterase and micrococcal nuclease. Incubation times and concentrations of the two enzymes need to be optimised for a given adduct, but can generally be taken to be 4-6 h at 37°C using enzyme to substrate ratios of 1:3 - 1:7.

2) Adduct Enrichment. There are several methods which can be used for adduct enrichment to increase the sensitivity of the adduct detection. The two most commonly used approaches are butanol enrichment (Gupta, 1985) and nuclease P_1 enrichment (Reddy and Randerath, 1986). The former preferentially extracts, with back-extraction for maximum enrichment, bulky aromatic and lipophilic nucleotides (PAH-DNA adducts) into a butanol phase from an acidic aqueous phase in the presence of a phase transfer agent, tetrabutyl ammonium chloride. The latter method uses the fact that dephosphorylation of normal nucleotides is preferentially carried out by the 3'-activity of the enzyme, and therefore in ^{32}P-postlabelling, the resulting normal nucleotides are not subjected to 5'-phosphorylation. This method is particularly suitable for small aromatic and bulky non-aromatic adducts (aromatic amines). Other methods of adduct enrichment are specific for particular adducts: postlabelling of small adducts (N7-alkyl, O^6-alkyl, malondialdehyde) has included a one- or two-step HPLC purification procedure (Shields *et al.*, 1990; Haque *et al.*, 1994; Vaca *et al.*, 1995), postlabelling of bulky human DNA adducts and O^6-methylguanine adducts have been developed which incorporate an immunoaffinity purification step (Widlak *et al.*, 1996; Cooper *et al.*, 1992; Povey and Cooper, 1995) and N7-methylguanine (7-MeGua) adducts have been postlabelled after purification using anion exchange chromatography (Mustonen, 1993).

3) ^{32}P-Labelling. Conversion of adducted-nucleoside 3'-monophosphates to 5'-^{32}P-labelled 3',5'-biphosphates (Figure 1.4.), is done by the incubation of the former with a 'hot mix' comprising of a buffer mix, T_4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. The catalyst, PNK, enzymatically transfers ^{32}P from [γ-^{32}P]ATP to the 5'-position of adducted nucleotides.

The [γ-^{32}P]ATP needs to be in molar excess to allow maximum transfer efficiency and the amount of PNK is also vital for phosphorylation. Labelling efficiencies have shown to be lower for N7-ethylguanine (7-EtGua) compared to 7-MeGua, and this has been attributed to concentration levels of PNK (Kovisto and Hemminki, 1990). Also, studies by Haque *et al.*, (1994) have shown that labelling of N7-methydeoxyguanosine 3'-monophosphate is not as efficient as for O^6-methyldeoxyguanosine 3'-monophosphate. This was attributed to differences in the PNK substrate specificity.

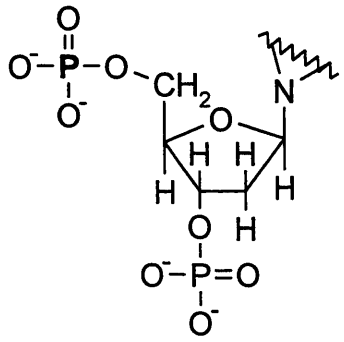


Figure 1.4. **P** denotes the radio-labelled atom on 3',5'-bisphosphatedeoxynucleosides.

4) Adduct Separation and Visualisation. After adduct labelling, samples are spotted on polyethyleneimine (PEI)-cellulose plates for multidirectional TLC. Radioactive normal nucleosides, unused [γ-^{32}P]ATP and other impurities are removed during the TLC procedure by using a high salt solution for the development buffer. Depending on the adduct of interest, varying buffers are used for separation. The labelled adducts are then detected by autoradiography.

Adduct Quantitation. Quantitation of adducts is generally carried out by employing one of two methods. The original method involved cutting the spots from the plates and then using liquid scintillation counting (Gupta *et al.*, 1982). A more recent procedure involves quantitation of adducts by storage phosphor imaging techniques (Reichert *et al.*, 1992; Povey and Cooper, 1995). After separation of adducts, the TLC plates are dried, wrapped in a clear

film and exposed to a phosphor screen for up to 2 h. Visualisation is carried out using a phosphorimager, and quantitation by measuring intensities of exposed areas.

1.4.3.3. An Overview of ^{32}P-Postlabelling

^{32}P is widely used in biological research but use of this isotope requires careful handling (Castegnaro *et al.*, 1993). ^{32}P is a high energy β-emitter with a mean energy of 695 KeV and with a maximum energy more than double its mean. Depending upon the area and size of irradiation doses, various effects can be observed; a decrease in the levels of leucocytes and platelets in the blood, epidermitis on irradiation of skin, cataracts in cases of eye irradiation, reduced sperm counts in men (reversible) on irradiation of testis and sterility results if women's ovaries are irradiated. Highest levels of exposure usually occur during the synthesis of [γ-^{32}P]ATP, TLC application and the cutting of the TLC plates (this risk has been reduced considerably, due to usage of storage phosphor imaging). Minimisation of exposure requires special needs, including adaptation of equipment and work environment, and special training for all those involved in the experimental work. The ^{32}P-postlabelling method is a very sensitive method and can detect as low as 1 adduct in 10^{10} normal nucleotides. Its optimum use is in the quantitation of bulky DNA adducts, but the method does not allow the identification of an adduct (unless a chemical standard is synthesised to use in co-chromatography to determine the nature of the adduct). The method has been shown to work for small adducts (alkyl adducts), as outlined above, but is very time consuming, labour intensive and studies have shown discrepancies in labelling efficiencies (Haque *et al.*, 1994; Kato *et al.*, 1993). It is known that small alkyl adducts play just as an important part in the multistage carcinogenesis process, as do the bulky adducts. Therefore, it is important to devise a technique to detect small alkyl adducts that is as sensitive, if not better than ^{32}P-postlabelling, that is generally applicable and not size-specific. The method should also be cheaper, safer and less time-consuming.

1.4.4. Immunological Detection and Quantitation of DNA Adducts

Antibodies recognising specific carcinogen-induced alkylated DNA adducts are becoming increasingly available (Poirier *et al.*, 1980; Wild, 1990), and in this section, some of the procedures that have incorporated antibodies to detect and quantify DNA damage, will be briefly discussed.

1.4.4.1. Radioimmunoassay (RIA)

In the RIA, the concentration of the antigen (adduct) in a sample is determined by measuring its ability to compete with a fixed amount of radio-labelled antigen for a limiting quantity of antibody, and sensitivity is shown to be in the range of 4 adducts/10^8 nucleotides (Farr, 1958; Müller and Rajewsky, 1980; Poirier, 1994). The antiserum competes for two forms of the original immunogen. The first is supplied in a constant amount and is radio-labelled. The second is the adduct (inhibitor). Inhibitor competition for antibody binding sites is concentration dependent over a range of concentrations and competition exists between the adduct and the radio-labelled antigen. The remaining steps of the assay focus on detection of the antibody bound to the tracer. The adduct-antibody complex is precipitated using a secondary antibody or reagents.

1.4.4.2. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA uses the same principles as RIA, but instead employs a solid phase bound antigen and a second antibody conjugated to an enzyme that cleaves a substrate to produce a colorimetric end-point. Essentially, an antibody is fixed to a plastic plate, and this immobilises the adduct in the sample. Sensitivity of the assay has shown to be able to measure 4 adducts/10^8 nucleotides (a more detailed description of ELISA will be presented in a later chapter).

1.4.4.3. Immunoslot-blot

In 1984, Nehls *et al.* reported the use of monoclonal antibodies against O^6-ethyldeoxyguanosine and O^4ethyldeoxythymidine to bind to ethylated single-stranded DNA immobilised on nitrocellulose filters (De Blas *et al.*, 1983). The assay requires as little as 1 μg of DNA to carry out the analysis. There have been reports of O^6-(2-hydroxyethyl)deoxyguanosine and imidazole ring-opened N7-methyldeoxyguanosine being assayed using this procedure (Wild, 1990). The assay is very sensitive, able to detect 1 adduct/10^8 nucleotides, and is becoming increasingly popular (Shuker and Benford, 1997).

1.4.4.4. Immunocytochemistry

In this assay, antibodies are applied to visualise adducts in individual cells by either enzyme substrates or fluorescent labels. The assay has been used to detect O^6-methyldeoxyguanosine (Den Engelse *et al.*, 1986), amongst others, at the single-cell level. Sensitivity is of the order of 1 adduct/10^{5-6} normal bases, but may be increased with the aid of confocal laser microscopy using fluorescent labels.

1.4.4.4. An Overview of Immunological Techniques

All the assays are sensitive, easy to use and have low costs, but a great deal of development work is needed for the production of antibodies. Prior knowledge of the structure of the DNA adduct is essential, as well as the ability to synthesise the appropriate antigen. A potential problem associated with the use of immunoassays is the possible cross-reactivity of the antibody with adducts of chemically related compounds (Müller and Rajewsky, 1981). However, in addition to direct detection of DNA adducts, antibodies have found a number applications for immunopurification (a more detailed discussion of immunoaffinity purification will be discussed in later chapters).

1.4.5. Fluorometric Methods for DNA Adduct Determination

Detection by a method that measures a property exhibited by the adduct of interest (*i.e.* fluorescence) and not exhibited by the solvent/mobile phase or other interfering species are inherently more sensitive (Johnson *et al.*, 1977). Therefore, fluorescence detection of adducts offers a high level of sensitivity in HPLC, potentially making the isolation of adducts unnecessary. Another advantage of detection by fluorescence (using HPLC) is that, whereas in detection by UV, for example, a slight change in flow rate, temperature or solvent composition would lead to drastically effected baseline stabilities and background noise levels, this is not observed as much with fluorescence detection. The major limitations to the use of fluorescence in the detection of carcinogen-DNA damage are that a prior knowledge of the chemistry of the adduct of interest is required and that the adduct be fluorescent (Weston, 1993). Hemminki (1980a; 1980b), showed that alkylguanine adducts (N7, O^6, and N2) could be detected and quantified by their native (but weak in the case of N7-adducts) fluorescence. The other three bases in DNA do not show native fluorescence properties

1.4.5.1. Synchronous Scanning Fluorescence Spectrophotometry (SSFS)

Some carcinogen-DNA adducts have physical properties which makes them stand out from other compounds. This is the case for some compounds where the carcinogen covalently attached to the DNA base is highly fluorescent, usually polycyclic aromatic hydrocarbons. Examples include the aflatoxin B_1 DNA adducts (Groopman *et al.*, 1991; Harris *et al.*, 1986), benzo[*a*]pyrene diol epoxide adducts (Weston *et al.*, 1990) and 3-hydroxy benzo[*a*]pyrene adducts (Ariese *et al.*, 1994). The technique of SSFS involves the generation of spectra of compounds by the scanning of both excitation and emission simultaneously with a fixed wavelength difference, greatly simplifying the spectrum obtained, and allowing the detection of specific adducts.

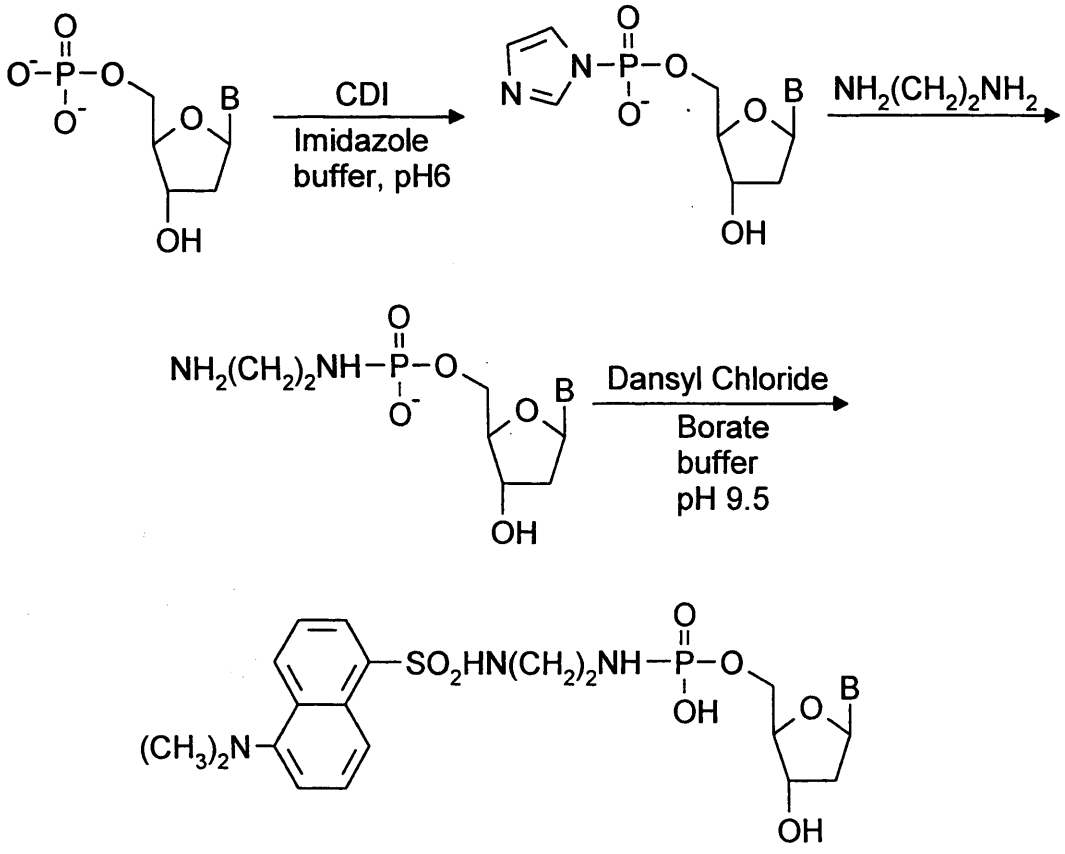
1.4.5.2. Fluorescent Postlabelling

Fluorescent postlabelling of compounds is a relatively new approach. There are pre- and post-column derivatisation methods using fluorogenic reactions which include fluorescence-generating and fluorescence-tagging. In the fluorescence-generating reactions, the reagents are generally non-fluorescent and react with target compounds to form conjugated ring molecules, resulting in the production of fluorescence. In the fluorescence-tagging reactions, the reagents are made up of highly fluorescent aromatic compounds and these are chemically attached to the analyte to form a fluorescence-tagged derivative. Some of these types of reactions are discussed below, all falling under the pre-column labelling category.

1.4.5.2.1. Dansyl Chloride

Work involving the tagging of fluorophores to the 5'-phosphate of adducted nucleosides of DNA has been employed, so that they can be detected by HPLC fluorescence. One tagging compound is 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride), used in a procedure which involves 5'-phosphoramidation with ethylenediamine followed by the conjugation of the free aliphatic amino group of the phosphoramidate with dansyl chloride (Scheme 3). The labelling procedure requires enzymatic digestion of the DNA and separation of the adducted nucleosides, for enrichment (Jain and Sharma, 1993; Sharma *et al.*, 1990a; 1990b; Kelman *et al.*, 1988). The assay is able to measure 1 adduct/10^6 normal nucleotides

from 100 μg of DNA. Some of the adducts that have been tagged and quantitated using this assay are *cis*-thymidine glycol monophosphate and 8-hydroxydeoxyguanosine 5'-monophosphate (from the X-irradiation of calf thymus DNA), N7-methyldeoxyguanosine and O^6-methyldeoxyguanosine (from calf thymus DNA exposed to N-methyl-N-nitrosourea), as well as the four 'normal' deoxynucleotides from calf thymus DNA. Quantitation can be unreliable as N7-alkyldeoxyguanosine 5'-monophosphates are unstable.

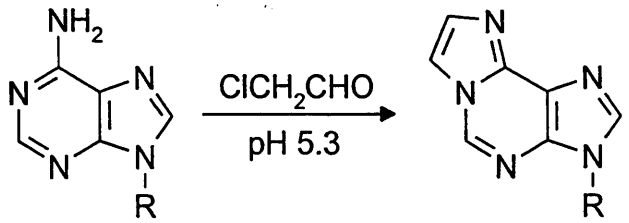


B=A, C, G, T.
CDI=1-ethyl-3,3-dimethylaminopropylcarbodiimide

Scheme 3. Scheme for the synthesis of dansyl chloride labelled nucleotides, (adapted from Azadnia *et al.*, 1994).

1.4.5.2.2. Haloacetaldehydes

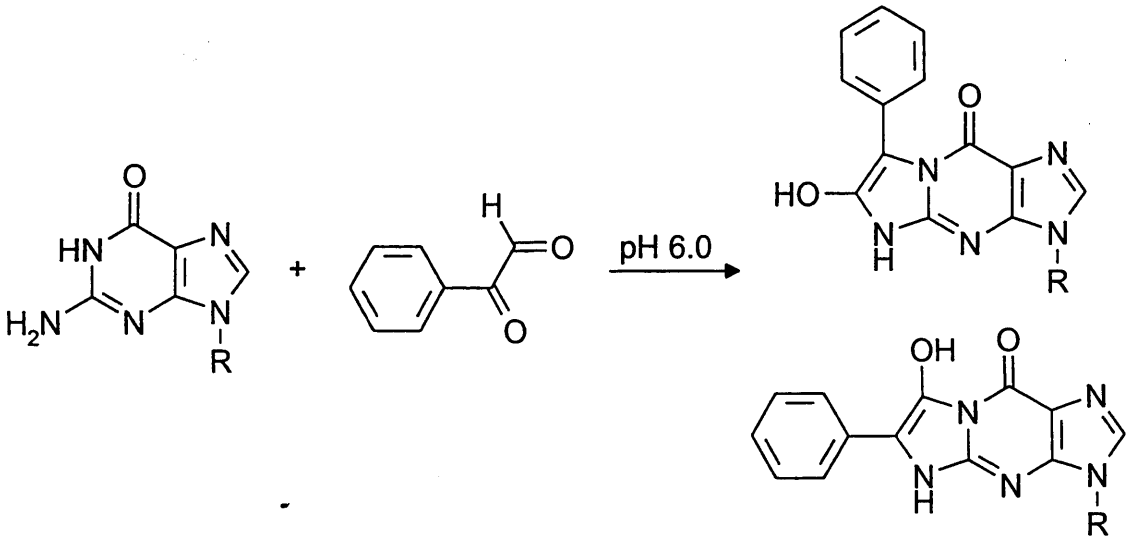
Work using chloroacetaldehyde and bromoacetaldehyde to convert adenine and cytosine adducts (and their nucleosides) to fluorescent etheno derivatives (Scheme 4) has also been carried out (McCann *et al.*, 1983; McClean *et al.*, 1987). But the studies were largely directed towards probing structural pertubations in duplex DNA (possible regions of single-strandedness), and have not been used for detection of adducted DNA bases.



Scheme 4. Fluorogenic reaction of adenine nucleosides with chloroacetealdehyde.

1.4.5.2.3. Phenylglyoxal

Some of the more recent research applicable to detection of guanine and its nucleosides and nucleotides, involves the cyclic reaction of phenylglyoxal with guanine, *etc.*, (Yonekura *et al.*, 1994; Yonekura *et al.*, 1993; Kai *et al.*, 1988). Derivatisation produces multiple products, but by altering reactions conditions (at 37°C for 15 min), optimum results may be obtained (Scheme 5). Initial experiments show that levels of under a picomole can be detected by HPLC with excitation at 360 nm and emission at 510 nm.



Scheme 5. Fluorogenic reaction of guanine nucleosides and nucleotides with phenylglyoxal.

1.4.5.3. An Overview of Fluorimetric Detection of DNA Adducts

Fluorescence detection provides a very specific and sensitive assay for adducts. Reviews by Lingeman *et al.*, (1985) and Ohkura *et al.*, (1994) describe many different fluorescence detection assays, but they are all for particular adducts. Therefore it is very important that the sensitivity and specificity that fluorescence detection provides, be implemented in assays that can be used for a homologous series of adducts.

1.5. Objectives of the Thesis

The main aim of this thesis is to present work carried out over a period of three years. The initial part of the study involved the modification and application of a novel fluorescent postlabelling assay for the detection and quantitation of 7-AlkGua adducts. Particular interest was applied to the study of N7-methyl- and N7-ethylguanine adducts from DNA exposed to direct-acting methylating and ethylating agents *in vitro*. The adducts were derivatised with a fluorescent postlabelling reagent, phenylmalondialdehyde (Phmal) to afford highly fluorescent tricyclic compounds which could then be detected by HPLC fluorescence. To increase sensitivity and selectivity of the adducts to be detected, an immunopurification procedure was incorporated. This involved the coupling of antisera (monoclonal antibody against a specific adduct) to immunoaffinity gels and the manufacture of immunoaffinity columns. Adducts could then be selectively isolated by loading samples onto the columns, washing them and eluting with appropriate buffer.

The production of a monoclonal antibody was also achieved in parallel with work into establishing a general method for the synthesis of 7-AlkGua haptens, for use in immunisation and as test antigens. Mice were immunised with a protein-bound hapten and tail-bleeds tested for antibody activity using ELISA. Eventual cloning of fused spleen cells and formation of hybridomas in mice lead to the production of a highly-specific monoclonal antibody.

The final part of the work included the application of the newly produced monoclonal antibody in conjunction with an attempt to identify a possible ethylating agent in tobacco smoke. It has been proposed that alanine (an amino acid in tobacco), undergoes nitrosation and decarboxylation to form a diazoethane generating agent, on the burning of tobacco. By synthesising a chemical analogue, ethylation of DNA *in vitro* was attempted and the detection of adducts was carried out by immunopurification HPLC fluorescent postlabelling. DNA was also exposed to tobacco smoke *in vitro* in an attempt to detect ethylation.

CHAPTER 2

Development and Application of a Fluorescent Postlabelling Assay for the Detection and Quantitation of N7-Alkylguanine Adducts

Chapter 2. Development and Application of a Fluorescent Postlabelling Assay for the Detection and Quantitation of N7-Alkylguanine Adducts.

2.1. Introduction

The work reported in this chapter describes attempts to modify and apply a novel fluorescent postlabelling assay for the detection of 7-AlkGua adducts. Specifically the study examines the use of Phmal as a fluorescent postlabelling reagent for 7-AlkGua adducts, which are readily available from the heat treated depurination of adducted DNA. Reaction of Phmal with N7-alkylated guanine bases affords highly fluorescent tricyclic compounds. It is this physical property that is utilised in a HPLC fluorescence detection method for the quantitation of 7-MeGua from calf thymus DNA (CT DNA) incubated with the direct-acting methylating agent, dimethylsulphate (DMS), and from CT DNA exposed to tobacco smoke. To isolate the required adduct prior to derivatisation, an immunoaffinity enrichment step is employed.

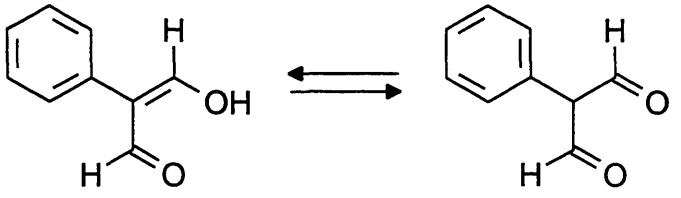
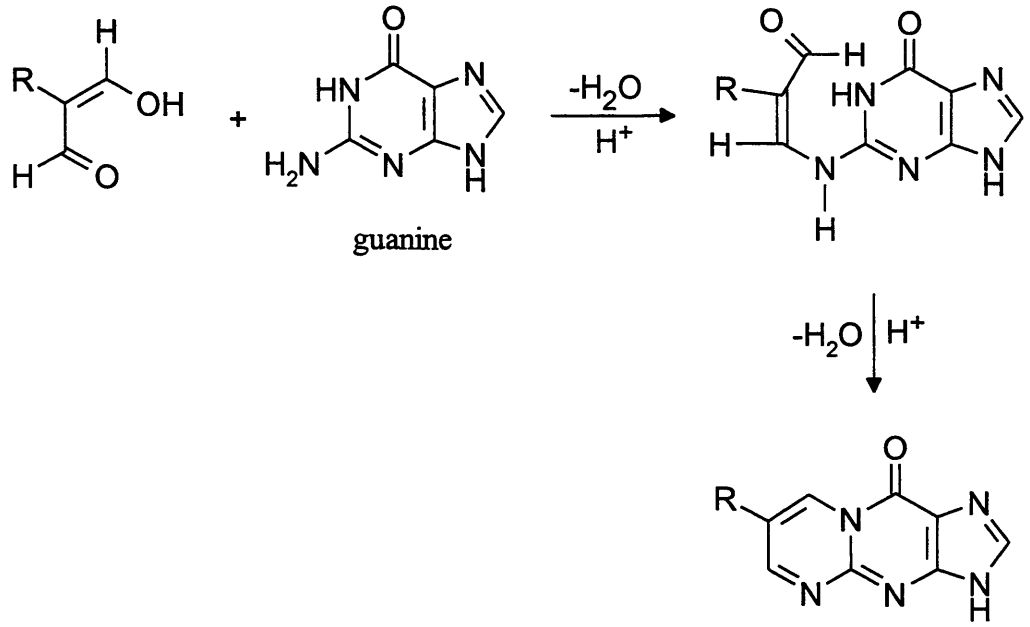


Figure 2.1. The two tautomeric forms of Phmal

2.1.1. Phenylmalondialdehyde as a Fluorescent Postlabelling Reagent

7-AlkGua do not posses high intrinsic fluorescence (Hemminki, 1980a; 1980b), but Moschel and Leonard (1976) found that 2-substituted malondialdehydes reacted with guanine, in an acid-catalysed cyclisation reaction, to afford highly fluorescent 1,N2-prop-2-en-2-yl-1-ylideneguanine derivatives (Scheme 6).



Scheme 6. Formation of a fluorescent 1,N2-prop-2-en-2-yl-1-ylideneguanine derivative from guanine and a 2-substituted malondialdehyde.

Subsequent work by Sabbioni *et al.* (1986) showed that the reaction proceeded just as well for 7-MeGua, using Phmal and pentafluorophenylmalondialdehyde (PFmal). These two reagents were studied in particular because from an analytical viewpoint the phenyl- and pentafluorophenyl- groups would have been very useful for detection by negative ion GC-MS or HPLC-ECD (Figure 2.2.).

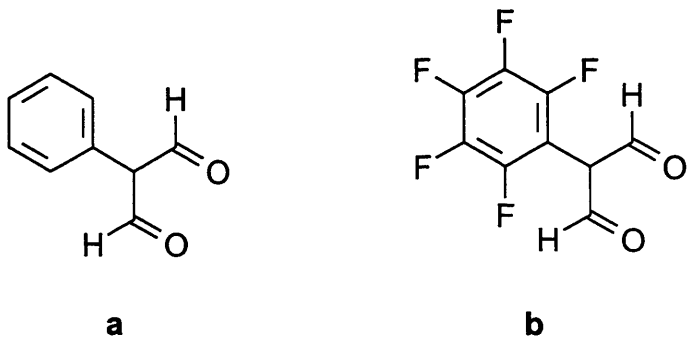


Figure 2.2. Structures of (a) Phmal and (b) pentafluorophenylmalon-dialdehyde.

Further work was carried out with the derivatisation of Phmal with a number of different N7-alkylated guanine standards (Shuker *et al.*, 1993), and the derivatives showed good chromatographic properties for reversed-phase (RP) HPLC analysis. Vaca *et al.* (1994) have also shown that it is possible to react a number of aromatic-substituted malondialdehydes with 7-MeGua. Their work showed that the intrinsic fluorescence of 7-MeGua can be increased by 10-20 fold by derivatisation with Phmal. All the work by the various groups carried out so far has indicated that the principle could be applied for the use in detecting a range of 7-AlkGua adducts, but a significant limitation of all the previous work was that it had not been applied to low levels of DNA adducts.

2.1.2. The Incorporation of Immunoaffinity Purification

Quantitation of very low levels of N7-alkylated guanine adducts can be hampered by the presence of interfering compounds (*i.e.* depurinated guanine in DNA hydrolysates). It is therefore necessary to incorporate an enrichment step prior to the fluorescent postlabelling to provide 'cleaner' samples. Immunoaffinity purification is used to isolate the 7-MeGua adducts from DNA hydrolysates.

Antibodies have been used extensively in methods to detect DNA adducts arising from a wide variety of agents (Wild, 1990). Depending on the specificity and affinity of the antibodies, they can either be used for direct detection of adducts in intact DNA using, for example, immunoslot-blot assays (Nehls et al., 1984), or in immunoaffinity purification of modified bases from hydrolysed DNA prior to quantitation, for example by gas chromatography-mass spectrometry (Shuker and Bartsch, 1994) or ^{32}P-postlabeling (Poirier and Weston, 1996). Immunoaffinity purification allows the highly selective purification and concentration of an analyte, and this means that whichever quantitative technique is used, very low levels of modified DNA bases can be detected in the presence of large excesses of unmodified base.

2.1.2.1. Choice of Affinity Gel and Antibody Type

For immunoaffinity to be achieved successfully, it is important that the immobilised antibody (binding protein) can still retain its specific binding affinity for an antigen. Substrates bound to the antibody must also be able to be eluted efficiently, once unbound material has been

washed away. Based on previous studies (Prevost *et al.*, 1990; Friesen *et al.*, 1991), bead-formed agarose gel was used to bind the protein ligand, which allowed good separation properties and stability under high and low pH values. Sepharose CL-4B was chosen for its open-pore structure, which accommodates the accessible coupling of proteins and for its low non-specific adsorption properties. Being cross-linked also limits the number of sites of attachment for the proteins. Protein A-Sepharose CL-4B gel was employed, as the protein A specifically binds with the F_C region of immunoglobulin G (IgG) type antibodies, allowing maximum exposure of the antibody binding site (Groding, 1986).

Antigens are passed over the immunoaffinity gels, and bound. The forces involved in binding the epitope of the antigen (methyl group at N7-position of guanine) to the paratope of the antibody are Van der Waal forces, Coulombic forces, dipole forces and hydrogen bonding. The unbound entities are washed away, and the antigen is then eluted by lowering the pH. Lowering of the pH allows protonation, which can either alter the degree of ionisation of the groups at the binding sites or alter the structure of the protein, which would then not be in a conformation to specifically bind the antigen. Lowering of salt concentration (ionic strength) and change in temperature can also elute antigens in some instances.

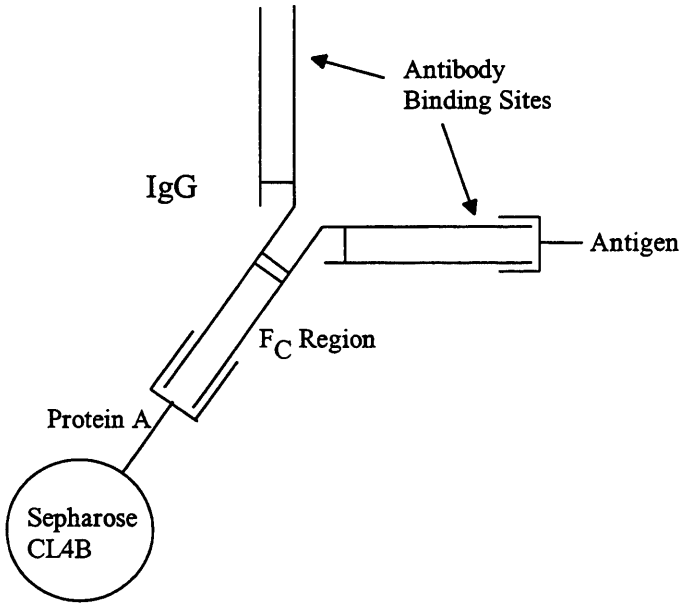


Figure 2.3. A diagram representing the principle of immunoaffinity gels based on immobilised Protein A.

2.1.2.2. *Structure of Immunoglobulin (Ig)*

All immunological active antibodies are immunoglobulins, which come from a family of glycoproteins. There are various classes of Ig, but they are all essentially made up of four chains (2 heavy and 2 light) and are bound by interchain disulphide bonds. The two heavy chains are connected to each other and each heavy chain has a light chain attached to it (Figure 2.3.). The most abundant class of Ig is IgG (comprising 75% of total serum), and has in itself four classes; IgG1, IgG2, IgG3 and IgG4. There is a hinge region in the middle of the molecule which allows some freedom to the two arms bearing the antigen binding sites. Cleaving Ig at the hinge region, with the enzyme papain, produces three fragments. Two of the fragments are identical, retaining their antigen binding properties, and the remaining larger fragment retains the effector functions (*e.g.* binding to surface receptors). As this larger fraction is crystallisable, it is termed the F_C (fragment crystallisable) fragment.

2.1.3. Effects of Tobacco Smoking on Levels of DNA Adducts

Tobacco smoke is considered to be one of the major causes of cancer accounting for about 30-40% of all cases worldwide (Doll and Peto, 1981). Up to 50 of the several thousands of compounds present in tobacco smoke are known to be human and/or animal carcinogens (IARC, 1986), belonging to the following classes: polycyclic aromatic hydrocarbons (PAH) [benzo(*a*)pyrene], aza-arenes, aromatic amines [4-aminobiphenyl, 2-naphthylamine], heterocyclic amines, N-nitrosamines, aldehydes, inorganic compounds [arsenic, chromium, polonium-210, cadmium] and miscellaneous organic chemicals [benzene, vinyl chloride]. *Note* - compounds in squared parentheses are either listed as grade 1 human carcinogens or are strongly suspected human carcinogens. Looking at studies involving tobacco smoking and levels of adducts detected in smokers compared to non-smokers, some show linear correlation between estimated total daily tobacco smoke exposure and adduct levels detected, whereas other studies do not give this correlation (Phillips *et al.*, 1988; Phillips *et al.*, 1990; Mustonen *et al.*, 1993; Ryberg *et al.*, 1994; Mustonen and Hemminki, 1992; Prevost and Shuker, 1996; Szyfter, 1996. Due to the large amount of studies carried out, only a few examples are stated). These inconsistencies can be put down to many factors; population size of the studies may not be large enough in many studies, occupational exposures may differ amongst subjects and test populations, self-reporting of smoking habits by test subjects may

not be entirely accurate and probably the most important being individual susceptibility. One interesting observation that has been noticed is that Ryberg *et al.*, (1994) showed statistically, that female smokers had higher levels of adducts than male smokers, indicating that maybe women are at a greater risk of tobacco-induced lung cancer. Female smokers are also at greater (two-fold or more) risk of developing cervical cancer than female non-smokers, as stated in a study by Phillips and Shé (1994).

2.1.3.1. Polycyclic Aromatic Hydrocarbon (PAH)-DNA Adducts from Exposure to Tobacco Smoke

Many human studies have detected large non-polar DNA adducts in the lung (especially the PAH-DNA adducts), using ^{32}P-postlabelling and have shown an increase in levels of adducts in smokers compared to non-smokers (Phillips *et al.*,1988; Phillips *et al.*, 1990). Phillips *et al.* (1988) showed in study using regression analysis, that in a group of patients undergoing thoracic surgery at Bradford Royal Infirmary (17 were smokers, 7 former smokers and 5 non-smokers) there was a linear relationship between the number of cigarettes smoked and the level of adducts observed in the DNA from non-tumourous lung tissue. This relationship was again observed by a different study carried out by Phillips *et al.*, (1990). In both studies, ^{32}P-postlabelling used as the method of detection, showed a band of adducts on the TLC plates (not discreet spots) which indicated that a large number of different compounds producing a complex mixture of adducts. This second study also showed that there was no significant differences between levels of adducts in peripheral blood leukocyte DNA in smokers compared to non-smokers. And yet, many studies have shown that white-blood cells from groups of workers (iron foundry workers, coke oven workers and roofers) occupationally exposed to airborne PAH show levels of adducts related to exposure (Phillips et al, 1990; Harris *et al.*, 1985; Phillips *et al.*, 1988). In these studies no effect of smoking on adduct levels was observed. A significant observation is that similar levels of adducts are detected using ^{32}P-postlabelling using the butanol extraction method and nuclease P_1 digestion method in lung tissue. This indicates that PAH are the major class of carcinogens responsible for the damage detected.

2.1.3.2. Alkylated-DNA Adducts from Exposure to Tobacco Smoke

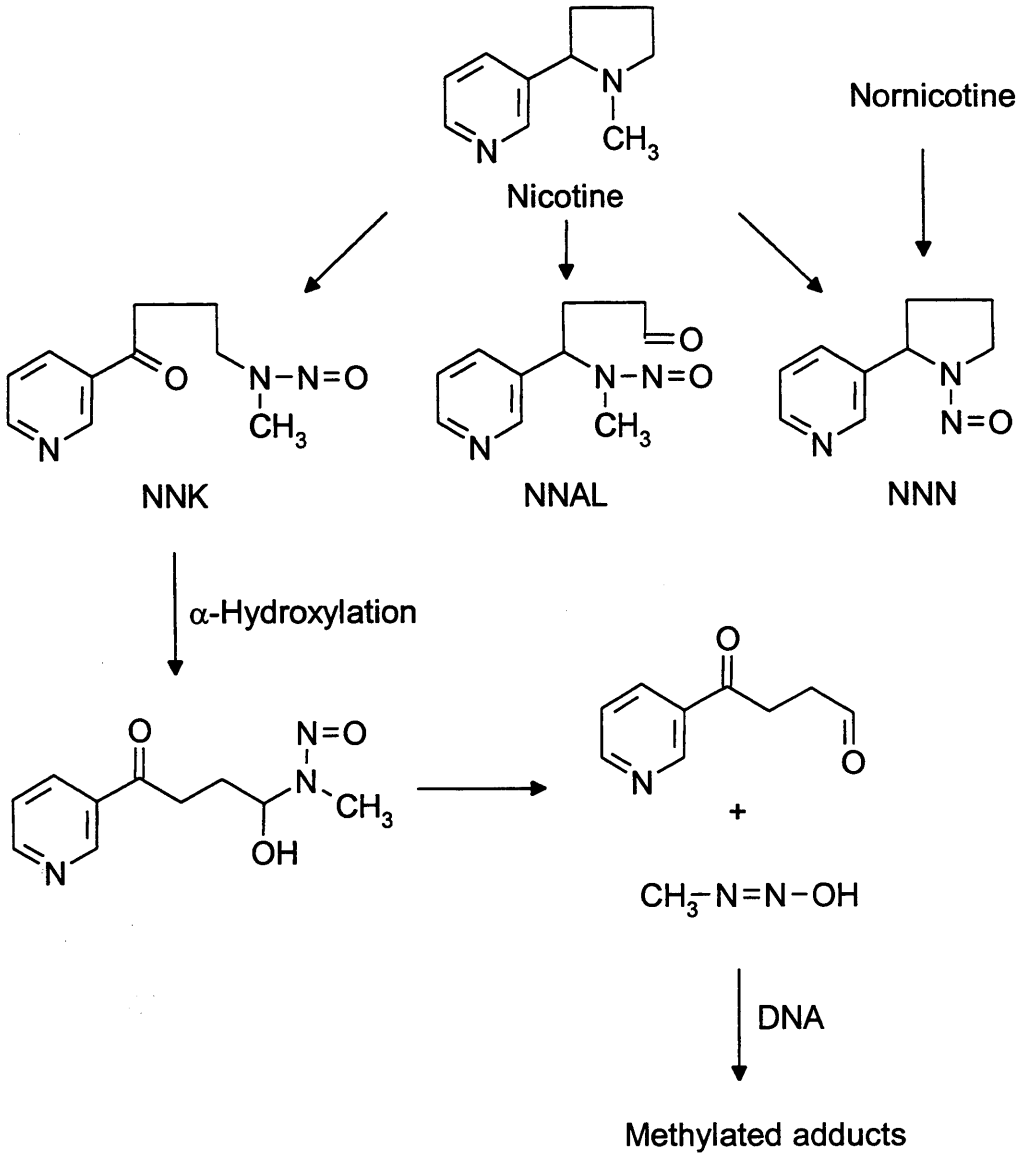
Nicotine, one of several tobacco alkaloids comprising ~1-2% of unburned tobacco, is a tertiary amine and is hypothesised to react with nitrosating agents (nitrite in saliva, nitrogen oxides in inhaled mainstream tobacco smoke) to give the three N-nitrosamines; 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosoamino)-1-(3-pyridyl)-butanal (NNAL) and N-nitrosonornicotine (NNN) (Hecht and Hoffman, 1988). Nicotine has been shown to react with nitrites at low pH to afford NNN and NNK. The strongest carcinogens amongst the tobacco-specific nitrosamines (TSNA) are NNK and NNN (Table 2.1.). In unburnt tobacco, there are very high amounts of tobacco-specific nitrosamines, up to mg/g concentrations (Gupta *et al.*, 1996), and in addition to the preformed nitroso compounds present in tobacco and tobacco smoke, nitrosamines can be formed endogenously from nitrosatable alkaloids and amines (pyrrolidine). Endogenous formation of nitrosamines from nitric oxide mediated reactions usually occurs during inflammatory processes via nitric oxide synthase, that accompany infections by *H. Pylori* bacteria, parasites or viruses.

Table 2.1. The variation in site of induction of tumours by NNN and NNK in different species.

	SPECIES		
TSNA	Mouse	Hamster	Rat
NNN	lung	trachea, nasal cavity	esophagus, nasal cavity
NNK	lung	lung, trachea, nasal cavity	lung, nasal cavity, liver

Nitrosamines require metabolic activation for binding to cellular DNA, and it is considered that they undergo α-hydroxylation, in part catalysed by cytochrome P450 enzymes. As can be seen from Table 2.1., NNK is a potent lung carcinogen, and α-hydroxylation leads to the formation of methane diazohydroxide, which reacts with DNA resulting in methylated bases;

O^6-methylguanine and 7-MeGua (Scheme 7 – *it must be noted that not all metabolites formed are represented in the scheme. Various demethylated and enzymatic oxidation products and corresponding metabolites are omitted*).



Scheme 7. Nitrosated products of nicotine, and subsequent metabolic activation of NNK to DNA binding species.

Studies have examined the relationship between dose of NNK and levels of methylated bases following treatment of rats. In the lung of rats it has been shown that NNK inhibits O^6-methylguanine repair by the enzyme O^6-methylguanine-DNA methyltransferase, allowing

the accumulation of the adduct in the lung. It has also been shown that cultured human lung tissue was able to α-hydroxylate NNK (Castonguay *et al.*, 1983). Therefore, results strongly suggest that O^6-methylguanine is formed in human lung due to NNK, and this brings about the question of NNK being a causative agent for lung cancer. Levels of 7-MeGua have been detected in humans (lung, white blood cells), formed possibly by NNK and other methylating species in tobacco smoke, and have shown to be higher in smokers compared to non-smokers (Mustonen *et al.*, 1993; Mustonen and Hemminki, 1992). Urinary excretion of 3-methyladenine and 3-ethyladenine has also shown to be significantly elevated in smokers compared to non-smokers (Kopplin *et al.*, 1995; Prevost and Shuker, 1996).

As well as forming tobacco-specific nitrosamines in tobacco smoke, the endogenous formation of tobacco-specific nitrosamines in rats treated with tobacco alkaloids and nitrosating agents was also shown to occur (Carmella *et al.*, 1997). The results in this study have a potential significance with respect to nitrosamine formation in people who use tobacco products (snuff, chewing tobacco) or even nicotine replacement therapy.

2.1.3.3. Effects of Environmental Tobacco Smoke

Environmental tobacco smoke is a mixture of cigarette sidestream smoke (85%) and mainstream smoke (15%). The sidestream smoke (generated at a lower burning temperature) contains carcinogens at higher concentrations than mainstream smoke. Rats subjected to environmental tobacco smoke were found to have an increase in lung tumour incidence and multiplicity (Witschi *et al.*, 1997), but no one carcinogen could be causally related to the tumour incidence. Unpublished data by Hecht *et al.*, (1997), has shown the presence of a metabolite of the lung carcinogen, NNK, in the urine of non-smokers exposed occupationally to environmental tobacco smoke.

