An Assessment of Benzene Genotoxicity: DNA Adduct Formation and its Consequence

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by

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Abstract

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by Margaret Gaskell

Benzene is a recognised human leukaemogen and rodent carcinogen, with exposure occurring from both environmental and occupational sources. The mechanism by which benzene exerts these effects is unknown and it was proposed that among other mechanisms, DNA adducts may play a role.

The aim of this study was to develop sensitive methods for the analysis of benzene-DNA adducts from benzene-exposed individuals and determine the consequence of such DNA adducts using the *supF* mutation assay.

Benzene-DNA adduct standards, which included a novel DNA adduct characterised and identified as (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, were synthesised and TLC and HPLC systems were developed for the analysis of these adducts following ^{32}P-postlabelling. Attempts were also made to produce an antibody to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the only benzene-DNA adduct so far identified following *in vivo* exposure, to be used in an enrichment step prior to ^{32}P-postlabelling. The benzene-DNA adducts formed by the benzene metabolite *para*-benzoquinone (*p*-BQ) and the damage induced by hydroquinone (HQ) were assessed for their mutagenic potential in repair proficient and deficient cell lines. Both treatments proved to be mutagenic with base substitution mutations predominating in each case. The majority of mutations following treatment with *p*-BQ were GC→TA transversions and GC→AT transitions, for HQ treatment GC→AT transitions predominated, for all cell lines investigated.

Application of the ^{32}P-postlabelling assay to the analysis of lymphocyte DNA from petroleum refinery workers resulted in no detectable benzene-DNA adducts, a possible reflection on the low benzene exposure (<2 ppm), the use of an inappropriate surrogate tissue or the detection limit of the assay. Further work in regard to improvement of the assay's sensitivity and selectivity would be necessary prior to any further analysis. The results from the mutation assay indicated that HQ may be the ultimate toxic metabolite, inducing the chromosomal aberrations observed in benzene exposed individuals.

Dedicated to the memory of my dad

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Abbreviations

Ad293	a human adenovirus transformed kidney cell line
AEC	3-amino-9-ethyl-carbazole
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
AMS	accelerator mass spectrometry
ANOVA	analysis of variance
AP	apurinic / apyrimidinic
ATP	adenosine triphosphate
BDS	base deactivated silica
BER	base excision repair
BFU-E	burst forming unit-erythroid
BO	benzene oxide
bp	base pair(s)
BPDE	benzo[*a*]pyrene diol epoxide
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CFU-E	colony forming unit-erythroid
Ci	curie(s)
CLL	chronic lymphocytic leukaemia
CMHT	Centre for the Mechanism of Human Toxicity
CML	chronic myeloid leukaemia
CPG	controlled-pore-glass
CPM	counts per minute
CS	Cockayne's syndrome
CSPD	calf spleen phosphodiesterase
ct-DNA	calf thymus DNA
D1-D4	TLC chromatography directions (1-4)
d	doublet
dd	doublet of doublets
dA	2'-deoxyadenosine
dAp	2'-deoxyadenosine 3'-monophosphate
DBU	diazabicyclo(5.4.0)undec-7-ene
dC	2'-deoxycytidine
DCM	dichloromethane
dCp	2'-deoxycytidine 3'-monophosphate
dG	2'-deoxyguanosine
dGp	2'-deoxyguanosine 3'-monophosphate
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNp	2'-deoxynucleoside 3'-monophosphate
dpm	disintegrations per minute
DTT	dithiothreitol
dXp	adducted 2'-deoxynucleoside 3'-monophosphate
ECACC	European Collection of Cell Cutures
EDTA	ethylene diamine tetraacetic acid

EGR1	early growth response 1
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
FCS	foetal calf serum
2-F-dI-CE	5'-dimethoxytrityl-5-fluoro-2'-deoxyinosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]
FDA	Food and Drug Administration
EPO	erythropoietin
GC	gas chromatography
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
Gp	guanosine 3'-monophosphate
GPA	glycophorin A
GSH	glutathione
GST	glutathione *S*-transferase
h	hour(s)
HAT	hypoxanthine-aminopterin-thymidine
HCR	host cell reactivation
HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
HL60	a human promyelocytic cell line
HPC	haematopoietic progenitor cells
HPLC	high performance liquid chromatography
HPRT	hypoxanthine guanine phosphoribosyl transferase
HQ	hydroquinone
HRP	horse radish peroxidase
*HYL*1	microsomal epoxide hydrolase
Hz	hertz
IAA	isoamyl alcohol
IAC	immunoaffinity chromatography
IARC	International Agency for Research on Cancer
Ig	immunoglobulin
IL	interleukin
i.p.	intra peritoneal
IPTG	isotropyl β-D-thiogalactoside
I-spot	indigenous spot
i.u.	international unit
J	joule
KLH	keyhole limpet haemocyanin
LB	luria broth
LC	liquid chromatography
M	molar
Max	maximum
m-CPBA	*meta*-chloroperbenzoic acid
M-CSF	monocyte-colony stimulating factor
MDS	myelodysplastic syndrome
MEM	minimal essential medium
$[M-H]^-$	deprotonated molecule ion
$[M+H]^+$	protonated molecule ion
Min	minimum

min	minute(s)
MLL	mixed lineage leukaemia
MN	micronuclei
MNU	methyl-nitrosourea
Mol	moles
MPO	myeloperoxidase
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cut off
m/z	mass to charge ratio
N	nucleoside
NER	nucleotide excision repair
NIOSH	National Institute for Occupational Safety and Health
NMR	nuclear magnetic resonance
Np	nucleotide
NQO1	NAD(P)H quinone oxidoreductase 1
NSO	a mouse myeloma cell line
NZW	New Zealand white
o-BQ	*ortho*-benzoquinone
OPD	o-Phenylenediamine dihydrochloride
OSHA	Occupational Safety and Health Administration
Ov	ovalbumin
PAH	polycyclic aromatic hydrocarbon
p-BQ	*para*-benzoquinone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PNACL	Protein and Nucleic Acid Chemistry Laboratory
^{32}pNp	^{32}P-labelled nucleoside bisphosphate
ppm	parts per million
^{32}pX	^{32}P-labelled-nucleoside 5'-monophosphate
^{32}pXp	^{32}P-labelled adducted nucleoside bisphosphate
^{32}pXpN	^{32}P-labelled dinucleoside 5'-monophosphate
QR	quinone reductase
RAL	relative adduct labelling
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
s	second(s)
s	singlet
s.c.	sub-cutaneously
SCE	sister chromatid exchange
SD	standard deviation
SPC	S-phenylcysteine
S-PMA	S-phenylmercapturic acid