2.2. Results and Discussion

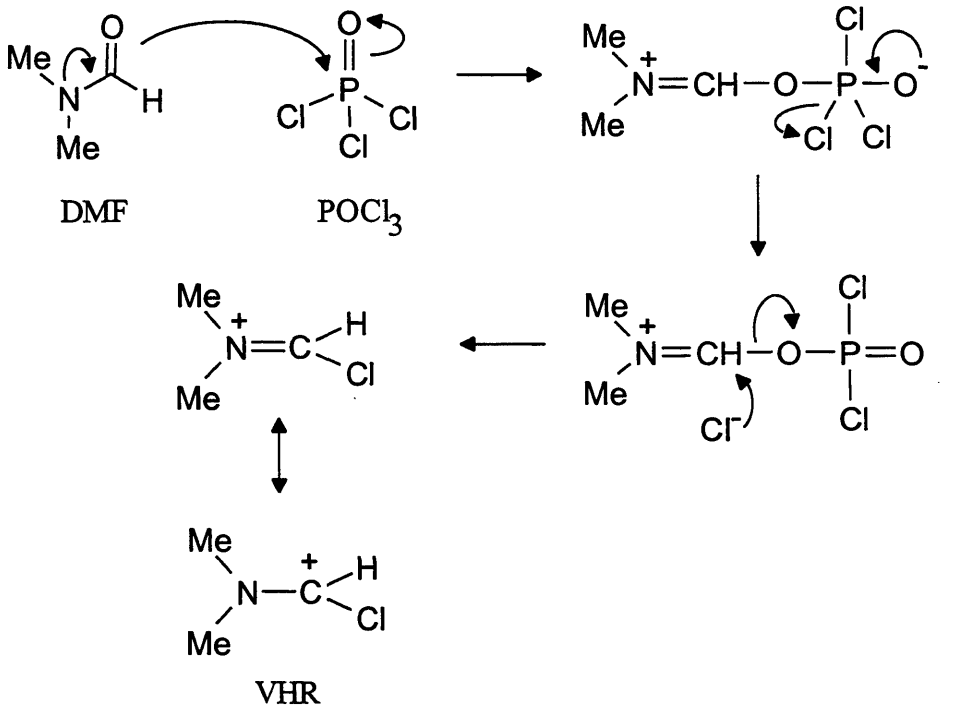
2.2.1. Fluorescent Postlabelling of N7-Alkylguanine Adducts

Prior to fluorescent postlabelling of 7-AlkGua adducts, the synthesis of Phmal was carried out. The approach was based on the hydrolysis of 2-phenyl-3-(dimethylamino)acrolein, which was first prepared by Arnold (1961; 1973). Later work by Coppola *et al.* (1974), showed some reactions of the acrolein, and it is the authors' methods that are used to synthesise Phmal. The fluorescent postlabelling reagent is then reacted with 7-MeGua using a method adapted from Sabbioni *et al.* (1986).

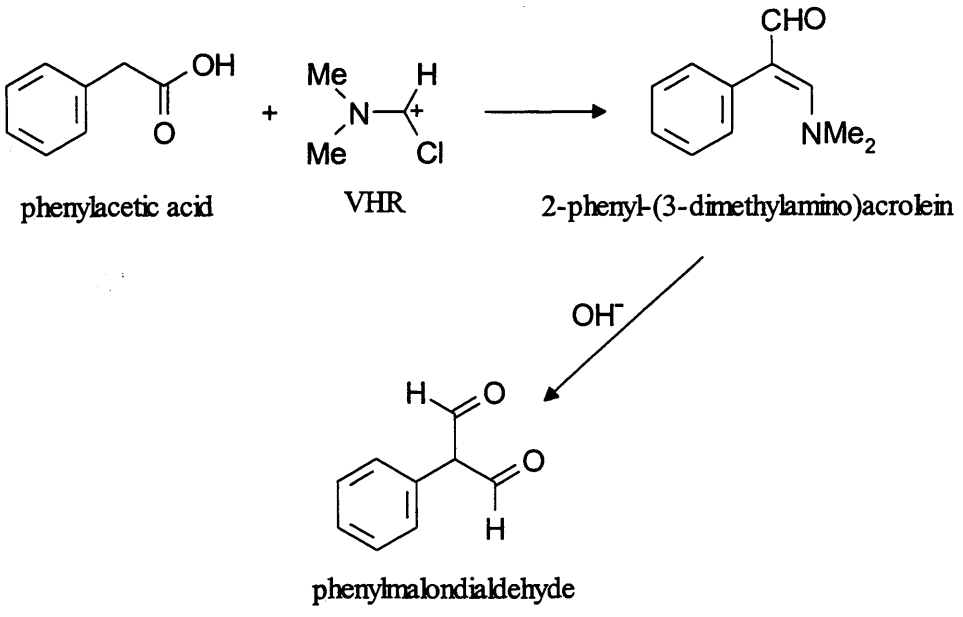
2.2.1.1. Synthesis of Phenylmalondialdehyde

Phmal was synthesised based upon an approach by Coppola *et al.* (1974), where the introduction of a carbon atom onto phenylacetic acid plays an integral part. Phenylacetic acid was reacted with the Vilsmeier-Haack reagent (VHR), which was formed *in situ* from the addition of phosphorus oxychloride and dimethylformamide, (Scheme 8), to afford 2-phenyl-3-(dimethylamino)-acrolein. This compound was successfully characterised by ^{1}H NMR and MS (FAB).

Hydrolysis of 2-phenyl-3-(dimethylamino)-acrolein provided the Phmal (Scheme 9), which was re-crystallised from water in a low yield. The purity of the compound was checked by ^{1}H NMR and MS.



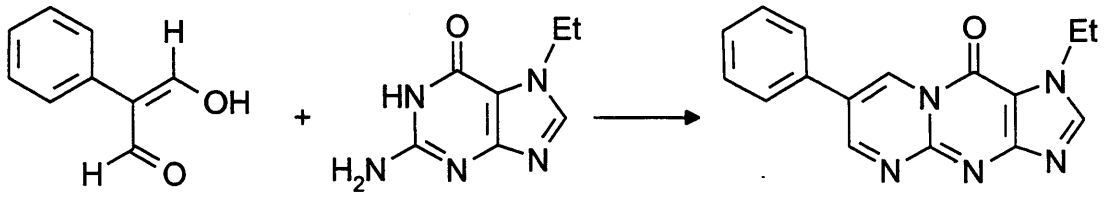
Scheme 8. Formation of the Vilsmeier-Haack reagent (VHR).



Scheme 9. Synthesis of Phmal.

2.2.1.2. Derivatisation of N7-Alkylguanine Adducts with Phenylmalondialdehyde

A general synthetic approach, adapted from Sabbioni *et al.* (1986), was applied to derivatise 7-AlkGua bases with Phmal, to provide standards. A solution of Phmal in glacial acetic acid was heated with 7-EtGua at 110°C for 1 h, to give a yellow solution of 7-phenyl-10-oxo-1-ethyl-9,10-dihydropyrimido[1,2-a]purine (Phmal-7-EtGua, Scheme 10). The product was re-crystallised from ethanol and water to give shiny yellow crystals, and purity checked by ^{1}H NMR and MS. All other standards of Phmal-7-AlkGua were supplied from earlier studies (Durand M.-J., 1993 Thesis).



Phmal 7-EtGua Phmal-7-EtGua

Scheme 10. Derivatisation of 7-EtGua with Phmal to give Phmal-7-EtGua.

2.2.1.3. Derivatisation of Picomole Quantities of N7-Methylguanine with Phenylmalondialdehyde

The general approach for fluorescent postlabelling was applied to the derivatisation of picomole quantities of 7-AlkGua. The derivatisation of 7-MeGua with Phmal was optimised to produce higher reaction efficiencies (~ 65%) than previously reported (Durand M.-J., 1993 Thesis), for picomole amounts of the base. Agarwal and Draper (1992) showed that the reaction product of malondialdehyde and deoxyguanosine was rapidly hydrolysed back to its two components on heating at 100°C in the presence of water. This reverse reaction caused by trace amounts of water in the reaction mixtures probably accounted for the low yields of derivatisation in initial studies. Therefore, water produced during the derivatisation reaction was removed by the addition of finely ground 4Å molecular sieve (sodium and calcium

aluminosilicates with cage-like crystal lattice structure) to the reaction mixture. The derivatised standards were then suitably prepared for HPLC analysis, and injected (Figure 2.4.). For samples to be derivatised, an immunoaffinity purification step was employed prior to derivatisation. This isolated the 7-AlkGua of importance from any interfering compounds.

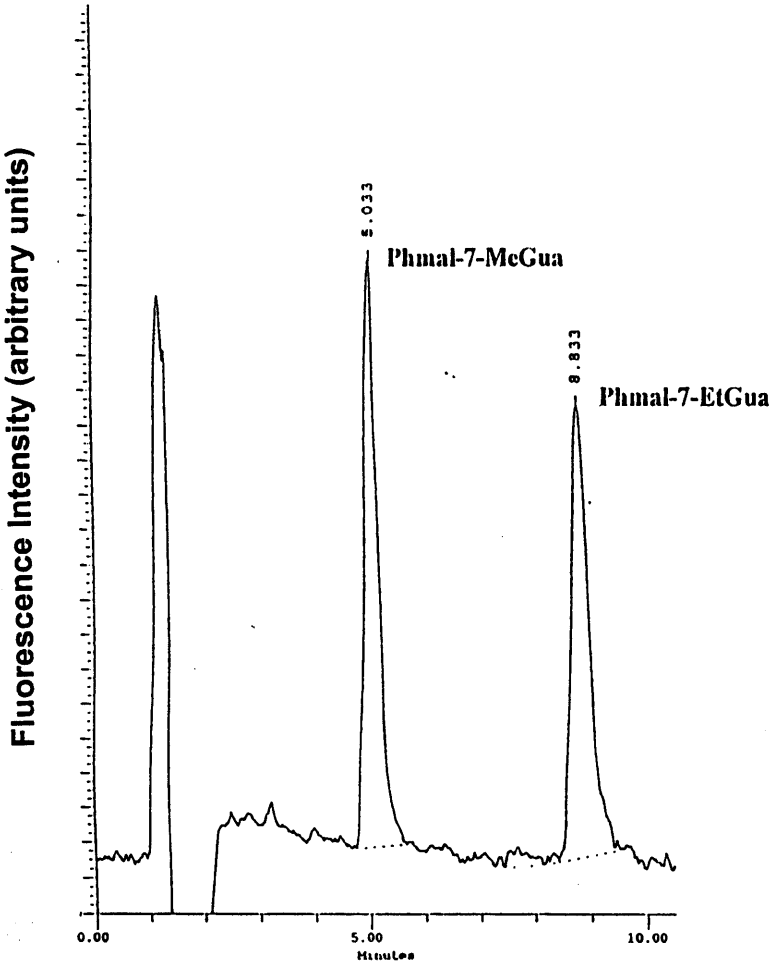


Figure 2.4. A typical HPLC chromatogram showing Phmal-7-MeGua (derivatised from 5 pmol of 7-EtGua) and Phmal-7-EtGua (derivatised from 5 pmol of 7-EtGua).

2.2.2. Immunoaffinity Purification

Immunoaffinity columns for 7-MeGua were prepared from antiserum containing antibodies against 7-MeGua. The capacity and retention of the columns was determined by passing standards of 7-MeGua through the column and derivatising the eluted base with Phmal. Quantification of the derivative was done by HPLC fluorescence.

2.2.2.1. Preparation of N7-Methylguanine Immunoaffinity Columns

Antiserum containing polyclonal antibodies against 7-MeGua (Durand and Shuker, 1994) was taken and the IgG fraction was precipitated by the addition of ammonium sulphate solution. The precipitated IgG fraction was coupled to protein A-Sepharose CL 4B gel as previously reported by Friesen *et al.* (1991). The immunoaffinity gel was then separated into polystyrene mini columns.

2.2.2.2. Capacity Determination of Immunoaffinity Columns

A saturation experiment was carried out to determine the capacity of the 7-MeGua immunoaffinity columns. The columns were loaded with varying concentrations of 7-MeGua, then washed with water and the bound base was then eluted with 1M acetic acid. These fractions were evaporated to dryness and derivatised with Phmal. The derivatives were then prepared for HPLC fluorescence detection. The capacity of the columns was shown to be *ca.* 800-900 pmol/mL gel (Figure 2.5.).

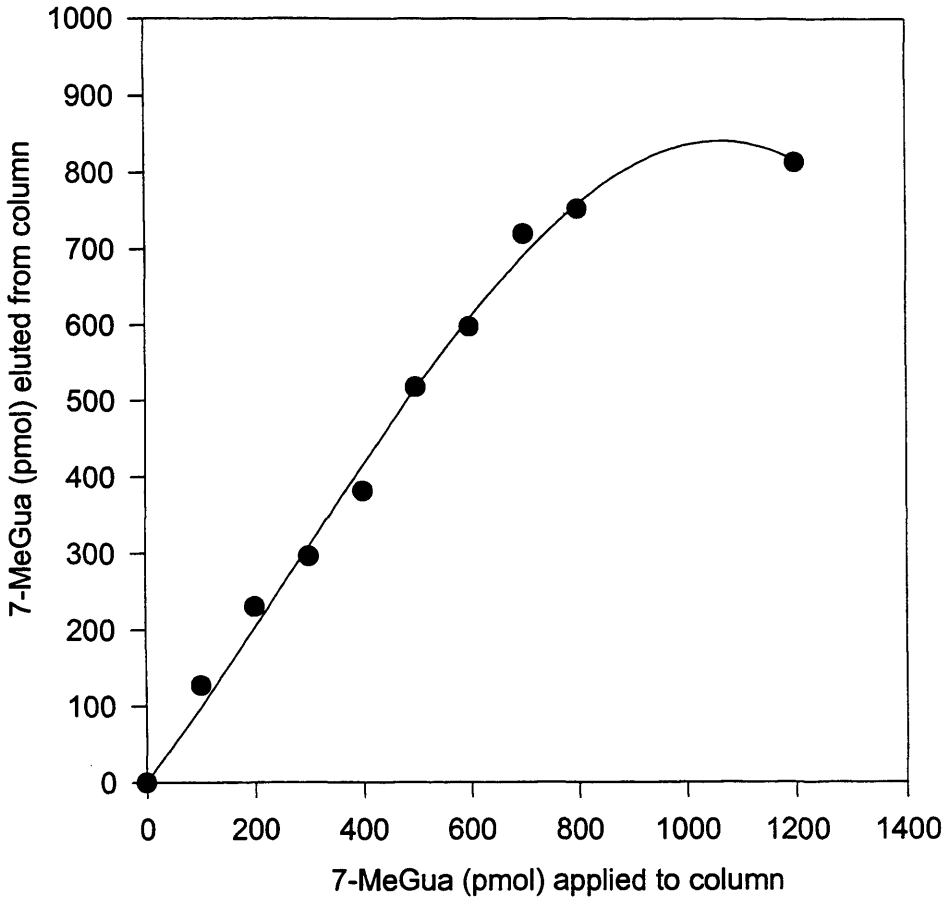


Figure 2.5. Plot showing the capacity of the gels in the immunoaffinity columns (1 mL) for 7-MeGua (n=2).

2.2.2.3. Percentage Recovery of N7-Methylguanine from the Immunoaffinity Columns

The percentage recovery of 7-MeGua from the columns was determined by passing standards of 7-MeGua through the columns and derivatising with Phmal, using 7-EtGua as an internal standard. A calibration graph was plotted and on the same axis a calibration line of standards derivatised with Phmal, that had not been passed through the columns, was also plotted (Figure 2.6.). The area ratio values on the vertical axis correspond to the peak area of standard divided by the peak area of the internal standard. By comparing the values of the

gradients' of the two lines, the percentage recovery of 7-MeGua from the immunoaffinity columns was calculated to be 94%. This virtually quantitative recovery of 7-MeGua from the columns meant that no correction factor for loss was necessary.

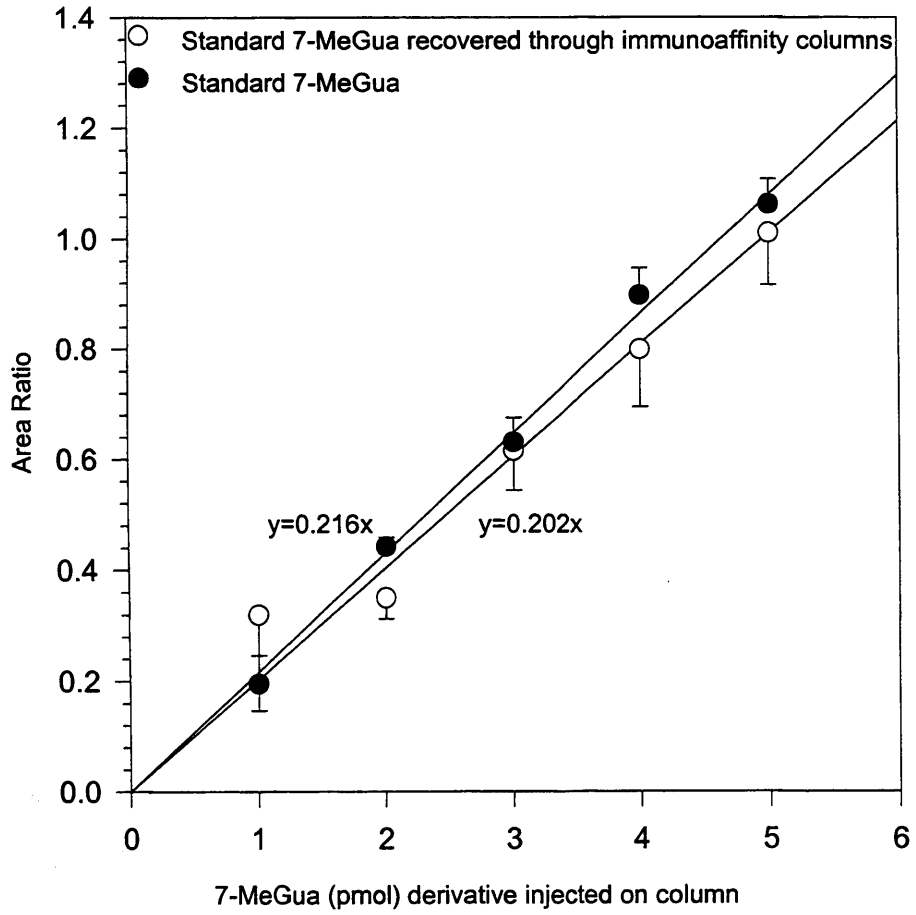


Figure 2.6. A plot showing the percentage recovery of 7-MeGua from the immunoaffinity columns (1 mL). A comparison of the two slopes indicates a 94% recovery.

2.2.3. Determination of N7-Methylguanine in Calf Thymus DNA Exposed to Dimethylsuiphate and Tobacco Smoke

To validate the fluorescent postlabelling assay incorporating immunoaffinity purification, two methylation studies were attempted. The first study involved reacting CT DNA with the direct-acting methylating agent, DMS, which preferentially forms methyl adducts at the N7-position of guanine (Beranek, 1990), and is also primarily used as a reagent to form N7-methylated guanine standards (Stillwell *et al.*, 1989). The second study attempted to observe the possibility of the formation of N7-methyl adducts from CT DNA exposed tobacco smoke. Increased levels of N7-methyl-2'-deoxyguanosine have been observed in lung tissues of smokers compared to non-smokers (Blömeke *et al.*, 1996; Haque *et al.*, 1994; Shields *et al.*, 1990), indicating the presence of a methylating agent(s), as discussed earlier.

2.2.3.1. Determination of N7-Methylguanine from Calf Thymus DNA Exposed to Dimethylsulphate

Incubation of DMS with CT DNA resulted in the formation of 7-MeGua, which was detected and quantified by the HPLC fluorescent postlabelling assay, incorporating the immunoaffinity purification step. The results can be seen in Table 2.1., and the level of 7-MeGua increases linearly with dose of DMS (Figure 2.7.). The increase in error observed, with increasing exposure to DMS, is a result of the amount of constant standard used. Serial dilutions of sample, prior to derivatisation with Phmal, may have reduced the size of the errors observed.

As can be seen from the results, the fluorescent postlabelling assay with the incorporation of immunoaffinity purification is very sensitive. The validity of using the Phmal-7-AlkGua derivative as a biomarker is substantiated, as low levels of modifications can be detected, with an absolute limit of detection for Phmal-7-AlkGua to be 0.5 pmol on-column. Allowing for the 94% recovery with the immunoaffinity columns and the 65% yield in derivatisation with Phmal, the limit of detection for this assay would be 0.8 pmol of 7-MeGua from a given sample of DNA

Table 2.1. A table showing the level of DMS exposure to CT DNA, and the corresponding amounts of 7-MeGua detected, using fluorescent postlabelling with immunoaffinity purification.

DMS (μM)	pmol of 7-MeGua/mg CT DNA (n=3)	Modification Level Adducts/10^6 nucleotides
0	1.86 ± 0.02	0.6
1	3.51 ± 0.88	1.1
10	29.6 ± 7.3	9.5
30	73.6 ± 17.4	23.6
50	146 ± 26	46.9
80	279 ± 83	89.6

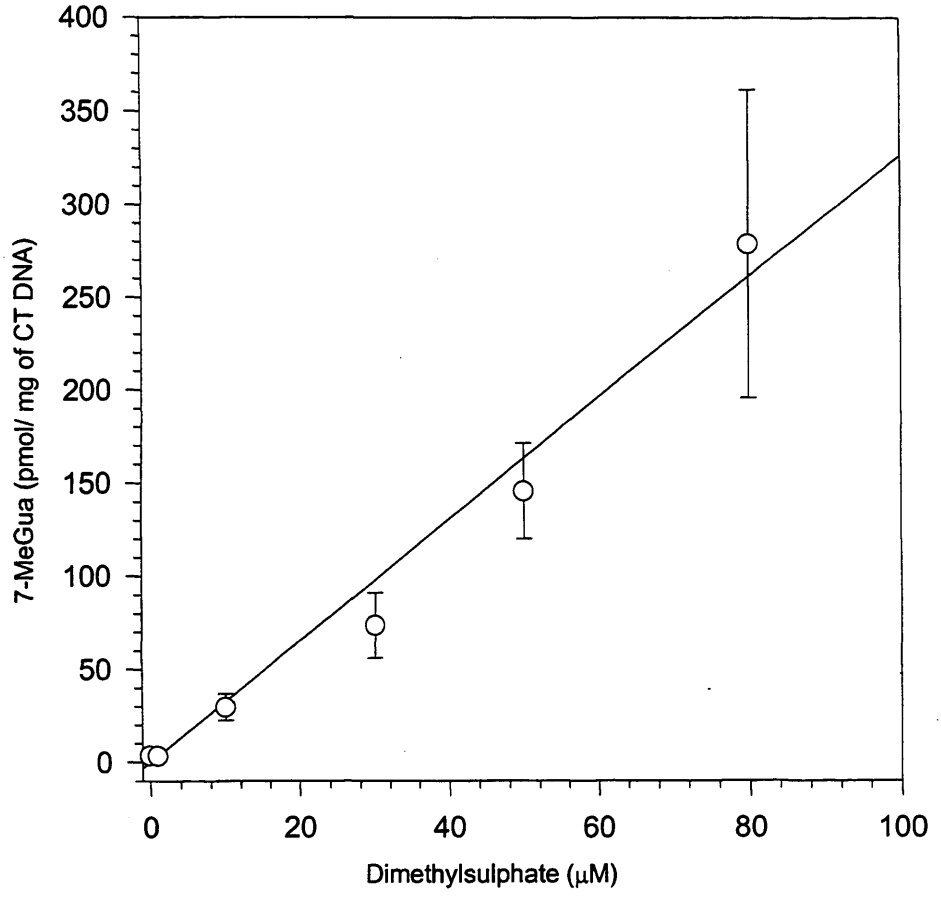


Figure 2.7. A plot showing a linear dose-response relationship between the levels of DMS exposure to CT DNA and the levels of 7-MeGua detected, using fluorescent postlabelling with immunoaffinity purification.

2.2.3.2. Determination of N7-Methylguanine from Calf Thymus DNA Exposed to Tobacco Smoke

As can be seen from the HPLC chromatograms obtained (Figure 2.8.), the levels of 7-MeGua formed from CT DNA exposed to tobacco smoke, could not be reliably quantified. The primary reason being the large excess of Phmal-7-EtGua internal standard detected. It seems that formation of 7-EtGua, from CT DNA exposed to tobacco smoke, also resulted as well as formation of 7-MeGua. The formed 7-EtGua, also seems to have been retained on the

immunoaffinity columns and then eluted with the 7-MeGua. (*Note*- subsequent work described in chapter 4 showed that the peak at ~8.5 min is more likely an artifactual compound which co-elutes with Phmal-7-EtGua, rather than Phmal-7-EtGua itself).

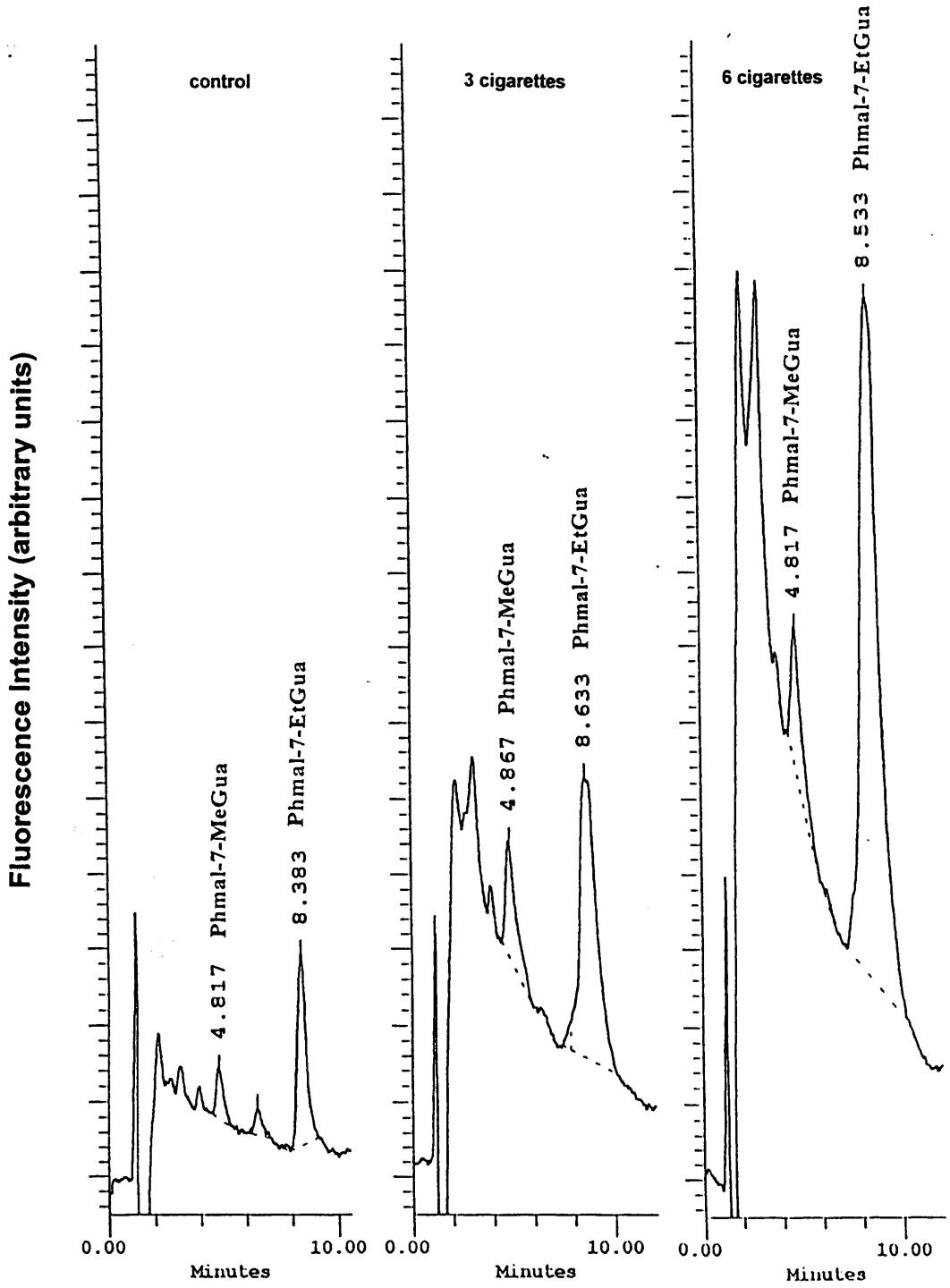


Figure 2.8. HPLC chromatograms showing the Phmal derivatives of DNA exposed to tobacco smoke (control, 3 cigarettes and 6 cigarettes) and passed through 7-MeGua immunoaffinity columns.

To assess whether 7-EtGua had indeed bound to the 7-MeGua immunoaffinity columns, standards of 7-MeGua and 7-EtGua (500 pmol of each) were combined and passed through the columns. The eluates were derivatised with Phmal and analysed using HPLC fluorescence detection. As can be seen from the resulting chromatogram (Figure 2.9.), binding of 7-EtGua to the immunoaffinity gel did occur. Therefore, this indicates that any 7-EtGua being formed in DNA exposed to tobacco smoke, would have been retained on the 7-MeGua immunoaffinity columns.

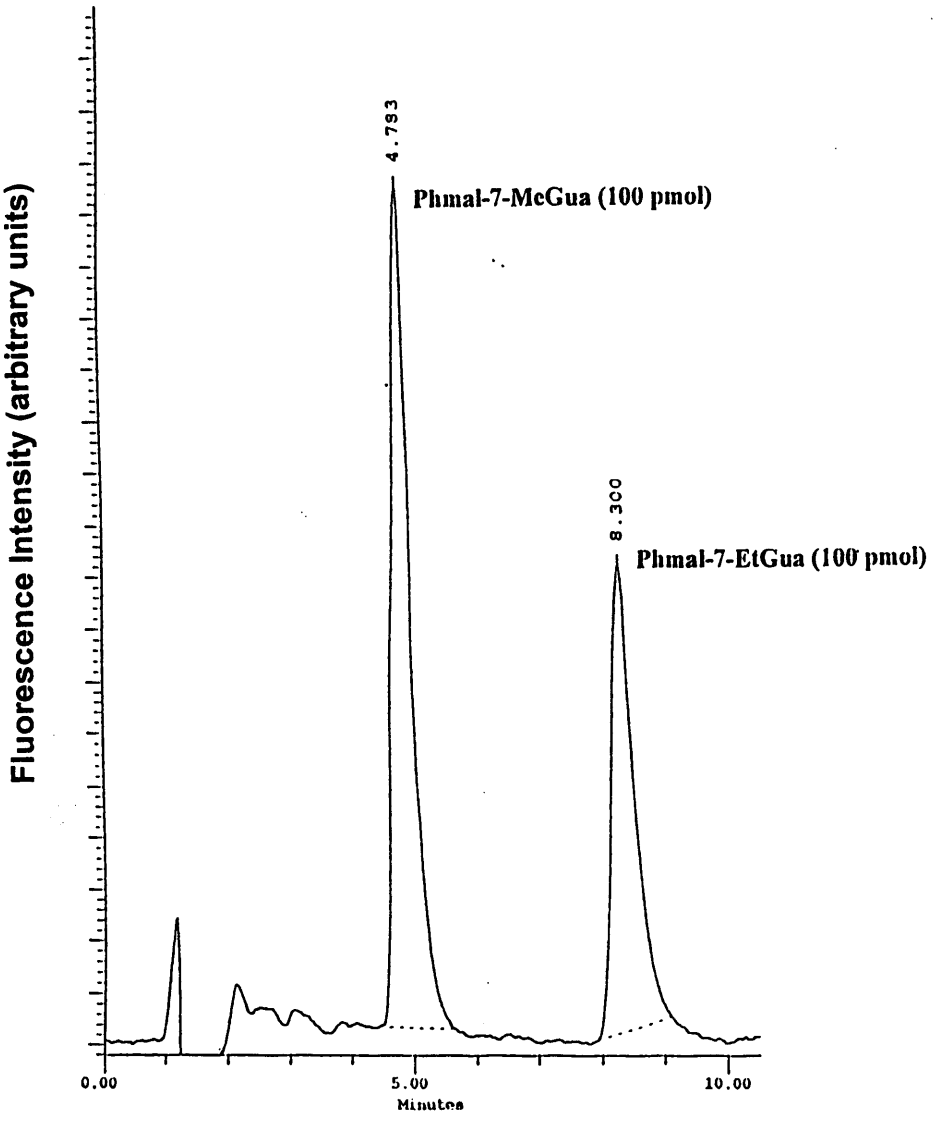


Figure 2.9. A typical HPLC chromatogram showing Phmal-7-MeGua and Phmal-7-EtGua, after the bases had been passed through a 7-MeGua immunoaffinity column and derivatised with Phmal.

These results had several implications on the rest of the study. Firstly, 7-EtGua could not be used as an internal standard for the assay, unless formed 7-EtGua could be removed prior to immunoaffinity purification of 7-MeGua and secondly, it was not known what type of competition for binding was occurring between 7-MeGua and 7-EtGua for the active sites on the immunoaffinity gel. In essence, it was not known whether all the 7-MeGua and 7-EtGua formed was actually being retained on the columns and being detected. It is surprising that 7-EtGua should be retained by an antibody site, specific for a smaller epitope such as 7-MeGua. Previous work on the cross-reactivity of the antibody with other related purines did not present any results that would have indicated that 7-EtGua was retained by the 7-MeGua antiserum (Durand and Shuker, 1994).

2.3. Conclusions

Fluorescent postlabelling of 7-AlkGua adducts with Phmal has shown to be a useful technique for measuring these adducts. The other fluorescent postlabelling methods discussed earlier (section 1.4.5.2.), relied on the 'tagging' of the adduct of interest with a fluorescent probe (dansyl chloride, *etc.*), and hence required the removal of excess reagent prior to quantitation. The major advantage of this method of fluorescent postlabelling over the other methods is the fact that the property of intense fluorescence is introduced into the molecules being detected by the reaction of the non-fluorescent Phmal with the weakly fluorescent alkylated base. Therefore, the removal of excess reagent is not necessary and background fluorescence is limited. The same argument may be applied to the use of phenylglyoxal as a fluorescent postlabelling reagent, but recent studies by Yonekura *et al.* (1993; 1994) showed that multiple products formed on reaction with guanine and its nucleotides and nucleosides, and may therefore may not be viable for derivatisation with a series of 7-AlkGua adducts.

The successful preparation of immunoaffinity columns allowed the selective isolation of the 7-MeGua from a solution of modified and unmodified bases, obtained from the thermal hydrolysis of CT DNA exposed to DMS. The fluorescent postlabelling assay coupled with the immunoaffinity purification step, not only allowed for greater selectivity for the adduct of interest, but also showed the assay to be very sensitive, being able to measure one 7-MeGua adduct per 10^6 normal nucleotides in 1 mg of CT DNA. Background levels of 7-MeGua were detected in untreated CT DNA to be approximately 6 adducts per 10^7 normal nucleotides in untreated CT DNA and this is consistent with results found by Kato *et al.* (1993), who extrapolated that untreated CT DNA contains levels of 7-MeGua adducts ranging from 0.3-0.6 adducts per 10^7 normal nucleotides. It is not too surprising that the number of adducts observed by Kato *et al.* are lower as their detection technique involved a two-step HPLC purification procedure, followed by ^{32}P-postlabelling of the small adduct. The combination of the three steps would have resulted in some loss of the adduct.

The second *in vitro* study was not as satisfactory, as the levels of 7-MeGua obtained from CT DNA exposed to tobacco smoke could not be quantified even though the immunoaffinity columns worked very well for the isolation of 7-MeGua adducts. Many studies have showed

the presence of elevated levels of 7-MeGua adducts in lungs of smokers, so it was disappointing not to have been able to quantify the adduct in this study (Kato *et al.*, 1993; Prevost and Shuker, 1996). Unfortunately it was found that the columns may have retained 7-EtGua from adducts produced from CT DNA exposed to tobacco smoke. This was a hindrance as 7-EtGua was being used as an internal standard. A simple solution would have been to adopt an alternative internal standard, but by doing this, the underlying problem would not have been solved; is there competitive binding between 7-MeGua and 7-EtGua for the immunoaffinity gel? Removal of 7-EtGua prior to isolation of 7-MeGua through the immunoaffinity columns would have rectified the problem, but not necessarily answer the question posed. An attempt at adopting different internal standards was not tried due to lack of time and the requirement of time for ongoing work.

CHAPTER 3

The Sensitivity of the Fluorescent Postlabelling Assay

Chapter 3. The Sensitivity of the Fluorescent Postlabelling Assay

3.1. Introduction

Having successfully developed a fluorescent postlabelling assay for the detection of 7-AlkGua adducts, able to detect 1 adduct per 10^6 normal deoxynucleotides from 1 mg DNA , the requirement to improve the sensitivity to enable the assay to be used for analysis of biological samples must now be addressed. This part of the work will examine the steps taken to improve the sensitivity of the assay, and other steps which may be employed after consideration of certain factors.

3.1.1. Optimisation of HPLC Conditions

The most important factors contributing to the sensitivity of a HPLC fluorescence assay are the chromatographic conditions, which include the instrumentation used, choice of column, choice of mobile buffers and the types of connections used. Some basic chromatographic theory, and the benefits of deciding on an appropriate column are discussed below.

3.1.1.1. Basic Chromatographic Theory

High performance liquid chromatography is a very efficient method of analytical separation. Separation of analytes occurs due to the difference in partitioning of the various sample constituents between the mobile and the tightly packed, small particulate stationary phases. The mode of action is called adsorption chromatography, and will concentrate on reverse-phase chromatography. Essentially, the stationary phase consists of a non-polar material with a high specific surface area and the mobile phase is relatively polar. In general, non-polar compounds elute later than polar compounds. The partition coefficient (K), the ratio of the concentration of the sample constituent in the stationary phase and the mobile phase, determines the separability. The differences in K need to be large enough for separability to occur and the K values can be altered by varying the temperature, stationary phase and/or the mobile phase.

3.1.1.2. Narrow-Bore HPLC Columns

The use of a narrow-bore column, compared to an analytical column, offers greater resolution of a mixture of components and because of the decrease in flow rate, allows for increased detectability and economical consumption of solvents. Using narrow-bore columns in comparison to standard analytical columns, much sharper peaks are obtained because of the smaller packaging particles that are used (3 μm) and also, the column diameter is reduced. The much reduced peak volumes of samples in narrow-bore columns mean that when the samples are eluted they are more concentrated. The observed fluorescent signal of a molecule is proportional to the radiant power incident on the molecule and the concentration of the molecule in the flow cell. The peak height corresponds to a maximum concentration of molecules in the flow cell, and since the components eluting from a narrow-bore column are less dilute, the maximum concentration and hence the peak height are greater. The increase in concentration is given by the ratio of the squares of the diameters of the columns; theoretically, a peak is

$$\frac{(4.6)^2}{(2.1)^2} = 4.8$$

times more intense on a 2.1 mm diameter column than on a 4.6 mm column with the same length and particle size.

3.1.2. Optimisation of Derivatisation Conditions

To increase yield efficiencies of reactions, a variety of techniques can be employed. They usually involve driving the equilibria of the reaction towards the product side. Techniques usually involve adding excess reagent, increasing temperature, increasing pressure, agitation or continuous removal of one of the products. In chapter 2, the use of excess reagent, elevated reaction temperature and removal of water product (by addition of a molecular sieve), showed to increase the reaction efficiency of the Phmal reaction with 7-AlkGua adducts.

3.1.3. Laser-Induced Fluorescence (LIF) Detection

The use of laser-induced fluorescence detectors is becoming a popular method for detecting analytes with HPLC analysis. Laser-induced fluorescence (LIF) detection has many advantages over conventional light sources (deuterium or xenon arc lamp). The

monochromatic light source, of a laser, gives accurate focusing and positioning, allowing for radiation to be efficiently used in a small detection volume. As mentioned earlier, the observed fluorescence from a molecule is directly proportional to the concentration of the molecule and the photon flux (radiant power), and comparison of photon fluxes for lasers *versus* standard light sources shows that lasers have 2-3 orders of magnitude of greater power in their output. This implies that limits of detection measured with LIF will be 2-3 orders of magnitude greater than standard light sources. This increase in photon flux also increases background fluorescence, therefore instrument design needs to allow for this (Yeung and Sepaniak, 1980; Green, 1983).

A recent evaluation of LIF detectors by Van de Nesse *et al.* (1995) showed that because lasers only have a discreet number of emission wavelengths, most commercially available LIF detectors only excite in the visibly region of the spectrum (> 400 nm). Deep-UV excitation (< 300 nm) is possible, but then the argon-ion or krypton-ion lasers used are only able to produce *ca.* 2.5 Watts of power for short lifetimes. It must be noted that too high a powered laser will force ground state depletion of molecules which would then make the fluorescent signal disproportional to the excitation power. In essence, fluorescence detection sensitivity cannot be improved indefinitely by increasing laser power.

As most commercially available fluorescence detectors only excite at wavelengths greater than 400 nm, it is conceivable that the compound to be detected by fluorescence may need to be altered, to enable commercially available fluorescence detectors to be used. A compound to be quantified can either be labelled with a fluorescent tag which may absorb radiation at greater than 400 nm, or as in the case of 7-AlkGua adducts where fluorescent postlabelling is done with Phmal, the labelling reagent may need to be altered. Essentially, a higher order of conjugation is required to increase the wavelength of excitation.

3.2. Results and Discussion

3.2.1. Optimisation of Derivatisation Reaction

As previously explained in section 2.2.1.3., the derivatisation of picomole quantities of 7-AlkGua with Phmal was optimised by the addition of 4Å molecular sieve, to abstract any water produced during the reaction (Figure 3.1.). Additionally, the interiors of the reaction vials were coated with a silanising reagent.

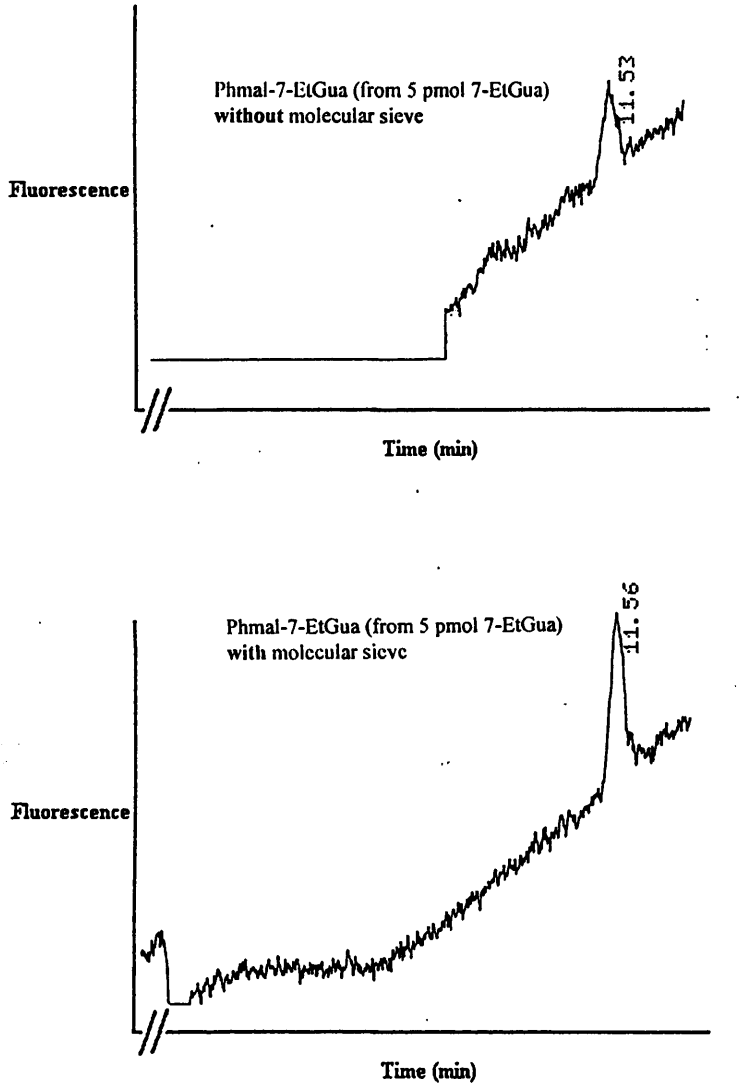


Figure 3.1. Chromatograms showing the efficiency of the Phmal derivatisation reaction with and without 4Å molecular sieve added.

3.2.1.1. Dry Phase Reaction

Maximum derivatisation yields were obtained using the silanised reaction vials and 4Å molecular sieve, but to increase the yields further and to keep the yields consistent, a dry phase reaction was attempted.

The use of the molecular sieve, having improved the yield of the derivatisation reaction, did not always provide consistent results (hence reactions carried out in at least triplicate). This can be attributed to many factors, which include pore size and quantity of sieve used. The pore size was ideal for trapping water, but it was not known to what extent it may have hindered the derivatisation reaction - were reagent molecules being trapped? The amount of sieve used was not consistent, as a weighable quantity could not be transferred to the reaction vial. Attempts to overcome this included making a suspension of the ground molecular sieve in glacial acetic acid, but attempts were unsuccessful, as the solution could not be made sufficiently homogenous.

The dry phase reaction allowed the inclusion of a molecular sieve to be omitted. As the reaction vessel was left uncovered, any water produced during the reaction would to all intent and purposes be boiled off. But one major problem with not using a solvent was that not all the reagent molecules were able to react. Essentially, solvation of reagent molecules was required to allow the reagents to mix and reactions to occur.

So even though the use of a molecular sieve did not always give consistent results, it was decided that it was the best option, and as long as an internal standard was incorporated, quantitation could be carried out.

3.2.2. Alteration of Chromatographic Conditions

As discussed previously, a variety of parameters can be altered to give improved peak shapes and retention times (pH of mobile phase, salt concentration, temperature, column length and width, *etc.*), but as optimum eluting conditions for Phmal-7-AlkGua had already been established (Shuker *et al.*, 1993), alteration of the HPLC column and modification of instrumentation to give improved sensitivity was decided.

Originally, a Jones Chromatography ODS (25.0 cm × 4.6 mm) reverse-phase column was employed for HPLC separations of Phmal-7-AlkGua compounds, and this was found to be adequate until it was realised that an increase in sensitivity was required for smaller samples of DNA. It was decided that a change of column may show an increased response in peak retentions and profiles. It is widely accepted that the chemical nature of the silica surfaces on the HPLC columns play an important part in the asymmetry of the eluted peaks. Packings that contain free silanol groups exhibit increased retention and broad peak tailing for basic samples. This is due to the result of two kinds of silanol interaction; hydrogen bonding and ion exchange. Therefore by blocking these free silanol groups (using BDS columns), the interactions are reduced or eliminated, giving sharper, well-resolved peaks. A Shandon Hypersil C_{18} base-deactivated silica [BDS] (25.0 cm × 4.6 mm) reverse-phase column was available and was therefore tested. There was an immediate improvement in the peak profile; sharper and well-resolved peaks were observed, but no increase in sensitivity was evident.

To improve the sensitivity of the assay further, without having to purchase a more sensitive fluorescence detector, it was decided that a narrow-bore column would be incorporated. Utilising a Shandon Hypersil C_{18} BDS (15.0 cm × 2.1 mm) reverse-phase column, at a flow rate of 0.2 mL/min, a three fold increase in peak-height was observed (Figure 3.2.), giving an improved signal-to-noise ratio. As the internal diameter of the column was decreased, the internal diameters of all the connecting tubing also needed to be decreased. This was done to ensure that a minimum dead volume was attained.

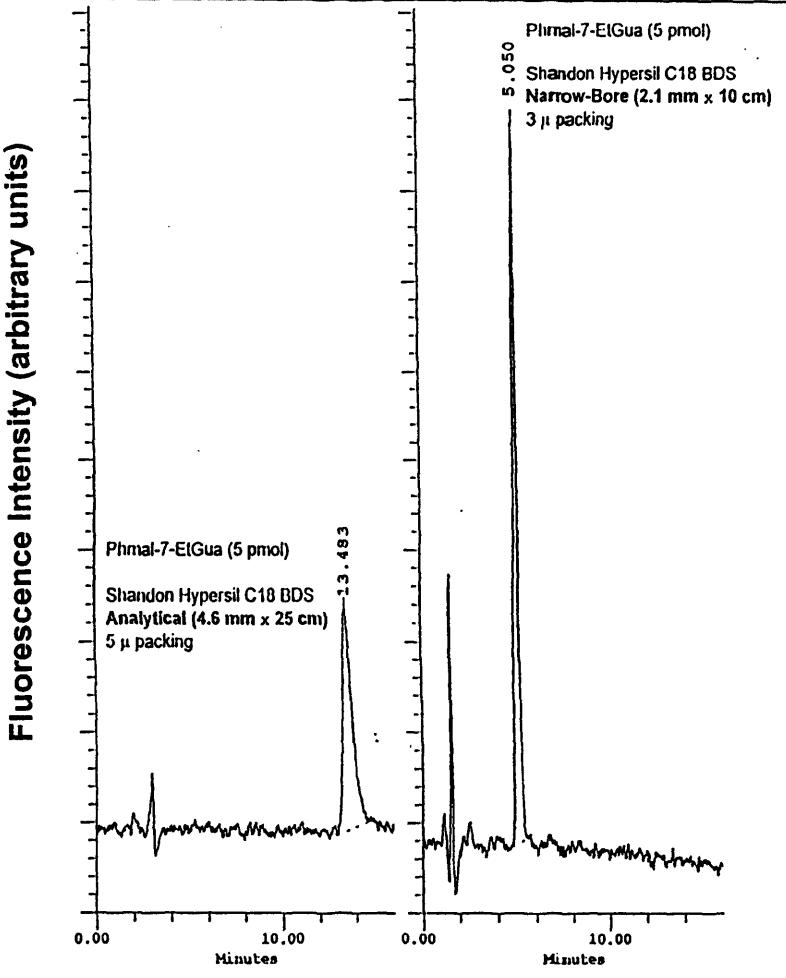


Figure 3.2. Chromatograms to show difference in peak shapes and intensities

A further increase in sensitivity could be observed by using micro-bore columns or even capillaries, where internal diameters are even smaller than used for narrow-bore columns. However, from a practical point of view, specialised HPLC equipment would be needed to cope with very low flow rates and also a major problem would be the small injection samples that would be required (Mills *et al.*, 1997).

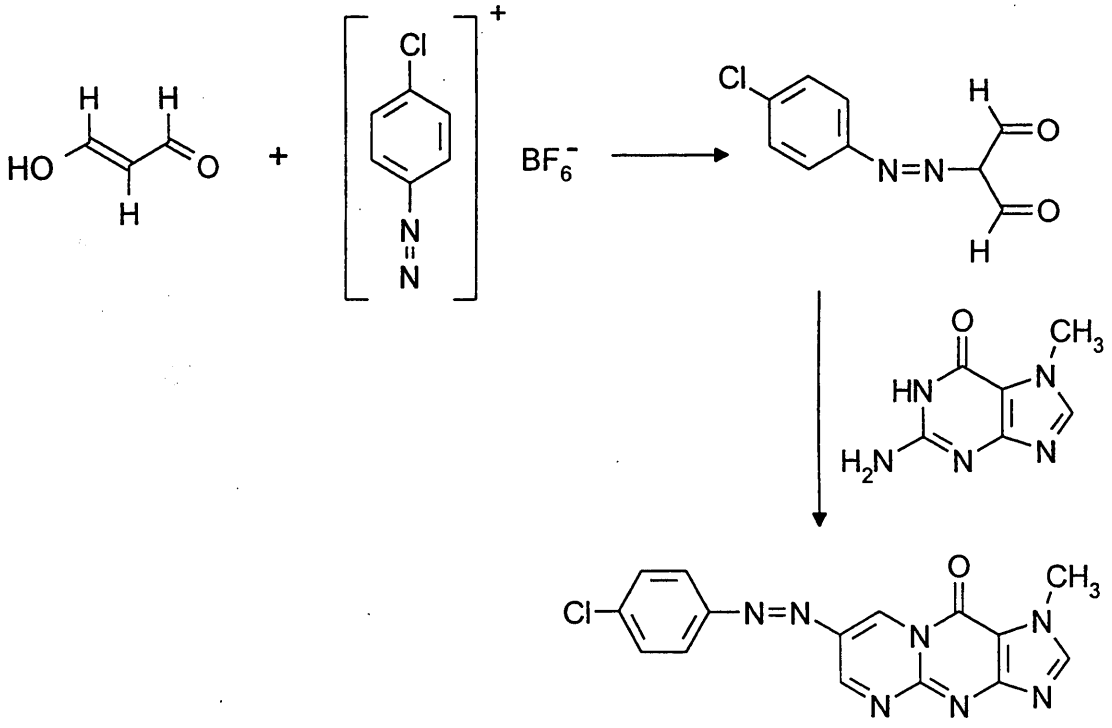
3.2.3. Attempts to Alter Chromophore

The assay to detect and quantify Phmal-7-AlkGua derivatives has been optimised as much as possible by improving the derivatisation technique and optimising the chromatographic conditions. The derivatives have an excitation wavelength of 280 nm, which do not make them amenable to detection by current commercially available laser-induced fluorescence detectors. Therefore it would be advantageous to be able to chemically modify the derivatives

to allow them to be excited at higher wavelengths. In order to do this, the conjugated π-electron system needs to be increased.

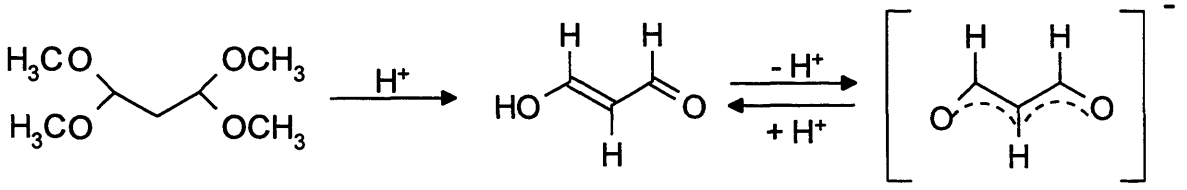
Molecular modelling studies on the Phmal-7-MeGua derivative by Vaca *et al.* (1994), showed that the phenyl substituent at the C2 position of malondialdehyde is not on the same plane as the tricyclic moiety of the fluorescent derivative and so therefore does not contribute to the conjugated π-electron system. The model was a generation of the lowest energy conformation for the Phmal-7-MeGua derivative. Therefore to increase the conjugated ring system further, the presence of an azo group between the phenyl ring and the tricyclic moiety may allow the molecule to lie in one plane. It would be of no advantage to alter the phenyl ring itself. Ideally this compound should also be modelled to determine its lowest energy conformation.

3.2.3.1. 4-Chlorobenzeneazomalondialdehyde as a Potential Fluorescent Postlabelling Reagent



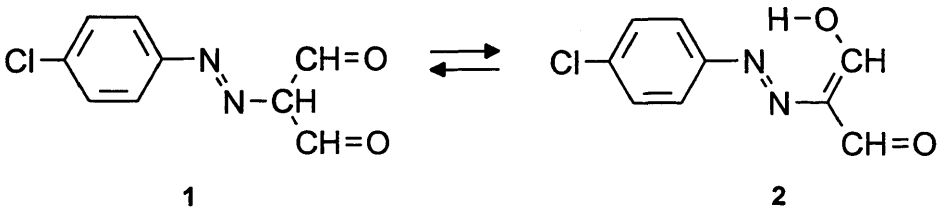
Scheme 11. Preparation of 7-(4-chlorobenzeneazo)-10-oxo-1-methyl-9,10-dihydropyrimido[1,2,a]purine (ClBmal-7-MeGua).

Based on work by Reichardt and Grahn (1970), who developed approaches for the preparation of substituted arylazomalondialdehydes by the coupling of aryldiazonium tetrafluoroborates with malondialdehyde, 4-chlorobenzenediazonium hexafluoroborate was reacted with malondialdehyde. It was envisaged that the conjugated system afforded by the Phmal-7-AlkGua derivative could be extended by coupling it with a diazo group attached to a phenyl ring. Initially, malondialdehyde was prepared by shaking 1,1,3,3-tetramethoxypropane with 0.5 M HCl.



Scheme 12. Formation of malondialdehyde.

The aqueous solution of malondialdehyde was added to a cold aqueous solution of 4-chlorobenzenediazonium hexafluoroborate. The product precipitated out after a couple of hours of stirring. The product was then filtered and re-crystallised, and characterised by MS (EI) and ^{1}H NMR and ^{13}C NMR. From the various tautomeric structures for 4-chlorobenzenemalondialdehyde, the NMR data suggests that it exists as the enolic tautomer, structure **2** (Scheme 13).



Scheme 13. 4-Chlorobenzenemalondialdehyde.

A standard of 4-chlorobenzeneazomalondialdehyde-7-methylguanine (ClBmal-7-MeGua) was synthesised and characterised by MS (FAB+) and ^{1}H NMR. The fluorescence properties of the derivative were also examined. Initially, the maximum UV absorbance was determined to be 368 nm. Ideally, this wavelength would be suited for use with commercially available laser-induced fluorescence detectors. Using a luminescence spectrophotometer and exciting at λ_{max} of 368 nm, an emission scan was carried out between 300 and 700 nm. Unfortunately, no emission peaks were observed. From these results it can be concluded that 4-chlorobenzeneazomalondialdehyde-7-methylguanine is not a fluorescent compound.

The fact that the 4-chlorobenzeneazo group may not lie in the plane of the tricyclic fluorescence group should not hinder the compound being fluorescent. In fact, the whole derivative, ClBMal-7-MeGua, may be planar and conjugated, but the presence of the chlorine group on the phenyl group may have lead to the fluorescence being quenched. It is known that the presence of atoms or groups such as the halogens, carboxylic, nitroso, keto, *etc.*, on an aromatic ring tends to eliminate fluorescence, because these electron-withdrawing groups delocalise the π-electrons on the conjugated ring system (Willard *et al.*, 1988).

3.3. Conclusions

There are many contributory factors which play an important part in the sensitivity of the HPLC fluorescent postlabelling assay. Three of the factors were dealt with in this study, with varying levels of success. The optimisation of the derivatisation reaction has been covered substantially, but it is worth pointing out that the higher the derivatisation efficiency the greater the fluorescent signal observed for a given amount of sample. Therefore, it is advantageous to optimise the derivatisation yields for the reaction. Presently, the incorporation of a molecular sieve (traps water produced during the reaction) allows a high yield of the Phmal-7-AlkGua derivative. If biological samples are to be analysed, results always need to be consistent. Therefore a procedure needs to be available which is reliable and does not necessarily require the derivatisation reaction to be carried out in triplicate, as there may not be enough sample to analyse in triplicate. The attempt at a dry phase reaction seemed logical, as it allowed water to be removed to enable the equilibrium to be shifted towards the products. Unfortunately, the reaction did not proceed as well as anticipated, probably due to the lack of solvation of the reactive species.

The chromatographic procedure was shown to be the most important of the contributing factors studied, in terms of increases in sensitivity attained. It was successfully shown that, going from a conventional analytical column (4.6 mm i.d.) to a narrow-bore column (2.1 mm i.d.), the sensitivity increased. Not only were the peaks well-resolved, but the peak height was also shown to have increased (*ca.* 3-fold). Incorporating a BDS narrow-bore column, to remove free hydroxyl groups on the silanols, less tail broadening was observed, resulting in sharper peaks. Theoretically, it should be possible to increase the sensitivity further by using even narrower columns, *e.g.* micro-bore (1 mm i.d.) columns or capillaries, but reducing the column diameters places limitations on the instrumentation that may be used. A conventional HPLC set-up could not be used for capillary or micro-bore columns (Mills *et al.*, 1997). The major limitation for a conventional HPLC set-up would be attaining the low flow rates required for micro-bore or capillary HPLC. Overcoming this problem by incorporating flow splitters would then lead onto sample injection problems, problems with tubing variations giving rise to high dead volumes and the requirement of specialised flow cells for the fluorescence detectors.

It is unfortunate that 4-chlorobenzeneazomalondialdehyde-7-methylguanine was not fluorescent. As discussed earlier, this was probably due to the presence of the chlorine atom, quenching the fluorescence produced. It may be appropriate to synthesise a standard without the presence of such fluorescence quenching groups, but due to the restrictions in time available, further work in this area could not be continued. Work by Reichardt *et al.* (1984), has shown the synthesis of a variety of 2-cycloalkylmalondialdehydes (ranging from cyclopropane to cyclopentane), but derivatisation with 7-AlkGua adducts would not lead to any further conjugation than that already provided by the fluorescent tricyclic moiety. The synthesis of 2-halomalondialdehydes has also been shown (Reichardt and Halbritter, 1970; 1975), but derivatisation to form a fluorescent tricyclic compound would probably result in fluorescence being quenched due to the presence of the halogens.

The use of LIF detectors in conjunction with HPLC is becoming very popular, and has been used to detect a variety of analytes, ranging from DNA-benzo[a]pyrene adducts, N-terminal prolyl peptides, DNA adducts of cisplatin and carboplatin, and dansylated DNA adducts (Wang and Laughlin, 1992; Toyo'oka *et al.*, 1994; Sharma *et al.*, 1995; Sharma and Freund, 1991), with very high sensitivity. Sharma and Freund (1991) showed the limit of detection for dansylated 5-methyldeoxycytidine-5'-monophosphate using LIF detection to be 200 times greater than using a conventional fluorescence source. The main advantage of using LIF is that for the same sensitivity as an analytical column, much less sample may be used. For example, a 200 times greater sensitivity using LIF means that 200 times less sample could be used; instead of 1 mg of DNA, 5 μg DNA could be used. Therefore by incorporating LIF with the immunopurification HPLC fluorescent postlabelling assay, a very sensitive and selective analytical procedure could be obtained.

CHAPTER 4

The Production of a Monoclonal Antibody Against N7-Ethylguanine and its Use

Chapter 4. The Production of a Monoclonal Antibody Against N7-Ethylguanine and its Use

4.1. Introduction

This chapter describes some further applications of the immunoaffinity HPLC fluorescent postlabelling assay highlighted in the previous chapters. As concluded in chapter 2, for the fluorescent postlabelling assay to be selective and sensitive, the immunoaffinity step needs to be incorporated and hence, this requires specific antibodies against the adduct. So in this study, a procedure to produce monoclonal antibodies against 7-EtGua is shown. This work involves the synthesis of a 7-EtGua hapten, which is coupled to a protein and used as a test antigen. Finally, the immunoaffinity HPLC fluorescent postlabelling assay is used to quantify 7-EtGua adducts from CT DNA exposed to diethylsulphate (DES) and tobacco smoke.

4.1.1. The Production of Monoclonal Antibodies

4.1.1.1. Background on Antibody Production

The objective of monoclonal antibody production is the formation of one cloned cell which produces one antibody of desired specificity, and the perpetual dividing of this one cell leads to a source of a single monoclonal antibody. The origin of the monoclonal antibody is a hybridoma, produced from the fusion of a plasma cell from an immunised spleen and a plasma cell from a myeloma cell-line. Utilising the research of scientists before them, it was the culmination of work by Köhler and Milstein (1975) that lead them to combine the nuclei of normal antibody-forming cells with cells of their malignant counterparts, and hence develop a technique to analyse and purify individual or classes of molecules from the highly complex mixtures experienced in biological material. Since this original work, large numbers of specific antibodies against many molecules have been produced and the procedure can be optimised to give high specificity, high affinity and physical properties tailored to suit individual needs. The technique involves many steps, including the immunisation of an animal, spleen cell fusion and cloning, hybridoma growth and the production of ascites, all which are outlined in Figure 4.1.

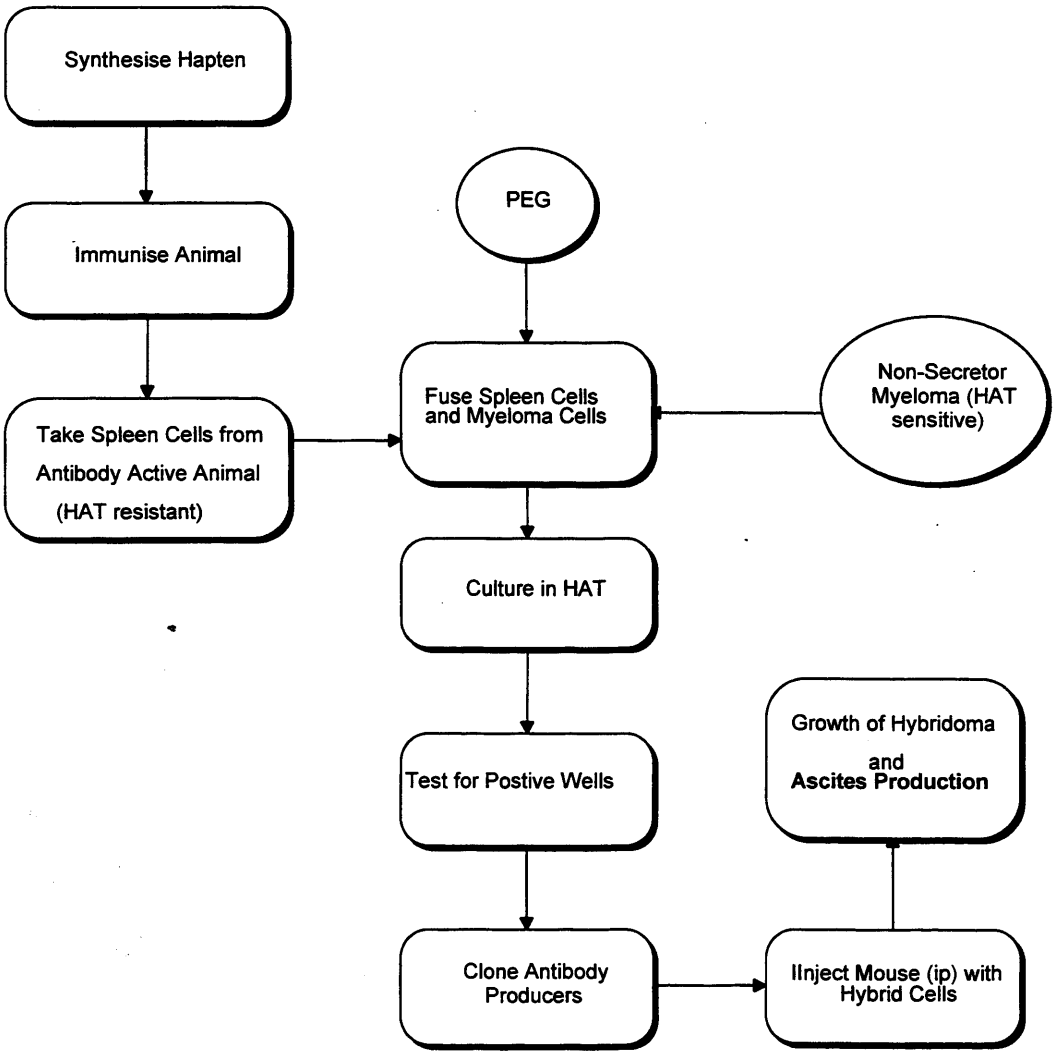


Figure 4.1. The various steps involved in the production of monoclonal antibodies (Feinberg and Jackson, 1983; Goding, 1986).

4.1.1.2. Immunisation of Animals

Antigens are introduced into animals (usually rats or mice because of their availability), and this immunisation of the animals provokes an immune response in the animal, which leads to the production of antibodies. These antibodies are a blood protein of the various classes of the globulin type (immunoglobulin) which are synthesised in the lymphoid tissue. To augment an immune response an adjuvant is utilised. Monomeric proteins are poorly immunogenic and tend to induce tolerance, therefore the mode of action of the adjuvant is thought to include a slow, prolonged release of the antigen in a highly aggregated form. The choice of adjuvant may also determine the class of immunoglobulin (Ig) antibody produced.

4.1.1.3. Cell Fusion and Cloning

Once the animals are shown to be making a good antibody response (tail bleeds tested using an ELISA study), the spleens are removed and a cell suspension prepared. These cells are then fused with a myeloma cell line by the addition of polyethylene glycol (PEG) which promotes the cell membranes to fuse producing multinucleate cells termed heterokaryons. These fusion events are poorly controlled and only a small proportion of the cells fuse successfully. The hybrid cells are genetically unstable and have strong tendencies to lose chromosomes. This loss of chromosomes is cell and species dependant, and later cloning may results in loss of antibody activity. Successfully fused cells are then used to produce a hybrid cell line. To do this selectively, and not to incorporate fused spleen-spleen and fused myeloma-myeloma cells, the fusion mixture is set up in a culture with medium containing hypoxanthine, aminopterin and thymidine (HAT). Aminopterin, a powerful toxin, is used to block a metabolic pathway, but if the cells are provided with the intermediate metabolites hypoxanthine and thymidine, this pathway can be bypassed. Therefore, spleen cells can grow in HAT medium, but the myeloma cells die in HAT medium because they have a metabolic defect and cannot use the bypass pathway. The fused spleen-spleen cells die in culture naturally after 1-2 weeks and the fused myeloma-myeloma cells are killed by the HAT. The fused spleen-myeloma cells, having the metabolic bypass of the spleen cells and the immortality of the myeloma cells, survive and some have the antibody producing capacity of the spleen cells. Feeder cells are added (a slow-growing or non-growing population of cells) to promote the growth of the lymphoid cells which tend not to grow well at low densities.

The name, feeder cell, implies that the cells produce something required for growth, and are usually peritoneal cells taken from the peritoneal cavity of the animal. Growing cells are then tested for antibody activity using ELISA, and those that test positive are cloned, *i.e.* plated out so that each well contains one cell. Cloning reduces the risk of overgrowth by nonproducer cells, ensuring that the antibodies are truly monoclonal. This produces a clone of cells which are again tested for antibody activity using a checkerboard ELISA and then tested for specificity using inhibition ELISA.

4.1.1.4. Hybridoma Growth and Production of Ascites

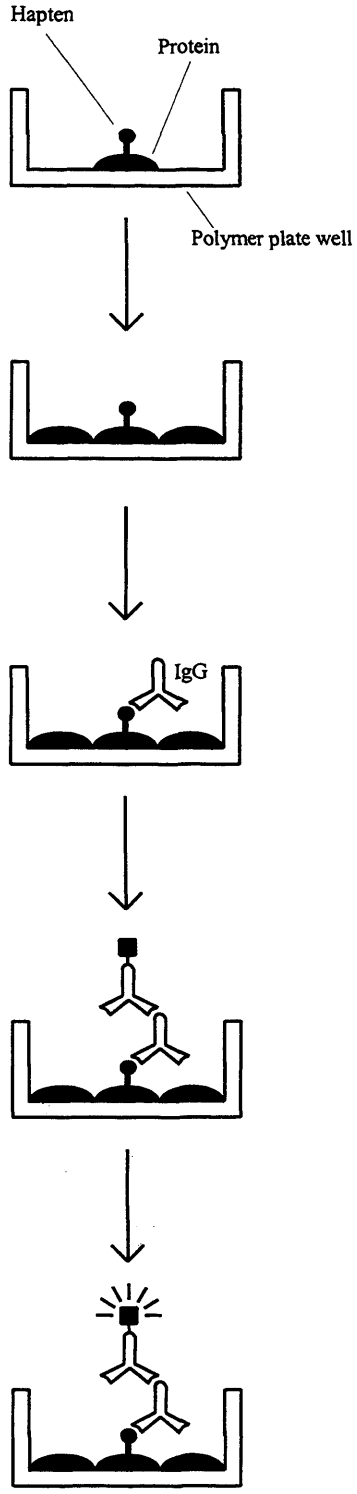
The antibody producing clones are taken and injected intraperitoneally into mice or rats. Tumours (or hybridomas) result usually after 2-4 weeks, and within the tumours, the ascites fluid produced contains the antibody. The antibody levels in ascites are similar to levels found in serum, ranging from 5-15 mg/mL, but larger volumes of ascites are usually obtained, 2-5 mL compared to 0.5-1 mL for serum. The ascites or serum are then taken and the antibody used in the appropriate manner, *i.e.* coupling immunoglobulin G fraction to protein A-Sepharose gel.

4.1.2. Production of Polyclonal Antibodies from Chicken IgY

The administration of an antigen with adjuvant, intravenously or intraperitoneally to a chicken, produces a high serum antibody 16-30 days after the first immunisation (Lösch *et al.*, 1986; Gassmann *et al.*, 1990). Polson *et al.* (1980) showed that it was possible to isolate antibodies from the egg yolks of immunised chickens, using polyethylene glycol (PEG). Antibodies produced in the maternal hen are passed through to their offspring, *i.e.* egg yolk. By using 3.5% PEG, the yolk and lipids in the yolk sack were shown to separate out in a 10 mM phosphate buffer solution at pH 7.5. The polyclonal antibody, immunoglobulin Y (IgY), was then precipitated with a 12% PEG solution. Unlike mammals who produce IgG antibodies, the chicken species (avian class) produce an IgY class of antibody, and the amount of IgY produced in one month is approximately 18 times greater than the amount of IgG produced in rabbits, for example (Gassmann *et al.*, 1990).

4.1.3. Use of ELISA in Testing Antibody Activity

Checkerboard ELISA and inhibition ELISA are both utilised to characterise antibody specificity. The procedure for the use of ELISA is illustrated in Figure 4.2. Polymer plate wells are coated with the protein-bound hapten and washed. The remaining active sites are blocked with protein, and plates washed once more. Antibody or antisera to be tested for activity is then added to each well, and left to bind with the hapten. Plates are washed, and horseradish peroxidase-linked goat anti-mouse IgG added to each well. This second antibody binds to the first antibody, and using an enzyme substrate, a colorimetric reaction is produced. Optical densities of the wells are then determined.



Well coated with protein-bound hapten.

Block active sites on plate with protein.

Antibody / Antisera added.

Horseradish peroxidase-linked goat anti-mouse IgG added to the well.

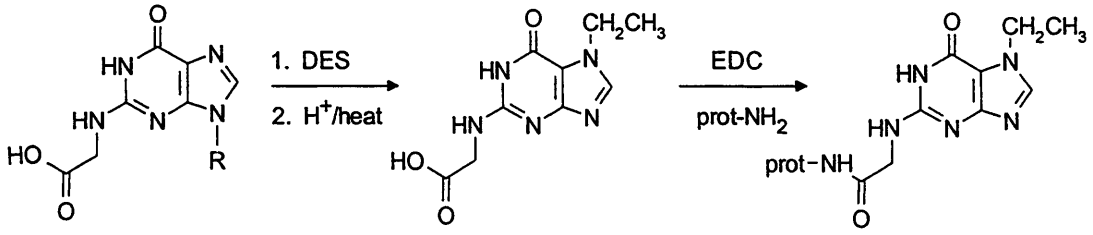
Enzyme substrate added to produce a colorimetric reaction. HCl added to stop reaction.

Figure 4.2. The procedure for an enzyme-linked immunosorbent assay (ELISA).

4.2. Results and Discussion

4.2.1. Monoclonal Antibody Production Against N7-Ethylguanine

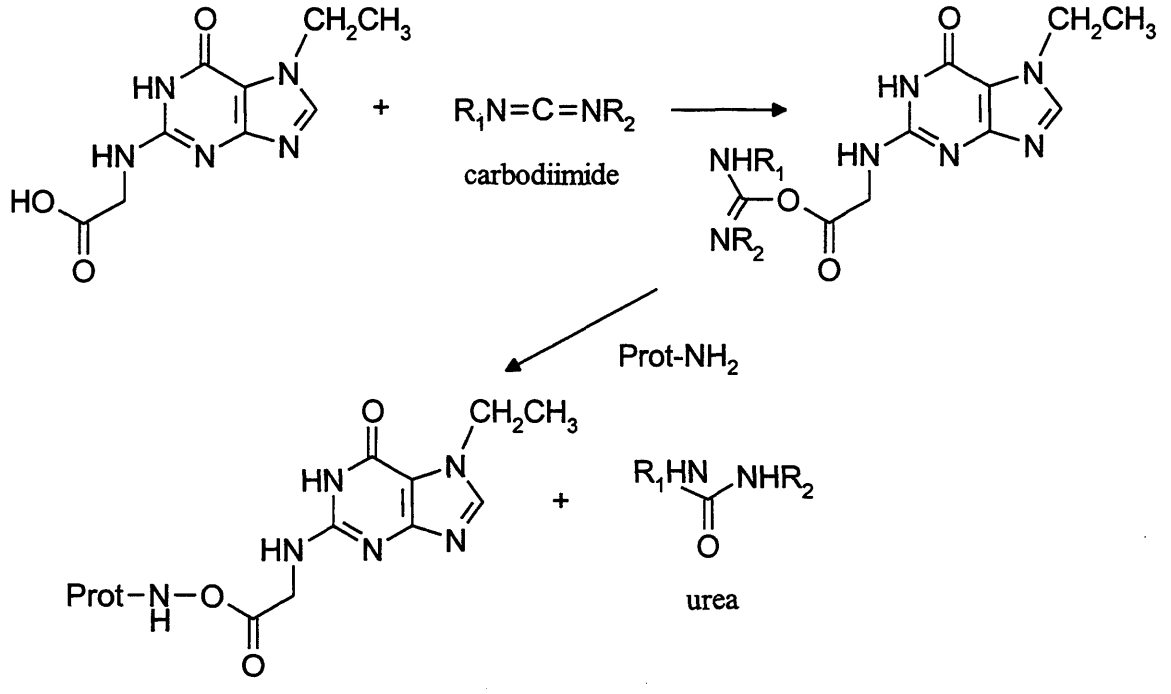
4.2.1.1. Protein Conjugation of Hapten



Scheme 14. Formation of protein coupled 7-EtGua haptens.

Ethylation of N^2-carboxymethylguanosine which was previously available (Durand and Shuker, 1994), afforded N^2-carboxymethyl-N7-ethylguanine which was successfully coupled to methylated bovine serum albumin (mBSA; Scheme 14). The 7-EtGua hapten was also coupled to ovalbumin (Ov). The actual coupling reactions were *via* a carbodiimide coupling procedure using 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC). The carbodiimides are useful tools as they work by promoting condensation between a free amino acid (on the protein) and free carboxyl group (on the hapten) to form a peptide link (Scheme 15).

On coupling with the respective proteins, the hapten was purified on a Sephadex G25 column. Separation on the column was by differences in molecular weight and the coupled protein was the first peak eluted. The second peak was the unbound hapten, which was also collected and kept for further coupling reactions (Figure 4.3.). The degree of modification for both protein conjugates was ascertained by UV spectrometry. Calibration graphs were plotted and modification levels were determined to be 4.3 mol hapten/mol protein and 1.6 mol hapten/mol protein for 7-EtGua-mBSA and 7-EtGua-Ov respectively. The former was used for immunising mice to produce the monoclonal antibody and the latter was used as the test antigen in preliminary enzyme-linked immunosorbent assay (ELISA) studies.



Scheme 15. The carbodiimide coupling reaction between the free amino group on protein and the free carboxyl group on the hapten.

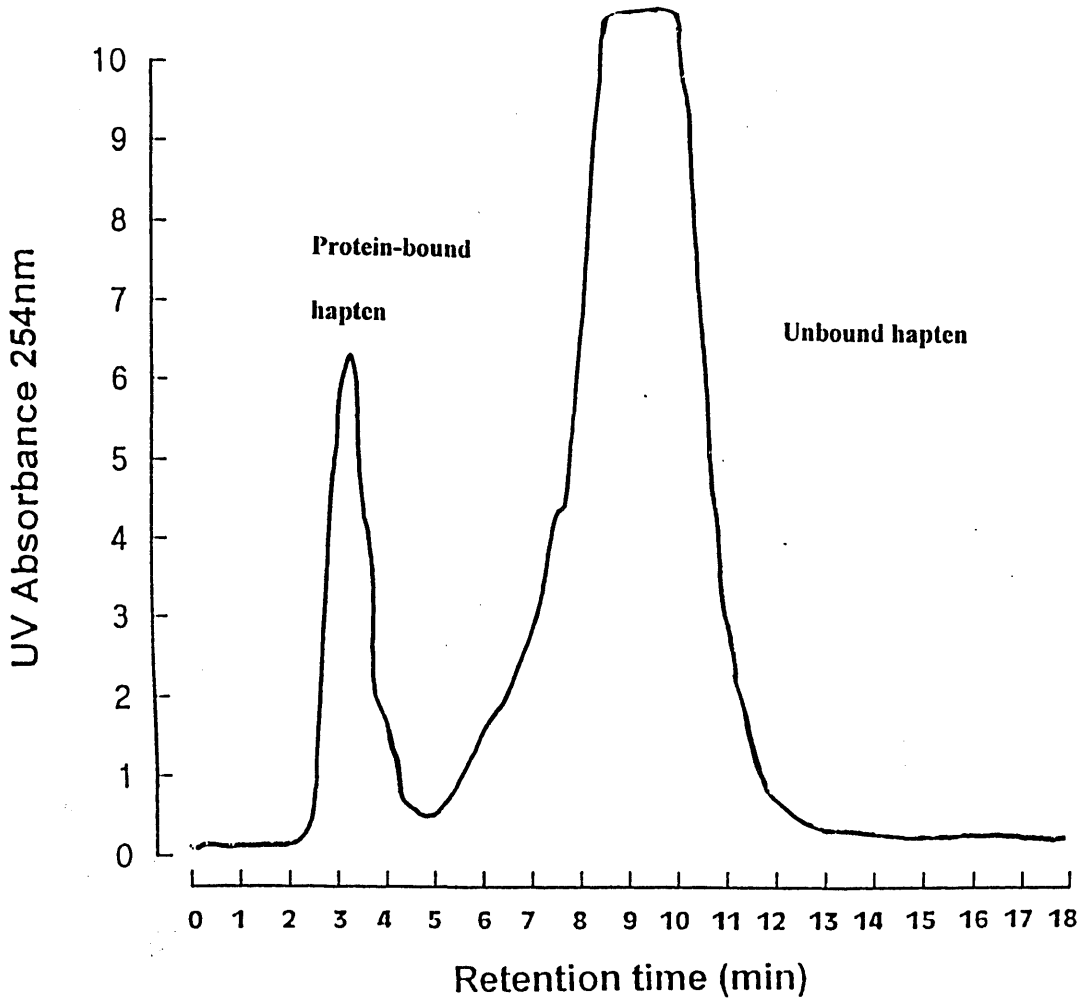


Figure 4.3. A typical UV trace of protein-bound hapten and unbound hapten.

4.2.1.2. *Immunisation Protocols*

Two protocols were attempted to provide the monoclonal antibody against 7-EtGua. Originally, three mice were injected subcutaneously with 100 μg of 7-EtGua-mBSA, but this was found to be too high, as no positive results were observed with ELISA, when cell supernatants were tested. In a second immunisation protocol, two mice were injected with 20 μg of the protein-bound hapten. Booster injections were given throughout the study. Tail bleeds from the mice were provided on a regular basis and tested using a checkerboard ELISA (Figure 4.4.). On some occasions it was found that background levels of optical density were very high, and this was attributed to available binding sites for the second antibody on the ELISA plates. So these were successfully blocked using 1% ovalbumin solution.

	7-EtGua-Ov						Ov					
Coating Antigen	undil.	1:10	1:100	1:500	1:1000	1:10000						
10 μg	0.742	0.719	0.375	0.109	0.050	0.058	0.282	0.141	0.061	0.050	0.059	0.042
1 μg	0.773	0.647	0.357	0.078	0.047	0.046	0.369	0.265	0.076	0.047	0.050	0.051
500 ng	0.643	0.628	0.252	0.078	0.045	0.046	0.386	0.191	0.076	0.046	0.044	0.052
100 ng	0.562	0.400	0.158	0.053	0.044	0.042	0.330	0.201	0.090	0.050	0.052	0.052
50 ng	0.467	0.335	0.101	0.067	0.042	0.043	0.331	0.209	0.059	0.053	0.048	0.040
10 ng	0.352	0.220	0.107	0.049	0.044	0.040	0.320	0.211	0.081	0.053	0.042	0.041
5 ng	0.340	0.230	0.085	0.048	0.039	0.043	0.324	0.215	0.082	0.044	0.048	0.038
1ng	0.373	0.230	0.083	0.046	0.042	0.040	0.331	0.195	0.073	0.043	0.048	0.047

Antibody dilution

Figure 4.4. A typical plate count showing the optical densities from a checkerboard ELISA for tail bleed from mouse (background levels of optical density are approximately 0.05).

Of the two mice, the mouse whose tail bleed showed the higher titre, was chosen for the remaining study. The spleen was taken and cells from it fused with a myeloma cell-line to produce heterokaryons. The fused cells were allowed to grow in culture medium with peritoneal macrophages as the feeder cells. Once a culture appeared to have grown, the

supernatants were tested using ELISA. The amount of coating antigen to be used was determined by looking at the previous checkerboard ELISA studies, and 500 ng/well of 7-EtGua-Ov gave a good overall response. Approximately 70 cell supernatants were tested and two were taken for cloning; cell supernatants 49 and 71 (Figure 4.5.). These two cell supernatants were tested on a checkerboard ELISA to verify that antiserum activity occurred substantially at various serum and coating antigen dilutions. Results were positive and discussions with Dept. of Surgery staff indicated that cell supernatants contained many types of cells, and were not monoclonal as suspected. This was advantageous, as better results would be obtained when the cells had been separated and cloned.

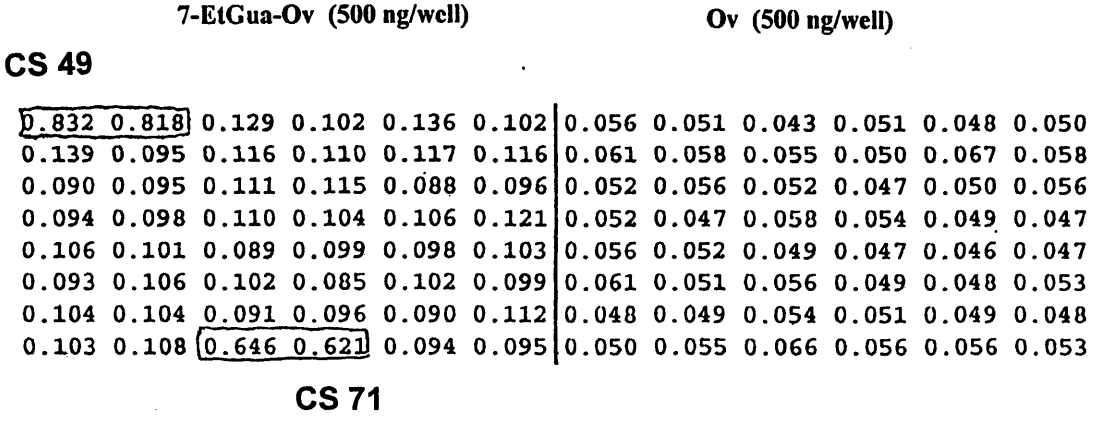


Figure 4.5. Typical plate counts showing the optical densities from an ELISA from checking cell supernatants.

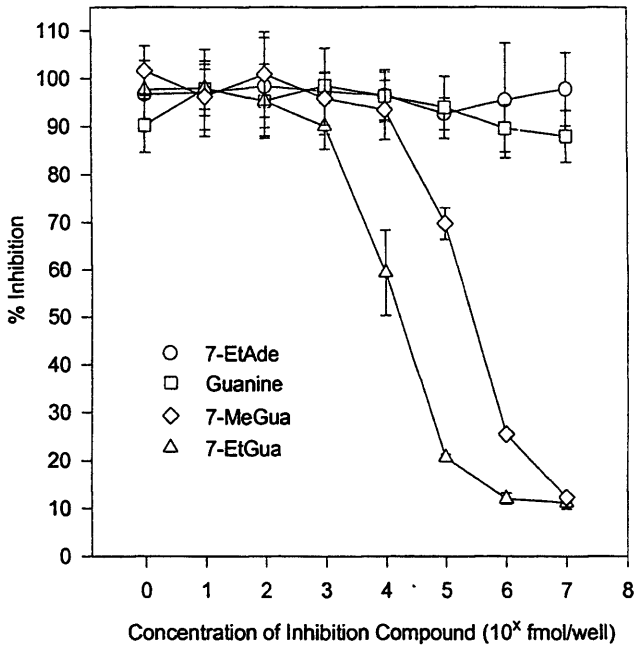
4.2.1.3. Characterisation of Monoclonal Antibody LDS99

The two cells were cloned, and the clones were tested using ELISA for antiserum activity. The clones were also tested for specificity with an inhibition ELISA using various alkylated and normal purine bases, and as can be seen from Figure 4.6., both of the clones tested showed very good antibody activity and also very good specificity for 7-EtGua (50%

inhibition [$I_{50\%}$] at 10 pmol/well). The clones also showed appreciable cross-reactivity towards 7-MeGua, $I_{50\%}$ at 100 pmol/well, but all other alkylated purines tested showed negligible cross-reactivity (Table 4.1.).

Some further ELISA studies showed that the cloned cell supernatant from clone #49 did not give antibody activity. This may have been due to some instability in the cells from emission of excess chromosomes. Therefore, the cloned cell supernatants from clone #71 were used to inject mice intraperitoneally to produce ascites fluid. After about 3 weeks, the ascites fluid, containing the monoclonal IgG antibody, was removed from the hybridomas. The isotype of the monoclonal antibody was determined to be IgG2a, and the antibody activity was finally tested using a checkerboard ELISA.

a



b

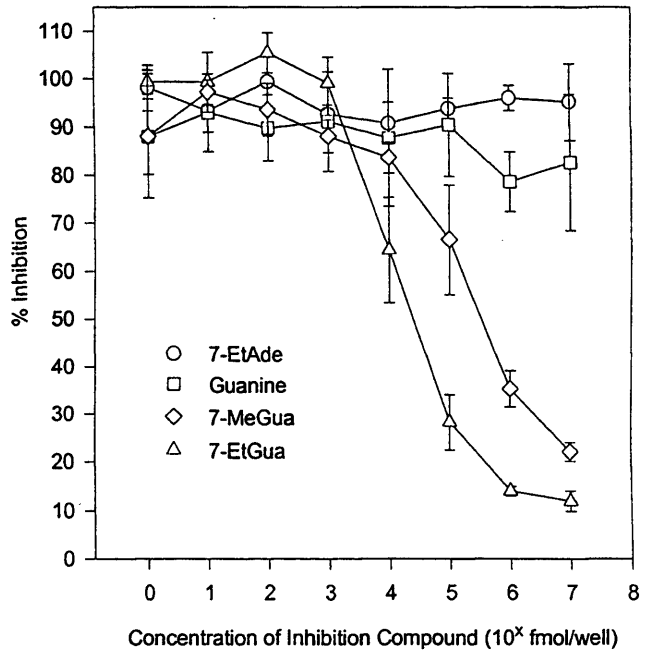


Figure 4.6. Inhibition curves for cloned cell supernatants from **a)** clone #49 and **b)** clone #71.