SSCC	sodium succinate, calcium chloride
SupF	*Escherichia coli* tyrosinase amber suppressor transfer RNA gene
T	time point
t-AML	therapy related-acute myeloid leukaemia
TBAC	tetrabutylammonium chloride
TLC	thin layer chromatography
Tp	thymidine 3'-monophosphate
TPO	thrombopoietin
t_R	retention time
TTD	trichothiodystrophy
TWA	time weighted average
Tween-20	polyoxyethylenesorbitan monolaurate
U	unit(s)
USERIA	ultrasensitive enzyme radioimmunoassay
UV	ultraviolet
v	volume
w	weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XP	*Xeroderma pigmentosum*
XpN	dinucleotide

Contents

Chapter 1
Introduction

1.0. General Introduction

1.1. Cancer

Cancer, defined as any malignant growth or tumour caused by abnormal and uncontrolled cell division is a common disease affecting a large number of people worldwide. In the UK alone, it has been estimated that one in three people will develop cancer whilst one in four will die from the disease.

Cancer development, a process more commonly referred to as carcinogenesis, is an evolutionary process involving multiple and highly complicated events which result in the accumulation of errors in vital regulatory pathways in a cell (King, 1996). Carcinogenesis can be divided into three main stages; initiation, promotion and progression. Initiation involves the exposure of a cell to a carcinogenic agent, which can be classed as either genotoxic or non-genotoxic and can be biological (eg. viruses), chemical (eg. benzene) or a physical agent (eg. ionising radiation). A genotoxic agent interacts directly with DNA, which may result in a mutation in a gene critical for cell survival. A non-genotoxic agent initiates carcinogenesis not by a direct interaction with DNA but by inducing changes in the control of cellular proliferation i.e. by disruption of gene function (Perera, 1996).

A single, inheritable, genetic alteration in the DNA of a cell is only the first step in the pathway of carcinogenesis. To develop into a cancerous growth or tumour the altered cell must multiply acquiring additional changes, a process referred to as promotion. Promotion allows the growth of the cell to become unregulated giving the cell a growth advantage over neighbouring normal cells.

A tumour, once formed, progresses by dedifferentiation into a more aggressive growth normally due to changes in growth regulatory mechanisms. These changes allow the tumour cells to grow outside their normal environment resulting in an invasion of surrounding normal tissue and also allows these cells to metastasize to distant organ sites (King, 1996).

1.2. Cancer incidence

Cancer incidence differs from country to country, and in a number of cases this has been linked to chemical exposure. In the UK for example, cigarette smoking plays a major role in a large number of cancer deaths having been linked to over 25 different cancers, ranging from cancer of the lung, which is the major cancer in UK males, to cancer of the blood, which includes myeloid leukaemia (Garner, 1998). Cigarette smoke contains more than 3500 chemicals, a number of which are known mutagens and carcinogens including benzo[*a*]pyrene, styrene, benzene and vinyl chloride (reviewed in Beach and Gupta, 1992, and IARC, 1986).

In parts of Africa and China, liver cancer incidence is common and this has been linked to exposure to aflatoxin (a mycotoxin contaminating such foods as peanuts, various other nuts, corn and rice) and hepatitis B infection. In the west however, where aflatoxin exposure is practically zero, liver cancer is associated with alcohol consumption (Garner, 1998).

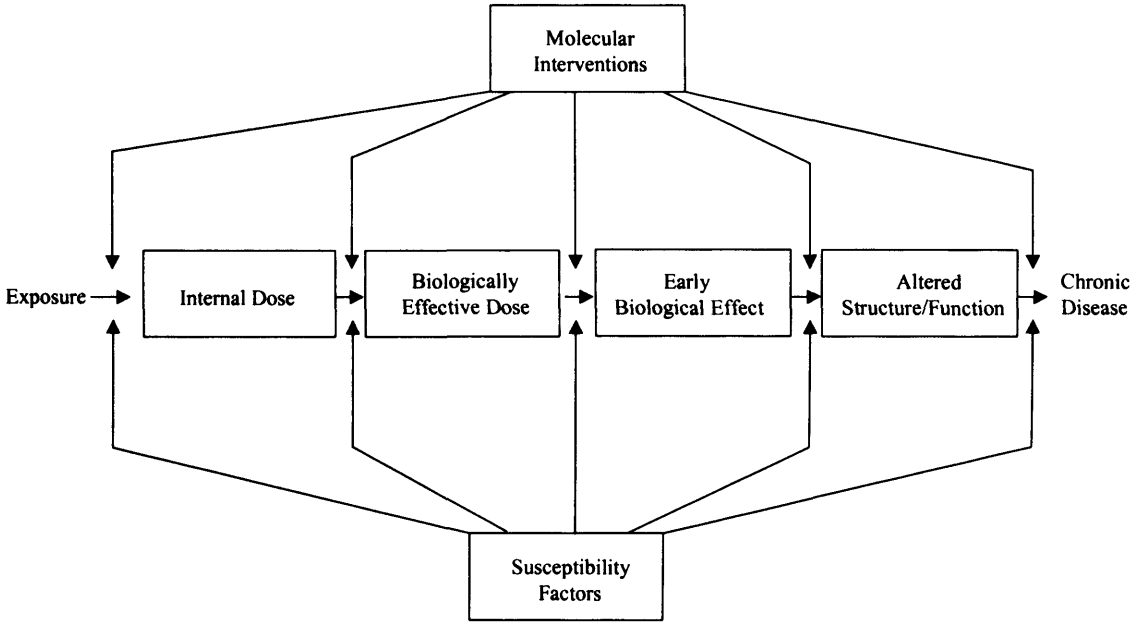
1.3. Molecular epidemiology

Epidemiology, the study of disease distributions in human populations, is important in identifying factors that influence specific types of human cancers. Molecular epidemiology involves research into the relationship between exposure to a cancer inducing agent and its biological effect, which can be strongly influenced by an individual's genetic and acquired susceptibility (Groopman *et al.*, 1995).