Table 4.1. Cross-reactivity of 7-EtGua monoclonal antibody with various alkylated and non-alkylated purines in a competitive ELISA.

Purine	$I_{50\%}$ (pmol/well)
7-EtGua	10
7-MeGua	100
7-HOEtGua	5000
7-HOPrGua	>10,000
7,9-diHOPrGua	>10,000
7-CEtGua	>10,000
7-CMeGua	>10,000
7-EtAde	>10,000
Guanine	>10,000
Adenine	>10,000

4.2.2. Preparation and Application of N7-Ethylguanine Immunoaffinity Columns

4.2.2.1. Capacity and Recovery from N7-Ethylguanine Immunoaffinity Columns

Immunoaffinity columns were prepared by covalently linking the IgG in the ascites fluid to protein A-Sepharose CL4B gel. The resultant gel was taken and separated into polystyrene mini columns. A saturation experiment was carried out to determine the capacity of the columns for 7-EtGua. Varying concentrations of 7-EtGua were loaded onto the columns and eluted with 1M acetic acid. Preliminary results indicated the capacity of the columns to be in excess of 1.5 nmol. Therefore, the gel from one of the columns was taken and diluted into five other columns with Sepharose CL4B gel. The saturation experiment was repeated by applying varying concentrations of 7-EtGua to these diluted columns, and then eluted with 1M acetic acid. The eluates were derivatised with Phmal, using 7-MeGua as an internal standard. Analysis was carried out by HPLC fluorescence detection. The capacity of the immunoaffinity gel was finally shown to be ~2.25-2.50 nmol/mL gel (Figure 4.7.).

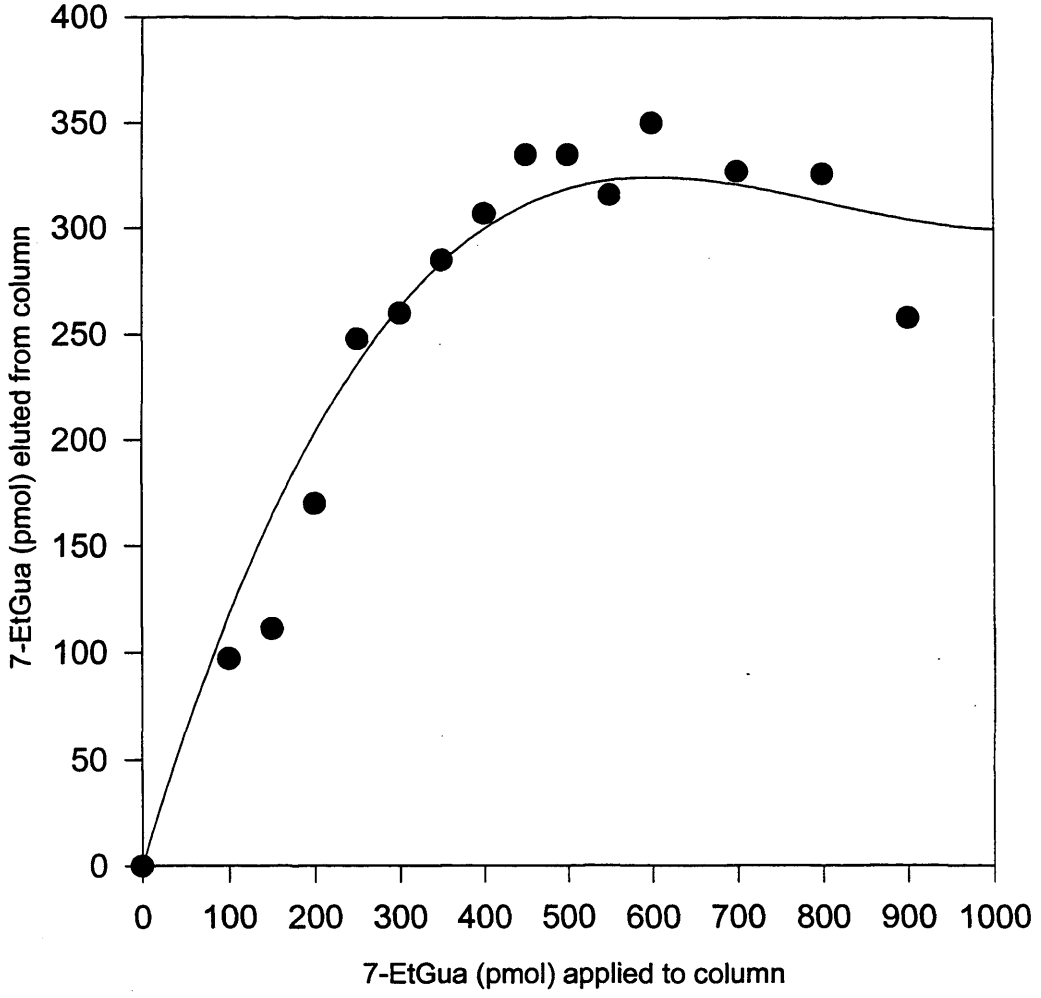


Figure 4.7. Plot showing the capacity of the diluted gels (1:5) in the immunoaffinity columns (1 mL) for 7-EtGua.

The percentage recovery of 7-EtGua from the immunoaffinity columns was determined by passing standards of 7-EtGua through the columns and derivatising with Phmal. Standards not passed through the columns were also derivatised with Phmal, and both sets of samples were analysed using HPLC fluorescence detection with 7-MeGua as an internal standard. From the two sets of data, two calibration lines were plotted on the same graph, and by comparison of the slopes, the recovery of 7-EtGua was determined to be 85% (Figure 4.8.).

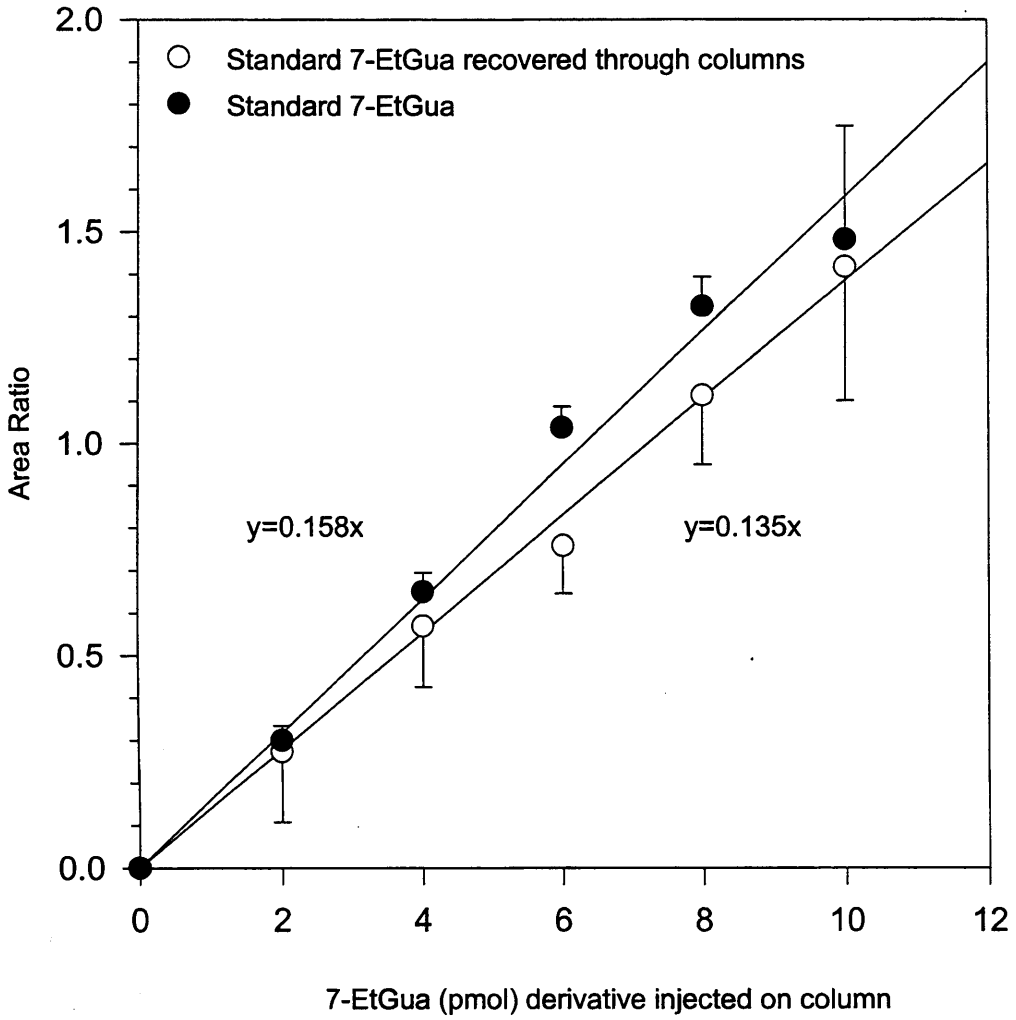


Figure 4.8. A plot showing the percentage recovery of 7-EtGua from the immunoaffinity columns. A comparison of the two slopes indicates an 85% recovery.

The recovery of 7-EtGua from the immunoaffinity columns was not quantitative, and therefore, the percentage loss in subsequent data relating to the calibration plots would have to be accounted for. To check the specificity of the immunoaffinity columns, standards of 7-MeGua (concentrations identical to 7-EtGua) were eluted through the columns as was done for standards of 7-EtGua. No peaks for Phmal-7-MeGua were observed, showing that 7-MeGua was not retained on the columns. Therefore, the columns could be applied to *in*

vitro DNA studies, with the assurance that any native 7-MeGua would be washed through the columns, allowing the use of 7-MeGua as an internal standard for Phmal derivatisations. This result is a little surprising as the antibody did show some cross reactivity towards 7-MeGua in the competitive ELISA studies and therefore, some 7-MeGua would have been expected to be retained. As discussed in Chapter 2, the immunoaffinity columns for 7-MeGua retained 7-EtGua which would not really be expected as the epitope (ethyl group) would be too big for the binding site on the antibody. Whereas in this study, the epitope (methyl group) is small enough to fit in the antibody binding site on the 7-EtGua antibody, and yet no binding of 7-MeGua was observed on the 7-EtGua immunoaffinity columns.

4.2.2.2. Quantitation of N7-Ethylguanine Produced in Calf Thymus DNA on Exposure to Diethylsulphate

Incubation of CT DNA with varying concentrations of DES resulted in the formation of 7-EtGua. Detection and quantitation of the 7-EtGua adduct was carried out by immunoaffinity purification of the DNA hydrolysates, followed by fluorescent postlabelling of the free modified base, using Phmal. Results were also provided, from an earlier study on the same samples, where detection and quantitation of the 7-EtGua adduct had been carried out by HPLC-HPLC-ECD. The two sets of results were compared and are summarised in Table 4.2. It must be noted that both sets of results are not corrected for losses in purification steps. HPLC purification gives an 80% recovery (*data not provided*), whereas recovery from immunoaffinity columns is 85%.

Table 4.2. Amounts of 7-EtGua (pmol/mg) from CT DNA exposed to DES, determined by two analytical methods.

DES (mM)	HPLC-HPLC-ECD	IA-HPLC-fluorescent postlabelling
	pmoles7-EtGua/ mg CT DNA	
1	62 ± 12	118 ± 51
5	425 ± 13	657 ± 32
10	883 ± 30	1412 ± 125
50	5995 ± 145	6669 ± 376

To obtain a better comparison of the results, the linear dose-response curves (Figure 4.9.) indicate that the immunoaffinity-HPLC fluorescent postlabelling assay is approximately 10% more sensitive (slightly higher results) than the HPLC-HPLC-ECD method.

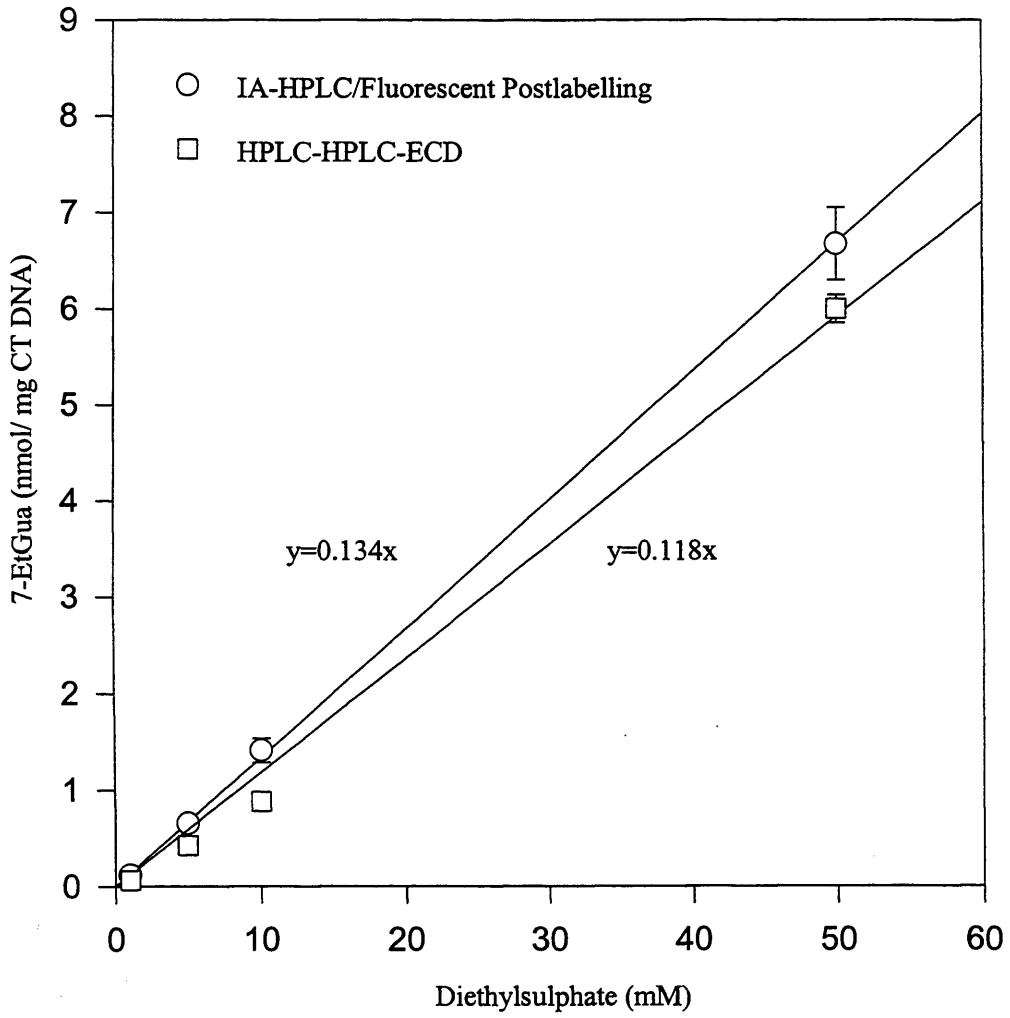


Figure 4.9. Linear dose-response curves for the determination of 7-EtGua adducts, from CT DNA exposed to varying concentrations of DES, by IA-HPLC fluorescent postlabelling and HPLC-HPLC-ECD.

4.2.2.3. *Quantitation of N7-Ethylguanine Produced in Calf Thymus DNA on Exposure to Tobacco Smoke*

As can be see from the chromatograms in Figure 4.10., no Phmal-7-EtGua peaks were observed. This strongly suggests that N7-ethylation of guanine in CT DNA exposed to tobacco smoke did not take place.

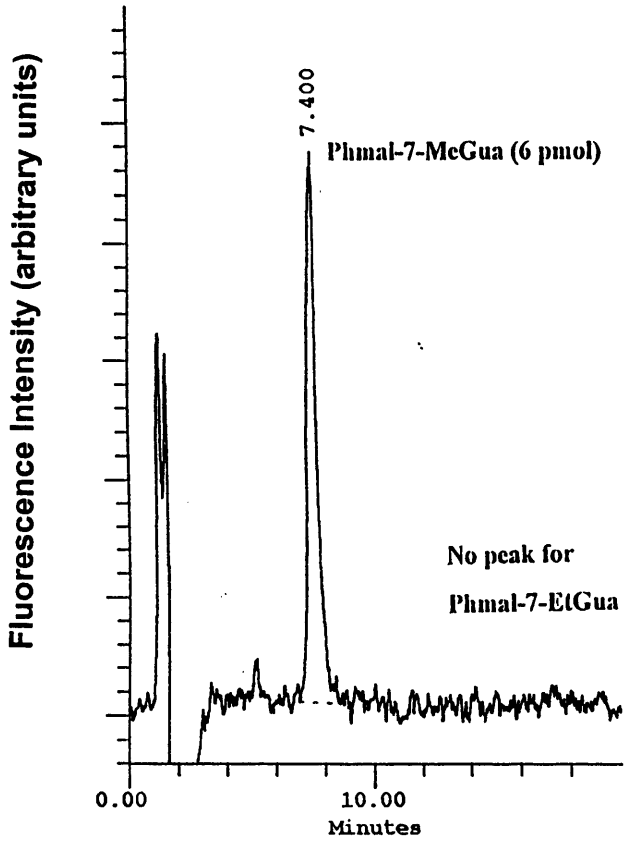
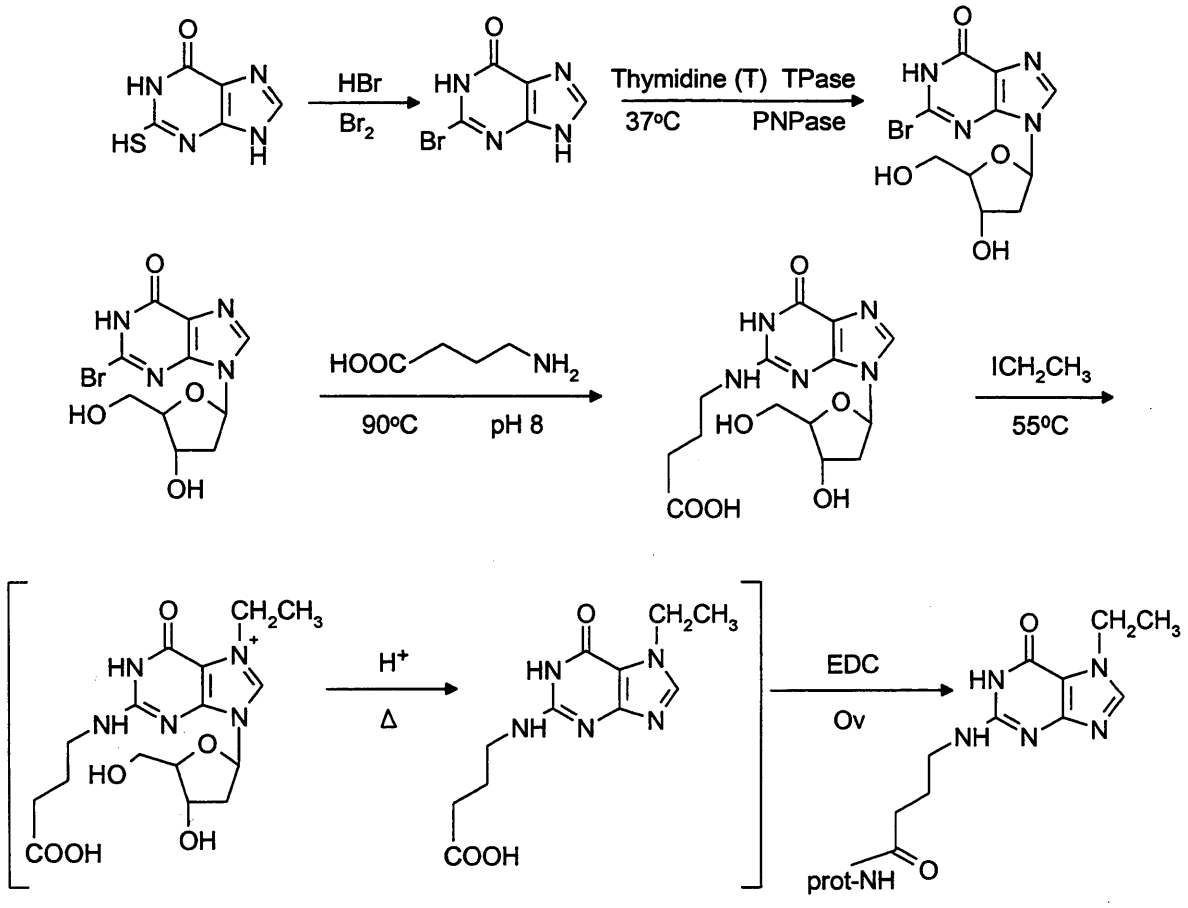


Figure 4.10. A typical chromatogram of bases, taken from CT DNA exposed to tobacco smoke, purified through an immunoaffinity column and derivatised with Phmal.

A previous study showed that N3-ethyladenine was formed in a linear dose-response relationship with the number of cigarettes used (Prevost and Shuker, 1994). Therefore, this was a surprising result, as 7-EtGua adducts were expected to be observed in detectable amounts, since our preliminary studies to detect 7-MeGua in CT DNA exposed to tobacco smoke (Chapter 2), suggested that 7-EtGua may have been formed.

4.2.3. The Improved Synthesis of an N7-Ethylguanine Hapten

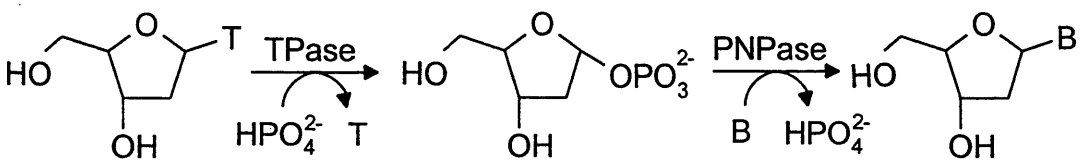
It became apparent at an early stage that the quantity of N^2-carboxymethyl-N7-ethylguanine-ovalbumin, that had been synthesised for use as a coating antigen in the ELISA studies, was insufficient. Due to the difficulty in synthesising this hapten (N^2-carboxymethylguanosine), an alternative synthesis of the hapten was successfully prepared, with the approach based on the displacement of halogens from the 2-position of bases.



Scheme 15. A diagramatic representation of the formation of a protein conjugated 7-EtGua hapten.

Briefly, 2-thioxanthine was converted to 2-bromo-6-hydroxypurine, using the method by Beaman *et al.* (1962), where the mercapto group was replaced by a bromine. The mechanism is thought to occur *via* a sulphonyl bromide intermediate which is displaced by a bromide ion in acid solution. Using a method by Pongracz and Bell (1996), the 2-bromo-6-hydroxypurine

was enzymatically converted to 2-bromo-2'-deoxyinosine. The enzyme, PNPase, catalysed the purine to displace the phosphate group from deoxyribose 1-phosphate, which was formed *in situ* by the phosphorolysis of thymidine (Chapeau and Marnett, 1991; Scheme 16).



Scheme 16. Mechanism of PNPase and TPase catalysed reaction to form 2-bromo-2'-deoxyinosine.

Reaction of 2-bromo-2'-deoxyinosine with 4-aminobutyric acid, afforded N^2-(3-carboxypropyl)-deoxyguanosine, which was then successfully ethylated at N7-position with an excess of iodoethane. N^2-(3-Carboxypropyl)-N7-ethyldeoxyguanosine depurinated *in situ* to give the required hapten. N^2-(3-carboxypropyl)-N7,N9-diethylguanine was also formed to a similar extent. Various conditions were studied at each stage to provide the final scheme (see below). The 7-EtGua hapten was then successfully coupled to ovalbumin and the degree of modification for the protein conjugate was determined to be 0.4 mol hapten/mol protein using UV spectrometry. This seemed a lot lower than previous experiments with coupling proteins, but all subsequent ELISA studies gave very good results.

4.2.3.1. Previous Attempts at Synthesising an N7-Ethylguanine Hapten

The conversion of the 2-thioxanthine to a 2-bromo-6-hydroxypurine compound is a standard synthetic procedure in the literature (Beaman *et al.*,1962), but the use of the subsequent steps to synthesise a hapten were novel in their approach.

Prior to the enzymatic conversion of 2-bromo-6-hydroxypurine to 2-bromo-2'-deoxyinosine and subsequent reaction with 4-aminobutyric acid, attempts were made to convert 2-bromo-6-hydroxypurine to N^2-carbamoylmethylguanine, using glycinamide hydrochloride, at pH 8. This reaction was based on work by Swinton *et al.*, (1983), who showed that glycinamide

reacted with 6-chloro-9-(β-D-2'-deoxyribofuranosyl)purine to give the unusual deoxynucleoside, α-N-(9-β-D-2'-deoxyribofuranosylpurin-6-yl)glycinamide (Figure 4.11.).

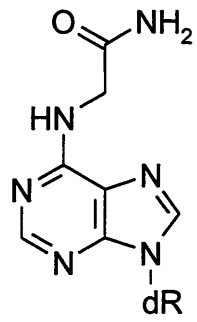


Figure 4.11. α-N-(9-β-D-2'-Deoxyribofuranosylpurin-6-yl)glycinamide.

The reaction was found not to work. This was probably due to the alkaline conditions of the reaction, with the proton at the N9-position being abstracted to give a delocalised negative charge. This delocalised charge, therefore, did not favour the nucleophilic substitution reaction.

To stabilise the N9-position of 2-bromo-6-hydroxypurine, the enzymatic conversion to 2-bromo-2'-deoxyinosine was utilised. Reaction of glycinamide with this compound was still found not to occur and it was then that the reactions with 4-aminobutyric and 5-aminovaleric acid were attempted. It was the former amino acid which finally gave the best results.

4.2.4. Attempt to Produce Polyclonal Antibodies Against N7-Ethylguanine

A decision to immunise chickens to produce polyclonal antibodies against a 7-EtGua hapten was undertaken as the first attempt to produce monoclonal antibodies from mice failed. Two hens (#50 and #51) were successfully immunised with the antigen, N7-ethyl-N^2-carboxymethylguanine, and given booster injections on day 12 and day 20. Eggs were collected prior to immunisation and from the last day of injection. The IgY was extracted from hen #50 (from two eggs laid on days 47 and 49) and hen #51 (from two eggs laid on days 48 and 49), using the procedure outlined in the Promega technical bulletin. The final IgY concentration in PBS was calculated for both hens and was shown to be 0.43 mg/mL and 0.51 mg/mL for hen #50 and hen #51 respectively. The solutions of IgY were then subjected to ELISA studies (Figure 4.12.), but were found to give optical density values too high for any conclusions to be drawn. Attempts at altering 2nd antibody dilutions were made, but ELISA results did not differ. Even the presence of a blocking agent (1% ovalbumin) did not prevent high readings.

	7-EtGua-Ov						Ov					
Coating Antigen	undil.	1:10	1:100	1:500	1:1000	1:10000	undil.	1:10	1:100	1:500	1:1000	1:10000
1 µg	1.034	0.969	1.159	1.122	1.129	0.791	1.079	0.962	0.943	0.940	0.952	0.947
500 ng	0.945	0.897	1.146	1.077	1.171	0.771	1.097	0.899	0.906	0.842	1.016	0.891
100 ng	1.093	0.960	1.092	0.904	1.095	0.997	1.038	0.941	0.913	0.916	0.969	0.948
50 ng	0.936	0.872	0.729	0.968	0.903	0.811	0.951	0.826	0.895	0.896	0.962	0.854
10 ng	1.031	0.995	1.162	1.041	0.961	0.923	0.970	0.786	0.868	0.899	0.806	1.059
5 ng	0.933	0.892	1.208	0.984	0.920	0.933	0.971	0.816	0.805	0.920	0.921	0.964
1ng	0.985	1.003	1.140	1.208	0.965	0.974	1.082	0.910	1.036	0.915	0.959	0.906
	0.039	0.040	0.039	0.039	0.039	0.042	0.038	0.040	0.043	0.043	0.041	0.033

Antibody dilution

Figure 4.12. A typical plate count showing the optical densities from a checkerboard ELISA from checking polyclonal antibody activity from chicken IgY.

4.3. Conclusions

The main aim of this study was achieved with great success, in that a mouse monoclonal antibody with high specificity was produced against 7-EtGua. The antibody was fully characterised, from using checkerboard ELISA to assess antibody activity in tail bleeds and cell supernatants, to inhibition/competitive ELISA for the specificity of the final antibody. The 7-EtGua antibody was coupled to protein A-Sepharose CL4B, and was shown to have a very high capacity of up to 2.5 ng of 7-EtGua/mg of gel and high specificity.

An important part of the production of the monoclonal antibody was the preparation of the 7-EtGua hapten. For the initial immunisation, the hapten was synthesised from previously available N^2-carboxymethylguanosine, but as this was in limited supply, the need for more hapten was required for use as a test coating antigen in ELISA studies. The supply of N^2-carboxymethylguanosine had been synthesised by Durand and Shuker (1994), adapted from a procedure by Robins and Gerster (1965). The yield of product had been very low, and the procedure very laborious. Therefore, an alternative approach was successfully established for the synthesis of an N7-alkylated guanine hapten, based on the displacement of a halogen from the 2-position of guanine by 4-aminobutyric acid. Since establishing this novel synthetic route for forming 7-AlkGua haptens, another procedure by Papoulis *et al.* (1995) has come to light. Their work showed the synthesis of N^2-(1-carboxyethyl)-9-methylguanine from methylglyoxal (pyruvaldehyde) and 9-methylguanine. This procedure could have possibly been adapted to react methylglyoxal with 7-AlkGua (or derivatives), to give N^2-(1-carboxyethyl)-7-alkylguanine. Therefore, if any haptens need to be synthesised in the future, maybe this alternate route could be investigated.

The production of polyclonal antibodies from chickens immunised with 7-EtGua-mBSA is a part of work which never came to fruition due the advent of the mouse monoclonal antibody. Successful extraction of IgY was obtained, but characterisation of the specificity of the antibodies was not achieved. This was due to irreproducible results from the ELISA studies. High optical density values were observed for background readings. To overcome the problem, a different coating antigen should have been attempted, to determine whether the problem lay therein. Due to the lack of time available to synthesise a new protein-bound

hapten, this was not done. If the polyclonal antibodies did indeed have specificity for 7-EtGua, then it may have been possible to combine them with the monoclonal antibody to form an ELISA assay for the detection and quantification of 7-EtGua adducts. The monoclonal antibody would be used for purification and the polyclonal antibody in the ELISA. The advantage of producing antibodies from chickens (though they are polyclonal), is that they are obtained in a very short space of time from first immunisation of the animal and collecting eggs, in contrast to bleeding animals, is non-invasive.

The successful preparation of immunoaffinity columns allowed the selective isolation of the 7-EtGua from a solution of modified and unmodified bases, obtained from the thermal hydrolysis of CT DNA exposed to DES. This assay was essentially carried out to show the effectiveness of the immunoaffinity columns, and was able to be compared to a HPLC-HPLC-ECD assay previously employed by Singh and co-workers (1997) to quantify the 7-EtGua adducts formed. Comparison of the two assays showed both assays were comparable in their levels of detection, but the HPLC-HPLC-ECD assay was already near to its limit of detection, whereas the fluorescent postlabelling assay was not. The HPLC-HPLC-ECD assay gave lower adducts levels than the postlabelling assay. The discrepancies between the two analytical methods may be in part attributed to losses of adduct in the HPLC purification step of the former. This shows the advantage of using an immunoaffinity purification method. Along with the high specificity for the adduct of interest, near maximum recovery is also obtained, virtually eliminating any losses in the purification step.

The *in vitro* study of tobacco smoke exposure to CT DNA did not give the expected results. Many previous studies have shown the formation of 7-EtGua in smokers' lungs (Blömeke *et al.*, 1996; Kato *et al.*, 1993), and the formation of N3-ethyladenine adducts in smokers, detected in urinary excretion (Prevost *et al.*, 1990; 1993; Prevost and Shuker, 1996). DNA exposed to tobacco smoke *in vitro* has shown dose-dependent levels of N3-ethyladenine (Prevost and Shuker, 1996), therefore it is somewhat surprising that 7-EtGua was not observed in this *in vitro* study. It has been shown that tobacco burned at different temperatures produces different carcinogens (Hoffmann and Hecht, 1990). Sidestream smoke (environmental tobacco smoke) is produced at a lower temperature, whereas mainstream smoke (inhaled by smoker) is produced at a higher temperature. Sidestream smoke has shown

to contain higher levels of identified carcinogens. Therefore, in this *in vitro* study the burning rate of the cigarettes should be monitored more closely, because at present the actual amount of exposure of tobacco smoke to CT DNA is highly variable. The only constant factors are the number of cigarettes used is known, the temperature of the DNA solutions are constant and amount of CT DNA used is known. Modification of the apparatus would be required to provide a constant amount of tobacco smoke to the CT DNA in solution, and raising the temperature of the DNA solution to physiological levels might be an important consideration.

Though this final *in vitro* study with tobacco smoke did not detect significant levels of 7-EtGua adducts, the work has shown that the fluorescent postlabelling assay has wide applicability. Once again, immunoaffinity purification affords selectivity and sensitivity when incorporated into the assay. The production of a monoclonal antibody is never an easy achievement, and this study has shown the time and patience required for a successful product.

CHAPTER 5

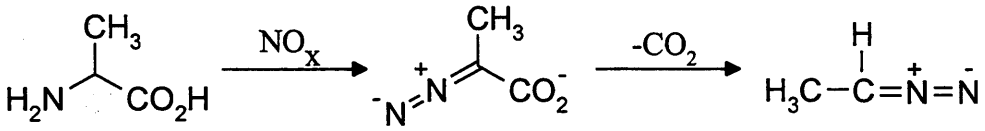
Synthesis and Reactions of 2-Diazopropanoic Acid (Diazoalanine)

Chapter 5. Synthesis and Reactions of 2-Diazopropanoic Acid (Diazoalanine)

5.1. Introduction

This part of the study will examine the effect of diazotisation on α-amino acids, in particular, L-alanine which is a common amino acid. The formation, stability and reactions of the diazotised alanine will be compared to the physical characteristics of diazotised glycine; diazoacetic acid.

From experimental studies it has been suggested that tobacco smoke contains a direct-acting ethylating agent (Prevost and Shuker, 1996). With analogy to diazotised glycine acting as a methylating agent on exposure to DNA (Shuker and Margison, 1997), this study is based on the hypothesis that alanine in burning tobacco undergoes nitrosation and decarboxylation to form an ethylating agent; diazoethane (Scheme 17). The fluorescent postlabelling assay, incorporating immuno-purification will be employed to detect possible 7-EtGua adducts formed on the exposure of DNA to diazopropanoic acid *in vitro*.



Alanine 2-Diazopropanoate Diazoethane

Scheme17. The proposed conversion of alanine, in tobacco on burning, to a diazoethane-generating agent.

5.1.1. Diazotisation of Amino Acids

Many carcinogenic alkylating agents are believed to owe their activity to conversion to highly reactive diazonium ions (Ford and Scribner, 1983). Among these are the N-alkyl-N-

nitrosoureas, N-alkyl-N-nitrosoguanidines and N,N-dialkylnitrosamines. Studies carried out on the nitrosation of peptides bonds by nitrogen oxides (Garcia *et al.*, 1984), showed the products formed, nitrosopeptides, reacted with amines and α-amino esters to afford diazo-compounds. The formation and decomposition of the diazoacetate ion has been studied in great detail (King and Bolinger, 1936; Kreevoy and Konasewich, 1970), and has been shown to be very unstable, readily decomposing to the alcohol and affording nitrogen. Diazoacetate has shown to form diazomethane and react with DNA *in vitro* to give methylated bases, of which O^6-methyl-2'-deoxyguanosine has been quantified (Shuker and Margison, 1997). It would be interesting to see if higher analogues yielded corresponding alkyl adducts. Reactions *in vitro* of N-nitroso(2-oxopropyl)propylamine showed its conversion to a methylating agent (diazomethane) using a microsomal, cytochrome P450-dependant mixed function oxidase from rat liver (Leung and Archer, 1985). An internal rearrangement occurs with loss of propionaldehyde, to afford diazomethane.

5.1.2. Alanine as a Potential Ethylating Agent in Tobacco Smoke

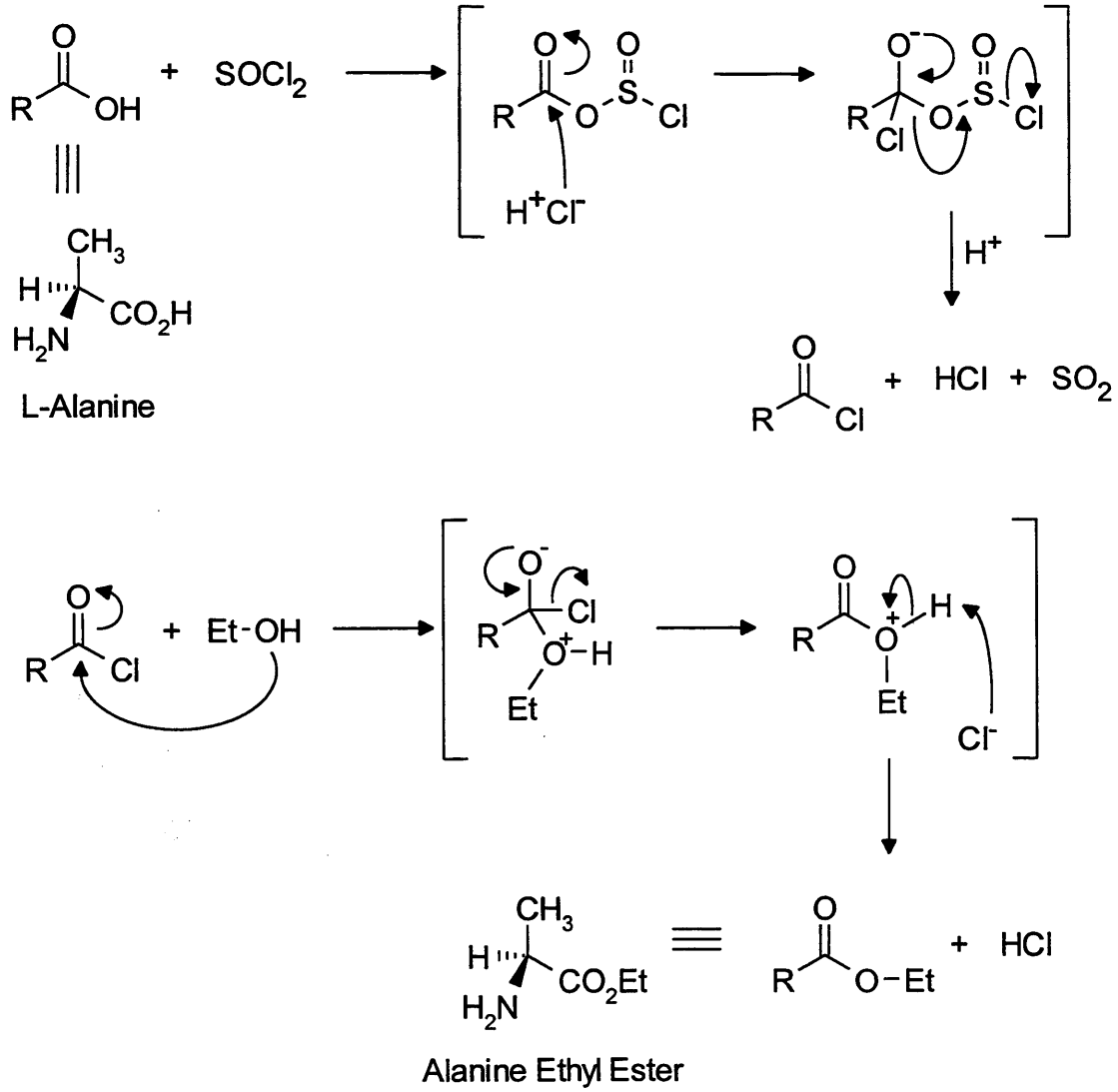
Studies on the urinary excretion of N3-alkyladenine adducts from smokers and non-smokers on controlled diets (Kopplin *et al.*, 1995; Prevost and Shuker, 1996; Stillwell *et al.*, 1991), have shown the presence of N3-methyladenine and N3-ethyladenine using RIA and GC-MS (of silanised derivatives). Prevost and Shuker's (1996) study on volunteers consuming a standardised diet low in preformed N3-methyladenine, showed a smoking-related increase in N3-methyladenine excretion. There are a number of compounds in tobacco smoke which are methylating agents (methyl chloride, dimethylnitrosamine), so the results were as expected. Higher alkyl analogues of N3-methyladenine have lower background levels in urine, and are only slightly affected by diet, as in the case of N3-ethyladenine (Prevost *et al.*, 1990). Therefore, when N3-ethyladenine and N3-(2-hydroxyethyl)guanine adducts were analysed from smokers, levels of N3-ethyladenine appeared to increase with number of cigarettes smoked and levels of N3-(2-hydroxyethyl)guanine seemed unchanged. Even though 2-hydroxyethyl protein and DNA adducts have been observed from exposures of ethylene oxide in tobacco smoke and pure ethylene oxide (Tavares *et al.*, 1994; Segerback, 1990), the lack of N3-(2-hydroxy-ethyl)adenine adducts observed was attributed to masking by an endogenous component (Prevost *et al.*, 1993). The observed increase in levels of N3-ethyladenine was definitely attributed to tobacco smoking, because on cessation of

smoking, the levels decreased. The possibility of preformed N3-ethyladenine in tobacco smoke was discounted as levels observed in cigarette condensates were not contributory to the excreted levels detected. Ethylating agents do exist in tobacco smoke (N-nitrosodiethylamine, 3 ng/cigarette; N-nitrosomethylethylamine, 3 ng/cigarette) but at too low a level to account for the excreted adducts. Prevost and Shuker (1996) also showed that DNA exposed to tobacco smoke *in vitro* contained dose-dependant levels of N3-ethyladenine and reported that levels of O^4-ethylthymine in liver DNA were shown to be higher in smokers compared to non-smokers from a study done by Kang and co-workers (1995). Furthermore, Kato and co-workers (1993) showed that lung DNA from smokers contains N7-ethyl and N7-methylguanine adducts, detected by P^{32}-postlabelling. As discussed earlier, glycine can be diazotised to form diazoacetic acid which has shown to methylate bases in DNA. Therefore by analogy, it can be supposed that alanine may be diazotised to the corresponding 2-diazopropanoic acid to give ethylation on exposure to DNA. Massey and Few (1983) presented a report on the mutagenicity effects of nitrogenous compounds in tobacco smoke. The report showed that by increasing levels of amino acids in cigarettes, the cigarettes smoke condensates were shown to be increasingly more mutagenic. Despite being unable to observe 7-EtGua adducts, in Chapter 4, on CT DNA exposed to tobacco smoke *in vitro*, it was decided to pursue the hypothesis that diazotisation of alanine could result in ethylation.

5.2. Results and Discussion

5.2.1. Attempted Synthesis of Ethyl-2-diazopropanoate

L-Alanine was converted to the alanine ethyl ester, *via* the acid chloride, using a method described by Blau and King (1993), as depicted in the scheme 18.



Scheme 18. Formation of L-alanine ethyl ester from the reaction of L-alanine with thionyl chloride and ethanol.

The product, shiny white crystals, was produced in a high yield; 97%. The structure of the compound was confirmed by MS and ^{1}H NMR. Interestingly, by mass spectrometry, alanine ethyl ester was detected as the hydrochloride salt.

The next step was to convert the alanine ethyl ester to the ethyl-2-diazopropanoate *via* the diazotisation of the primary amine group (Figure 5.1.). Three approaches were attempted and these are discussed below:

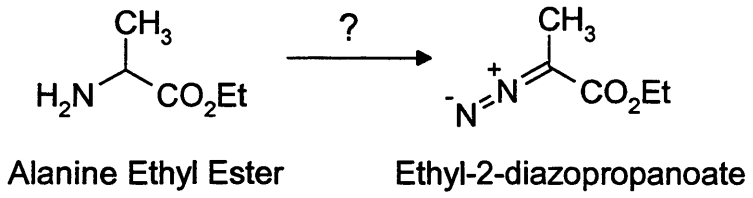
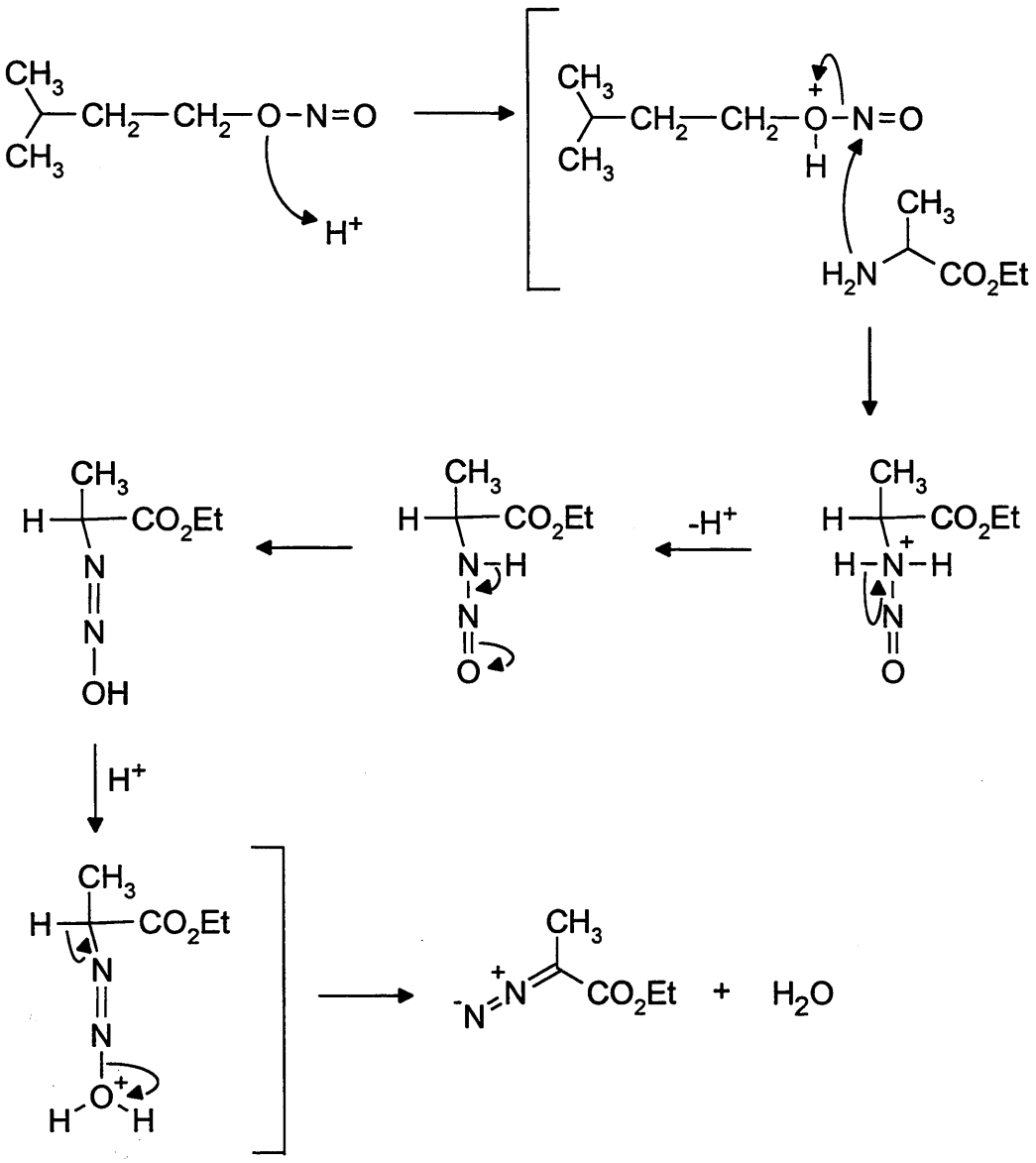


Figure 5.1. Diazotisation of alanine ethyl ester.

5.2.1.1. Diazotisation by Isopentyl Nitrite

Diazotisation of alanine ethyl ester was first attempted with isopentyl nitrite (isoamyl-nitrite) as outlined in a procedure by Takamura *et al.* (1975). Takamura *et al.* showed that it was possible to prepare the α-substituted α-diazo-esters of amino acid esters, by refluxing the amino acid ester in chloroform with isopentyl nitrite and a little acid catalyst. Despite the fact that the diazotisation was shown to occur in an acid solution, the actual species attacked is the small amount of free amine present and not the salt of the amine (Challis and Ridd, 1962), as depicted in the scheme 19.



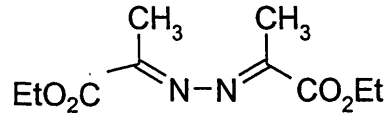
Scheme 19. Proposed scheme for the diazotisation of alanine ethyl ester with isopentyl nitrite.

Acetic acid was added to the alanine ethyl ester in chloroform, which was followed by the addition of a slight excess of isopentyl nitrite. Immediately, the colourless solution became yellow. The reaction was followed by TLC and spots visualised with ninhydrin. The completion of the reaction was indicated by the disappearance of the spot for the starting material, alanine ethyl ester. Subsequent washing with acid (to remove any remaining alanine ethyl ester), base (to remove acid) and water, extraction into chloroform and drying over

anhydrous sodium sulphate was carried out. Analysis of the chloroform solution using mass spectrometry (FAB; glycerol matrix) did not give a mass ion profile for the parent compound (MW 128) or daughter products. There was evidence of starting material, with a mass ion profile at 118 *m/z* units (alanine ethyl ester, MW 117). As the washings of the product were being carried out, the intense yellow colour of the compound appeared to diminish, until the final volume of liquid was almost colourless. It seems that the product may have decomposed, and on evaporation of the chloroform under a stream of nitrogen no product was present. Takamura *et al.* showed the successful diazotisation of many amino acid esters to give the α-substituted α-diazo esters, but interestingly they did not show a reaction of any alanine ethyl esters (except for an alanine benzyl ester).

5.2.1.2. Diazotisation by Sodium Nitrite

The second attempt at the diazotisation of alanine ethyl ester was based on previous work by Challis and Latif (1990) and involved the same mechanistic approach as for isopentyl nitrite, but this time using sodium nitrite as the diazotising reagent. Challis and Latif had shown that glycylglycine ethyl ester hydrochloride could be diazotised using sodium nitrite in a study highlighting the synthesis of some new diazopeptides. Ethyl glycine ester hydrochloride has also shown to be diazotised using sodium nitrite to ethyl diazoacetate (Searle, 1963). Therefore, it was supposed that the more recent procedure by Challis and Latif could be applied to the alanine ethyl ester hydrochloride. An excess of aqueous sodium nitrite was reacted with alanine ethyl ester in 2 M sodium acetate. The solution was then left to stir in dichloromethane with some glacial acetic acid and the reaction was followed by TLC. After a couple of hours of stirring at 5°C, the colourless solution became yellow and the starting material spot no longer appeared on a TLC plate. The dichloromethane layer, containing the yellow product was taken and washed with dilute base, and dried over anhydrous sodium sulphate. The majority of the dichloromethane solvent was evaporated off under a stream of nitrogen, and the remaining solution was analysed by mass spectrometry. Analysis of the dichloromethane solution did not give a mass ion profile for the parent compound (MW 128), nor any profiles corresponding to any daughter compounds. Interestingly though, a predominant mass ion profile was observed at 229 *m/z* units, which could possibly correspond to a dimeric structure;



Dimeric structure with a MW 228.

Figure 5.2. Possible formation of a dimeric structure.

Evaporation of the remaining dichloromethane, once again, afforded no product. The yellow colour had diminished until there was nothing left in the vial. It can be assumed that whatever had formed, was not very stable. Diazopeptides are relatively labile in acidic media (Challis *et al.*, 1990), readily losing molecular nitrogen to give an alcohol. Therefore, a procedure would be required for diazotisation of the alanine ethyl ester to be carried out in neutral conditions.

5.1.2.3. Diazotisation by Dinitrogen Tetroxide (N_2O_4)

Based on work carried out by Challis and Latif (1990), the third attempt at the diazotisation of alanine ethyl ester involved dissolving the alanine ethyl ester in dichloromethane and reacting it with N_2O_4 at -40°C. An excess of triethylamine was added to keep the conditions non-acidic and anhydrous sodium sulphate was added to absorb the water produced by the reaction. The organic phase was washed with dilute base and water, and allowed to dry over anhydrous sodium sulphate. The dichloromethane layer was evaporated off and the yellow oily product purified on a flash column. A pale yellow oil was afforded on evaporation of the eluting solvent. Analysis by mass spectrometry did not show any mass ion profiles corresponding to the product compound and by comparing the ^1H NMR spectrum for alanine ethyl ester with that for ethyl-2-diazopropanoate (Figure 5.3.), it can be seen that they are almost identical, except for the quadruplet peak corresponding to the single proton. In the spectrum of alanine ethyl ester, the quadruplet peak is at 4.1 ppm and in the spectrum for the ethyl-2-diazopropanoate, the quadruplet peak is at 5.4 ppm.

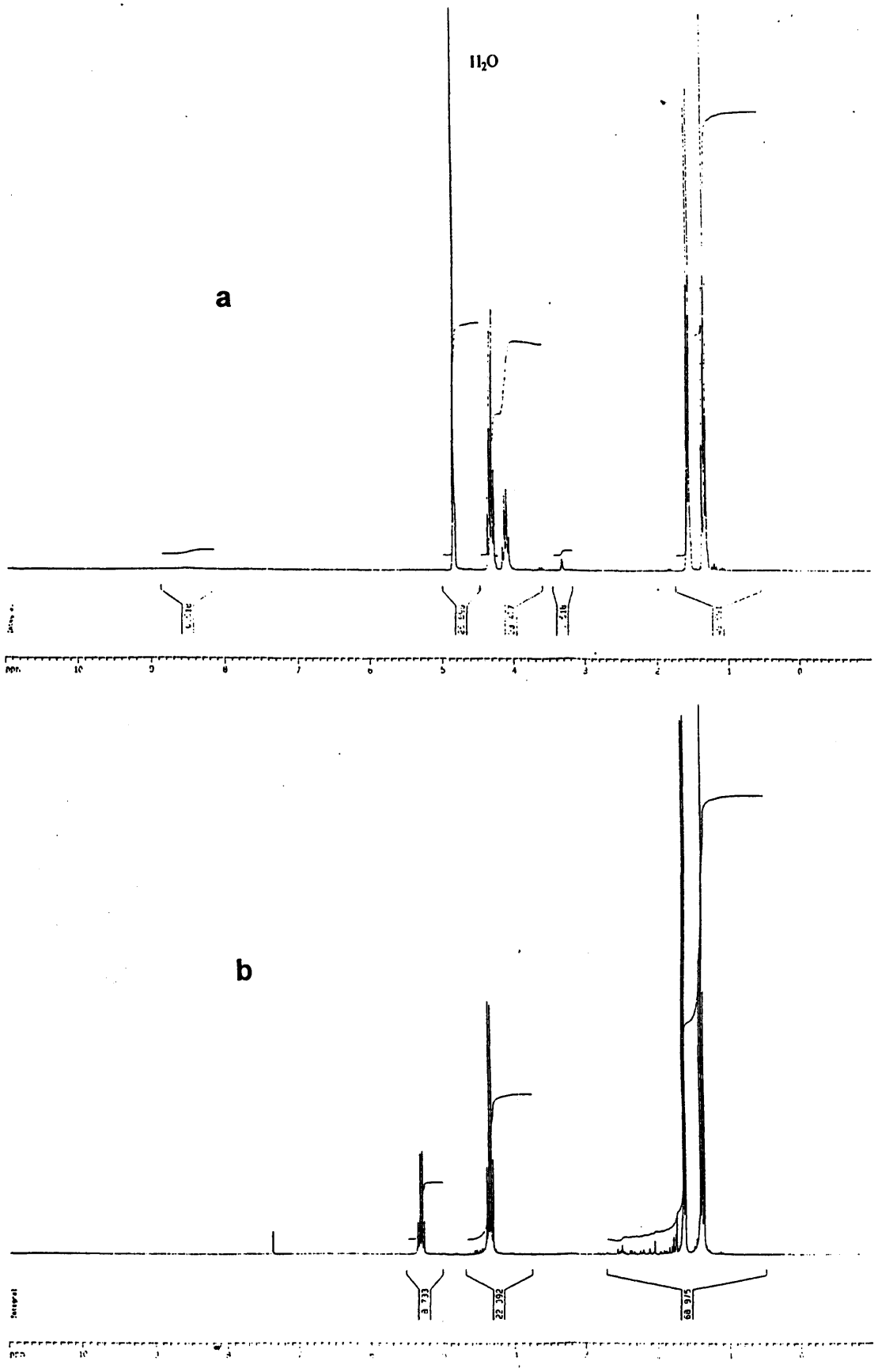


Figure 5.3. ^{1}H NMR spectra for a) alanine ethyl ester hydrochloride and b) N_2O_4 reaction product.

This shift downfield indicates that the proton has become electronically deshielded (*i.e.* the electron cloud surrounding the proton has been partially withdrawn by a electron withdrawing moiety), which would correlate with the introduction of a diazo group. But if a diazo group had been introduced, the proton singlet would no longer be there and the doublet corresponding to the methyl group would have become a singlet at ~1.9 ppm (Garcia *et al.*, 1984). It is obvious though, that a transformation has occurred to cause a shift downfield for the single proton, and that the proton is still present. Alanine ethyl ester hydrochloride is acidic in nature, but when reacted with N_2O_4 and subsequently washed with dilute base and water, remaining alanine ethyl ester is in a neutral from, which may account for the downfield shift. This was tested by taking a solution of alanine ethyl ester hydrochloride in dichloromethane and washing it with dilute base and water, and then analysing the compound using ^1H NMR. No peaks were observed, suggesting that any excess alanine ethyl ester would have been washed away, and that the spectrum for the assumed ethyl-2-diazopropanoate is not alanine ethyl ester in a neutral form, but in fact a product compound.

By taking into consideration the various components added into the reaction mixture; a diazotising reagent, a sodium salt, the proposed product compound is probably the sodium salt of the diazoate ion (Figure 5.4.). Nitrosation is possibly observed, without the eventual diazotisation.

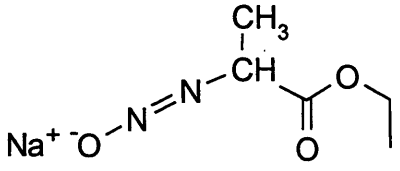


Figure 5.4. Ethyl-2-(sodium diazoate)propanoate

Alkanediazoates are reported to be reactive intermediates in the carcinogenic and chemotherapeutic activity of a wide range of N-nitroso compounds, and recent studies on the decomposition of alkanediazoates do not use the above methodology to synthesise the alkanediazoates (Ho and Fishbein, 1994; Finneman and Fishbein, 1996). Instead the authors generate the alkanediazoates from the potassium ethoxide stimulated cleavage of the parent

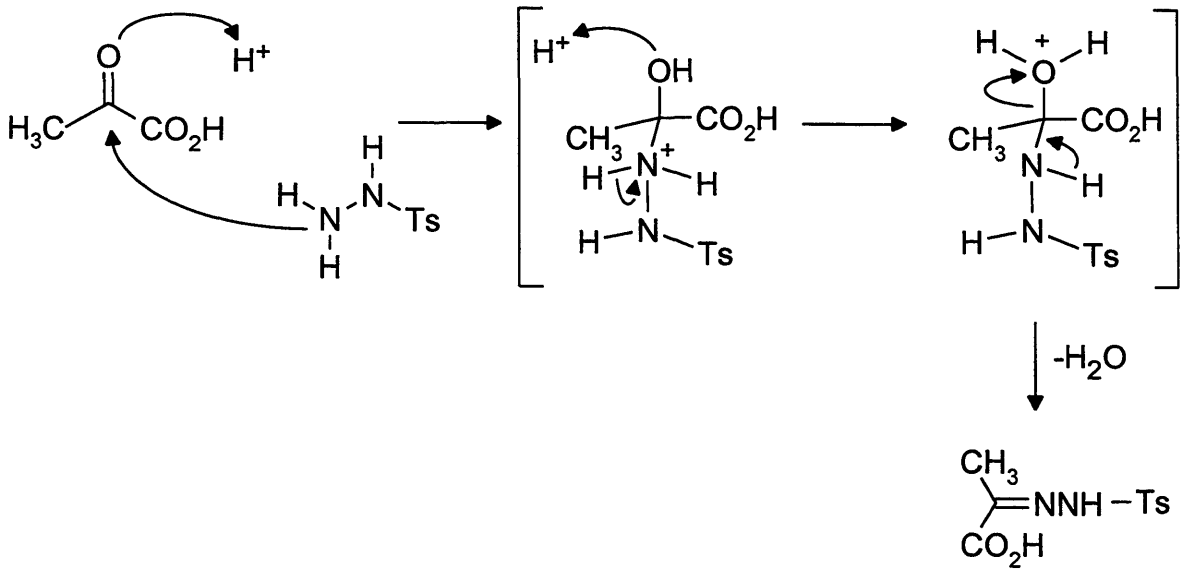
nitrosourethanes or from the reaction of the appropriate hydrazine with butyl nitrite and sodium ethoxide. Further studies on the characterisation of the proposed diazoate salt were not carried out and validated by carrying out decomposition studies due to lack of time.

5.2.2. Synthesis of Potassium 2-Diazopropanoate from Pyruvic Acid

Attempts to form ethyl-2-diazopropanoate by nitrosation under various conditions had proven to be very difficult, possibly due to the instability of the compound. Therefore the following approach was adopted which used pyruvic acid as the starting material. Pyruvic acid is the natural precursor to alanine, formed from a bacterial enzymatic (*Bacillus subtilis*) reaction of pyruvic acid and ammonia.

5.2.2.1. Pyruvic Acid p-Toluenesulphonyl Hydrazone

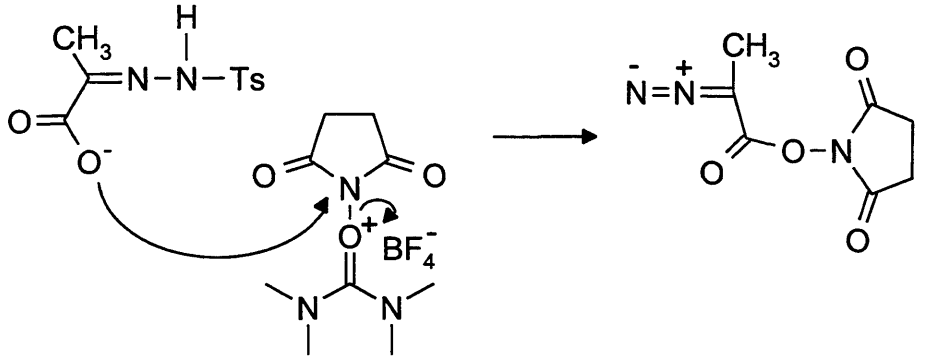
Recent work by Ouihia *et al.*, (1993) has shown the synthesis of a new diazoacylating agent, succinimidyl diazoacetate, from the reaction of N-hydroxysuccinimide with glyoxylic acid p-toluenesulphonyl hydrazone (tosylhydrazone). Therefore, it was believed that the same methodology could be applied to form a pyruvic acid tosylhydrazone. Adapting a procedure by Blankley *et al.*, (1973), who showed the formation of glyoxylic acid tosylhydrazone from glyoxylic acid and p-toluenesulphonyl hydrazine, and combining it with work by Bertz and Dabbagh (1983), who described preparations of aldehyde and ketone tosylhydrazones, pyruvic acid was reacted with the tosyl hydrazine (Scheme 20).



Scheme 20. The formation of the tosyl hydrazone from pyruvic acid and tosyl hydrazine.

5.2.2.2. Formation of O-(N-Succinimidyl)-2-diazopropanoate

The pyruvic acid tosylhydrazone was reacted with O-(N-succinimidyl)-N,N,N,N-tetramethyluronium tetrafluoroborate (TSTU), to afford a yellow crystalline solid of O-(N-succinimidyl)-2-diazopropanoate (Scheme 21). The product was characterised by ^{1}H NMR, MS (FAB) and IR. A characteristic infrared absorption band for N=N at 2100 cm^{-1} was present for the compound, along with the characteristic C=O absorption band at 1750 cm^{-1}.

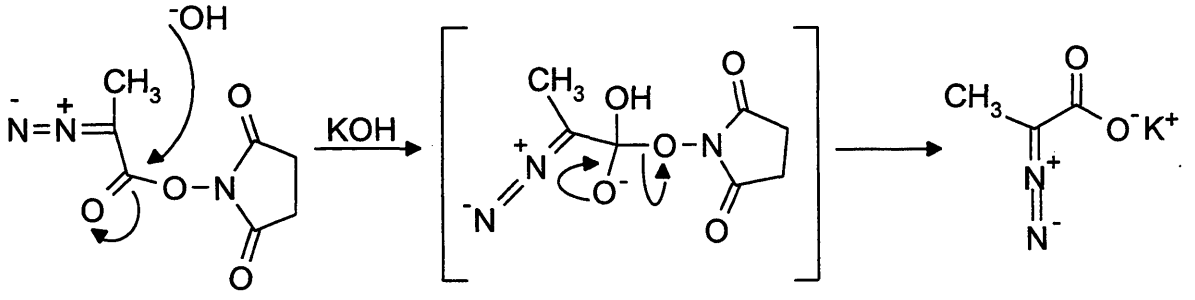


Scheme 21. Reaction of tosylhydrazone with TSTU to give O-(N-succinimidyl)-2-diazopropanoate.

The reaction proceeded with the electropositive nitrogen atom undergoing a nucleophilic substitution reaction from the carboxylate ion on the deprotonated pyruvic acid group. The reaction occurred *via* the loss of the p-toluenesulphonyl (tosylate) group which is a good leaving group.

5.2.2.3. Formation of Potassium 2-Diazopropanoate

A method based on studies by King and Bolinger (1936) was adapted to synthesise potassium 2-diazopropanoate (Scheme 22). It was formed from the saponification of O-(N-succinimidyl)-2-diazopropanoate with 1M KOH, to give potassium 2-diazopropanoate, and was followed by TLC.



Scheme 22. Formation of potassium 2-diazopropanoate.

The nucleophilic addition of the hydroxide ion to the ester carbonyl gives the tetrahedral oxo-succinimidyl intermediate. Elimination of the oxo-succinimidyl group generates the carboxylic acid, which leads to the abstraction of the acidic proton by the oxo-succinimidyl group to form a carboxylate ion. Presence of the potassium ion enables formation of the potassium salt. Attempts to isolate and characterise the potassium salt were made and are discussed below.

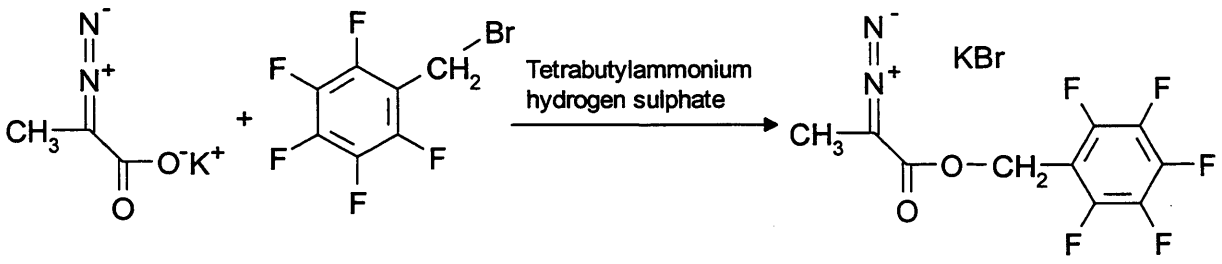
5.2.2.4. Attempts at Isolation and Characterisation of 2-Diazopropanoate Salt

Saponification of the O-(N-succinimidyl)-2-diazopropanoate with KOH gave the potassium salt, which remained at the origin when spotted on a TLC plate. Other than this observation,

it could not be confirmed that it was indeed the potassium salt of 2-diazopropanoate that had been formed. So therefore, a chemical derivatisation was attempted for characterisation.

5.2.2.4.1. Formation of a Pentafluorobenzyl Salt

An attempt to form pentafluorobenzyl-2-diazopropanoate was carried out using the potassium salt, pentafluorobenzyl bromide and a phase transfer catalyst, tetrabutylammonium hydrogen sulphate (Scheme 23). The procedure was adapted from Shuker *et al.*, (1984), where extractive alkylation was carried out on an 7-MeGua derivative using pentafluorobenzyl bromide.

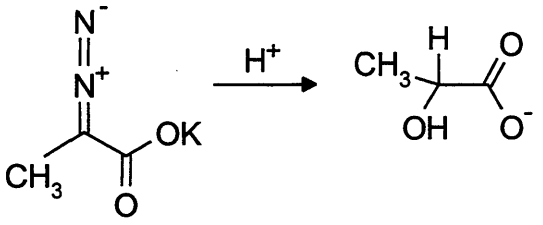


Scheme 23. Attempted production of pentafluorobenzyl-2-diazopropanoate.

The extracted dichloromethane layer was analysed by MS (FAB+), but no mass ion profiles were observed that indicated presence of the organic salt (MW 280). The spectra showed a strong signal for the tetrabutylammonium (MW 241) and pentafluorobenzyl (MW 180) groups. None of the mass spectra showed presence of any brominated species (no characteristic isotope pattern for Br^{79} and Br^{81}). Back-extraction with dichloromethane made no difference to the mass spectra observed. A mass ion profile at 100 *m/z* units was present which could have been attributed to the 2-diazopropanoate ion (MW 99), but on addition of acid to the solution (which would have decomposed the 2-diazopropanoate to the alcohol), the same mass ion profile remained on re-analysis.

5.2.3. A Kinetic Study of the Decomposition of Potassium 2-Diazopropanoate

Salts of diazoacetic acid, stable in alkaline conditions, are found to rapidly decompose on neutralisation with acid, with evolution of nitrogen (King and Bolinger, 1936; Kreevoy and Konasewich, 1970). The same is true of phenyldiazoacetic acid (Kresge *et al.*, 1995). Therefore the stability of potassium 2-diazopropanoate was examined by carrying out a decomposition study of the salt at various pH values (Scheme 24).



Scheme 24. Decomposition of potassium 2-diazopropanoate.

The stock solution of potassium 2-diazopropanoate was taken and dilutions were made with sodium citrate (SSC) buffer, and the UV absorbance scanned over 150 nm to 450 nm. The compound was found to have a maximum absorbance at 265 nm (λ_{max}). Buffers of SSC were made up in the pH range of 2.0 to 10.0, and the potassium 2-diazopropanoate stock solution was diluted using these buffers. The absorbance of 100 μM solutions at various pH values was recorded every 15 min at 37°C for a period of up to 900 min. Typical UV traces can be seen in Figure 5.5.

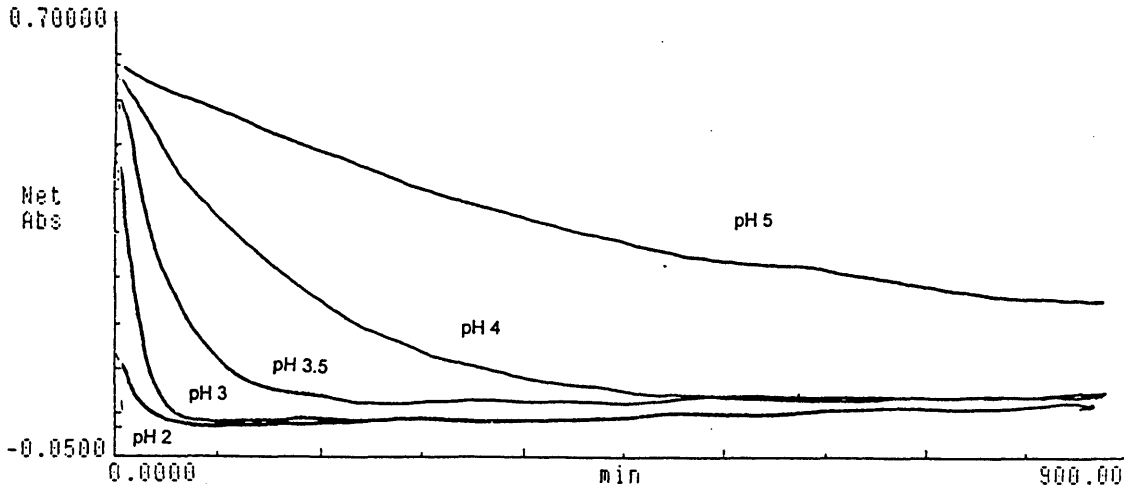


Figure 5.5. UV traces for the decomposition of potassium 2-diazopropanoate at various pH values.

The decomposition followed first-order kinetics. By plotting graphs of the natural log values of absorbance at time=0 divided by absorbance at time=t against time, t (min), the rate constants, k, were determined and the half lives of the decomposition of potassium 2-diazopropanoate were calculated (Table 5.1.), using the following equation;

$$t_{1/2} = \ln 2/k \quad \text{where } k = \text{rate constant (gradient)}$$

Table 5.1. The rate constants and half-lives for the decomposition of 2-diazopropanoate in various pH buffers at 37°C (n=3).

pH	$t_{1/2}$ / min
2.0	0.7 (40 s)
3.0	10
3.5	30
4.0	120
5.0	380
6.0	670
7.0	590
8.0	480
9.0	500
10.0	360

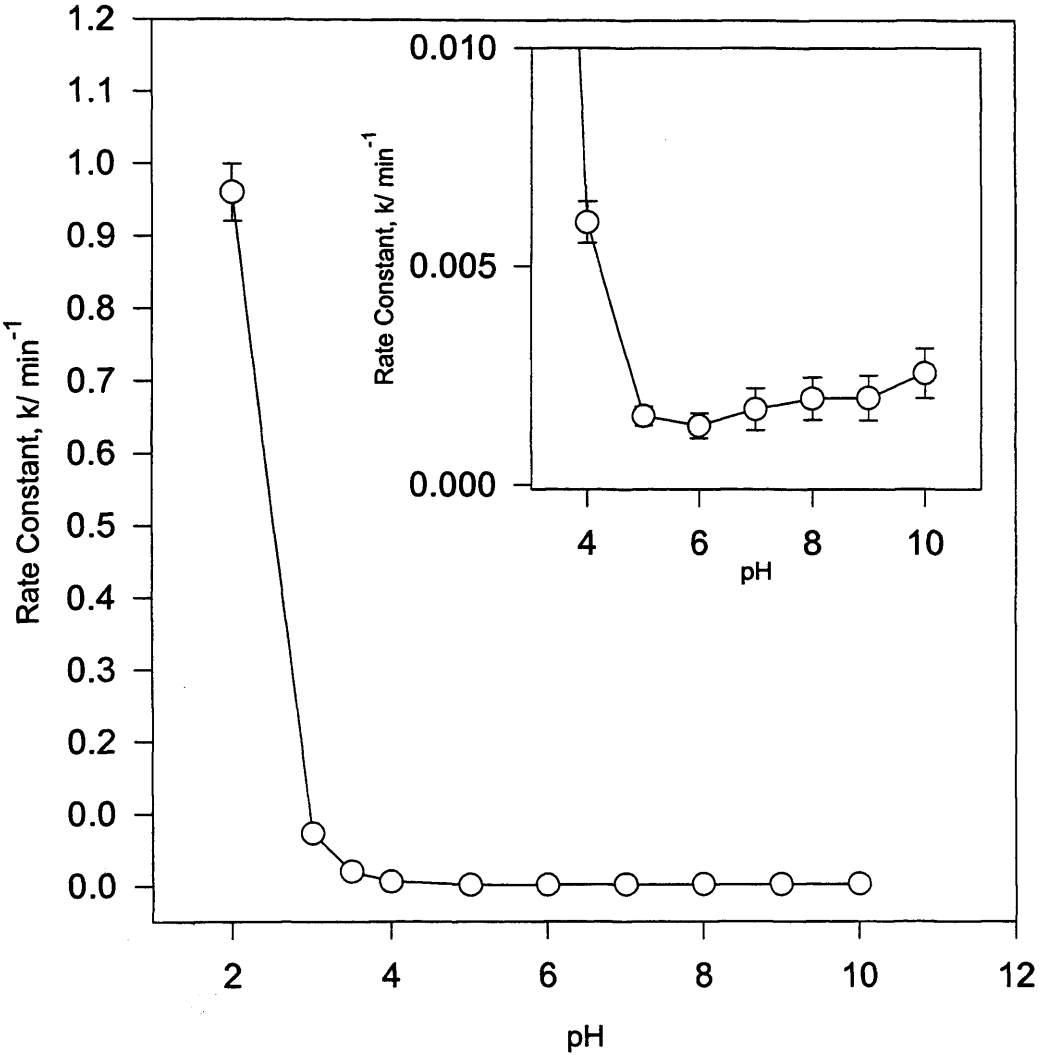
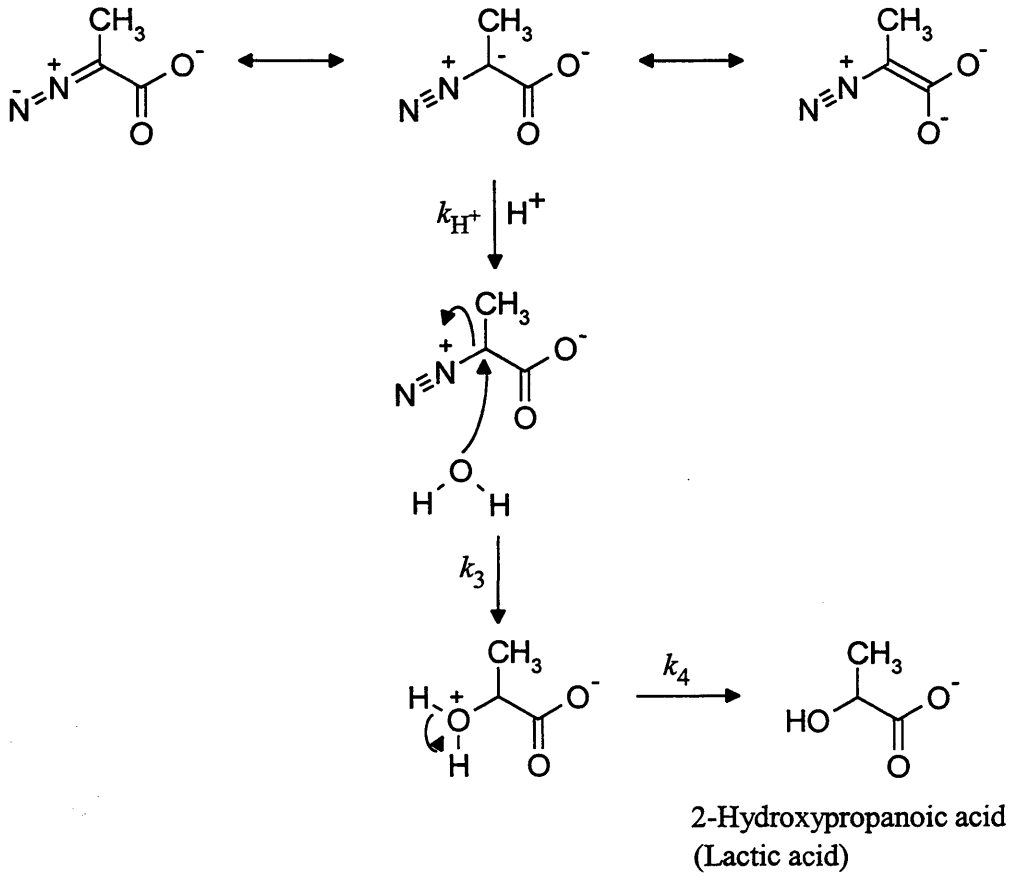


Figure 5.6. A plot of rate constant versus pH for the decomposition of potassium 2-diazopropanoate.

From the plot of rate constant versus pH for the decomposition of potassium 2-diazopropanoate (Figure 5.6.), it can be seen that the compound is very stable over a large pH range (5-10). The rate profile consists of two linear portions which are connected by a curved region, known as an upward bend, and this upward bend generally signifies a change in the species undergoing reaction (Kresge *et al.*, 1995). Various reactions take place during

the decomposition of potassium 2-diazopropanoate and these can be seen in Scheme 25. Studies on the decompostion of phenyldiazoacetic acid and potassium diazoacetate indicate that the protonation of the diazo carbon is the rate-limiting step and therefore determines the overall rate of the decomposition (King and Bolinger, 1936; Kreevoy and Konasewich, 1970; Kresge *et al.*, 1995). The rate of carbon protonation is governed by the negative charge density at the diazo carbon atom.



Scheme 25. Mechanism of decomposition of potassium 2-diazopropanoate.

5.2.4. Reaction of Potassium 2-Diazopropanoate with Calf Thymus DNA

Incubation of CT DNA with varying concentrations of potassium 2-diazopropanoate was carried out. Detection and quantitation of the 7-EtGua adduct which should have formed was carried out by immunoaffinity purification of the DNA hydrolysates, followed by fluorescent postlabelling of the free modified base, using Phmal. As can be seen from the chromatogram

below (Figure 5.7.) no peaks for Phmal-7-EtGua were observed. Repeated experiments provided the same results. Interestingly, there is a peak present just before the Phmal-7-MeGua internal standard, but this has not been identified. The position of the unknown peak relative to Phmal-7-MeGua, indicates that the unknown compound is more polar than 7-MeGua, maybe with either a hydroxy- or carboxy- group attached.

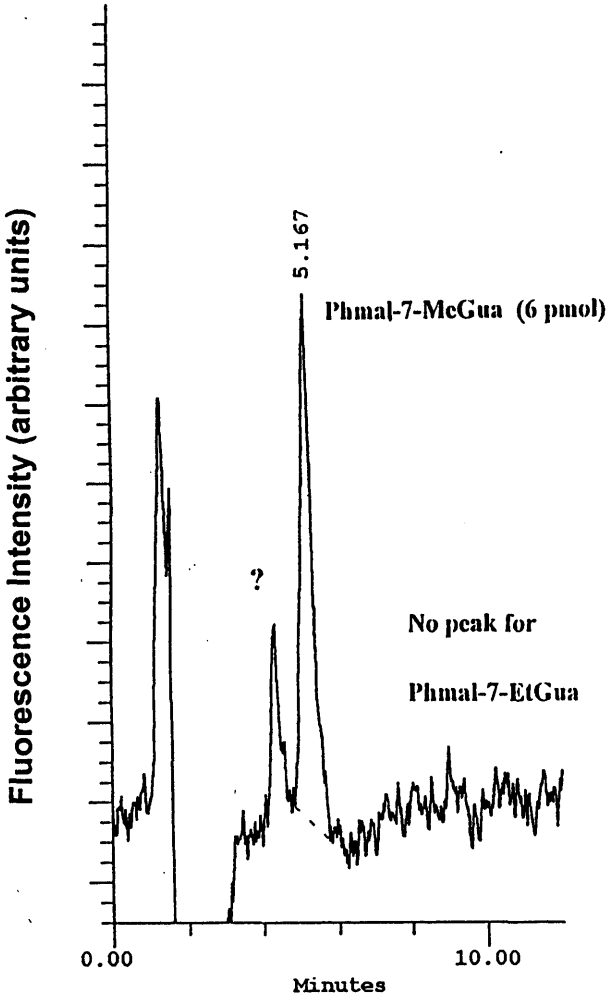
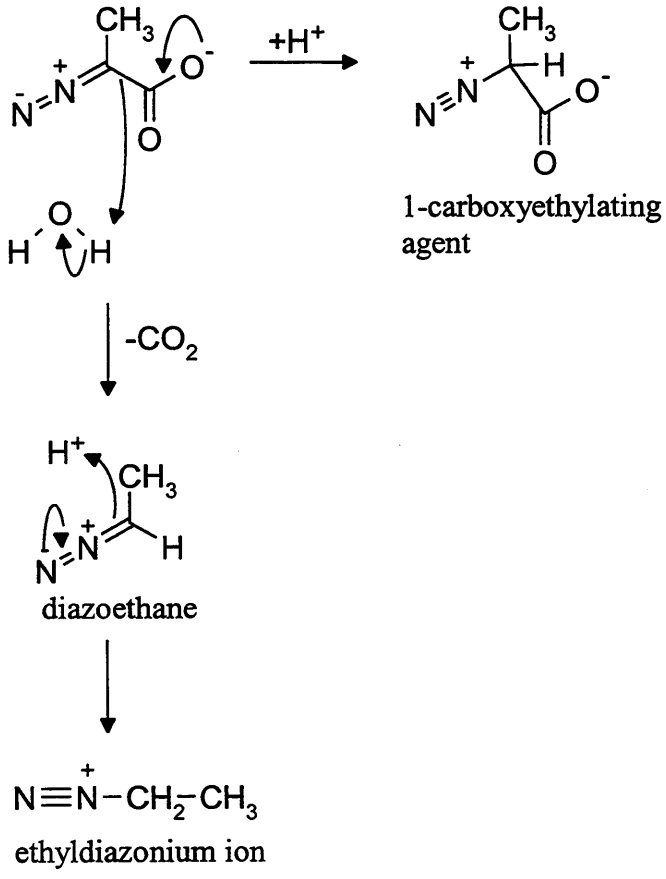


Figure 5.7. A typical chromatogram of modified bases derivatised with Phmal after being passed through 7-EtGua immunoaffinity columns from CT DNA incubated with potassium 2-diazopropanoate.

What are the possible products of the reaction between potassium 2-diazopropanoate and DNA? As the primary interest lay in the determination of 7-AlkGua adducts, these will be discussed. Looking at scheme 26, it can be seen that the formation of diazoethane (ethylating agent) is possible, as well as a 1-carboxyethylating agent. Therefore, the peak observed just before Phmal-7-MeGua on the chromatogram may be due to the Phmal derivative of N7-(1-carboxyethyl)guanine. The fact that no ethylation was observed, may be due to the high stability of potassium 2-diazopropanoate at pH 7, it maybe actually necessary to pyrolyse potassium 2-diazopropanoate to allow the reaction with DNA to occur in the gas-phase.

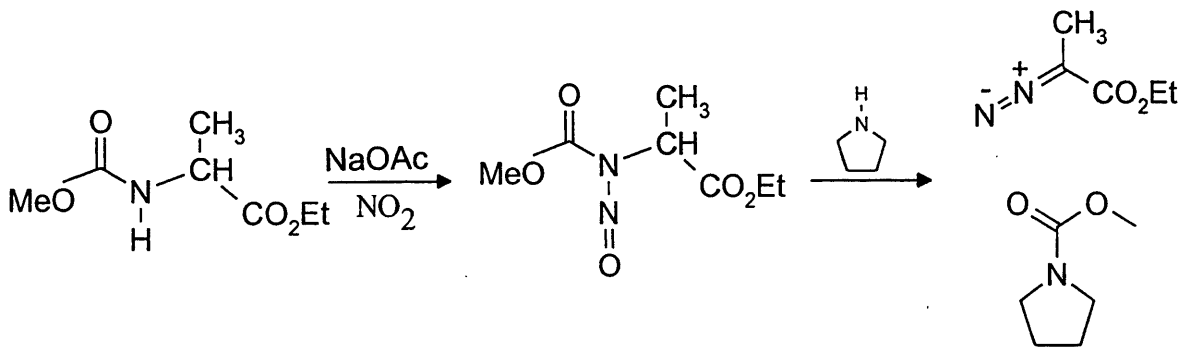


Scheme 26. Possible formation of diazoethane and a 1-carboxyethylating agent from 2-diazopropanoate.

5.3. Conclusions

It is surprising that ethylation was not observed as energy calculations by Ford and Scribner (1983) showed that ethylation by the diazonium reagent is favoured over methylation by its diazonium reagent. Also methylation of DNA by diazoacetate has been readily observed by other groups (Ford and Scribner, 1990; Harrison *et al.*, 1997). The only conclusions that can be drawn from this are that either diazoethane was not generated from 2-diazopropanoate and/or that the 1-carboxyethylating agent was preferentially formed. In the case of the latter, it would be very difficult to confirm without synthesising a standard of N7-(1-carboxyethyl)guanine, derivatising with Phmal and comparing HPLC results with samples. Diazoethane not being generated is a likely possibility as the 2-diazopropanoate has shown to be a very stable compound over a large pH range. The half life at pH 7 (*in vitro* study carried out at this pH) was shown to be approximately 10 hours, whereas the half life of diazoacetate ion is considerably less, at ~ 6 min (Kreevoy and Konasewich, 1970).