Molecular epidemiology focuses on the use of biological or biochemical markers (biomarkers) which are measurable events occurring in a biological system, for example, the human body. In epidemiology, the measurable event is generally considered to be an early change that could later lead to clinical disease (Grandjean, 1995). Biomarkers can be subdivided into 3 main groups: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. Figure 1.1. highlights the route taken following exposure to a carcinogen to the development of cancer in relation to molecular epidemiology. The predictive value for identifying the chemical involved in a particular exposure decreases the further down the process from exposure to tumour

formation one goes, although the ability to predict risk increases as one goes further down the process (Farmer *et al.*, 1996).

Figure 1.1. : Flow chart highlighting the route of cancer development in relation to molecular epidemiology (adapted from Groopman and Kensler, 1999).



1.3.1. Biomarkers of exposure

Exposure to a chemical carcinogen can occur from a number of sources including environmental, occupational, medicinal and life style sources (i.e. diet). Exposure to a chemical carcinogen would normally be assessed by measurement of the agent of interest in media i.e. air, water, food etc and would normally rely on the use of questionnaires and personal air monitoring in the case of environmental exposure (Groopman *et al.*, 1995).

A biomarker of exposure would indicate both the presence and extent of exposure to a chemical carcinogen and can be assessed by monitoring for the presence of the parent compound or a metabolite of the compound in bodily fluids, for example, urine, blood or serum (ECETOC, 1989). In the case of volatile compounds, for example benzene,

the compound can be monitored in exhaled breath if exposure occurred recently. A biomarker of this type gives a clear demonstration that a chemical has been absorbed and distributed in the body and hence takes into account individual differences in absorption, metabolism, excretion and distribution but does not provide evidence that toxicological damage has occurred, such evidence is obtained from biologically effective dose measures.

The covalent interaction between the parent compound or metabolite with an endogenous component i.e. DNA, RNA or protein is termed an adduct, and it is these chemical-DNA and chemical-protein adducts which are referred to as biologically effective dose measures. These give a measure of exposure at the individual level and a relevant measure of exposure at the target molecule (DNA) or valid surrogate molecule (protein) (Wild and Pisani, 1998).

1.3.1.1. DNA adducts

The formation of a DNA adduct is considered to be one of the earliest events in the initiation stage of cancer. For a carcinogen to bind to DNA the carcinogen needs to be chemically reactive or be metabolised to an activated electrophilic species, normally by oxidation or reduction reactions (ECETOC, 1989). The electrophilic species will react with nucleophilic (electron rich) centres in DNA forming different adducts. The possible sites of reaction in DNA, which are shown in Figure 1.2, include the O^6 of guanine, the nitrogen atoms in the purine and pyrimidine bases, the phosphate oxygens and quite commonly the C-8 position of guanine (Farmer, 1994). In general, the nitrogen atoms are more reactive than the oxygen atoms, with the N-7 of guanine and the N-3 of adenine being the most reactive. Carcinogens with an affinity towards DNA are divided into 2 main groups based on the Swain-Scott constant. Carcinogens with a low Swain-Scott constant tend to react with the less nucleophilic centres such as the O^6 of guanine whereas those with a high Swain-Scott constant, react with the more nucleophilic sites, which include the nitrogen atoms (reviewed by Friedberg *et al.*, 1995).

DNA adduct formation can be affected by DNA conformation (bases located within the major groove of DNA are more accessible to reaction compared to bases located within

the minor groove) and sequence context (the electrostatic potential of the N-7 position of guanine is enhanced if the base is flanked by other guanine bases). DNA damage, and hence DNA adducts, can occur not just from exogenous sources but also from endogenous processes which include oxidative stress leading to the production of 8-oxo-deoxyguanosine and lipid peroxidation (Wild and Pisani, 1998). A wide variety of chemicals have been studied, and their ability to form DNA adducts in animal models have been recorded. Such chemicals include alkylating and arylating agents, aldehydes, vinyl chloride, epoxides, hydrazines, polycyclic aromatic hydrocarbons, nitroaromatics and aromatic amines and metals (ECETOC, 1989). More specific compounds which can give rise to DNA adducts, the tissues in which adducts have been observed and the method of detection are shown in Table 1.1.

Figure 1.2 : Representation of DNA with the nucleophilic centres highlighted in red with base identification (adapted from Friedberg *et al.*, 1995).

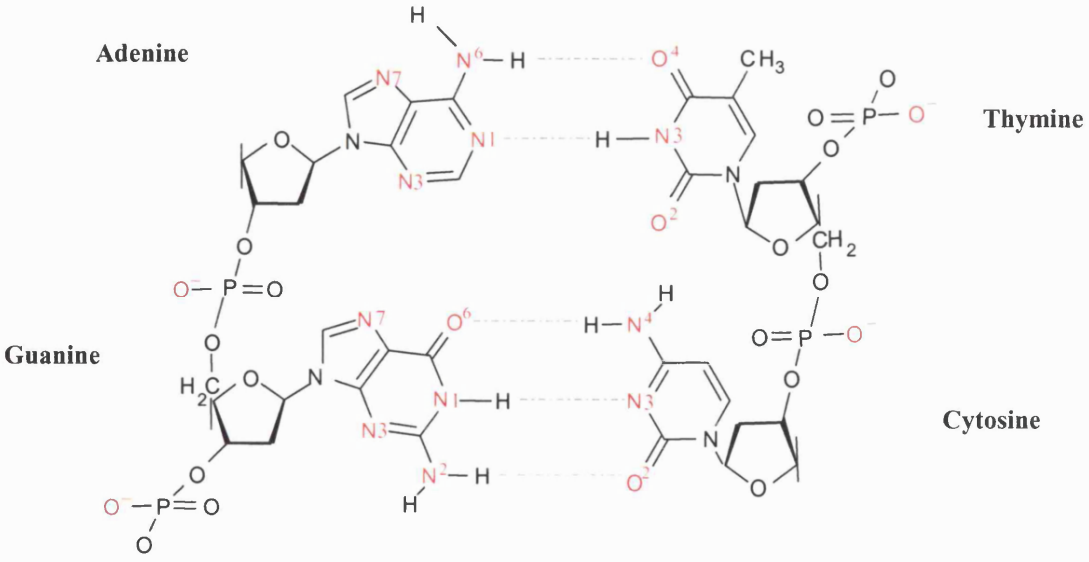


Table 1.1.: Examples of some of the chemicals or chemical mixtures which have been shown to form DNA adducts in human tissues and their method of detection (adapted from Garner, 1998).