As a full characterisation of potassium 2-diazopropanoate could not be achieved (elemental analysis would have been ideal) due to a lack of material, it cannot be stated for definite that saponification of O-(N-succinmidyl)-2-diazopropanoate yielded the required potassium salt, even though decomposition studies would suggest so. Further investigation should be attempted with the synthesis of ethyl 2-diazopropanoate using a method described by Garci *et al.* (1984), where a protected ethyl alaninate was nitrosated and reacted with pyrrolidine (nucleophilic cleavage) to afford ethyl 2-diazopropanoate in high yields (Scheme 27).



Scheme 27. Synthesis of ethyl 2-diazopropanoate using a method by Garcia *et al.*, 1984.

Although ethylation of guanine at the N7-position was not observed, this work has definite potential for studying the reactions of diazo compounds with DNA. If time had permitted further studies may have been attempted. Ford and Scribner (1983) have shown that gas-phase ethylation occurs more favourably than methylation by their diazonium ions. Therefore, it would be interesting to attempt gas-phase reactions of potassium 2-diazopropanoate with DNA *in vitro*. The salt would need to be pyrolysed and the gaseous phase passed through a solution of DNA. This procedure may produce the reactive diazonium species that do not seem to be formed in aqueous solution. Recent studies have shown that the gas phase of environmental tobacco smoke is a potential carcinogen (Witschi *et al.*, 1997). Mice exposed to filtered and unfiltered tobacco smoke produced a statistically higher number of lung tumours than air-exposed controls. The study concluded that although the gas phase of environmental tobacco smoke is as carcinogenic as full environmental tobacco smoke (particulate and gaseous matter), the carcinogenicity of the gas phase may be due to some as yet unidentified carcinogens.

To further identify alanine as a possible ethylating agent in tobacco smoke, an interesting experiment would be to spike cigarettes with alanine or deuterated-alanine. A solution of the amino acid could be sprayed or injected onto the tobacco and the cigarettes left to dry (Massey and Few, 1983.). By exposing the spiked tobacco smoke to DNA, it may be possible to isolate any 7-EtGua adducts formed by using immunopurification. Subsequent derivatisation with Phmal and HPLC fluorescence analysis (or MS for deuterated analogues) may give quantitation (and structural analysis).

CHAPTER 6

Materials and Experimental Methods

Chapter 6. Materials and Experimental Methods

6.1. Instrumentation and Chemicals

All ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 250 MHz spectrometer. Chemical shifts for ^{1}H and ^{13}C are expressed in parts per million downfield from tetramethylsilane (0.00 ppm). All mass spectroscopy was carried out on a VG 70-SEQ mass spectrometer (Micromass, Manchester, UK) and the samples were run in a glycerol matrix, using fast atom bombardment (positive ion mode), unless otherwise stated.

All chemicals and reagents were obtained from Sigma (Sigma Chemical Co. St. Louis, MO, USA), Aldrich (Aldrich-Chemical Co. Ltd., Poole, Dorset, England) and Fisher (Fisher Scientific UK Ltd., Loughborough, UK), unless otherwise stated. Solvents were all of analytical grade or higher (HPLC analysis) and obtained from Fisher Scientific UK Ltd. Deuterated solvents for NMR studies were obtained from Fluka Chemika, Switzerland.

6.2. Experimental Methods for Chapter 2

6.2.1. Chemicals and Reagents

Dimethylformamide (Sigma), 0.1 M Tris-(hydroxymethyl)-methylamine (pH 7.4) (Sigma, Tris, 12.114 g dissolved in 1 L of water), 0.2 M Triethanolamine (pH 8.2) (Sigma, 37.14 g dissolved in 1 L of water), 20 mM Dimethylpimelimidate dihydrochloride (Sigma, 518.36 mg dissolved in 100 mL triethanolamine buffer), 20 mM Ethanolamine (Sigma, 120 mL added to 100 mL of triethanolamine buffer), Phosphate Buffered Saline (Unipath Ltd., Basingstoke, England; PBS), PBS-azide (0.02% NaN_3 in PBS), Phosphorus oxychloride (Aldrich), Sodium hydroxide (Fisher), Ammonium Sulphate (Fisher), Protein A Sepharose CL4B (Pharmacia Biotech, Sweden), 7-MeGua (Sigma), Dimethylsulphate (Aldrich), Potassium carbonate (Fisons), Anhydrous sodium sulphate (Fisons), Calf thymus DNA (Sigma).

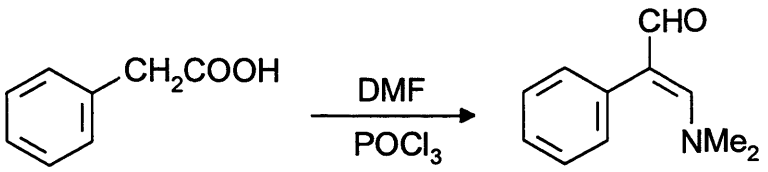
6.2.2. HPLC Conditions

All injection volumes were 10 μL.

A Waters/Millipore HPLC system coupled to a Waters 470 fluorescence detector was used to run the samples, through a Shandon Hypersil C_{18} BDS column (2.1 mm x 10 cm) fitted with a guard frit, at a flow rate of 0.2 mL/min. The lamp excitation wavelength was set to 280 nm and the emission wavelength was set to 527 nm. The samples were analysed using an isocratic programme using two buffered solvents at a flow rate of 0.2 mL/min; Solvent A consisted of 20 mM KH_2PO_4 (pH 7.1) and Solvent B was the same salt solution, but in 60% acetonitrile. Solvent A was run at 58% and Solvent B at 42%.

6.2.3. Synthesis of Phenylmalondialdehyde

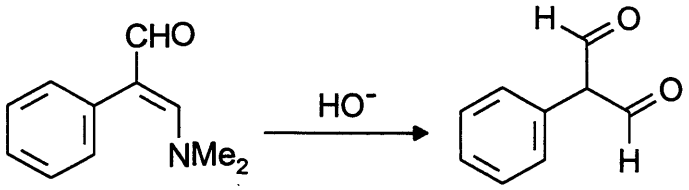
6.2.3.1. Formation of 2-phenyl-3-(dimethylamino)-acrolein



Arnold (1961; 1973)

Dimethylformamide (38.7 ml, 36.5 g, 0.5 mol) was added dropwise to phosphorus oxychloride ($POCl_3$, 28 ml, 46 g, 0.3 mol) whilst maintaining the temperature at 30°C. The mixture was then stirred for five minutes. Phenylacetic acid (13.6 g, 0.1 mol) was dissolved in DMF (40 ml) and poured into the $POCl_3$ solution. The mixture was stirred at 70°C and the reaction was monitored by TLC ($MeOH:CHCl_3$/2:5). The reaction was stopped after 6 h. The mixture was then poured over ice (ca. 450 g) and neutralised with saturated potassium carbonate solution (*ca.* 50 mL). Sodium hydroxide solution (50% w/v *ca.* 50 mL) was added to hydrolyse the intermediate, and this was followed by TLC. Precipitated salts were removed by filtration and washed with toluene (3 x 20 mL). The liquid portion was also extracted with toluene (4 x 25 mL). The toluene extracts and washings were combined and dried overnight with anhydrous sodium sulphate. Vacuum distillation afforded a pale yellow oil (9.38 g) was collected at 175-177°C (0.1 mm Hg). Purity of the compound before and after distillation was checked by TLC, NMR and MS. Yield, 9.38 g (54%); ^{1}H NMR 2.70-2.90 (6 H, $N(CH_3)_2$), 7.20-7.45 (m, 5 H, Ar), 9.2 (s, 2 H, -CHO), > 11, (d, 1 H, ArCH); MS *m/z* 176 $(M+H)^+$

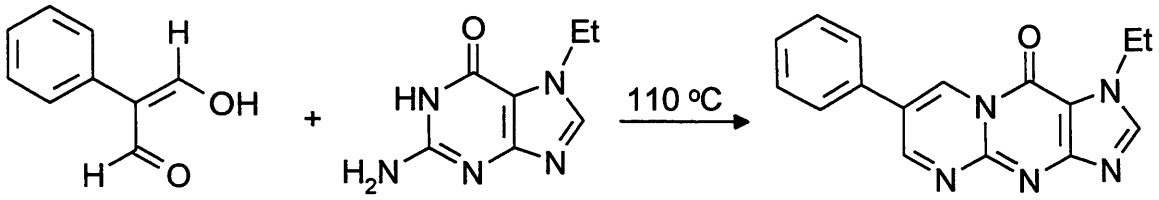
6.2.3.2. *Hydrolysis of 2-phenyl-3-(dimethylamino)-acrolein to phenylmalondialdehyde*



Coppola *et al.* (1974)

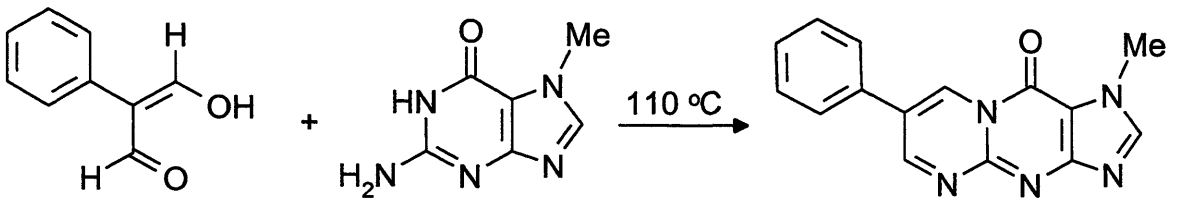
2-Phenyl-3-(dimethylamino)-acrolein (9.38 g) in ethanol (82 mL) was transferred to a 500 mL round-bottomed flask. The mixture was refluxed with 20 % sodium hydroxide (108 mL) for 3.5 h (monitored by TLC), and then allowed to cool. The organic matter was extracted with dichloromethane (3 x 25 mL) and the remaining colourless aqueous layer was taken and acidified to pH 2 with 5 M hydrochloric acid (dropwise addition). The solution was allowed to cool at room temperature. White crystals formed as the solution cooled. The solution was stored overnight at 4°C to allow maximum crystallisation. The crystals were then filtered and allowed to dry over phosphorus pentoxide (P_2O_5). The structure and purity of the Phmal were checked by NMR and MS. Yield, 0.5 g (7%); ^{1}H NMR 7.20-7.45 (m, 5 H, Ar), 8.65 (s, 2 H, -CHO), > 11 (d, 1 H, ArC<u>H</u>(CHO)$_2$) ppm; MS *m/z* 149 $(M+H)^+$

6.2.4. Synthesis of Phenylmalondialdehyde-N7-ethylguanine



Phmal (50.01 mg, 33.8 μmol) was dissolved in glacial acetic acid (3.0 mL). 7-EtGua (21.03 mg, 11.7 μmol) was added, and the mixture heated at 110°C for 1.5 h. A yellow solution resulted. The solution was allowed to cool and then the excess acetic acid was evaporated off to leave afford a yellow oil. Upon cooling at 4°C for 2 days, crystals of Phmal-7-EtGua formed. The crystals were dissolved in a minimum volume of hot ethanol, and water was added until cloudiness started. The yellow solution was filtered and then allowed to cool in ice water. Yellow/shiny crystals started to form within 2 h. These were filtered, washed with cold water and left to dry over P_2O_5. Yield, 32.14 mg (94%); ^{1}H NMR 1.6-1.7 (t, 3 H, -$CH_2\underline{C}H_3$), 4.5-4.6 (q, 2 H, -$\underline{C}H_2CH_3$), 7.5-7.8 (m, 5 H, Ar), 8.2 (s, 1 H, -CH), 9.2 (d, 1 H, -CH), 9.4 (d, 1 H, -CH) ppm; MS *m/z* 292 $(M+H)^+$

6.2.5. Derivatisation of Picomole Quantities of N7-Methylguanine with Phenylmalondialdehyde



7-MeGua (0.825 g, 5 mmol) was dissolved in 0.1 M HCl to give a 5 mM solution. A 10 μL aliquot was made up into 1 mL of water, to give a 50 μM solution of 7-MeGua. Five separate dilutions were made by taking 10, 20, 30, 40 and 50 μL into 1 mL of water to give 0.5, 1.0, 1.5, 2.0, 2.5 pmol/μl of 7-MeGua. Aliquots (10 μL) were added to silanised 100 μL reacti-vials. Aliquots (10 μL) of 2.5 μM 7-EtGua (25 pmol) were also added as an internal standard. The solutions were evaporated to dryness and powdered 4Å molecular sieve (~1mg) was added. Aliquots (20 μL) of Phmal (5 mg/mL) in glacial acetic acid were added to each of the reacti-vials. The mixtures were allowed to heat at 110°C for 40 min. The solutions were allowed to cool and then evaporated to dryness. The derivatives were re-dissolved in acetonitrile (10 μl) followed by the addition of mobile phase (40 μL) buffer. The solutions were then filtered (to remove the 4Å molecular sieve), using nylon non-sterile microfuge microfiltration centrifuge filters (Alltech, UK). All derivatisations were carried out in triplicate. Aliquots (10 μL) were analysed using HPLC fluorescence as described in section 6.2.2.

6.2.6. Preparation of N7-Methylguanine Immunoaffinity Columns

6.2.6.1. Preparation of Immunoglobin G (IgG) from Antiserum

Antiserum (5 mL) was placed in a small a small beaker with a stirrer. Saturated ammonium sulphate solution (*ca.* 3.3 mL) was added whilst stirring to give a final concentration of 40% v/v. The solution was allowed to stir for a further 5 min. The solution was then transferred to a 50 mL polypropylene tube and centrifuged at 3000 *g* for 15 min. The supernatant was discarded and the IgG pellet washed with 50% v/v saturated ammonium sulphate solution

(x2). The pellet was then resuspended in PBS (5 mL) and dialysed against PBS (3 L) overnight. The suspension was then transferred to a 15 mL polypropylene tube and centrifuged at 3000 *g*. The pellet was discarded.

6.2.6.2. Preparation of IgG-Protein A Sepharose CL 4B

Protein A Sepharose CL 4B (5 mL) was washed in a tapped-column with Tris buffer (2 x 20 mL) and then suspended in Tris buffer (10 mL). The IgG solution was added and the mixture made up to 50 mL with Tris buffer (30 mL). The mixture was stirred, end-over-end, for 30 min at room temperature in a stoppered chromatography column (2 cm x 30 cm) with a glass sinter at one end. The liquid was then drained and the excess IgG washed with Tris buffer (2 x 50 mL), with stirring for 10 min each washing. The gel was then washed with triethanolamine buffer (2 x 50 mL, 10 min each washing) and resuspended in dimethylpimelimidate solution (100 mL). The solution was allowed to stir end-over-end for 45 min. The liquid was then drained off and the gel resuspended in 20 mM ethanolamine solution (100 mL) and stirred for 5 min. The gel was then drained and washed with PBS-azide (5 x 20 mL) for 10 min each time. The gel was washed into a 50 mL polypropylene tube using 10 mL aliquots (50 mL). The suspension was centrifuged at 3000 *g* and some of the PBS-azide removed to leave *ca.* 25 mL. The gel was suspended and partitioned equally amongst five polystyrene fritted mini-columns. The gel was then washed down with PBS-azide (2 x 1 mL). Frits were added onto the top of the gels, and again they were washed with PBS-azide (2 x 1 mL). The columns were stored at 4°C.

6.2.7. Determination of N7-Methylguanine Capacity of the Immunoaffinity Columns

6.2.7.1. Protocol for the Use of Immunoaffinity Columns

A 50 μM stock solution of 7-MeGua (50 pmol/μL) was taken and dilutions were made to give a range from 1 pmol/μl to 12 pmol/μL. Aliquots (100 μL) were taken up into PBS (2 mL) and applied to the immunoaffinity columns. The columns were then washed with PBS-azide (3 mL), followed by water (10 mL). The 7-MeGua was eluted by the addition of 1 M acetic acid (2 mL) and the fraction (2 mL) was collected in a 15 mL polypropylene tube. The columns were washed with a further aliquot of 1 M acetic acid (3 mL). The columns were then re-conditioned by adding PBS-azide (15 mL). The collected fractions were evaporated down to dryness.

6.2.7.2. Phenylmalondialdehyde Derivatisation of Dried Fractions

The dried fractions were dissolved in 0.01 M HCl (200 μL). Aliquots were take in duplicate and derivatisation was carried out as described in section 6.2.5.

6.2.8. Determination of N7-Methylguanine Recovery from Immunoaffinity Columns

Standards of 7-MeGua (10 μL from stocks of 0.5, 1.0, 1.5, 2.0, 2.5 μM) were added to PBS solutions (2 mL). These were applied to the immunoaffinity columns and eluted (see section 6.2.7.1.). The dried fractions were dissolved in 0.01 M HCl (70 μL) and transferred to 100 μL reacti-vials. An aliquot (10 μL) of 2.5 μM 7-EtGua (25 pmol) was added as an internal standard. Derivatisation with Phmal was carried out (see section 6.2.6.). Standards were derivatised in triplicate. Standards of 7-MeGua (10 μL from stocks described above) were derivatised without passing through immunoaffinity columns (see section 6.2.6.), using 7-EtGua as an internal standard. Standards were derivatised in triplicate. All samples were analysed by HPLC fluorescence as described in section 6.2.2.

6.2.9. Determination of N7-Methylguanine Produced in Calf Thymus DNA on Incubation with Dimethylsulphate

6.2.9.1. Methylation of Calf Thymus DNA by Dimethylsulphate

Aliquots of CT DNA solution (1 mL; 5 mg/mL of 1.5 M sodium chloride/ 150 μM trisodium citrate buffer/ 1 μM EDTA; SSC buffer) were incubated at 37°C for 30 minutes with five different concentrations of DMS; 1 μM, 10 μM, 30 μM, 50 μM and 80 μM. The incubations were done in triplicate. Control solutions of untreated CT DNA were also incubated under the same conditions. After incubating, the solutions were cooled in ice and the DNA precipitated by the addition of ice-cold 2-propanol (2 mL). The CT DNA was pelleted and the supernatants removed. The CT DNA pellets were re-dissolved in SSC buffer (1 mL) and the solutions heated at 100°C for 30 min to depurinate the DNA at alkylated sites. The solutions, were cooled in ice and the DNA re-precipitated (*ca.* 2 hours at -20°C) by the addition of 1/10 volume of ice-cold 3M sodium chloride solution followed by the addition of 2 volumes of ice-cold 2-propanol. The DNA was pelleted and the supernatants were transferred to 15 mL polypropylene tubes and evaporated to dryness.

6.2.9.2. Isolation of N7-Methylguanine Using Immunoaffinity Purification and Derivatisation with Phenylmalondialdehyde

The dried residues were re-dissolved in PBS-azide (2 mL) and the PBS solutions applied to the immunoaffinity columns. Fractions of 7-MeGua were collected (see section 6.2.7.1.). To test background levels of the columns, blank runs were done before applying the DNA samples, which consisted of running just PBS (2 mL) through the columns and eluting with 1 M acetic acid. Acetic acid (1 M, 3 mL) was also placed into two polypropylene tubes as a background test. All these fractions were evaporated to dryness. The dried fractions were re-dissolved in 0.01 M HCl (70 μL) and derivatised with Phmal (see section 6.2.6.) using 7-EtGua as an internal standard. The samples were analysed using HPLC fluorescence as described in section 6.2.2.

6.2.10. N7-Methylguanine Produced in Calf Thymus DNA on Exposure to Tobacco Smoke

Aliquots of CT DNA solution (4 mL; 5 mg/mL of sodium chloride/ trisodium citrate buffer; SSC buffer) were added to a 10 mL round-bottomed flask. The CT DNA was then exposed to the tobacco smoke from 3 and 6 cigarettes (Silk Cut Ultra), using the apparatus shown in Figure 6.1.

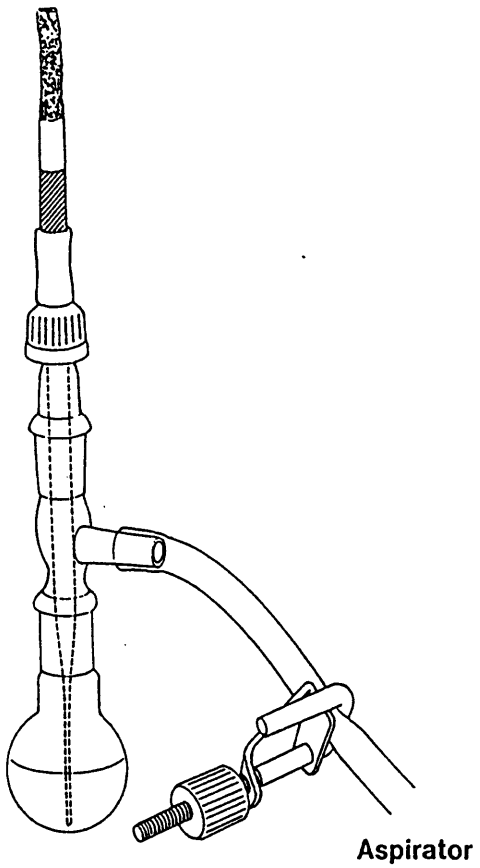


Figure 6.1. Apparatus used to trap cigarette smoke. (Adapted from Leanderson and Tagesson, 1989).

Aliquots (3 x 1 mL) were taken separately and the CT DNA was precipitated by the addition of 2 volumes of ice-cold 2-propanol. The liquid was decanted off and the CT DNA was rinsed with two further aliquots of 2-propanol (2 x 3 mL). The CT DNA was re-dissolved in SSC buffer (3 x 1 mL) by end-over-end stirring. The solutions were heated at 100°C for 30

min to depurinate the DNA at alkylated sites. The solutions, were again cooled in ice and the DNA re-precipitated (*ca.* 2 hours at -20 °C) by the addition of 1/10 volume of ice-cold 3M sodium chloride solution followed by the addition of 2 volume of ice-cold 2-propanol. The DNA was pelleted and the supernatants were transferred to 15 mL polypropylene tubes and evaporated to dryness. The dried residues were re-dissolved in PBS (2 mL) and the PBS solutions applied to the immunoaffinity columns. Fractions of 7-EtGua were collected (see section 6.2.8.1.) and allowed to evaporate down to dryness. Unexposed CT DNA was used as controls. The dried fractions were re-dissolved in 0.01 M HCl (70 μL) and derivatised with Phmal (see section 6.2.6.) using 7-EtGua as an internal standard (10 μL of a 2.5 μM solution), and the samples were analysed using HPLC fluorescence.

6.3 Experimental Methods for Chapter 3

6.3.1. Chemicals and Reagents

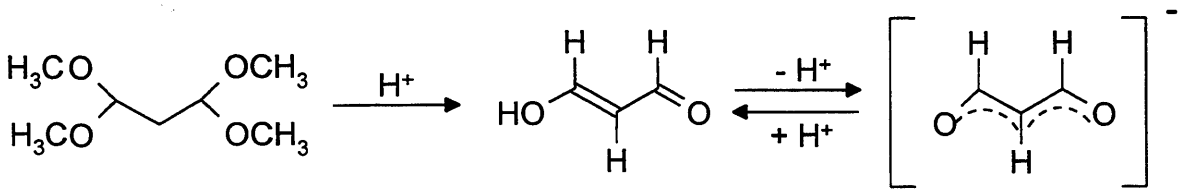
1,1,3,3-Tetramethoxypropane (Aldrich), 4-Chlorobenzodiazonium hexafluoroborate (Acros Organics, Fisher Scientific UK Ltd.), Acetic acid (Fisher).

6.3.2. Dry-Phase Reaction for the Derivatisation of N7-Methylguanine with Phenylmalondialdehyde

The procedure was repeated as in section 6.2.5., but the addition of a 4Å molecular sieve was omitted. An aliquot of Phmal (20 µL) in glacial acetic acid (5 mg/mL) was added to the dried residue of 7-MeGua and the solvent was evaporated off in a rotary evaporator. The dried residual mixture was heated at 110°C for 40 min without placing the lid on the reacti-vial. This allowed any water produced during the reaction to boil off. The contents were prepared for HPLC analysis after cooling.

6.3.3. Synthesis of 7-(4-Chlorobenzeneazo)-10-oxo-1-methyl-9,10-dihydro-pyrimido[1,2,a]purine (4-Chorobenzeneazomalondoaldehyde-7-methylguanine)

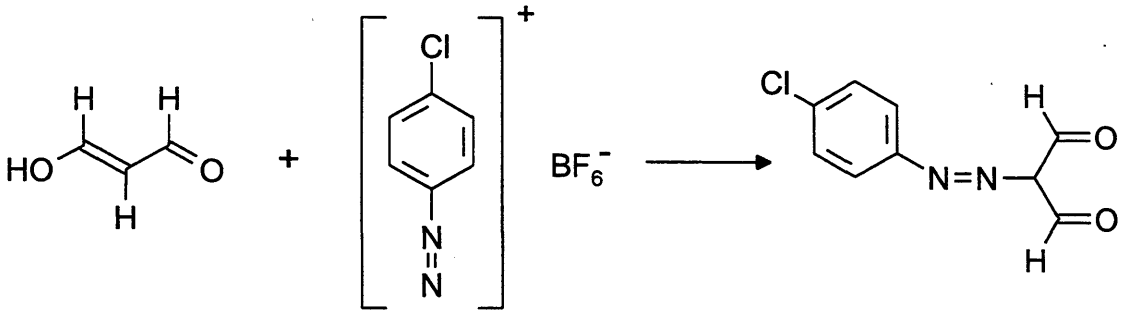
6.3.3.1. Preparation of the Malondialdehyde



Reichardt and Grahn (1970)

1,1,3,3-Tetramethoxypropane (3.08 mL, 19 mmol) was taken in 0.5 M HCl and shaken for 1-2 h, to produce the malondialdehyde.

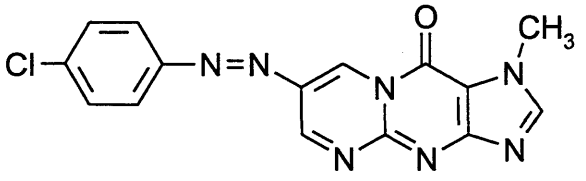
6.3.3.2. Preparation of the 4-Chlorobenzeneazomalondaldehyde



Reichardt and Grahn (1970)

4-Chlorobenzodiazonium hexafluoroborate (5 g, 17.6 mmol) was dissolved in ice-cold water (60 mL). With stirring, the malondialdehyde solution was added quickly. A yellow/gold precipitate appeared. The suspension was allowed to stir for a further 2 h, until production of deep yellow precipitate ceased. The solution was then filtered, washed with ice-cold water and left to dry overnight. It was re-crystallised from a minimum amount of hot acetic acid, filtered and left to dry in a vacuum. Yield, 0.874 g (22.3%); ^{1}H NMR (*d*-DMSO) 7.65-7.96 (m, 4H, Ar), 9.65 (s, 1H, CCH), 9.95 (s, 1H, CHO), 14.20 (s, 1H, OH), 2.60 (DMSO), 3.55 (H_2O) ppm; ^{13}C NMR (*d*-DMSO) 120 (2 AR), 130 (2 Ar), 186 (C=CH), 191 (CHO); MS (EI) *m/z* 210 $(M)^+$

6.3.3.3. Characterisation of 4-Chlorobenzeneazomalondaldehyde-N7-methyguanine



4-Chlorobezeneazomalondialdehyde (48.10 mg, 2.23 mmol) was dissolved in glacial acetic acid (1.5 mL) and placed in a capped 5 mL Dupont tube. 7-MeGua (23.14 mg, 0.12 mmol) was added and the solution heated at 110°C for 1 h. The solution was then allowed to cool, and immediately, crystals started to form. The crystals were filtered, washed with acetic acid and dried overnight under vacuum. Yield, 27.71 mg (58%); ^{1}H NMR 4.2 (s, 3 H, -CH_3), 7.5-

7.9 (m, 4 H, Ar-H), 8.15 (s, 1 H, -NCHN-), 9.5 (d, 1 H, -CCHN-), 9.8 (d, 1 H, -CCHNCO) ppm; MS *m/z* 340 $(M+H)^+$, 372 $(M+MeOH+H)^+$

To assess the product's fluorescence properties, a serious of dilutions were prepared in water. The product (1.695 mg) was dissolved in DMSO (1 mL) to give a 5 mM stock. Dilutions were made to give 500 μM, 50 μM and 5 μM solutions. A UV absorbance scan was carried out of the 5 μM solution, in a quartz cuvette, using a KONTRON UVIKON 860 UV spectrophotometer. An LB 50 Perkin Elmer Luminescence spectrophotometer, was used to scan the emission of the 3 dilutions of product.

6.4. Experimental Methods for Chapter 4

6.4.1. Chemicals and Reagents

Ovalbumin (Grade VII; Sigma): coating antigens (0.25 mg of Ov-hapten and Ov dissolved in 1 mL of water and subsequent dilutions made), Methylated bovine serum albumin (Sigma), Balanced Salt Solution (Solution A: $CaCl_2.2H_2O$ 0.0074 g/L, Glucose 1.0 g/L, $MgCl_2$ 0.1992 g/L, KCl 0.4026 g/L, Tris 17.565 g/L, Solution B: NaCl 8.19 g/L. Mix 1 volume of solution A with 9 volume of solution B), 0.05 M citrate buffer (tri-sodium citrate; Fisher), Enzyme substrate (1 mg of 3'3'5'5'-Tetramethylbenzidine [Sigma] dissolved in 100 µL of dimethylsulphoxide, which was added to 10 mL of citrate buffer pH 5.3, followed by addition of 2 mL of 30% w/w solution of H_2O_2), Tween 20 (Sigma; polyoxyethylene-sorbitan monolaurate), Ammonium formate (Fisher), Sodium hydrogen carbonate (Fisher), Horseradish peroxidase-linked goat anti-mouse IgG (Sigma), Kieselgel 60 (Fluka Chemika, Swizerland), Thymidine phosphorylase (EC 2.4.2.4., TPase, Sigma), Purine nucleoside phosphorylase (EC 2.4.2.1., PNPase, Sigma), 4-Aminobutyric acid (Sigma), Iodoethane (Sigma), EDC (Sigma).

6.4.2. HPLC Conditions

All injection volumes were 10 µL, unless otherwise stated.

System 1: A Gilson HPLC system coupled to an Applied Biosystems 1000S diode array detector was used to run the samples. Aliquots (10 µL) were injected onto a Shandon Hypersil C_{18} BDS column (4.6 mm x 25 cm), fitted with a Shandon Hypersil C_{18} BDS guard cartridge, and eluted with Solvent A, 50 mM ammonium formate (pH 6.5) and Solvent B, methanol (0 min-0%B, 15 min-30%B, 20 min-0%B). The flow rate was 1 mL/min, and the UV absorbance was monitored at 278 nm.

System 2: A Gilson HPLC system coupled to an Applied Biosystems 1000S diode array detector was used to run the samples. Compounds were separated using a Shandon Hypersil C_{18} BDS semi-preparative column (10.0 mm x 25 cm), and eluted with Solvent A, 50 mM ammonium formate (pH 6.5) and Solvent B, methanol (0 min-0%B, 25 min-35%B, 30 min-

0%B) using a flow rate of 5 mL/min. The UV absorbance was monitored at 278 nm. Injection volumes were 500 μL.

System 3: A Waters/Millipore HPLC system coupled to a Shimadzu SPD-6AUV detector was used to run the samples. Compounds were separated using a Shandon Hypersil C_{18} BDS column (4.6 mm x 25 cm) fitted with a guard cartridge. The reaction mixture (10 μL) was added to water (990 μL) and 10 μL aliquots were eluted with Solvent A, 50 mM ammonium formate (pH 5.4) and Solvent B, methanol (0 min-0%B, 30 min-35%B, 35 min-60%B, 40 min-0%B). The flow rate was 1 mL/min, and the UV absorbance monitored at 254 nm.

System 4: A Waters/Millipore HPLC system coupled to a Shimadzu SPD-6A UV detector was used to run the samples. Compounds were separated using a Shandon Hypersil C_{18} BDS column (10.0 mm x 25 cm), and eluted with Solvent A, 50 mM ammonium formate (pH 5.4) and Solvent B, methanol (0 min-0%B, 20 min-35%B, 25 min-60%B, 30 min-0%B). The flow rate was 1 mL/min, and the UV absorbance monitored at 254 nm.

System 5: A Waters/Millipore HPLC system coupled to a Waters 470 fluorescence detector was used to run the samples. The lamp excitation wavelength was set to 280 nm and the emission wavelength was set to 527 nm. The samples (10 mL aliquots) were separated using a Shandon Hypersil C_{18} BDS column (2.1 mm x 10 cm) fitted with a guard frit, with an isocratic program using 50 mM ammonium formate (pH 7.1) and acetonitrile (75:25, v/v) at a flow rate of 0.2 mL/min.

6.4.3. Production of Monoclonal Antibodies Against N7-Ethylguanine

6.4.3.1. Synthesis of N7-Ethyl-N^2-carboxymethylguanine

N^2-Carboxymethylguanosine [Durand and Shuker, 1994] (24.34 mg, 0.07 mmol) was dissolved in THF (1 mL) and DES (18.34 μL, 0.14 mmol) added. The solution was heated, in a capped 5 mL Dupont tube, at 55°C. The reaction was monitored hourly by HPLC (section 6.4.2., system 1). Aliquots (10 μL) were taken up into water (1 mL) and injections (10 μL) made. After 3 h, a further aliquot of DES (9 μL, 0.07 mmol) was added. The reaction was stopped at 6 h. The reaction mixture was evaporated down to an oil and extracted with chloroform (2 x 0.75 mL) to afford a white/yellow solid. This was taken up into water (1 mL) and 0.1M HCl (20 μL) added. The mixture was heated at 115°C and the depurination was monitored by HPLC (section 6.4.2., system 1). The depurination was stopped after 3 h. On cooling, a white precipitate formed which was re-dissolved on the addition of dilute $NaCHO_3$ (20 mL). The N7-ethyl-N^2-carboxymethylguanine was collected by passing aliquots of the reaction mixture (100 μL) through a semi-preparative column (section 6.4.2., system 2). The fractions were pooled and lyophilised. Yield 6.43 mg (39%); ^{1}H NMR (D_2O) 1.4 (t, 3 H, -$CH_2\underline{CH_3}$), 3.9 (q, 2 H, -$\underline{CH_2}CH_3$), 4.3 (s, 2 H, -CH_2-), 8.4 (s, 1 H, -NCHN-) ppm; MS *m/z* 238 $(M+H)^+$

6.4.3.2. Protein Conjugation of N7-Ethyl-N^2-carboxymethylguanine with Methylated Bovine Serum Albumin and Ovalbumin

Methylated bovine serum albumin (mBSA, 25 mg) was dissolved in water (2 mL) and the pH adjusted to pH 6.5 by the dropwise addition of saturated $NaHCO_3$ solution. 1-Ethyl-3-dimethylamino-propylcarbodiimide (EDC, 9.3 mg) was then added. A solution of N7-ethyl-N^2-carboxymethylguanine (5.63 mg in 0.5 mL H_2O) was added in 20 μL aliquots over 1.5 h, whilst maintaining the pH at 6.5. The experiment was carried out in the dark and the solution left to stir overnight.

Ovalbumin (5 mg) was dissolved in water (0.5 mL) and the pH adjusted to 6.5 by dropwise addition of saturated $NaHCO_3$ solution. EDC (1.5 mg, *ca.* 6.5 μmol) was then added. A solution of N7-ethyl-N^2-carboxymethylguanine (0.75 mg, 3.16 μmol in 200 μL H_2O) was

added in 20 μL aliquots over 45 min, whilst maintaining the pH at 6.5. The experiment was carried out in the dark. The solution was left to stir overnight.

Purification of both protein bound haptens was carried out identically. The cloudy solution was treated with 0.01 M HCl (100 μL). The resulting clear solution was then applied onto the top of a Sephadex G25 column (10 mm x 200 mm, Gilson UV detector and Gilson pump), which had previously been conditioned with 0.05 M HCl. The protein bound hapten was then eluted by passing 0.05 M HCl through the column at a rate of 2 mL/min, and collecting the appropriate fractions. Detection was carried out by UV at 254 nm. The pooled fractions were then lyophilised to give pure protein bound hapten.

6.4.3.3. Quantification of Protein Bound Hapten

UV absorbances were measured on a Beckman DU-7000 at 278 nm, using water as a blank. Protein (mBSA or Ov) was dissolved in water to give a 100 μg/mL solution. The molecular weight of protein was taken to be 66000 amu (100 μg = 1.52 nmol) and the solutions were spiked with N7-ethyl-N^2-carboxymethylguanine (hapten) to give:

2 mol hapten/ mol mBSA	1 mol hapten/ mol Ov
5 mol hapten/ mol mBSA	2 mol hapten/ mol Ov
7 mol hapten/ mol mBSA	3 mol hapten/ mol Ov
10 mol hapten/ mol mBSA	4 mol hapten/ mol Ov
15 mol hapten/ mol mBSA	5 mol hapten/ mol Ov

Stock solution of hapten = 0.9 mg/mL (3.8 μmol/mL).

mBSA

60 μL into 1 mL of water gives 228 nmol/mL

40 μL into 1 mL of water gives 152 nmol/mL

28 μL into 1 mL of water gives 106 nmol/mL

20 μL into 1 mL of water gives 76 nmol/mL

8 μL into 1 mL of water gives 30 nmol/mL

Of each of these dilutions, a 100 μL aliquot was added to 0.9 mL of the mBSA solution, and the UV absorbance measured.

Ov

20 μL into 1 mL of water gives 76 nmol/mL

16 μL into 1 mL of water gives 60.8 nmol/mL

12 μL into 1 mL of water gives 45.6 nmol/mL

8 μL into 1 mL of water gives 30 nmol/mL

4 μL into 1 mL of water gives 15 nmol/mL

Of each of these dilutions, a 100 μL aliquot was added to 0.9 mL of the Ov solution, and the UV absorbance measured. The UV absorbance of the protein bound hapten (100 μg/mL) was also measured and compared to the calibration data, to give amount of protein conjugation.

6.4.3.4. Immunisation Protocol for the Production of Mouse Monoclonal Antibodies (1)

All procedures were carried out by staff in the animal house, CMHT, MRC Toxicology Unit, University of Leicester, Leicester.

Animals and Housing

Number/sex:	3/F	**Species/strain:**	Mouse/BALB/c
Number per cage:	3	**Identification:**	Tail marking

Protocol

Day 0: Antigen (mBSA-N7-ethyl-N^2-carboxymethylguanine, 0.35 mg) was dissolved in water (175 mL) and adjuvent (Hunter's Titremax, 90 μL) added. The solution was mixed thoroughly and a further aliquot of adjuvent added (85 μL). Each mouse received two subcutaneous injections (2 x 50 μL).

Day 14: Booster injection. Each mouse was treated as Day 0.

Day 28: Blood sample removed from tail vein of each mouse. Blood clots were homogenised in balanced salt solution (250 μL). The supernatants were then assayed for antibody production using ELISA.

Day 31: Booster injection. Each mouse treated as Day 14.

Day 68: Blood from tail vein from each mouse tested as on Day 28.

Mouse #2 was given booster injection as on Day 14, and spleen aseptically removed from mouse #2, frozen in liquid nitrogen and supplied to Tissue Culture Service (CMHT, MRC Toxicology Unit, University of Leicester, Leicester) for fusion and cloning.

6.4.3.5. *Immunisation Protocol for the Production of Mouse Monoclonal Antibodies (2)*

All procedures were carried out by BMS staff, Dept. of Surgery, Leceister Royal Infirmary, Leicester. The procedure was as described in section 6.4.3.4., except that the mice were immunised with 20 μg of antigen and Titremax Research adjuvent employed.

6.4.3.6. *Protocol for Checkerboard ELISA*

- A 96 well (8 x 12) polystyrene microtitre plate (NUNC, Nalge NUNC Products, Denmark) was filled with a solution of coating antigen (Ov-N7-ethyl-N^2-carboxymethylguanine or Ov-N7-ethyl-N^2-(3-carboxypropyl)guanine, 40 μL/well), at increasing dilutions down the plate and dried overnight at 40°C. The first half of the plate was coated with Ov-hapten and the second half with Ov (Figure 6.2.).

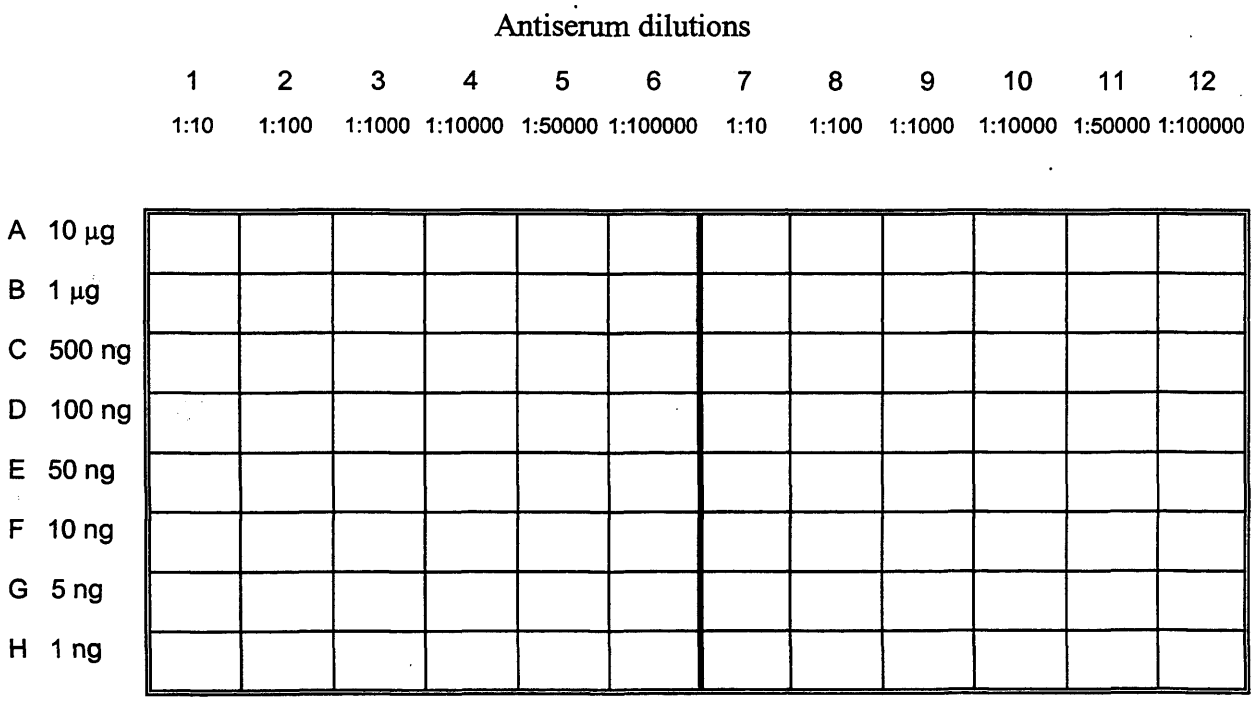


Figure 6.2. Coating concentrations for Checkerboard ELISA

- The plates were then stored at room temperature (wrapped to protect from the dust and kept in the dark) or used immediately.

- The plates were washed with PBS/0.005% Tween (6 times, first time submerging the plates in the bath and leaving for 2 minutes). They were then dried by tapping onto absorbent towel.
- **Optional Blocking Step:** To each well was added aliquots (25 μL) of 1% ovalbumin solution. The plates were covered and allowed to incubate at RT for 40 min. The plates were the emptied by inversion and washed with PBS/0.05% Tween (6 times). They were then dried by tapping onto absorbent towel.
- To each well was added PBS (25 μL), followed by the serum (or monoclonal antibody) at various dilutions (25 μL). The plate was covered and allowed to incubate at RT for 90 min.
- Each well was filled with PBS/0.005% Tween, and the plate emptied by inversion. This step was repeated once more and the plate washed in a bath a further 4 times.
- Horseradish peroxidase-linked goat anti-mouse immunoglobin G (Sigma, 50 mL of a 1:10000 dilution in PBS) was added to each well; the plates were covered and allowed to incubate at RT for another 90 min.
- The plates were then emptied by inversion and washed 6 times with PBS/0.005% Tween and once with distilled water. Dried by tapping onto absorbent towel.
- Enzyme substrate in 0.05 M citrate buffer pH 5.3 (50 μL) was added to each well and the plates were allowed to incubate at RT on a plate shaker for 15 min to allow a blue colour to develop.
- 1 M HCl (50 mL) was added to each well, and the optical density is measured of each well is measured at 450 nm on an automatic plate reader (Labsystems Multiskan Plus).

6.4.3.7. ELISA Protocol for Assaying Antibody Activity of Cell Supernatants

- A 96 well (8 x 12) polystyrene microtitre plate was filled with a solution of coating antigen (Ov-N7-ethyl-N^2-carboxymethylguanine or Ov-N7-ethyl-N^2-(3-carboxypropyl)guanine, 500 ng/40 μL/well) and dried overnight at 40°C.
- The plates were then stored at room temperature (wrapped to protect from the dust and kept in the dark) or used immediately.

- The plates were washed with PBS/0.005% Tween (6 times, first time submerging the plates in the bath and leaving for 2 minutes). They were then dried by tapping onto absorbent towel.
- To each well was added PBS (25 μL), followed by the cell supernatants and tissue culture medium (25 μL). The plate was covered and allowed to incubate at RT for 90 min (Figure 6.3.).

	1	2	3	4	5	6	7	8	9	10	11	12
A	CS1	CS1	CS2	CS2	CS3	CS3	CS4	CS4	CS5	CS5	CS6	CS6
B	CS8	CS8	..	..	..	..	..	..	..	..	CS12	CS12
C												
D												
E												
F												
G												
H									TCM	TCM	TCM	TCM

Figure 6.3. ELISA for checking cell supernatants

(CS, cell supernatants; TCM, tissue culture medium)

The rest of the protocol was carried out as described in section 6.4.3.6.

6.4.3.8. Protocol for Inhibition ELISA

This protocol applies to all samples received: cell supernatants and monoclonal antibodies.

- The first four rows of a 96 well (8 x 12) polystyrene microtitre plate were filled with a solution of coating antigen (Ov-N7-ethyl-N^2-(3-carboxypropyl)guanine, 100 ng/40 μL/well) and dried overnight at 40°C.
- The plates were then stored at room temperature (wrapped to protect from the dust and kept in the dark) or used immediately.

- The plates were washed with PBS/0.005% Tween (6 times, first time submerging the plates in the bath and leaving for 2 minutes). They were then dried by tapping onto absorbent towel.
- To each well was added aliquots (25 μL) of 1% ovalbumin solution. The plates were covered and allowed to incubate at RT for 40 min. The plates were the emptied by inversion and washed with PBS/0.05% Tween (6 times). They were then dried by tapping onto absorbent towel.
- To each well was added an alkylated base (or normal base) in PBS (25 mL), control (25 μL of PBS), or blank (50 μL of PBS). Into all the wells, except for the blank's column, were added a 1:10 dilution of cell supernatants or monoclonal antibody (25 μL). The plates were covered and left to incubate at RT for 90 min (Figure 6.4.).

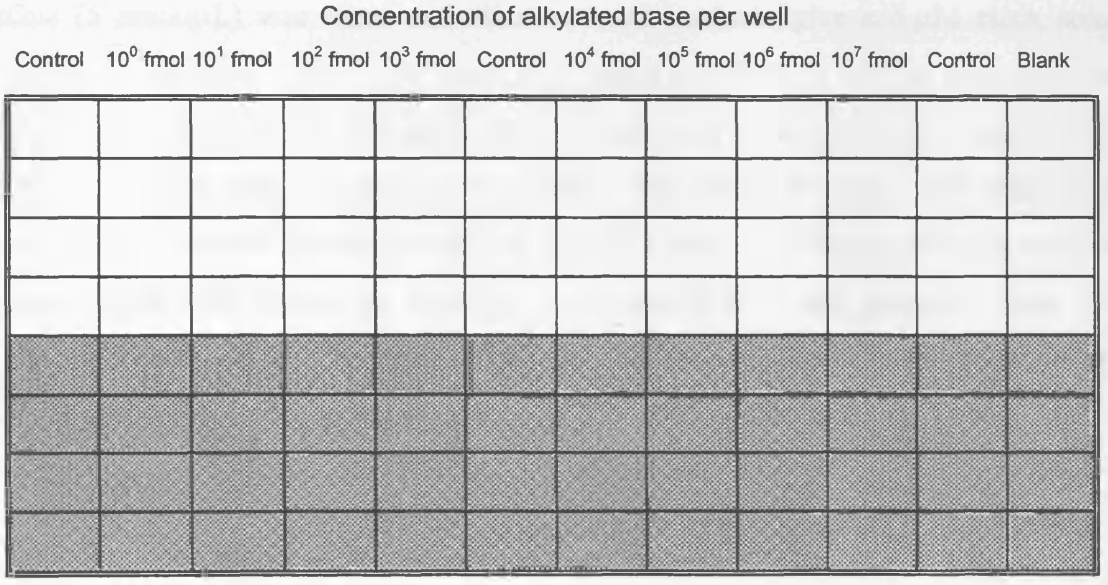


Figure 6.4. Coating concentrations of alkylated bases for inhibition ELISA (shaded areas represent wells not used).

The rest of the protocol was carried out as described in section 6.3.3.5. From the optical density data obtained, inhibition curves were plotted

6.4.4. Preparation and Application of N7-Ethylguanine Immunoaffinity Columns

6.4.4.1. Preparation of N7-Ethylguanine Immunoaffinity Columns

The procedure was identical to that described for the preparation of 7-MeGua immunoaffinity columns in section 6.2.6.2. The only difference being that ascites fluid (2.5 mL, containing IgG at 10 mg/mL) was added to the washed Protein A-Sepharose CL4B gel, and not the IgG fraction. Also, the mixture was made up to 50 mL with Tris buffer (32.5 mL).

6.4.4.2. Determination of the Capacity of the Columns

The capacity of the columns was determined by diluting the gel from one of the columns into five further columns (1:5 dilution), using Sepharose CL4B gel. A 5 mM stock solution of 7-EtGua (5 nmol/μL) was taken and dilutions were made to give a 5 μM stock solution (5 pmol/μL). Aliquots of the 5 μM stock were taken into PBS (2 mL) to give solutions of 7-EtGua ranging from 100 to 900 pmol. These solutions were applied to the immunoaffinity columns and eluted with 1 M acetic acid (2 mL). The eluted fractions were evaporated to dryness, and the residues re-dissolved in 0.01 M HCl (100 μL). Aliquots (25 mL) were taken and derivatised with Phmal as described in section 6.2.5., and analysed using HPLC fluorescence (section 6.4.2., system 5). The procedure was repeated for 7-MeGua., to check for binding.

6.4.4.3. Determination of N7-Ethylguanine Recovery from the Columns

The procedure was identical to that described in section 6.2.8., and samples analysed using HPLC fluorescence (section 6.4.2., system 5). Standards of 7-EtGua (10 μL from stocks of 1, 2, 3, 4, 5 μM) were added to PBS solutions (2 mL). An aliquot (10 μL) of 3 μM 7-MeGua (30 pmol) was added as an internal standard.

6.4.4.4. *N7-Ethylguanine Produced in Calf Thymus DNA on Exposure to Diethylsulphate*

Stock solutions of CT DNA incubated with varying concentrations of DES were supplied by Dr. R. Singh and were prepared as follows: (taken from PhD Thesis, 1997, Singh R.) Stock solutions (5.0ml) of 1, 5, 10, 50 and 100mM DES dissolved in 0.5M sodium phosphate buffer (pH 6.0), were incubated with CT DNA (50mg) for 8 h at room temperature. The CT DNA was then precipitated out by the addition of ice-cold 3M sodium acetate (0.5mL) followed by ice-cold 2-propanol (4.0mL), subsequently washed with absolute ethanol followed by 70% ethanol, and dissolved in SSC buffer (1.5mM NaCl, 150μM trisodium citrate, 1.0μM EDTA, pH 7.3). The concentration of CT DNA was calculated by determining the absorbance at 260nm (assuming that $A_{260nm} = 1$ is equivalent to 50μg/mL) against SSC buffer as the blank.

6.4.4.4.1. Quantitation by HPLC-HPLC-ECD

Quantitation of 7-EtGua adducts was carried out using HPLC-HPLC-ECD by Dr. R. Singh as follows: (taken from PhD Thesis, 1997, Singh R.) The CT DNA (100μg) was lyophilised, re-dissolved in 100μl of 0.1 M formic acid (pH 2.3) and heated at 70°C for 1h. The total hydrolysed reaction volume was injected onto a Gilson HPLC system fitted with a Jones Chromatography Apex C_{18} column (4.6 mm x 25 cm) and a HPLC Technology Techsphere C_{18} guard column (3.0 mm x 10.0 mm) with UV absorption monitored at 254nm an Applied Biosystems 1000S Diode array detector. The sample was initially eluted with 100% 50mM ammonium formate (pH 5.4) and was reduced to 80% with methanol over 15 min and taken back to 100% over the final 5 min, at flow rate of 1ml/min. The fraction corresponding to 7-EtGua was collected, lyophilised and re-dissolved in water. This sample was then subjected to isocratic reverse phase HPLC with electrochemical detection to determine the level of 7-EtGua. The HPLC-ECD system consisted of a Gynkotek Model 300 pump and a pulse damper which were connected to an Antec EC-detector containing a VT-03 electrochemical detector flow cell that was fitted with a 50μm spacer. The cell and HPLC column were housed in a Faraday cage. The optimum oxidation potential of 1.10V for the detector cell was determined by chromatographing a standard sample of 7-EtGua at range of voltage settings. An aliquot of the DNA sample was injected onto the above described HPLC system fitted with a Shandon Hypersil C_{18} column (4.6 mm x 25 cm) and eluted using 25mM H_3PO_4-KOH (pH 6.0)/ methanol (90:10, v/v) at a flow rate of 1mL/min. The mobile phase buffer was

prepared by adjusting the pH of a 25mM solution of phosphoric acid (ECD grade) to pH 6.0 with 5 M KOH. The level of 7-EtGua in each DNA sample was determined from a calibration line constructed by plotting the signal response (peak height) obtained from a series of 7-EtGua standards. The 7-EtGua standards were prepared by the serial dilution (with water) of a stock solution of 7-EtGua which was dissolved in 0.1 M formic acid (pH 2.3).

6.4.4.4.2. Quantitation by Immunoaffinity-HPLC Fluorescent Postlabelling

Aliquots (500 μL) of each of the five CT DNA solutions, incubated with varying concentrations of DES, were taken and heated at 100°C for 30 min to depurinate the CT DNA at alkylated sites. The solutions were then cooled and CT DNA precipitated by addition of ice-cold 3M NaCl (50 μL) and ice-cold 2-propanol (1 mL). The CT DNA was pelleted and the supernatants quantitatively transferred to 15 mL polypropylene tubes. The supernatants were allowed to evaporate to dryness. The dried residues were dissolved in PBS (2 mL) and passed through 7-EtGua immunoaffinity columns. Elution of the retained base was by 1 M acetic acid. The eluted fractions were collected and allowed to evaporate to dryness. The residues were re-dissolved in 0.01 M HCl (100 μL) and aliquots (10 μL) transferred to reacti-vials with 7-MeGua as an internal standard. Derivatisation with Phmal was carried out and the samples prepared for HPLC fluorescence. Samples were run on a Waters/Millipore HPLC system as described earlier (section 6.4.2., system 5), with injection volumes of 10 μL.

6.4.4.5. Quantitation of N7-Ethylguanine Produced in Calf Thymus DNA on Exposure to Tobacco Smoke

Aliquots of CT DNA solution (2 mL; 5 mg/mL of sodium chloride/ trisodium citrate buffer/ 1 μM EDTA; SSC buffer) were added to a 10 mL round-bottomed flask. A further aliquot of SSC buffer (1 mL) was added to increase the volume of the reaction medium. The CT DNA was then exposed to the tobacco smoke from 5 cigarettes (Benson and Hedges; 12 mg Tar, 0.9 mg Nicotine), using the apparatus shown in Figure 6.1. The CT DNA was precipitated by the addition of ice-cold 2-propanol (2 x vol., 6 mL). The liquid was decanted off and the CT DNA was rinsed with two further aliquots of 2-propanol (2 x 3 mL). The CT DNA was re-dissolved in SSC buffer (0.75 mL) by end-over-end stirring. The solutions were heated at 100°C for 30 min to depurinate the DNA at alkylated sites. The solutions, were again cooled

in ice and the DNA re-precipitated (*ca.* 2 hours at -20°C) by the addition of 1/10 volume of ice-cold 3 M sodium chloride solution followed by the addition of 2 vol of ice-cold 2-propanol. The DNA was pelleted and the supernatants were transferred to 15 mL polypropylene tubes and evaporated to dryness. The dried residues were re-dissolved in PBS (2 mL) and the PBS solutions applied to the immunoaffinity columns. Fractions of 7-EtGua were collected (see section 6.2.8.1.) and allowed to evaporate down to dryness. Unexposed CT DNA was used as controls. The dried fractions were re-dissolved in 0.01 M HCl (70 μL) and derivatised with Phmal (see section 6.2.7.) using 7-MeGua as an internal standard (10 μL of a 3 μM solution), and the samples were analysed using HPLC fluorescence (section 6.4.2., system 5).

6.4.5. Production of Polyclonal Antibodies in Chickens Against N7-Ethylguanine

6.4.5.1. Immunisation Protocol for the Production of Polyclonal Antibodies

All procedures were carried out by staff in the animal house, CMHT, MRC Toxicology Unit, University of Leicester, Leicester.

Animals and Housing

Number/sex:	2/F	**Species/strain:**	Chicken
Number per cage:	1	**Identification:**	unknown

Protocol

Before Day 0: Collect eggs

Day 0: First injection set. Antigen (mBSA-N7-ethyl-N^2-carboxymethylguanine, 40 μg) was dissolved in 50% 10 mM phosphate buffer (pH 7.2) and 50% Complete Freund's adjuvent (750 μL). Two intramuscular injections (2 x 750 μL) in the pectoral muscle of each chicken were made.

Day 12: Second injection set. Two im injections (210 mg/750 mL) in the pectoral muscle of each chicken were made.

Day 20: Third injection set. Each chicken treated as Day 12.

Eggs collected from last day of injection, and stored at 4°C.

6.4.5.2. Immunoglobulin Y (IgY) Extraction from Chicken Eggs

Reagents supplied by Promega, and protocol taken from Promega technical bulletin.

Eggs (2 x 2) from each hen were taken:

Hen 1: eggs laid on Day 46 and Day 48
Hen 2: eggs laid on Day 47 and Day 48

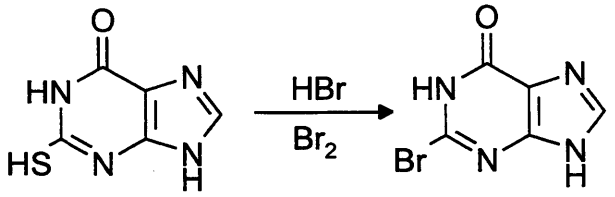
- The eggs were allowed to warm to room temperature. A 100 mL beaker, with stir bar, was tared.
- Each egg was cracked and the egg white poured off using an egg separator. The residual egg white was removed with a gauze sheet, taking care not break the yolk sack. The yolk sack was then ruptured with a pipette and the contents were allowed to drip into the tared beaker. The yolk sack was then discarded, and the weight of the egg yolk measured (Hen 1: 31.8 g, Hen 2: 24.4 g).
- The yolks were stirred at RT and 3 volumes of Precipitation Solution A was added slowly (assuming 1 g of yolk equal to 1 mL). The yolk mixture was continuously stirred for 5 min to precipitate the lipids. The mixture was then centrifuged at 4°C for 10 min at 10000 G.
- The supernatant was collected into a measuring cylinder, filtering it through a filter disc. The volume of the supernatant was measured and then it was transferred to a clean beaker containing a stirrer. The pellet was discarded.
- The supernatant was stirred at RT and 1/3 volume of Precipitation Solution B was slowly added. The solution was continuously stirred for 5 min to precipitate the IgY. The mixture was then centrifuged at 4°C for 10 min at 10000 G.
- The supernatant was poured off and discarded. To increase the IgY purity, it was resuspended in a further portion of Precipitation Solution B, stirred and re-centrifuged.
- The IgY pellet was resuspended in PBS equal to the original volume of the egg yolk, and stored at -20°C.

6.4.5.3. Determination of Chicken IgY in PBS

The IgY solution was taken from -20°C, and allowed to warm to room temperature. A 100 μL aliquot was diluted in 1 mL of water and the absorbance measured at 280 nm. The concentration was determined using 13.3 mL/mg as the extinction coefficient, (A_{280}/13.3 = concentration in mg/mL). The chicken IgY was tested for antibody activity by running an ELISA as described in previous sections.

6.4.6. A General Synthesis of N7-Alkylated Guanine Haptens

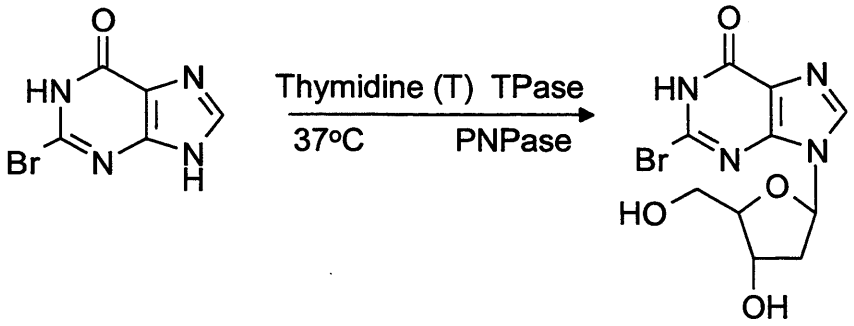
6.4.6.1. Synthesis of 2-Bromo-6-hydroxypurine



Beaman *et al.*, 1962.

2-Thioxanthine (5 g, 30 mmol) was poured into a cooled solution of 48% aqueous HBr (50 mL) and methanol (20 mL). The solution was stirred in an ice-bath and bromine (10 mL) was added *via* a syringe over a period of 45 min, whilst maintaining a low temperature. The solution was stirred at 5-6°C for a further 4 h. The suspension was filtered and the moist solid washed with cold acetone. The solid was then slurried with cold water, filtered and washed with acetone and water. The dried solid was then transferred into a beaker with water (40 mL). 1 M NaOH was added until no more solid dissolved. The solution was filtered and the residue washed with cold water. The filtrate was made acidic by dropwise addition of 5 M HCl, which afforded the product as a pale yellow precipitate. This was filtered and allowed to dry over P_2O_5. Yield, 1.46 g (23%); MS *m/z* 215 and 217 $(M+H)^+$

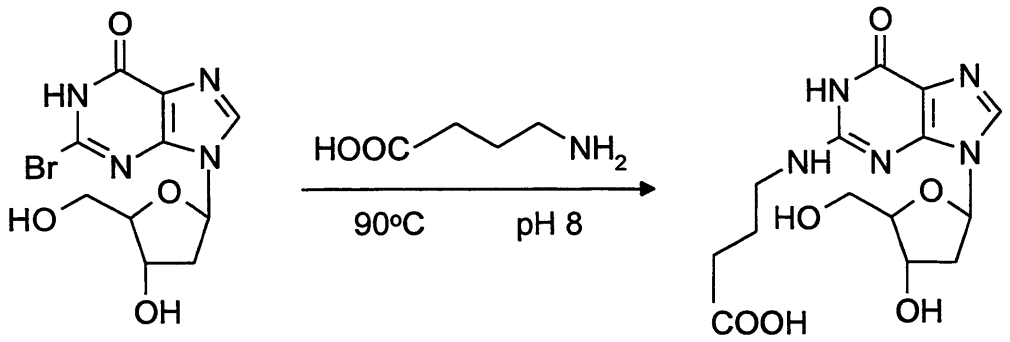
6.4.6.2. Formation of 2-Bromo-2'-deoxyinosine



Pongracz and Bodell, 1996.

2-Bromo-6-hydroxypurine (216 mg, 1.01 mmol), and thymidine (242 mg,) were dissolved in 20 mM potassium phosphate buffer pH 7.4 (100 mL) at pH 7.4. The solution was incubated overnight at 37°C with 60 units of thymidine phosphorylase (EC 2.4.2.4., Sigma) and 90 units of purine nucleoside phosphorylase (EC 2.4.2.1., Sigma). The solution was evaporated to dryness and the residue dissolved in water (50 mL). The solution was placed in a flask with 1 g of silica gel (Keiselgel 60) and evaporated to dryness. A silica gel column (2.0 cm x 20 cm) was prepared with chloroform/methanol/triethylamine (80:15:5) as the eluting solvent. The silica gel containing the product was placed on top of the column, and the product was eluted as the third fraction. Yield, 155.38 mg (47%); MS *m/z* 331 and 333 $(M+H)^+$

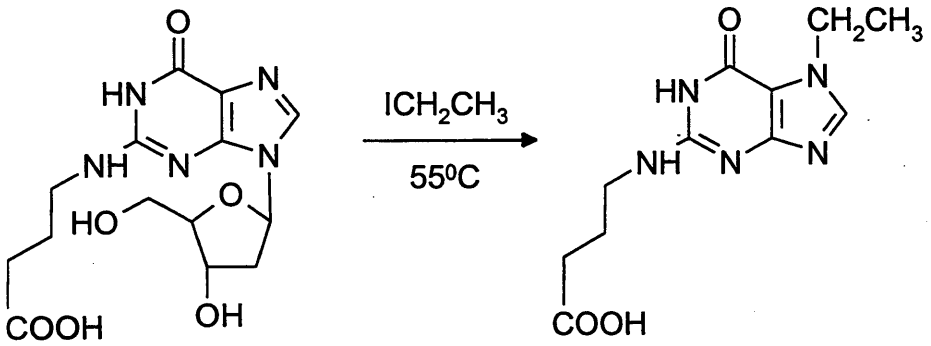
6.4.6.3. Synthesis of N^2-Carboxypropyl-deoxyguanosine



An aqueous solution (1 ml) of 2-bromo-2'-deoxyinosine (100 mg, 0.30 mmol) was added to an aqueous solution (4 mL) of 4-aminobutyric acid (625 mg, 6.06 mmol) and sodium

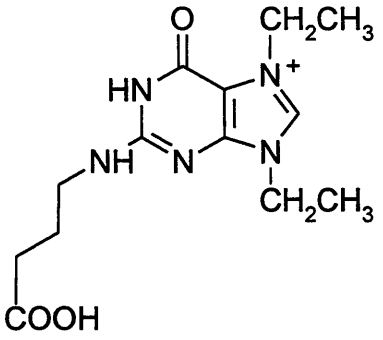
bicarbonate (509 mg, 6.06 mmol). The pale yellow solution was allowed to heat for *ca.* 42 h at 85-90°C. The reaction was followed by HPLC/UV (section 6.4.2., system 3). An aliquot (10 µL) was taken into water (90 µL) and 10 µL used for injecting. The product was isolated by separation on a semi-preparative column, using the same solvent conditions as aforementioned (section 6.4.2., system 4). Yield, 55.9 mg (52%); ^{1}H NMR (D_2O) 1.9 (m, 2H, CCH_2C), 2.45 (t, 2H, $CC\underline{H}_2N$), 2.55 and 2.95 (m, 2H, H-2'), 3.5 (t, 2H, $OOCC\underline{H}_2C$), 3.8 (m, 2H, H-5'), 4.15 (m, 1H, H-4'), 4.65 (m, 1H, H-3'), 6.35 (t, 1H, H-1'), 8.0 (s, 1H, $C_8\underline{H}$), 4.8 (H_2O), 0.65 (*impurity*), 2.25 (*impurity*); MS *m/z* 354 $(M+H)^+$

6.4.6.4. Synthesis of N7-Ethyl-N^2-(3-carboxypropyl)-guanine



N^2-(3-carboxypropyl)-guanine (34.78 mg, 0.099 mmol) was dissolved in DMF (4 mL). Pyridine (79.6 µL, 0.99 mmol) and iodoethane (613.37 µL, 9.9 mmol) were added, and the mixture heated at 55°C for 3.5 h. The reaction was followed by HPLC/UV (section 6.4.2., system 4, UV absorbance monitored at 278 nm). The product was purified using semi-preparative HPLC (section 6.4.2., system 4, UV abs. monitored at 278 nm). Yield, 5.67 mg (21%); ^{1}H NMR (D_2O) 1.45 (t, 3H, $-CH_2C\underline{H}_3$), 1.9 (m, 2H, CCH_2C), 2.3 (t, 2H, $CC\underline{H}_2N$), 3.35 (t, 2H, $OOCC\underline{H}_2C$), 4.3 (q, 2H, $-C\underline{H}_2CH_3$), 7.9 (s, 1H, $C_8\underline{H}$), 4.8 (H_2O) ppm; ^{13}C nmr (D_2O) 16.7 ($-CH_2\underline{C}H_3$), 26.1 ($C\underline{C}H_2C$), 35.2 ($C\underline{C}H_2N$), 40.9 ($00C\underline{C}H_2C$), 42.3 ($-\underline{C}H_2CH_3$), 143.2 (C-8), 163.9 (-COOH) ppm; MS *m/z* 268 $([M-d_2]+H)^+$ (*note: mass spectral analysis carried out in D_2O*).

6.4.6.4.1. N7, N9-Diethyl-N^2-(3-carboxypropyl)guanine; A Side Product



The above reaction also afforded the diethyl product in approximately the same quantity as the monoethylated hapten, and was isolated as described above. Yield 7.41 mg (26%); ^{1}H NMR (D_2O) 1.45 (t, 3H, -$CH_2C\underline{H}_3$), 1.9 (m, 2H, CCH_2C), 2.3 (t, 2H, $CC\underline{H}_2N$), 3.35 (t, 2H, $OOCC\underline{H}_2C$), 4.3 (q, 2H, -$C\underline{H}_2CH_3$), 7.9 (s, 1H, $C_8\underline{H}$), 4.8 (H_2O) ppm; MS *m/z* 294 $(M)^+$

6.4.7. Protein Conjugation of N7-Ethyl-N^2-Carboxypropylguanine with Ovalbumin and Quantitation of Hapten Bound to Protein

Ovalbumin (25.25 mg, grade VII) was dissolved in water (2.0 mL) and the pH adjusted to 6.5 by dropwise addition of saturated $NaHCO_3$ solution. EDC (6.5 mg, *ca.* 66 μmol) was then added. A solution of N7-ethyl-N^2-(3-carboxypropyl)guanine (4.36 mg, 33 μmol in 400 μL H_2O) was added dropwise over a period of 1 h, whilst maintaining the pH at 6.5. The experiment was carried out in the dark. The solution was left to stir overnight. The protein bound hapten was then purified using a Sephadex G50 column (flow rate 2.0 mL/min) as described in section 6.3.3.2. UV absorbance was carried out on a KONTRON UVIKON 860 at 278 nm. The blank was a solution of ovalbumin (100 μg/mL). Ov was dissolved in water to give a 100 μg/mL solution. Molecular weight of protein was taken to be 45000 amu (100 μg = 2.22 nmol). This solution was spiked with N7-ethyl-N^2-(3-carboxypropyl)guanine (hapten) to give

1 mol hapten/ mol Ov
2 mol hapten/ mol Ov
3 mol hapten/ mol Ov
4 mol hapten/ mol Ov
5 mol hapten/ mol Ov

Stock solution of hapten = 0.78 mg/mL (2.93 μmol/mL). A 37.9 μL aliquot was made up to 1 mL in Ov solution to give a 29.6 μg/mL solution of the hapten. Aliquots of this stock solution were taken into 1 mL Ov solution for UV analysis.

20 μL aliquot gives 2.22 nmol/mL
40 μL aliquot gives 4.44 nmol/mL
60 μL aliquot gives 6.66 nmol/mL
80 μL aliquot gives 8.88 nmol/mL
100 μL aliquot gives 11.11 nmol/mL

The UV absorbance of the protein bound hapten (100 μg/mL) was also measured and compared to the calibration data, to give amount of protein conjugation.

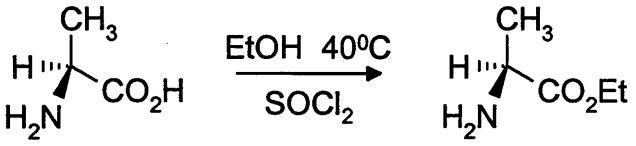
6.5. Experimental Methods for Chapter 5

6.5.1. Chemicals and Reagents

Thionyl chloride (Aldrich), Indanetrione hydrate (Hopkin and Williams Ltd., Chadwell Heath, England), Pyruvic acid (Aldrich), p-Toluenesulphonyl hydrazide (Aldrich), O-(N-Succinimidyl)-N,N,N,N-tetramethyluronium tetrafluoroborate (Aldrich), Diisopropylethyl-amine (Aldrich), L-Alanine (Sigma).

6.5.2. Synthesis of Ethyl-2-diazopropanoate from L-Alanine

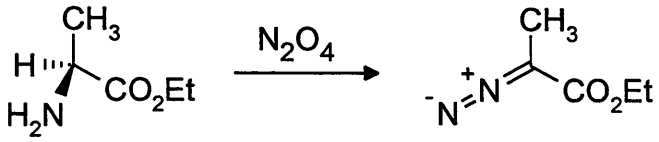
6.5.2.1. Esterification of L-Alanine



Blau and King, 1993.

L-Alanine (1 g, 11.2 mmol) was added to ethanol (8 mL) in a 25 mL round-bottomed flask. The solution was cooled in a CO_2-acetone bath. Thionyl chloride (842 μL, 11.5 mmol) was added in 100 μL aliquots whilst shaking the flask. The mixture was allowed to come to room temperature and then heated at 40°C with stirring. The reaction was followed by TLC (MeOH:$CHCl_3$/1:1), using ninhydrin (5% w/v indanetrione hydrate in acetone) for development. A stream of nitrogen was used to blow off the SO_2 gas in solution and also the solvent. The ester crystallised out to give a white mass. The solid was re-crystallised from ethanol and water, to afford shiny white crystals of L-alanine ethyl ester hydrochloride. Yield, 1.66 g (97%); ^{1}H NMR (d-MeOH) 1.35 (t, 3 H, -$CH_2\underline{CH_3}$), 1.6 (d, 3 H, -CH_3), 4.1 (q, 1 H, -CH-), 4.3 (q, 2 H, -$\underline{CH_2}CH_3$), 4.85 (H_2O), 3.3 (*impurity*) ppm; MS *m/z* 118 $(M+H)^+$, 235 $(2M+H)^+$, 388 $(3M+HCl)^+$, 424 $(3M+2HCl)^+$

6.5.2.2. *Diazotisation of L-Alanine Ethyl Ester by Dinitrogen Tetroxide*



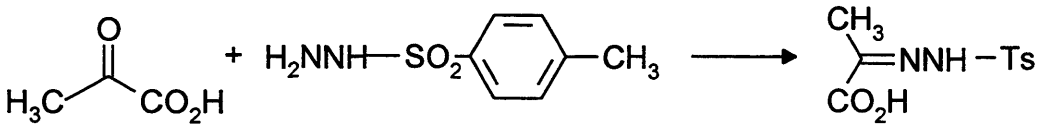
Challis and Latif, 1990.

Alanine ethyl ester hydrochloride (499.03 mg, 3.26 mmol), was dissolved in dichloromethane (20 mL). Triethylamine (1.18 mL, 8.54 mmol) was added with anyhydrous sodium sulphate (250 mg). The solution was cooled to -40°C ($CO_{2(s)}$-acetonitrile) with stirring. An ice/$CO_{2(s)}$/water slurry was prepared, in which N_2O_4 gas (brown) was carefully liquefied. The N_2O_4 liquid [blue] (0.27 mL, 4.3 mmol) was then added to cold dichloromethane (10 mL). The dichloromethane solution was gradually added to the solution of alanine ethyl ester. The resulting yellow solution was allowed to warm to room temperature, and then washed with water (5 mL) and 0.1 M $NaHCO_3$ (5 mL). The dichloromethane layer was allowed to dry overnight with anhydrous sodium sulphate, at 4°C in the dark. The solvent was evaporated off on a rotary evaporator to afford a yellow oil, which was purified on silica gel column (1.0 cm x 10 cm).

Eluting solvent: petroleum ether 40-60°C:Diethyl ether/1:1. Fractions were pooled, and solvent evaporated off, to afford a small amount of yellow oil. ^{1}H NMR ($CDCl_3$) 1.35 (t, 3 H, -$CH_2C\underline{H}_3$), 1.6 (d, 3 H, -CH_3), 4.3 (q, 2 H, -$C\underline{H}_2CH_3$), 5.4 (q, 1 H, -CH-), 7.35 (*impurity*) ppm.

6.5.3. Synthesis of Potassium 2-Diazopropanoate from Pyruvic Acid

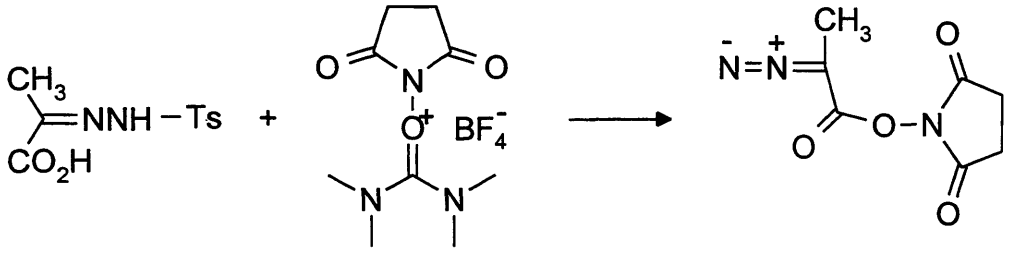
6.5.3.1. Synthesis of Pyruvic Acid p-Toluenesulphonyl Hydrazone



Ouihia *et al.*, 1993.

Pyruvic acid (1.264 mL, 18.4 mmol) was added to water (25 mL) in a 100 mL conical flask. This was heated to 60-70°C and then treated with a warm solution of p-toluenesulphonyl hydrazide (4.23 g, 22.7 mmol) in 12 mL of aqueous 2.5M HCl. The resulting mixture was heated in an oil bath with continuous stirring until all the hydrazone, which initially separated as an oil, solidified (this occurred almost immediately). The reaction mixture was allowed to come to RT and then filtered. The white solid was washed with cold water (15 mL) and left to dry over P_2O_5. The white solid (*ca.* 4 g) was re-crystallised from ethyl acetate, and the product washed with cold chloroform:ethyl acetate/2:1. Yield, 3.34 g (71%); ^{1}H NMR (D_2O) 2.04 (s, 3 H, $NCCH_3$), 2.48 (s, 3 H, Ar-CH_3), 7.48 (d, 2 H, H_a), 7.96 (d, 2 H, H_b), 4.96 (H_2O) ppm; MS *m/z* 257 $(M+H)^+$, 101 $(CH_3C(COOH)NNH)^+$, 91 $(Ar\text{-}CH_3)^+$

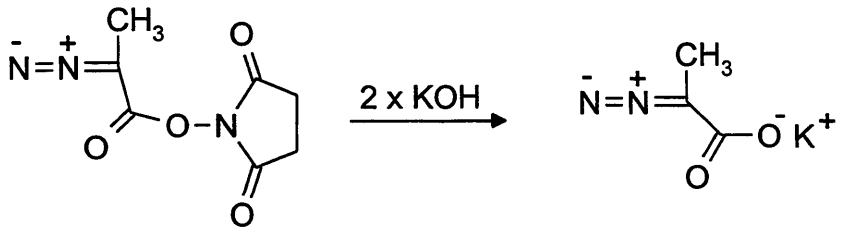
6.5.3.2. Formation of O-(N-Succinimidyl)-2-diazopropanoate



The pyruvic acid tosyl hydrazone (0.9947 g, 3.9 mmol) was dissolved in DMF (60 mL) and stirred at room temperature. O-(N-Succinimidyl)-N,N,N,N-tetramethyluronium tetrafluoroborate (1.29 g, 4.29 mmol) and diisopropylethylamine (747 μL, 4.29 mmol; Aldrich) were also added. The reaction was monitored by TLC; ethyl acetate:petroleum ether

(60-80°C)/3:2. After 4.5 h, the reaction was stopped, and the solution cooled. It was then passed through a basic alumina column (1.0 cm x 20 cm), to remove acidic starting materials. The column was then washed with ethyl acetate (15 mL). The solution was concentrated down to a smaller volume (*ca.* 1 mL). The solution was purified on a silica gel column (3.0 cm x 12 cm), eluting with ethyl acetate:petroleum ether (60-80°C)/3:2, to afford a yellow crystalline solid. Yield, 118 mg (15%); ^{1}H NMR (D_2O) 2.5 (s, 3 H, -CH_3), 2.85 (s, 4 H, -CH_2CH_2-), 4.85 (H_2O) ppm; MS *m/z* 257 $(M+H)^+$, 349 $(M+Glyerol+1)^+$; IR (nujol mull) 1750 (C=O), 2100 (N=N) cm^{-1}

6.5.3.3. Formation of Potassium 2-Diazopropanoate



The O-(N-succinimidyl)-2-diazopropanoate (13.5 mg, 68.5 μmol) was dissolved in THF (135 μL) in a 1 mL reacti-vial. The solution was allowed to stir, and 1 M KOH (137 μL, 137 μmol) was added. The reaction was monitored by TLC (ethyl acetate:petroleum ether (60-80°C)/3:2). After 40 minutes, the yellow potassium salt (product spot) was observed at the origin. The reaction solution was kept as a stock (250 mM) at -20°C.

6.5.4. A Kinetic Study of the Decomposition of Potassium 2-Diazo-propanoate Related to pH at 37°C

A KONTRON UVIKON 860 UV spectrophotometer set at 278 nm, was employed for this study.

SSC buffer (1 mL aliquots) was taken and the pH altered to give a range from pH 2.0 to pH 10.0, by addition of 0.01 M HCl or 0.01 M NaOH. From the stock of 250 mM potassium 2-diazopropanoate, a 40 μL aliquot was added to 0.96 mL of water, to give 10 mM solution. Aliquots (10 μL) of this 10 mM stock were added to the buffered SSC (0.99 mL). The solutions were placed in quartz cuvettes, and placed in the uv spectrophotometer, at 37°C. The absorbance of the buffered solutions was measured at a sampling rate of 5 sec/sample every 15 min for a period of 900 min, except for the buffered solution at pH 2.0, where the measurements were made every 30 sec for 15 min.

6.5.5. Reaction of Potassium 2-Diazopropanoate with Calf Thymus DNA

Aliquots of CT DNA solution (6 x 200 μL; 5 mg/mL of 1.5 M sodium chloride/ 150 μM trisodium citrate buffer/ 1 μM EDTA; SSC buffer) were incubated overnight at 37 °C with an aliquot (22.2 μL) of 125 mM potassium 2-diazopropanoate. The incubations were done in triplicate. After incubating, the solutions were heated at 100°C for 30 min to depurinate the modified bases. The solutions, were cooled in ice and the DNA re-precipitated (*ca.* 2 hours at -20 °C) by the addition of 1/10 volume of ice-cold 3 M sodium chloride solution followed by the addition of 2 volumes of ice-cold 2-propanol. The DNA was pelleted and the supernatants were transferred to 1.5 mL Eppendorf tubes and evaporated to dryness. The residues were re-dissolved in PBS (2 mL)of which three were passed through 7-EtGua immunoaffinity columns and the other three passed through 7-MeGua columns. The eluant was collected and derivatised with Phmal. The derivatives were analysed using HPLC fluorescence (section 6.4.2., system 5).

Summary

Summary

As the title of this thesis explains, the aim of this work was to develop and apply a fluorescent postlabelling assay to detect and quantify 7-AlkGua adducts. In order to successfully achieve the objective, various determinants had to be considered, and these were primarily dealt with in the early stages of the work. As the possibility of using Phmal derivatives of 7-AlkGua adducts as biomarkers of recent exposure to alkylating agents was to be considered, it was important to be able to efficiently derivatise the adducts at picomole levels. This was achieved with the incorporation of a molecular sieve to trap water produced during the reaction. For enhanced sensitivity of the assay, the use of narrow-bore HPLC columns was employed to give greater peak heights, reducing signal-to-noise ratio. A further technique employed to improve the sensitivity of the fluorescent postlabelling assay, was to include an adduct isolation step prior to derivatisation, namely immunoaffinity purification. Quantitation of very low levels of N7-alkylated guanine adducts can be hampered by the presence of interfering compounds (*i.e.* depurinated guanine in DNA hydrolysates), therefore, not only did this step increase sensitivity by reducing background interference, but also allowed for greater selectivity. This novel fluorescent postlabelling assay has the potential for wide applicability for a range of 7-AlkGua adducts, and coupling with immunoaffinity purification allows the selectivity of a particular adduct, ensuring good sensitivity. Potential 100-1000 fold increases in sensitivity are possible with laser-induced fluorescence (LIF) detection, as highlighted earlier, but an attempt to synthesise a fluorescent postlabelling reagent to be used with commercially available LIF detectors was unsuccessful.

The immunoaffinity fluorescence postlabelling assay was shown to be very sensitive and was exemplified by the detection and quantitation of 7-MeGua from DNA treated *in vitro* with DMS. The immunoaffinity columns were prepared from previously available antiserum against 7-MeGua. But for this assay to have a wider applicability, the need for antibodies against other adducts was necessary. Some work has been carried out on the production of polyclonal antibodies against the Phmal derivative, where the tricyclic ring moiety is the epitope (Shuker *et al.*, 1993), but this has the disadvantage of not having the selectivity that using a monoclonal antibody against the adduct has. Incorporation of interfering peaks would still be observed.

Much research has been carried out on the detection of N7-alkylated guanine adducts (especially for 7-MeGua and a little for 7-EtGua) in DNA exposed to tobacco smoke, both *in vitro* and *in vivo*. It was decided that the fluorescent postlabelling assay could be used to detect and quantitate 7-EtGua adducts in DNA exposed to tobacco smoke *in vitro* and possibly identify an ethylating agent in tobacco smoke. Therefore, a major part of this research was the production of a mouse monoclonal antibody against 7-EtGua. Mice were immunised with N^2-carboxymethyl-7-EtGua coupled with methylated bovine serum albumin, and after a couple of failed attempts, monoclonal antibodies were finally received. Tails-bleeds, cell supernatants and ascites fluid were all tested for antibody activity using ELISA studies. A method for the preparation of suitably functionalised 7-AlkGua derivatives for use in preparing and for testing antibody activity of monoclonal antibodies was successfully established based on the displacement of a halogen from the 2-position of guanine by 4-aminobutyric acid. Parallel work into the production of polyclonal antibodies against 7-EtGua from chickens was also carried out, with little success. It could not be confirmed that a polyclonal antibody had indeed been produced as an appropriate test coating antigen for use in ELISA studies was not available. As stated earlier the advantage in producing polyclonal antibodies from chickens is that they are obtained in a very short space of time and collecting eggs, in contrast to bleeding animals, is non-invasive.

The success observed in the production of mouse monoclonal antibody was not repeated in the subsequent detection and quantitation of 7-EtGua adduct in DNA exposed *in vitro* to tobacco smoke, using the immunoaffinity fluorescent postlabelling assay. This was attributed to experimental design and variation in cigarette composition. More recent work hypothesised that alanine in burning tobacco undergoes nitrosation and decarboxylation to form an ethylating agent, therefore variation in concentrations of this amino acid could explain why N7-ethylation of guanine was not observed. Quantification of 7-EtGua formed in CT DNA exposed to diazopropanoic acid (diazoalanine) was attempted using the immunoaffinity fluorescent postlabelling assay, but the experiment was unsuccessful. This was attributed to the stability of the diazopropanoate salt over a wide pH range. Pyrolysing the salt and attempting a gas-phase reaction would have been a more viable experiment to attempt.

A successful exemplification of the fluorescent postlabelling assay, incorporating the mouse monoclonal antibody against 7-EtGua, was the detection and quantitation of the adduct in DNA treated *in vitro* with diethylsulphate. The results were compared with a published assay, HPLC-ECD with HPLC pre-purification of the adduct. Both assays gave comparable results, with the fluorescent postlabelling assay showing higher recoveries. But it was the fluorescent postlabelling assay which was the more sensitive, as the HPLC-HPLC-ECD assay was as its limit of detection.

The immunoaffinity fluorescent postlabelling assay has shown to be a selective and sensitive method for the detection of 7-AlkGua adducts. Presently, the assay is not sensitive enough for the detection of adducts in DNA exposed *in vivo* to alkylating agents, unless it is known that the levels of adduct to be detected are very high (*e.g.* chemotherapeutic drugs). It may be modified in many ways to improve sensitivity and these include changing the chromatographic conditions to narrower columns, *i.e.* capillary electrophoresis and most importantly the incorporation of a LIF detector. Although, the assay was only applied successfully in two *in vitro* studies, the use of various monoclonal antibodies would allow a wider applicability for the assay.

Appendix

Appendix

Publications

Rana R.R., Singh R., Larkins A., James R. and Shuker D.E.G. (1998) Determination of N7-ethylguanine by immunoaffinity-fluorescence postlabeling. *Submitted to Chem. Res. Toxicol.*

Durand M.-J., Molko D., Bowman E., Weston A., Lamb J., Rana R.R. and Shuker D.E.G. (1998) Detection and quantitation of N7-alkylguanines by fluorescence postlabeling using phenylmalondialdehyde. *Submitted to Chem. Res. Toxicol.*

Poster Presentations

Rana R.R. and Shuker D.E.G. (1995) Detection of N7-alkylated guanines by a fluorescence post-labelling assay. UKEMS Meeting, 5-7 July, Leicester, UK.