Chemical	Tissue	Method of Detection
4-Aminobiphenyl	Bladder	Postlabelling, GC/MS*
4,4'-Methylenebis(2-chloroaniline)	Bladder	Postlabelling
Methylating agents	Lymphocytes, Liver	IAC**/Postlabelling
Aflatoxin B_1	Urine, lung, kidney, liver	Immunoassay
Cisplatin	Lymphocytes	HPLC***/Immunoassay
Benzo[*a*]pyrene	White blood cells, lung	Fluorescence
Cigarette smoke	Lymphocytes, Lung, Bladder, Breast, Cervix, Placenta, Buccal Mucosa	Postlabelling
Foundry fumes	Lymphocytes	Postlabelling
Air Pollution	Lymphocytes	Postlabelling
MeIQx	Colon, Kidney	Postlabelling
PhIP	Colon	GC/MS
Aristolochic Acid	Kidney	Postlabelling
Malondialdehyde	Lymphocytes, liver	GC/MS
Styrene Oxide	Lymphocytes	Postlabelling
4-Hydroxynonenal	Lymphocytes, liver	IAC/Postlabelling

* GC/MS (gas chromatography/mass spectrometry)
** IAC (Immunoaffinity chromatography)
*** HPLC (High performance liquid chromatography)

1.3.1.2. The consequence of DNA adducts

If adducted nucleotides are not released from DNA either spontaneously (depurination or depyrimidation), giving rise to abasic sites, a process which normally occurs for N-7 guanine and N-3 adenine adducts, or promptly repaired prior to DNA replication, miscoding may occur which can lead to mutations, oncogene activation and altered expression or inactivation of regulatory and tumour suppressor genes. The presence of a DNA adduct in a critical gene provides the potential for a mutagenic event i.e. deletion, insertion or base substitution mutations, which can result in subsequent alteration in gene expression and a loss of growth control. The potency of an adduct is affected by its sequence context, whether it is on the transcribed or non-transcribed

strand of DNA and its ability or failure to be repaired (hot spots for mutations are sites which have cold spots for repair).

Work has been carried out to determine the association of DNA adducts and cancer risk, which varies depending on chemical class. For aflatoxins, aromatic amines and polycyclic aromatic hydrocarbons, tumour induction in animal models is associated with the major DNA adduct formed, and for aflatoxin the major N-7-guanine adduct has been found to be significantly predictive of liver cancer in a population study (Bonassi and Au, 2002). With *N*–nitrosamines however, tumour formation is associated with the minor DNA adducts (Beland and Poirier, 1993).

For specific carcinogens further work is required to determine the relationship between DNA adducts and cancer and whether the adducts observed in non-target tissues reflect what would be observed in target tissue. In terms of the quantity of adducts observed there are no certainties in regard to adduct level in relation to biological significance, particularly in regard to human exposure. Work carried out using animal models has estimated that an adduct level ranging between 50 and 8000 adducts per 10^8 nucleotides has been associated with a 50 % tumour incidence (Phillips *et al.*, 2000). However, the level below which there is no measurable biological effect, is unknown (Phillips *et al.*, 2000). DNA adducts found in humans are often chemically identical to those found in experimental animals and hence data from animal models can be used to improve our understanding of human cancer risk (Beland and Poirier, 1993).

1.3.1.3. Protein adducts

Protein adducts, although not directly involved in carcinogenesis are considered to be useful as surrogate biomarkers of exposure when DNA is unavailable (Wild and Pisani, 1998). No repair processes exist for protein adducts and therefore they may not accurately reflect the effects of exposure in the target organ. In regard to biomarker analysis however, the lack of repair processes is advantageous as protein adducts exhibit longevity and can be detected in accessible tissues, for example blood where protein is in plentiful supply. Protein binding is however dependent on the protein and class of compound under investigation, for example chemical binding to either haemoglobin or albumin does not happen to the same extent (Hemminki *et al.*, 1995).

1.3.1.4. Analytical methods for adduct analysis

The analytical methods used to measure biomarkers of exposure (chemical DNA and protein adducts), must be sufficiently sensitive and specific in order to quantify levels in a limited sample from an individual (Groopman and Kensler, 1999). Ideally, analysis of DNA adducts would occur in the target tissue for toxicity but as such tissue is normally inaccessible for these studies, surrogate tissues/cells would normally be employed which include white blood cells, buccal cells, cells in urine (exfoliated from the bladder), and cells from bronchial lavage. Urine can also be used to assess for the presence of DNA bases following depurination. These surrogate tissues are used routinely, and are thought to reflect adduct levels in the target tissue, although this may not always be the case (ECETOC, 1989, Groopman and Kensler, 1999, Wild and Pisani, 1998).

A number of methods exist for the determination and measurement of DNA and protein adducts, whether from target or surrogate tissues or as excretion products in urine. An overview of the main methods employed is outlined in Table 1.2. Prior to the development of sensitive methods such as ^{32}P-postlabelling or mass spectrometry (MS), DNA adduct detection was reliant on the administration of ^{14}C or ^{3}H radiolabelled carcinogens to animals. Binding was routinely analysed by scintillation counting, but this method was fraught with disadvantages and contamination with RNA, protein or unbound radiolabelled metabolite gave false positive results.

More recently, administration of radiolabelled compounds to humans at very low levels has become a possibility with the introduction of accelerator mass spectrometry (AMS), a nuclear physics technique historically used in the measurement of radiocarbon (^{14}C) to date archaeological samples. The technique involves the use of a tandem van de Graaff accelerator to generate positively charged atoms, which can then be separated using a conventional mass spectrometer. Three carbon isotopes (^{14}C, ^{13}C and ^{12}C) are separated and the isotope ratios are used to determine the presence of ^{14}C in samples compared to control samples. AMS is significantly more sensitive than liquid scintillation counting being capable of measuring concentrations one thousandth of the natural level found in living tissue (Young *et al.*, 2001) and has successfully been applied to the determination of drug (i.e. tamoxifen (White *et al.*, 1997)) and other

chemical (eg. heterocyclic amines and benzene (Mani *et al.*, 1999, Robertson-Creek *et al.*, 1997 and Turteltaub *et al.*, 1993)) disposition at low, human relevant doses (Young *et al.*, 2001), detecting levels as low as 1-10 adducts /10^{12} nucleotides.

Administration of unlabelled compounds followed by analysis using ^{32}P-postlabelling, mass spectrometry, immunochemical methods, electrochemical detection or synchronous fluorescence spectroscopy (Phillips *et al.*, 2000) are methods also employed. Each method has advantages and disadvantages and these are highlighted in Table 1.2. ^{32}P-postlabelling and immunochemical methods are discussed in more detail in Chapters 3 and 4, respectively. Mass spectrometry is an analytical tool that allows the determination of molecular mass. Many different types of mass spectrometer exist, but each are based on a common principle. Samples are introduced into the mass spectrometer into a region, known as the source, and are ionised (positively or negatively) into the gas phase. Gas-phase ions then pass into a second region called the analyser where they are separated depending on their mass to charge ratio (*m/z*). Ions then pass into the final region, the detector, which measures the *m/z* and ion intensity.

Table 1.2. : Brief assessment of the main methods utilised for the analysis of adducts (Biomarkers of Exposure)

Method	Limit of detection	Main Advantages	Main Disadvantages	Reference
Administration of radiolabelled compound followed by scintillation counting of DNA	Dependent on specific activity of radiocompound but levels as low as 1 adduct in 10^9 nucleotides have been determined	Straight forward determination	^{3}H likely to lose radiolabel through exchange processes ^{14}C compounds difficult to prepare and expensive High specific activity required Radiohazard disposal problem Presence of radioactivity in sample does not prove adduct formation as metabolic incorporation may have occurred Requires mg of DNA	Reviewed in Phillips *et al.*, 2000 and Nestmann *et al.*, 1996
Administration of radiolabelled compound followed by isotope ratio measurements (AMS)	6 adducts per 10^{12} nucleotides	AMS has high selectivity, sensitivity and precision	Measures isotope ratios only, proving no information on nature or chemical form of isotope RNA, protein and unbound metabolite contamination can give false positive results Instrumentation large and expensive Requires mg of DNA	Reviewed in Phillips *et al.*, 2000 and Robertson-Creek *et al.*, 1997.
^{32}P-postlabelling	1 to 100 adducts per 10^9 nucleotides (aromatic adducts) 1 adduct per 10^5 to 10^6 nucleotides (non aromatic adducts)	Highly sensitive Only requires 1-10 µg DNA Can be used for the detection of adducts from complex mixtures	Does not provide structural information of adducts Assay could give false negative results due to loss of adducts False positive results due to presence of indigenous (I) spots Lack of selectivity Adduct underestimation (incomplete DNA digestion, labelling efficiency etc)	Randerath and Randerath, 1991
Immunoassays	1 adduct per 10^8 nucleotides	Highly sensitive with good relative specificity Inexpensive, easy to perform High number of samples can be analysed at once	Requirement to immunise animals and characterise antiserum Need to develop antibodies for each adduct of interest Prior knowledge of test compound /exposure required Large amounts of DNA required	Reviewed in Poirier, 1991 and Hemminki *et al.*, 1995.
Mass Spectrometry	Levels as low as 1 adduct per 10^9 nucleotides has been achieved	Characterisation of the DNA adduct can be carried out including detailed structural information Low background	Decreased sensitivity Unable to screen mixtures of unknown adducts mg of DNA required	Reviewed in Hemminki *et al.*, 2000 and Phillips *et al.*, 2000.
HPLC with fluorescence detection or electrochemical detection	1 adduct per 10^7 bases for fluorescence detection	Highly sensitive for specific adduct types	Limited to compounds possessing a fluorophore or an electrochemically active group	Reviewed in Phillips *et al.*, 2000 and Garner, 1998.

1.3.2. Biomarkers of effect

A biomarker of effect may be the presence of a biological response to exposure as measured by the functional capacity of the affected system. Such a biomarker may be the unusual presence or absence of an endogenous component as dictated for example by the altered expression of a particular gene (Groopman *et al.*, 1995). Biomarkers of effect are generally considered to be preclinical indicators of abnormalities, the most common of which are cytogenetic endpoints and include loss and gain of whole or part of chromosomes, chromosomal breaks, gaps and gene mutations.

Biomarkers of effect can be measured using various techniques which include analysis of sister chromatid exchange, an assessment of micronuclei formation (which measures loss of part or whole of chromosomes), the comet assay (which measures strand breaks, DNA crosslinking, alkali labile sites and incomplete excision repair sites) and fluorescence *in situ* hybridisation (which is capable of measuring a whole range of chromosomal abnormalities by the use of fluorescent probes).

1.3.3. Biomarkers of susceptibility

A biomarker of susceptibility can be inherited or induced and indicates an individuals sensitivity to the effects of a particular xenobiotic (Grandjean, 1995). Such biological markers include markers of metabolism (i.e. polymorphisms in cytochrome P450 genes) or DNA repair capacity (Groopman and Kensler, 1993) and the risk of cancer can be strongly influenced by age, gender, ethnic origin, genetics, diet (antioxidants), immune function or pre-existing disease (Perera, 1996 and Vineis and Perera, 2000).