Rana R.R. and Shuker D.E.G. (1996) Detection of N7-alkylated guanines by immunoaffinity purification, followed by HPLC-fluorescence postalbelling. BTS Spring Meeting, *The Bases for Selective Toxicity: Human Implications*, 1-3 April, York, UK.

Rana R.R. and Shuker D.E.G. (1997) Detection of N7-alkylated guanines by immunoaffinity purification, followed by HPLC-fluorescence postalbelling. ACS Meeting, *Symposium of the Division of Chemical Toxicology*, 7-11 September, Las Vegas, USA.

References

References

Agarwal S. and Draper H.H. (1992) Isolation of a malondialdehyde-deoxyguanosine adduct from rat liver DNA. *Free Rad. Biol. Med.*, **13**, 695-699.

Aitio A.A., Anderson D., Blain P., Bond J., Buratti M., Calder I., Chahoud I., Fowle J.R., Gerhardsson L., Henderson R., Koetër H.B.W.M. and Nishikawa A. (1992) In: *Biomarkers and Risk Assessment: Concepts and Principles*, Environmental Health Criteria 155, Switzerland.

Ariese F., Verkaik M., Hoornweg G.P., van de Nesse R.J., Jukema-Leenstra S.R., Hofstraat J.W., Gooijer C. and Velthorst N.H. (1994) Trace analysis of 3-hydroxy benzo[*a*]pyrene in urine for the biomonitoring of human exposure to polycyclic aromatic hydrocarbons. *J. Anal. Toc.*, **18**, 195-204.

Arnold Z. (1961) Synthetic reactions of dimethylformamide. XII. Formylation of some carboxylic acids and their derivatives. *Coll. Czech. Chem. Commun.*, **26**, 3051-3057.

Arnold Z. (1973) Synthetic reactions of dimethylformamide. XXV. Preparation of dimethylaminomalondialdehyde, 2-methoxy-, 2-ethoxy-, and 2-dimethylaminotrimethinium salts. *Coll. Czech. Chem. Commun.*, **38**, 1168-1172.

Azadnia A., Campbell R. and Sharma M. (1994) The scope of dansyl vs fluorescein label in fluorescence postlabelling assay for DNA damage. *Anal. Biochem.*, **218**, 444-448.

Beach A.C. and Gupta R.C. (1992) Human biomonitoring and the ^{32}P-postlabelling assay. *Carcinogenesis*, **13**, 1053-1074.

Beaman A.G., Gerster J.F. and Robins R.K. (1962) Potential purine antagonists. XVIII. The preparation of various bromopurines. *J. Org. Chem.*, **27**, 986-989.

Beranek D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res.*, **231**, 11-30.

Bianchini F. and Wild C.P. (1994) 7-Methyldeoxyguanosine as a marker of exposure to environmental methylating agents. *Tox. Lett.*, **72**, 175-184.

Bertz S.H. and Dabbagh G. (1983) Improved preparations of some arenesulphonylhydrazones. *J. Org. Chem.*, **48**, 116-119.

Blankley C.J., Sauter F.J. and House H. (1973) Crotyl diazoacetate. *Org. Syn.*, **V**, 258-263.

Blömeke B, Greenblatt M.J., Doan V.V., Bowman E.D., Murphy S.E., Chen C.C., Kato S. And Shields P.G. (1996) Distribution of 7-alkyl-2'-deoxyguanosine adduct levels in human lung. *Carcinogenesis,* **17**, 741-748.

Brookes P. and Lawley P.D. (1960) The reaction of mustard gas with nucleic acid *in vitro* and *in vivo*. *Biochem. J.*, **77**, 478-484.

Brookes P. and Lawley P.D. (1961) The reaction of mono- and di-functional alkylating agents with nucleic acids. *Biochem. J.*, **80**, 496-503.

Brown K., Burns P.A. and Balmain A. (1995) Transgenic approaches to understanding the mechanisms of chemical carcinogenesis in mouse skin. *Tox. Lett.*, **82/83**, 123-130.

Butlin H.T. (1892) Cancer of the scrotum in chimney-sweeps and others. II. Why foreign sweeps do not suffer from scrotal cancer. *Brit. Med. J.*, **2**, 1-6.

Carmella S.G., Borukhova A., Desai D. and Hecht S.S. (1997) Evidence for endogenous formation of tobacco-specific nitrosamines in rats treated with tobacco alkaloids and sodium nitrite. *Carcinogenesis*, **18**, 587-592.

Castegnaro M., Bresil H. and Manin J.P. (1993) Some safety procedures for handling ^{32}P during postlabelling assays. In: *Postlabelling Methods for the Detection of DNA Adducts.* (Phillips D.H., Castegnaro M. and Bartsch H., Eds.), IARC Scientific Publications No. 124, Lyon, IARC, 87-92.

Castonguay A., Lin D., Stoner G.D., Radok P., Furuya K., Hecht S.S., Schut H.A.J. and Klaunig J.E. (1983) Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of N'-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and their analogues. *Cancer Res.*, **43**, 1223-1229.

Challis B.C. and Ridd (1962) In: *Advanced Organic Chemistry. Reactions, Mechanism and Structures.* (March J. Ed.) John Wiley and Sons, New York, 1985.

Challis B.C. and Latif F. (1990) Synthesis and characterisation of some new diazopeptides. *J. Chem. Perkin Trans. 1*, 1005-1009.

Chang L.W., Hsia S.M.T., Chan P.-C. and Hsieh L.-L. (1994) Macromolecular adducts: biomarkers for toxicity and carcinogenesis. *Ann. Rev. Pharmacol. Toxicol.*, **34**, 41-47.

Chetsanga C.J., Bearie B. and Makaroff C. (1982) Alkaline opening of imadazole ring of 7-methlguanosine. 1. Analysis of the resulting pyrimidine derivatives. *Chem. -Biol. Interact.*, **41**, 217-233.

Chetsanga C.J., Lozon M., Makaroff C. and Savage L. (1981) Purification and characterisation of *Escherichia coli* formamidopyrimidine-DNA glycosylase that excises damaged 7-methylguanine from deoxyribonucleic acid. *Biochemistry*, **20**, 5201-5207.

Chetsanga C.J. and Makaroff C. (1982) Alkaline opening of imidazole ring of 7-methylguanosine. 2. Further studies on reaction mechanisms and products. *Chem. -Biol. Ineracts.*, **41**, 235-249.

Colburn N.H., Richardson R.G. and Boutwell R.K. (1965) Studies of the reaction of ß-propiolactone with deoxyguanosine and related compounds. *Biochem. Pharmacol.*, **14,** 1113-1118.

Cooper D.P., Griffith K.A. and Povey A.C. (1992) Immunoaffinity purification combined with ^{32}P-postlabelling for the detection of O^6-methylguanine in DNA from human tissue. *Carcinogenesis*, **13**, 469-475.

Cooper D.P., O'Connor P.J., Povey A.C. and Rafferty J.A. (1995) Cell and molecular mechanisms in chemical carcinogenesis. In: *Oxford Textbook of Oncology*, **1**, (Peckham M., Pinedo H and Veranesi U., Eds.), Oxford University Press, Oxford, 135-147.

Coppola G.M., Hardtmann G.E. and Huegi B.S. (1974) Synthesis and reactions of 2-aryl-3-(dimethylamino)acroleins. *J. Heterocycl. Chem.*, **11,** 51-56.

De Blas A.L. and Cherwinski H.M. (1983) Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies. *Anal. Biochem.*, **133**, 214-219.

Decaprio A.P. (1997) Biomarkers: coming of age for environmental health and risk assessment. *Environ. Sci. Tech.*, **31**, 1837-1849.

Den Engelse L., Menkveld G.J., De Brij R.-J. and Tates A.D. (1986) Formation and stability of alkylated pyrimidines and purines (including imidazole ring-opened 7-alkylguanine) and alkylphosphotriesters in liver DNA of adult rats treated with ethylnitrosourea or dimethylnitrosamine. *Carcinogenesis*, **7**, 393-403.

Dennisenko M.F., Pao A., Tang M.-S. and Pfeifer G.P. (1996) Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hotspots in *P53*. *Science*, **274**, 430-432.

Doll R. and Peto R. (1981) Avoidable risks of cancer in the US. *J. Nat. Can. Inst.*, **66**, 1194-1265.

Durand M.-J. and Shuker D.E.G. (1994) Preparation of compound-specific and group-specific antibodies to 7-methylguanine and related 7-alkylguanines and their use in immunoaffinity purification. *Carcinogenesis,* **15,** 957-961.

Farmer P.B. (1994) Carcinogen adducts: use in diagnosis and risk assessment. *Clin. Chem.*, **40,** 1438-1443.

Farmer P.B. (1995) Monitoring of human exposure to carcinogens through DNA and protein adduct determination. *Tox. Lett.*, **82/83,** 757-762.

Farmer P.B., Sepai O., Lawrence R., Autrup H., Nielsen P.S., Vestergård A.B., Waters R., Leuratti C., Jones N.J., Stone J., Baan R.A., van Delft J.H.M., Steenwinkel M.J.S.T., Kyrtopoulos S.A., Souliotis V.L., Theodorakopoulos N., Bacalis N.C., Natarajan A.T., Tates A.D., Haugen A., Andreassen Å., Øvrebø S., Shuker D.E.G., Amaning K.S., Schouft A., Ellül A., Garner R.C., Dingley K.H., Abbondandolo A., Merio F., Cole J., Aldrich K., Beare D., Capulas E., Rowley G., Waugh A.P.W., Povey A.C., Haque K., Kirsch-Volders M., Van Hummelen P. and Castelain P. (1996) Biomonitoring human exposure to environmental carcinogenic chemicals. *Mutagenesis*, **11,** 363-381.

Farmer P.B. and Sweetman G.M.A. (1995) Mass spectrometric detection of carcinogen adducts. *J Mass Spec.*, **30,** 1369-1379.

Farr R.S. (1958) A quantitative immunochemical measure of the primary interaction between I BSA and antibody. *J. Infect. Dis.*, **103,** 239-262.

Feinberg G. and Jackson M.A. (1983) *The Chain of Immunology*, 1ed., Blackwell Scientific Publications, Oxford, 141-213.

Finneman J.I. and Fishbein J.C. (1996) Mechanisms of decomposition of (Z)-2,2,2-trifluoro-1-arylethanediazoates in aqueous media. *J. Am. Chem. Soc.*, **118**, 7134-7138.

Ford G.P. and Scribner J.D. (1983) Theoretical study of gas-phase methylation and ethylation by diazonium ion and rationalisation of some aspects of DNA reactivity. *J. Am. Chem. Soc.*, **105**, 349-354.

Ford G.P. and Scribner J.D. (1990) Prediction of nucleoside-carcinogen reactivity. Alkylation of adenine, cytosine, guanine and thymine, and their deoxynucleosides by alkanediazonium ions. *Chem. Res. Tox.*, **3**, 219-230.

Foulds L. (1954) The experimental study of tumour progression: a review. *Cancer Res.*, **14**, 327-339.

Friedberg E.F. (1985) In: *DNA Repair*, Freeman, San Francisco, USA.

Friesen M.D., Garren L., Prevost V. and Shuker D.E.G. (1991) Isolation of urinary 3-methyladenine using immunoaffinity columns prior to determination by low-resolution gas chromatography-mass spectrometry. *Chem. Res. Toxicol.* **4**, 102-106.

Garcia J., Gonzalez J., Segura R. and Vilarrasa J. (1984) Nitrosation of peptide bonds. Cleavage of nitrosated peptides by pyrrolidine and α-amino esters. *Tetrahedron*, **30**, 3121-3127.

Gassmann M., Thömmes P., Weiser T. and Hübscher U. (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J.*, **4**, 2528-2532.

Gerster J.F. and Robins R.K. (1965) Purine nucleosides. X. The synthesis of certain naturally occurring 2-substituted amino-9-β-D-ribofuranosylpurin-6(1H)-ones (N^2-substituted guanosines). *J. Am. Chem. Soc.*, **87**, 3752-3759.

Giese R.W. (1997) Detection of DNA adducts by electron capture mass spectrometry. *Chem. Res. Toxicol.*, **10**, 255-270.

Goding J.W. (1986) *Monoclonal Antibodies: Principles and Practice*, 2ed. Academic Press Inc., London.

Green R.B. (1983) Laser: Practical detectors for chromatography? *Anal. Chem.*, **55**, 20A-32A.

Groopman J.D., Donahue P.R, Zhu J., Chen J. and Wogan, G.N. (1985) Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography. *Proc. Natl. Acad. Sci. (U.S.A.),* **82,** 64926496.

Groopman J.D., Sabbioni G. and Wild C.P. (1991) Molecular dosimetry of human aflatoxin exposures, In: *Molecular Dosimetry and Human Cancer.* (Groopman J.D., Ed.), CRC Press, Boca Raton, 303-324.

Gupta R.C. (1985) Enhanced sensitivity of ^{32}P-postlabelling analysis of aromatic carcinogens: DNA adducts. *Cancer Res.*, **45**, 5656-5662.

Gupta R.C., Murti P.C. and Bhonsle R.B. (1996) Epidemiology of cancer by tobacco products and the significance of tobacco specific nitrosamines. *Crit. Rev. Toxicol.*, **26**, 183-198.

Haque K., Cooper D.P. and Povey A.C. (1994) Optimisation of ^{32}P-postlabelling assays for the quantitation of O^6-methyl and N7-methyldeoxyguanosine-3'-monophosphates in human DNA. *Carcinogensis*, **15**, 2485-2490.

Haque K., Cooper D.P., van Delft J.H.M., Lee S.M. and Povey A.C. (1997) Accurate and sensitive quantitation of N7-methyldeoxyguanosine-3'-monophosphate by ^{32}P-postlabelling and storage-phosphor imaging. *Chem. Res. Toxicol.*, **10**, 660-666.

Harris C.C. (1989) Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. *Carcinogenesis*, **10**, 1563-1566.

Harric C.C. (1995) 1995 Deichmann lecture - p53 tumour suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology and cancer risk assessment. *Tox. Lett.*, **82/83**, 1-7.

Harris C.C., LaVeck G., Groopman J.D., Wilson V.L. and Mann D. (1986) Measurement of aflatoxin B_1, its metabolites, and DNA adducts by synchronous fluorescence spectroscopy. *Canc. Res.*, **46**, 3249-3253.

Harrison K.L., Fairhurst N., Challis B.C. and Shuker D.E.G. (1997) Synthesis, characterisation, and immunochemical detection of O^6-(carboxymethyl)-2'-deoxyguanosine: a DNA adduct formed by nitrosated glycine derivatives. *Chem. Res. Tox.*, **10**, 652-659.

Hecht S.S. and Hoffmann D. (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis*, **9**, 875-884.

Hecht S.S., Parsons W.D., Akerkar S., Pullo D., Murphy S.E., Bonilla L.E. and Carmella S.G. (1997) Metabolite of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the urine of people exposed occupationally to environmental tobacco smoke. *ACS Abstract*, **1**, abstract no. 011.

Hemminki K. (1980a) Identification of guanine-adducts of carcinogens by their fluorescence. *Carcinogenesis*, **1**, 311-316.

Hemminki K. (1980b) Fluorescence properties of alkylated guanine derivatives. *Acta Chem. Scand. B.*, **34**, 603-605.

Henderson R.F. (1995) Strategies for use of biological markers of exposure. *Tox. Lett.*, **82/83**, 379-383.

Ho J. and Fishbein J.C. (1994) Rate-limiting formation of diazonium ions in the aqueous decomposition of primary alkanediazoates. *J. Am. Chem. Soc.*, **116**, 6611-6621.

Hoffmann G.R. and Fuchs R.P.P. (1997) Mechanisms of frameshift mutations: insight from aromatic amines. *Chem Res. Toxicol.*, **10**, 347-359.

Hoffmann G.R. and Hecht S.S. (1990) Advances in tobacco carcinogenesis. In: *Chemical Carcinogenesis and Mutagenesis I*, (Cooper C.S. and Grover P.L., Eds.), Springer-Verlag, Berlin, Germany, 63-102.

Jain R. and Sharma M. (1993) Fluorescence postlabelling assay of DNA damage induced by N-methyl-N-nitrosourea. *Cancer Res.*, **53**, 2771-2774.

Jansen J.G., De Groot A.J.L., Van Teijlingen C.M.M., Lohman P.H.M., Mohn G.R. and Van Zeeland A.A. (1994) Formation and persistence of DNA adducts in pouch skin fibroblasts and liver tissue of rats exposed *in vivo* to the monofunctional alkylating agents N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. *Mut. Res.*, **307**, 95-105.

Johnson E., Abu-Shumays A. and Abbott S.R. (1977) Use of fluorescence detection in high-performance liquid chromatography. *J. Chrom.*, **134**, 107-119.

Jones J.W. and Robins R.K. (1963) Purine nucleosides. III. Methylation studies of certain naturally occurring purine nucleosides. *J. Am. Chem. Soc.*, **85**, 193-201.

Kai M., Ohkura Y., Yonekura S. and Iwasaki M. (1988) Selective determination of guanine and its nucleosides and nucleotides by reaction with phenylglyoxal as a fluorogenic reagent. *Anal. Chem.*, **207**, 243-249.

Kang H.-I., Konishi C., Bartsch H., Kuroki T. and Huh N.-H. (1995) Detection of smoking-related O^4-ethylthymine in human lung tissue (abstract). *Proc. Am. Assoc. Cancer Res.*, **36**, 136.

Kato S., Petruzzelli S., Bowman E.D., Turteltaub K.W., Blömeke B., Weston A. and Shields P.G. (1993) 7-Alkyldeoxyguanosine adduct detection by two-step HPLC and the ^{32}P-postlabeling assay. *Carcinogenesis*, **14**, 545-550.

Keith G. and Dirheimer G. (1995) Postlabelling: A sensitive method for studying DNA adduct and their role in carcinogenesis. *Curr. Opin. Biotech.*, **6**, 85-100.

Kelman D.J., Lilga K.T. and Sharma M. (1988) Synthesis and application of fluorescent labelled nucleotides to assay DNA damage. *Chem. Biol. Interact.*, **66**, 85-100.

King C.V. and Bolinger E.D. (1936) The catalytic decomposition of diazoacetate ion in aqueous solution. *J. Am. Chem. Soc.*, **58**, 1533-1542.

Koivisto P. and Hemminki K. (1990) ^{32}P-postalbelling of 2-hydroxyethylated, ethylated and methylated adducts of 2'-deoxyguanosine 3'-monophosphate. *Carcinogenesis*, **11**, 1389-1392.

Kopplin A., Eberle-Adamkiewicz G., Glüsenkamp K.-H., Nehls P. and Kirstein U. (1995) Urinary excretion of 3-methyladenine and 3-ethyladenine after controlled exposure to tobacco smoke. *Carcinogenesis*, **16**, 2637-2641.

Kreevoy M.M. and Konasewich D.E. (1970) The mechanism of hydrolysis of diazoacetate ion. *J. Phys. Chem.*, **74**, 4464-4472.

Kubo K., Ide H., Wallace S.S. and Kow Y.W. (1992) A novel, sensitive, and specific assay for abasic sites, the most commonly produced DNA lesion. *Biochemistry*, **31**, 3703-3708.

Lawley P.D. (1974) Some chemical aspects of dose-response relationships in alkylation mutagenesis. *Adv. Cancer Res.*, **23**, 283-295.

Lawley P.D. (1976) Methylation of DNA by carcinogens: Some applications of chemical analytical methods. In: *Screening Tests in Chemical Carcinogenesis.* (Montesano R., Bartsch H., Tomatis L. and Davis W., Eds.) IARC Scientific Publications No. 12 pp 181-208, International Agency for research on Cancer, Lyon, France.

Lawley P.D. and Brookes P. (1963) Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem. J.*, **89**, 127-138.

Lawrence J.F., Leduc R. and Ryan J.J. (1981) The determination of O-methyl and 7-methlguanine in mouse liver DNA from dimethylnitrosamine-treated mice by high-performance liquid chromatography with UV absorbance detection. *Anal. Biochem.*, **116**, 433-438.

Leung K.-H. and Archer M.C. (1985) Mechanism of DNA methylation by N-nitroso(2-oxopropyl)propylamine. *Carcinogenesis*, **6**, 189-191.

Lindahl T. (1982) DNA repair enzymes. *Ann. Rev. Biochem.*, **51**, 61-87.

Lindahl T., Sedgwick B., Sekiguchi M. and Nakabeppu Y. (1988) Regulation and expression of the adaptive response to alkylating agents. *Annu. Rev. Biochem.*, **57**, 133-157.

Lingeman H., Underberg W.J.M., Takadate A. and Hulshoff A. (1985) Fluorescence detection in high performance liquid chromatography. *J. Liq. Chrom.*, **8**, 789-874.

Loeb L.A. and Preston B.D. (1986) Mutagensis by apurinic/apyridiminic sites. *Ann. Rev. Genet.*, **20**, 210-230.

Loechler E.L. (1994) A violation of the Swain-Scott principle, and *not* S_N1 versus S_N2 reaction mechanisms, explain why carcinogenic alkylating agents can form different proportions of adducts at oxygen versus nitrogen in DNA. *Chem. Res. Toxicol.*, **7**, 277-280.

Losch U., Schranner I., Wanke R. And Jurgens L. (1986) The chicken egg, and antibody source. *J. Vet. Med.*, **33**, 609-619.

Magee P.N., Montesano R. and Preussman R. (1976) N-Nitroso compounds and related carcinogens. In: *Chemical Carcinogens*, (Searle C.E., Ed.), ACS Monograph Series No. 173, ACD, Washington, DC, USA, 491-625.

Massey E. and Few G. (1983) Tobacco smoke mutagenicity: the influence of nitrogenous compounds. Status report. *http://www.library.ucsf.edu/tobacco/doc/htm/1135.01/index.html.*

Mattes W.B., Lee C.-S., Laval J. and O'Connor T.R. (1996) Excision of DNA adducts of nitrogen mustards by bacterial and mammalian 3-methyladenine-DNA glycosylases. *Carcinogenesis*, **17**, 643-648.

McCann W.P., Hall L.P. and Nonldez W.K. (1983) Preparation, titration, and storage of chloroacetaldehyde for fluorimetric determination of adenine and its derivatives. *Anal. Chem.*, **55**, 1454-1455.

McClean M.J., Larson J.E., Wohlrab F. and Wells R.D. (1987) Reaction conditions affect the specificity of bromoacetaldehyde as a probe for DNA cruciforms and B-Z junctions. *Nuc. Acids. Res.*, **15**, 6917-6935.

McGee J. O'D., Isaacson P.G. and Wright N.A. (1992) *Oxford Textbook of Pathology.*, 1ed., Oxford University Press, Oxford, 571-678.

Miller E. (1978) Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. *Cancer. Res.*, **38**, 1479-1496.

Miller E. and Miller J.A. (1947) The presence and significance of bound aminoazo dyes in the livers of rats fed *p*-dimethylaminoazobenzene. *Cancer Res.*, **7**, 468-480.

Miller E. and Miller J.A. (1981) Mechanisms of chemical carcinogenesis. *Cancer*, **47**, 1055-1064.

Miller J.A. (1970) Carcinogenesis by chemicals: an overview - G.H.A. Clowes memorial lecture. *Cancer. Res.*, **30**, 559-576.

Mills M.J., Maltas J. And Lough W.J. (1997) Assessment of injection volume limits when using on-column focusing with microbore liquid chromatography. *J. Chrom. A.*, **759**, 1-11.

Moschel R.C. and Leonard N.J. (1976) Fluorescent modifications of guanine. Reactions with substituted malondialdehydes. *J. Org. Chem.* **41**, 294-300.

Müller R. and Eisenbrand G. (1985) The influence of N7 substituents on the stability of N7-alkylated guanosines. *Chem. -Biol. Interacts.*, **55**, 173-181.

Müller R. and Rajewsky M.F. (1980) Immunological quantification by high-affinity antibodies of O^6-ethyldeoxyguanosine in DNA exposed to N-ethyl-N-nitrosourea. *Cancer. Res.*, **40**, 887-896.

Müller R. and Rajewsky M.F. (1981) Antibodies specific for DNA components structurally modified by chemical carcinogens. *J. Canc. Res. Clin. Oncol.*, **102**, 99-113.

Mustonen R., Försti A., Hietanen P. and Hemminki K. (1991) Measurement by ^{32}P-postlabelling of 7-methylguanine levels in white blood cell DNA of healthy individuals and cancer patients treated with dacarbazine and procarbazine. Human data and method development of 7-alkylguanines. *Carcinogenesis,* **12,** 1423-1431.

Mustonen R and Hemminki K. (1992) 7-Methylguanine levels in DNA of smokers' and non-smokers' total white blood cells, granulocytes and lymphocytes. *Carcinogenesis*, **13**, 1951-1955.

Mustonen R., Schoket B. and Hemminki K. (1993) Smoking-related DNA adducts: ^{32}P-Postlabelling analysis of 7-methylguanine in human bronchial and lymphocyte DNA. *Carcinogenesis*, **14**, 151-154.

Nehls P., Adamkiewicz J. and Rajewsky M.F. (1984) Immuno-slot-blot: a highly sensitive immunoassay for the quantification of carcinogen-modified nucleosides in DNA. *J. Cancer Res. Clin. Oncol.*, **108,** 23-29.

Ouihia A., René L., Guilhem J., Pascard C. and Badet B. (1993) A new diazoacylating reagent: preparation, structure, and use of succinimidyl diazoacetate. *J. Org. Chem.*, **58**, 1641-1642.

Papoulis A., Al-Abed Y. And Bucala R. (1995) Identification of N^2-(1-carboxyethyl)guanine (CEG) as a guanine advanced glycosylation end product. *Biochem.*, **34**, 648-655.

Park J.-W., Cundy K.C. and Ames B.N. (1989) Detection of DNA adducts by high-performance liquid chromatography with electrochemical detection. *Carcinogenesis*, **10**, 827-832.

Pegg A.E. (1984) Methylation of the O^6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Canc. Invest.*, **2**, 223-231.

Phillips D.H. (1996) DNA adducts in human tissues: biomarkers of exposure to carcinogens in tobacco smoke. *Environ. Health. Perspec.*, **104**, 453-458.

Phillips D.H. and Castegnaro M. (1993) Results of an interlaboratory trial of ^{32}P-postlabelling. In: *Postlabelling Methods for Detection of DNA Adducts.* (Phillips D.H., Castegnaro M. and Bartsch H. Eds.), IARC Scientific Publication No. 124, International Agency for Research on Cancer, Lyon, France, pp. 35-50.

Phillips D.H., Hewer A., Martin G.N., Garner R.C. and King M.M. (1988) Correlation of DNA adduct levels in human lung with cigarette smoking. *Nature*, **336**, 790-792.

Phillips D.H., Schoket B., Hewer A., Bailey E., Kostic S. and Vincze I. (1990) Influence of cigarette smoking on the levels of DNA adducts in human bronchial epithelium and white blood cells. *Int. J. Cancl.*, **46**, 569-575.

Phillips D.H. and Shé M. Ni. (1994) DNA adducts in cervical tissue of smokers and non-smokers. *Mut. Res.*, **313**, 277-284.

Pitot H.C. and Dragan Y.P. (1991) Facts and theories concerning the mechanism of carcinogenesis. *The FASEB J.*, **5**, 2280-2286.

Poirier M.C. (1981) Antibodies to carcinogen-DNA adducts. *JNCI.*, **67**, 515-519.

Poirier M.C. (1990) Human exposure monitoring, dosimetry and cancer risk assessment: the use of antisera specific for carcinogen-DNA adducts and carcinogen-modified DNA. *Drug Metab. Rev.*, **26**, 87-109.

Poirier M.C. and Weston, A. (1996) Human DNA adduct measurements: State of the art. *Environ. Health Perspect.*, **104**, 883-893.

Polson A., Barbara von Mechmar M. and Fazakerley G. (1980) Antibodies to proteins from yolk of immunised eggs. *Imm. Comm.*, **9**, 495-514.

Pongracz K. and Bodell W.J. (1996) Synthesis of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-phosphate: comparison by ^{32}P-postlabelling with the DNA adduct formed in HL-60 cells treated with hydroquinone. *Chem. Res. Toxicol.*, **9**, 593-598.

Povey A.C. and Cooper D.P. (1995) The development, validation and application of a ^{32}P-postlabelling assay to quantify O^6-methylguanine in human DNA. *Carcinogensis*, **16**, 1665-1669.

Prevost V. and Shuker D.E.G. (1996) Cigarette smoking and urinary 3-alkyadenine excretion in man. *Chem Res. Tox.*, **9**, 439-444.

Prevost V., Shuker D.E.G., Bartsch H., Pastorelli R., Stillwell W.G., Trudel L.J., and Tannenbaum S.R. (1990) The determination of urinary 3-methyladenine by immunoaffinity chromatography-monoclonal antibody-based ELISA: use in human biomonitoring studies. *Carcinogenesis* **11**, 1747-1751.

Prevost V., Shuker D.E.G., Friesen M.D., Eberle G., Rajewsky M.F. and Bartsch H. (1993) Immunoaffinity purification and gas chromatography-mass spectrometric quantification of 3-alkyladenines in urine: metabolism studies and basal excretion levels in man. *Carcinogenesis*, **14**, 199-204.

Prevost V. and Shuker D.E.G. (1996) Cigarette smoking and urinary 3-alkyladenine excretion in man. *Chem. Res. Toxicol.*, **9**, 439-444.

Reddy M.V. (1993) ^{32}P-Postlabelling analysis of small aromatic and bulky non-aromatic DNA adducts. In: *Postlabelling Methods for Detection of DNA Adducts* (Phillips D.H., Castegnaro M. and Bartsch H. Eds.), IARC Scientific Publication No. 124, International Agency for Research on Cancer, Lyon, France, pp 25-34.

Randerath K., Reddy M.V. and Gupta R.C. (1981) ^{32}P-Labelling test for DNA damage. *Proc. Natl. Acad. Sci. USA.*, **78**, 6126-6129.

Randerath, K. and Randerath, E. (1993) Postlabelling methods - an historical review. In: *Postlabelling Methods for Detection of DNA Adducts.* (Phillips D.H., Castegnaro M. and Bartsch H. Eds.), IARC Scientific Publication No. 124, International Agency for Research on Cancer, Lyon, France, pp 3-9.

Reddy M.V. and Randerath K. (1986) Nuclease P_1-mediated enhancement of sensitivity of ^{32}P-postlabelling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543-1551.

Reichardt C., Ferwanah A.-R., Preβler W. And Yun K.-Y. (1984) Darstellung und eigenschaften von cycloalkylmalonaldehyden. *Liebegs Ann. Chem.*, 649-679.

Reichardt C. and Grahn W. (1970) Zur darstellung und struktur von arylazomalondialdehyden (mesoxaldehyd-2-phenylhydrazonen). *Chem. Ber.*, **103**, 1065-1071.

Reichardt C. and Halbritter K. (1970) Darstellung von fluor- und jodmalondialdehyd. *Liebigs Ann. Chem.*, **737**, 99-107.

Reichardt C. and Halbritter K. (1975) Preparation, structure and reactions of halomalondialdehydes. *Angew. Chem. Internat. Edit.*, **14**, 86-94.

Reichert W.L., Stein J.E., French B., Goodwin P. and Varanasi U. (1992) Storage phosphor imaging technique for the detection and quantitation of DNA adducts measured by the ^{32}P-postlabelling assay. *Carcinogenesis*, **13**, 1475-1479.

Roe R., Paul J.S. and Montgomery P.O'B. (1973) Synthesis and PMR spectra of 7-hydroxyalkylguanosinium acetates. *J. Heterocycl. Chem.*, **10**, 849-857.

Ryberg D., Hewer A., Phillips D.H. and Haugen A. (1994) Different susceptibility to smoking induced DNA damage among male and female lung cancer patients. *Cancer Res.*, **54**, 5801-5803.

Sabbioni G., Tannenbaum S.R. and Shuker D.E.G. (1986) Synthesis of volatile, fluorescent 7-methylguanine derivatives via reaction with 2-substituted fluorinated malondialdehyde. *J. Org. Chem.* **51**, 3244-3246.

Saffhill R., Margison G.P. and O'Connor P.J. (1985) Mechanisms of carcinogenesis induced by alkylating agents. *Biochim. Biophys. Acta.*, **823**, 111-145.

Samson L.D. (1992) The repair of DNA alkylation damage by methyltransferases and glycosylases. In: *Essays Biochem.*, **27**, Biochemical Society, 69-78.

Schulte-Hermann R., Bursch W., Grasl-Kraupp B., Török L., Ellinger A. and Müllauer L. (1995) Role of active cell death (apoptosis) in multi-stage carcinogenesis. *Tox. Lett.*, **82/83**, 143-148.

Searle N.E. (1963) Ethyl diazoacetate. *Org. Synth.*, **IV**, 424-426.

Segerbäck D. (1990) Reaction products in haemoglobin and DNA after *in vitro* treatment with ethylene oxide and N-(2-hydroxyethyl)-N-nitrosourea. *Carcinogenesis*, **11**, 307-312.

Sharma M., Box H.C. and Paul C.R. (1990a) Detection and quantitation of 8-hydroxydeoxyguanosine-5'-monophosphate in X-irradiated calf-thymus DNA by fluorescence postlabelling. *Biochem. Biophys. Res. Comm.*, **167**, 419-424.

Sharma M., Box H.C. and Kelman D.J. (1990b) Fluorescence postlabelling assay of *cis*-thymidine glycol monophosphate in X-irradiated calf thymus DNA. *Chem. -Biol. Interactions*, **74**, 107-117.

Sharma M. and Freund H.G. (1991) Development of laser-induced fluorescence detection to assay DNA damage. In: *Proceedings of the Society of Photo-Optical Instrumentation Engineers on Optical Methods for Ultrasensitive Detection and Analysis*, (Feary B.L. Ed), Los Angeles, CA, **SPIE 1435**, 280-291.

Sharma M., Jain R., Ionescu E. And Slocum H.H. (1995) Capillary electrophoretic separation and laser-induced fluorescence detection of the major DNA adducts of cisplatin and carboplatin. *Anal. Biochem.*, **228**, 307-311.

Shields P.G. and Harris C.C. (1991) Molecular epidemiology and the genetics of environmental cancer. *J. Am. Med. Assoc.*, **266**, 681-687.

Shields P.G., Povey A.C., Wilson V.L., Weston A. and Harris C.C. (1990) Combined high-performance liquid chromatography/^{32}P-postlabeling assay of N^7-methyldeoxyguanosine. *Canc. Res.*, **50**, 6580-6584.

Shuker D.E.G., Bailey E., Gorf S.M., Lamb J. and Farmer P.B. (1984) Determination of N-7-[$^2H_3$]methyl guanine in rat urine by gas chromatography-mass spectrometry following administration of trideuteromethylating agents or precursors. *Anal. Biochem.*, **140**, 270-275.

Shuker D.E.G. and Bartsch H. (1994) Detection of human exposure to carcinogens by measurement of alkyl-DNA adducts using immunoaffinity clean-up in combination with gas chromatography-mass spectrometry and other methods of detection. *Mutat. Res.*,**313**, 263-268.

Shuker D.E.G. and Benford D.J. (1997) DNA modification in carcinogen risk assessment in relation to diet: recent advances and some perspectives from a MAFF workshop. *Biomarkers*, **2**, 265-278.

Shuker D.E.G., Durand M.-J. and Molko D. (1993) Fluorescent postlabelling of modified DNA bases. In: *Postlabelling Methods for Detection of DNA Adducts.* (Phillips D.H., Castegnaro M. and Bartsch H. Eds.), IARC Scientific Publication No. 124, International Agency for Research on Cancer, Lyon, France, pp. 227-232.

Shuker D.E.G. and Margison G.P. (1997) Nitrosated glycine derivatives as a potential source of O^6-methylguanine in DNA. *Cancer Res.*, **57**, 366-369.

Singer B. (1985) *In vivo* formation and persistence of modified nucleosides from alkylating agents. *Environ. Health Persp.*, **62**, 41-48.

Singer B. and Essigman J.M. (1991) Site-specific mutagenesis: retrospective and prospective. *Carcinogenesis*, **12**, 949-955.

Singer B. and Grunberger D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, USA.

Singer B. and Hang B. (1997) What structural features determine repair enzyme specificity and mechanism in chemically modified DNA? *Chem. Res. Toxicol.*, **10**, 713-731.

Singh R., Sweetman G.M.A., Farmer P.B., Shuker D.E.G. and Rich K.J. (1997) Detection and characterisation of two major ethylated deoxyguanosine adducts by high performance liquid chromatography, electrospray mass spectrometry, and ^{32}P-postlabeling. Development of an approach for detection of phosphotriesters. *Chem. Res. Toxicol.*, **10**, 70-77.

Skipper P.L. and Tannenbaum S.R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis*, **11**, 507-518.

Sorsa M., Hemminki K. and Vainio H. (1982) Biologic monitoring of exposure to chemical mutagens in the occupational environment. *Terat. Carc. Mutag.*, **2**, 137-150.

Stillwell W.G., Glogowski J., Xu H.-X., Wishnok J.S., Zavala D., Montes G., Correa P. and Tannenbaum S.R. (1991) Urinary excretion of nitrate, N-nitrosoproline, 3-methyladenine and 7-methylguanine in a Columbian population at high risk of gastric cancer. *Cancer Res.*, **51**, 190-194.

Stillwell W.G., Xu H.-X., Adkins J.A., Wishnok J.S. and Tannenbaum S.R. (1989) Analysis of methylated and oxidised purines in urine by capillary gas chromatography-mass spectrometry. *Chem. Res. Toxicol.*, **2**, 94-99.

Swain C.G. and Scott C.B. (1953) Quantitative correlation of reaction rates. Comparison of hydroxide ion with other nucleophilic reagents towards alkyl halides, esters, epoxides and acyl halides. *J. Am. Chem. Soc.*, **75**, 141-147.

Swenson D.H. (1983) Significance of electrophilic reactivity and especially DNA alkylation in carcinogenesis and mutagenesis. In: *Developments in the Science and Practice of Toxicology*, (Hayes A.W., Schnell R.C. and Miya T.S., Eds.), Elsevier, Amsterdam, 247-254.

Szyfter K., Hemminki K., Szyfter W., Szmeja Z., Banaszewski J. and Pabiszczak M. (1996) Tobacco smoke-associated N7-alkylguanine in DNA of larynx tissue and leucocytes. *Carcinogenesis.*, **17**, 501-506.

Takamura N., Mizoguchi T., Koga K. And Yamada S. (1975) A simple and convenient method for preparation of α-substituted α-diazo esters. *Tetrahedron*, **31**, 227-230.

Takeda Y., Ohlendorf D.H., Anderson W.F. and Mathews B.W. (1983) DNA-binding proteins. *Sciences*, **221**, 1020-1026.

Talpaert-Borlé M. and Liuzzi M. (1983) Reaction of apurinic/apyridiminic sites with [^{14}C]methoxyamine. A method for the quantitative assay of AP sites in DNA. *Biochim. Biophys. Acta*, **740**, 410-416.

Tavares R., Ramos P., Palminha J., Bispo M.A., Paz I., Bras A., Farmer P.B. and Bailey E. (1994) Transplacental exposure to carcinogens. Evaluation in haemoglobin of hydroxyethylvaline adduct levels in smoking and nonsmoking mothers and their newborns. *Carcinogenesis*, **15**, 1271-1274.

Thomas L., Yang C.-H. and Goldthwait D.A. (1982) Two DNA glycosylases in *Escherichia coli* which release primarily 3-methyladenine. *Biochemistry*, **21**, 1162-1169.

Toyo'oka T., Ishibashi M. and Terao T. (1994) Sensitive determination of N-terminal prolyl peptides by high-performance liquid chromatography with laser-induced fluorescence. *J. Chrom. A.*, **661**, 105-112.

Uziel M. and Houck K. (1991) Direct labelling of DNA-adducts formed from carcinogenic diol-epoxides with a fluorescent reporter molecule compound specific for the *cis*-vic diol group. *Biochem. Biophys. Res. Comm.*, **180**, 1233-1240.

Vaca C.E., Conradi M., Sievertzon M. and Bergman J. (1994) Synthesis of fluorescent derivatives of 7-methylguanine through reaction with 2-aryl-substituted malondialdehydes: analysis by HPLC with fluorescence detection. *Chem. Bio. Interactions.* **93**, 235-249.

Vaca C.E., Fang J.-L., Mutanen M. and Valsta L. (1995) ^{32}P-Postlabelling determination of DNA adducts of malondialdehyde in humans: total white blood cells and breast tissue. *Carcinogenesis*, **16**, 1847-1851.

Van Delft J.H.M., van Weert E.J.M., Scellekens M.M., Claassen E. and Baan R.A. (1991) The isolation of monoclonal antibodies selected for the detection of imidazole ring-opened N7-ethylguanine in purified DNA and in cells *in situ*. Cross reaction with methyl, 2-hydroxyethyl and sulphur mustard adducts. *Carcinogenesis*, **12**, 1041-1049.

Van de Nesse R.J., Velthorst H.H., Brinkman U.A.Th. and Gooijer C. (1995) Laser-induced fluorescence detection of native-fluorescent analytes in column liquid chromatography, a critical evaluation. *J Chrom. A.*, **704**, 1-25.

Wang R. and O'Laughlin W.O. (1992) Determination of DNA-benzo[a]pyrene adducts by high-performance liquid chromatography with laser-induced fluorescence. *Environ. Sci. Technol.*, **26**, 2294-2297.

Watson W.P. (1987) Post-radiolabelling for detecting DNA damage. *Mutagenesis*, **2**, 319-331.

Wattenberg L.W. (1978) Inhibition of chemical carcinogenesis. *J. Natl. Cancer Inst.*, **60**, 11-18.

Weinberg R.A. (1991) Tumour suppressor genes. *Science*, **254**, 1138-1146.

Weinstein I.B. (1981) Current concepts and controversies in chemical carcinogenesis. *J. Supramol. Struct. Cell. Biochem.*, **17**, 99-120.

Weston A., Bowman E.D., Manchester D.K. and Harris C.C. (1990) Fluorescence detection of lesions in DNA. In: *DNA Damage and Repair in Human Tissues.* (Sutherland B.M. and Woodhead A.D., Eds.), Plenum, New York, 63-82.

Weston A. (1993) Physical methods for the detection of carcinogen-DNA adducts in humans. *Mut. Res.*, **288**, 19-29.

Widlak P., Gryzbowska E., Hemminki K., Santella R. and Chorazy M. (1996) ^{32}P-Postlabelling of bulky human DNA adducts enriched by different methods including immunoaffinity chromatography. *Chem. Biol. Inter.*, **99**, 99-107.

Wild C.P. (1990) Antibodies to DNA alkylation adducts as analytical tools in chemical carcinogenesis. *Mut. Res.*, **233**, 219-233.

Wild C.P., Smart G., Saffhill R. and Boyle J.M. (1983) Radioimmunoassay of O^6-methyldeoxyguanosine in DNA of cells alkylated *in vitro* and *in vivo*. *Carcinogenesis,* **4,** 1605-1609.

Willard H.H., Merrit L.L., Dean J.A. and Settle F.A. (1988) *Instrumental Methods of Analysis*, 7ed., Wadsworth Publishing Co., USA.

Witschi H., Espiritu I., Peake J.L., Wu K., Maronpot R.R. and Pinkerton K.E. (1997) The carcinogenicity of environmental tobacco smoke. *Carcinogenesis*, **18**, 575-586.

Witschi H., Espiritu I., Peake J.L., Wu K., Maronpot R.R., Pinkerton K.E. and Jones A.D. (1997) The carcinogenic potential of the gas phase of environmental tobacco smoke. *Carcinogenesis*, **18**, 2035-2042.

Wogan G.N. and Gorelick N.J. (1985) Chemical and biochemical dosimetry of exposure to genotoxic chemicals. *Environ. Health Perspec.*, **62**, 5-18.

Venitt S. and Parry J.M. (1984) Background to mutagenicity testing. In: *Mutagenicity Testing: A Practical Approach*. IRL Press, Oxford, 1-24.

Yeung E.S. and Sepaniak M.J. (1980) Laser fluorometric detection in liquid chromatography. *Anal. Chem.*, **52**, 1465A-1481A.

Yonekura S., Iwasaki M., Kai M. and Ohkura Y. (1993) High performance liquid chromatography of guanine and its nucleosides and nucleotides by pre-column fluorescence derivatisation with phenylglyoxal reagent. *J. Chrom.*, **641**, 235-239.

Yonekura S., Iwasaki M., Kai M. and Ohkura Y. (1994) High performance liquid chromatography of guanine and its nucleosides and nucleotides in biospecimens with precolumn fluorescence derivatisation using phenylglyoxal. *Anal. Sci.*, **10**, 247-251.