1.4. DNA repair

Repair of DNA damage by cellular mechanisms, restoring DNA to its correct sequence is critical in modulating the effects of DNA adducts, as unrepaired lesions may induce mutations during DNA replication (reviewed by Friedberg *et al.*, 1995). Repair of DNA adducts is dependent on a number of factors including the position of the lesion; adducts in a functional gene or lesions on the transcribed strand are removed/repaired faster than adducts in a non coding region close to the gene or on a non transcribed strand (Hemminki, 1993). A number of methods are involved in repair of DNA damage and these include direct reversal, recombinational repair, mismatch repair, base

excision repair and nucleotide excision repair (Bohr, 1995). The latter two methods are discussed in more detail in Chapter 6.

1.5. Benzene: history and background information

Benzene was first isolated in 1825 by Michael Faraday from an oily film that was deposited from the gas used for lighting. He discovered that the new compound had an equal number of carbons and hydrogens and called it 'carbureted hydrogen'. In 1834, Mitscherlich discovered he could produce the same substance by heating a chemical that had been isolated from gum benzoin, and hence renamed the compound benzin. This name was rejected by other chemists, and in France and England the name benzene was used instead. In 1845 Hoffman isolated benzene from coal tar and in 1865 Kekulé proposed the first structure of benzene (reviewed by Parke, 1989).

1.6. Benzene toxicity

The toxicity of benzene was first noted in 1897 by Santesson and much work was carried out by Selling in the period 1910-1916 (Selling, 1916). Selling was the first to describe benzene as a powerful leucotoxin based on his observations following treatment of rabbits with benzene (Selling, 1916). His observations not only included the effects of benzene on circulating white blood cells, parenchymal cells in the bone marrow and the overall effect on the entire haematopoietic system but also how the bone marrow underwent successful regeneration upon cessation of benzene treatment (Selling, 1916), an effect often seen in exposed individuals (Rangan and Snyder, 1997). More recently a number of long-term studies have found benzene to be carcinogenic at a number of sites in mice and rats, sites that include the zymbal gland, forestomach and adrenal gland (Huff *et al.*, 1989, Maltoni and Scarnato, 1979 and Snyder *et al.*, 1980).

1.6.1. Benzene toxicity in humans

In the early half of the 20th century, acute and chronic toxicity among workers exposed to high levels of benzene was reported by a number of clinicians. At this time however, the observed toxicity was thought to be due to impurities in the benzene rather than the benzene itself (reviewed by Parke, 1989). In the latter half of the 20th century these observations were corroborated by epidemiological studies among workers from a number of different occupations, including shoe workers in Turkey (Aksoy *et al.*, 1974) and rubber workers in Ohio (Rinsky *et al.*, 1987).

Benzene toxicity can be described as either acute or chronic. Acute toxicity is related to the central nervous system resulting in muscle tremors, convulsions, salivation, nystagmus (involuntary, rapid, rhythmic movement of the eyeball) and phenomena of very intense asphyxiation due to paralysis of the medullary respiratory centre (Cohen *et al.*, 1978). Chronic benzene toxicity is predominantly a disorder of the haematopoietic system although central nervous system, cardiovascular and gastrointestinal effects have been reported (Cohen *et al.*, 1978). Benzene induces a range of haematotoxic effects in exposed individuals. These effects include a depression in numbers of one blood cell type (leucopenia, thrombocytopenia and anaemia), an effect that was reversible upon cessation of exposure (Rangan and Snyder, 1997) or a depression in all blood cell types (pancytopenia), an effect associated with irreversible bone marrow aplasia or aplastic anaemia, a disorder which in most cases is fatal (Aksoy, 1989 and Rangan and Snyder, 1997).

Individuals that survive aplasia, have bone marrow with abnormal marrow architecture, inadequate haematopoiesis and many cells demonstrating chromosomal damage, a condition termed myelodysplastic syndrome (MDS). MDS is often considered to be an early stage of acute myeloid leukaemia (AML) (Rangan and Snyder, 1997), a leukaemia type that has been shown to have a strong association with benzene exposure although other types of leukaemia have not been ruled out.

1.7. Bone marrow specific toxicity

1.7.1. Background information

All blood cell types, which can be seen in Figure 1.3, originate from a pluripotent stem cell in the bone marrow, a process referred to as haematopoiesis (Kuby, 1994). Pluripotent stem cells are undifferentiated cells with the capacity to divide and give rise to precursors of any of the cells shown in Figure 1.3. When a pluripotent stem cell divides it can regenerate the stem cell line or in the presence of the appropriate growth factor it can become committed to a particular developmental pathway, a crucial line of development for the maintenance of the finite proportion of haematopoietic cells. The initial division can give rise to lymphoid stem cells, which generate T and B progenitor lymphocytes, or a myeloid stem cell that gives rise to progenitor cells for erythrocytes,

neutrophils, eosinophils, basophils, monocytes, mast cells and platelets (Vander *et al.*, 1990).

Areas of bone marrow in the human body include the vertebrae, sternum, ribs, skull, scapulae, pelvis and proximal limb bones, collectively termed, flat and irregular bones. A diagrammatical cross section of the structure of bone marrow can be seen in Figure 1.4. In adult bone marrow the haematopoietic cells grow and mature on a meshwork of stromal cells. Stromal cells are non-haematopoietic cells, which are predominantly fibroblasts and macrophages and also include fat cells and endothelial cells. Stromal cells provide both a structural and functional role, influencing haematopoietic stem cell differentiation by providing membrane bound and diffusible growth factors called cytokines. Cytokines have overlapping functions which can act individually or in combination to regulate haematopoiesis (Irons and Stillman, 1996a and Tavassoli and Yoffey, 1983); for example, interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor (GM-CSF) are produced by fibroblasts and are required to both sustain and stimulate the growth of stem cells or early haematopoietic progenitor cells (HPC) (Irons and Stillman, 1996a and 1996b).

1.7.1.1. Targets for benzene toxicity within the bone marrow

Any agent (including benzene) that is toxic to pluripotent stem cells can cause alterations in two important capabilities of these cells, which are self-renewal and differentiation. A block or disturbance in self-renewal or differentiation could cause aplastic anaemia or leukaemia, respectively (Aksoy, 1989). An imbalance in blood cell production for whatever reason can lead to a number of haematological disorders which include anaemia, leucopenia, thrombocytopenia, pancytopenia and perhaps even aplastic anaemia and leukaemia; conditions mentioned previously as occurring in benzene exposed individuals. Possible targets therefore within the bone marrow for benzene toxicity include both the developing haematopoietic stem cells and stromal cells.

Of the two main cell types in the stroma, macrophages have been shown to be more sensitive to the toxic effects of benzene compared to fibroblastoid cells mainly due to

the high level of active peroxidase enzymes (fibroblastoid cells have none), which are involved in the metabolism of hydroxylated benzene metabolites to more reactive species, and low level of the detoxifying enzyme, NAD(P)H: quinone oxidoreductase 1 (NQO1) (Ganousis *et al.*, 1992, Thomas *et al.*, 1989b, Trush *et al.*, 1996).

Macrophages play a critical role in haematopoiesis, producing the growth factor interleukin-1 (IL-1), which in turn stimulates fibroblastoid cells to produce the colony-stimulating growth factors IL-3 and GM-CSF, which are required for myelopoiesis. In the presence of the benzene metabolite, hydroquinone (HQ), a decreased production of interleukin-1 (IL-1) has been demonstrated in macrophages (Thomas *et al.*, 1989a) and an increased level of metabolite-covalent binding to macromolecules in this same cell type compared to fibroblastoid cells has also been observed (Thomas *et al.*, 1989b).

Other targets for benzene toxicity include granulocytes (eosinophils, basophils and neutrophils), pluripotent stem cells and myeloblasts. Following benzene exposure granulocytes demonstrated a decreased phagocytic function and a decrease in lipid content, alkaline phosphatase and myeloperoxidase and an increase in acid phosphatase and β-glucuronidase (Aksoy, 1989). Pluripotent stem cells or early progenitor cells which carry the CD34 surface antigen have been shown to be susceptible to aneusomy (increased levels of trisomy and monsomy) of chromosomes 7 and 8 following exposure to the benzene metabolite, HQ. Monosomy 7 and trisomy 8 are two common clonal aberrations found in myeloid leukaemias (Smith *et al.*, 2000). HQ has also been shown to promote the cellular proliferation and differentiation of the myeloblast to the myelocyte but inhibits both maturation to the neutrophil (Hazel *et al.*, 1996b) and apoptosis, resulting in expansion of the clone of myelocytes (Hazel *et al.*, 1996a) and hence promoting the development of leukaemia.

1.7.1.2. Benzene induced toxicity in the blood cells of exposed animals

Benzene-exposed animals often show an imbalance in blood cell numbers which include a decrease in both leukocyte (Lezama *et al.*, 2001) and more specifically, lymphocyte number and an increase in neutrophil, monocyte, and eosinophil cell

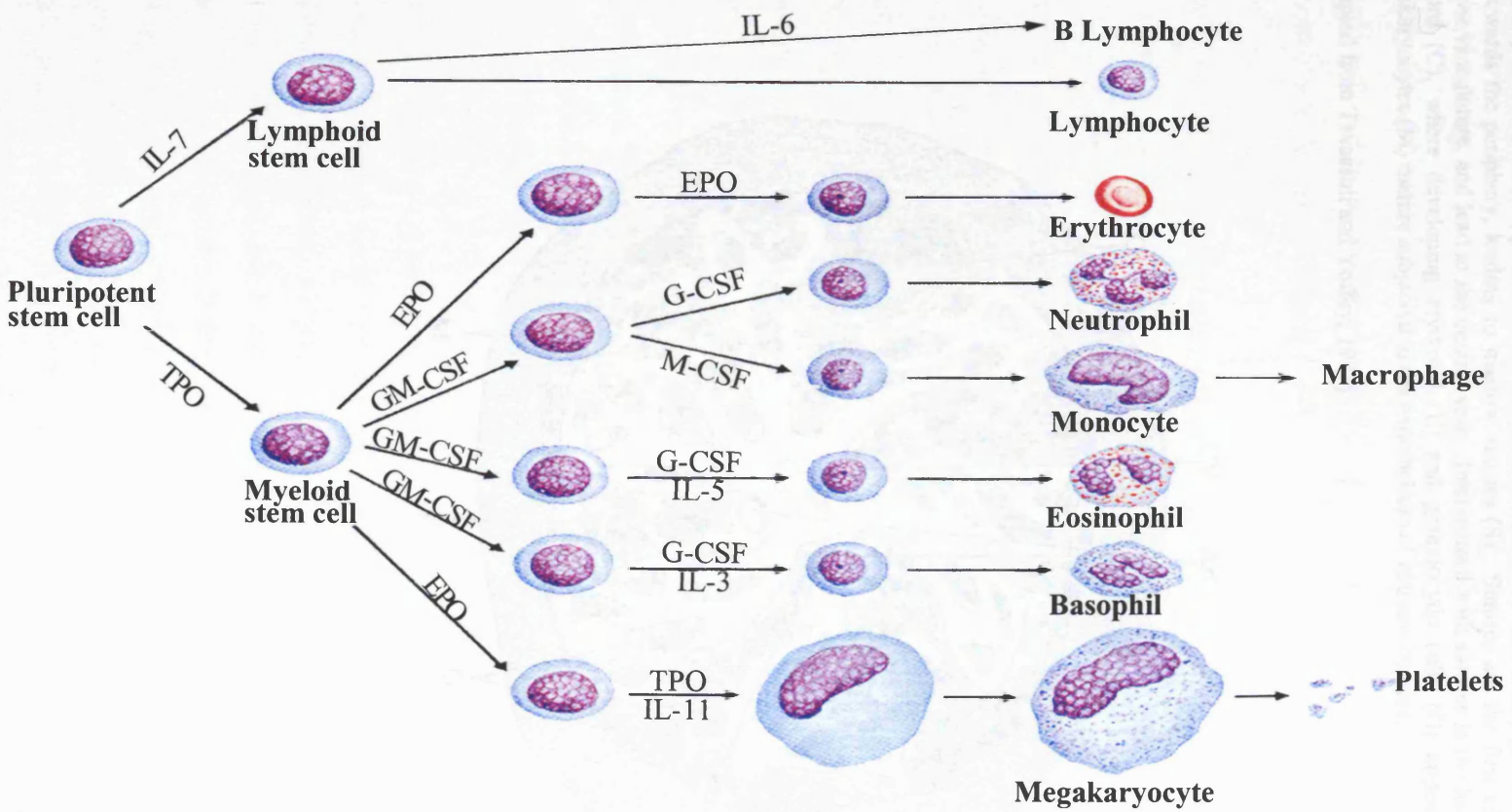
numbers, thought to be due to the stimulation of granulocyte/macrophage progenitor cells by the benzene metabolite, HQ (Henschler *et al.*, 1996).

Male B6C3F1 mice exposed by inhalation to benzene for 6 h/day, 5 days week for 8 weeks showed a rapid reduction in splenic (T and B), thymic (T) and femoral (B) lymphocytes at doses over 100 ppm. Removal of exposure led to a recovery in lymphocyte numbers within approximately 11 days (Farris *et al.*, 1997). A similar study carried out in C57Bl mice (exposed for 6 h/day for 6 days) observed a similar depression in lymphocyte numbers even at doses as low as 10 ppm. Erythrocyte numbers were elevated at 10 ppm, were similar to control levels at 30 ppm and depressed at doses over 100 ppm (Rozen *et al.*, 1984). For longer exposure times (up to 178 days), at 10 ppm benzene a significant decrease in circulating red cells, lymphocytes, marrow burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) colonies were observed in this mouse strain, neutrophil numbers were unaffected (Baarson *et al.*, 1984).

Mouse bone marrow stromal cells have been shown to be significantly more susceptible to the benzene metabolites, HQ and *para*-benzoquinone (*p*-BQ) than stromal cells from rats when comparing the induction of cytotoxicity (Zhu *et al.*, 1995). This difference in susceptibility is thought to be linked to the levels of the detoxifying enzyme, quinone reductase and the cellular antioxidant, glutathione (GSH), which protects cells against oxidative damage, both of which are higher in rat bone marrow stromal cells compared to mouse bone marrow stromal cells (Zhu *et al.*, 1995).

Male Balb/c mice treated with benzene (880 mg/kg) or a combination of metabolites (50mg/kg phenol and HQ) subcutaneously once a day for three days resulted in a 30-40 % decrease in bone marrow cells although this was not selective towards any particular subpopulation of cell. The treatment however did activate phagocytes (macrophages and granulocytes), producing superoxide anions, which are capable of reacting with water and other molecules i.e. metals, to produce hydroperoxy- and hydroxyl radicals. These radicals can induce membrane lipid peroxidation reactions and induce direct damage to cellular macromolecules (Laskin *et al.*, 1989).

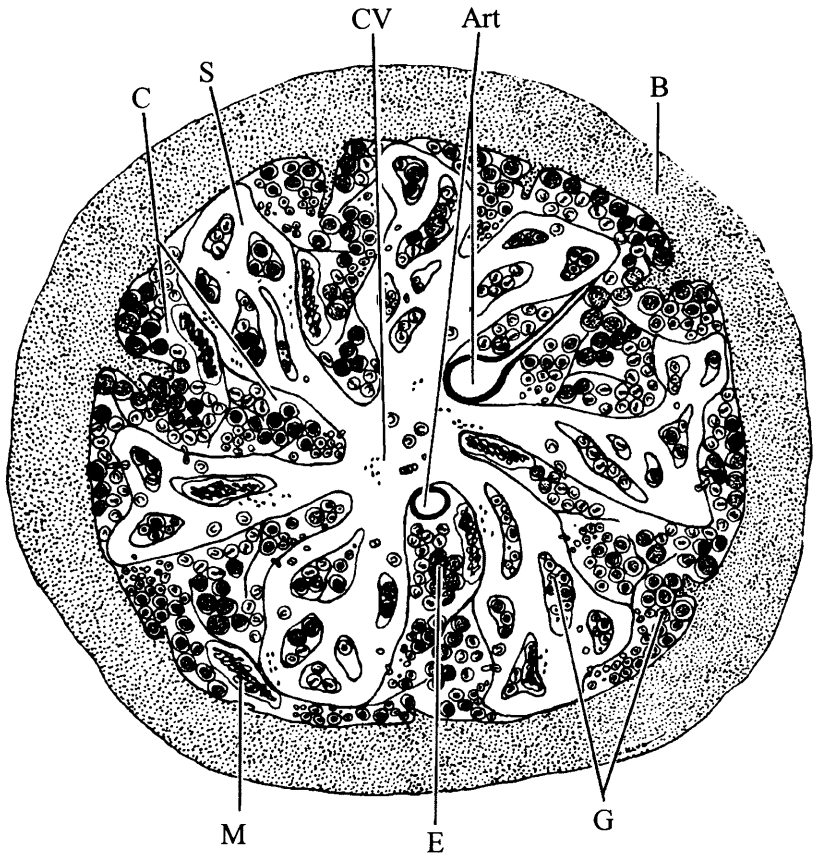
Figure 1.3. : Production of Blood Cells by the Bone Marrow



Adapted from Vander *et al.*, 1990
IL : Interleukin
GM-CSF : granulocyte-monocyte colony stimulating factor
TPO : thrombopoietin
EPO : erythropoietin
M-CSF : Monocyte colony stimulating factor

Figure 1.4: Diagrammatical representation of the organizational layout of bone marrow as seen in the cross section of a tubular bone. The surrounding bone is identified as B. The central artery (Art) and vein (CV) run at the centre, parallel to the long axis of the bone. The artery gives out branches, which run towards the periphery, leading to marrow sinuses (S). Sinuses are the first efferent elements of marrow vasculature, and lead to the central vein. Interspersed with sinuses is the haematopoietic space or cord (C), where developing erythroid (E) and granulocytic cells (G) appear in distinct foci. Megakaryocytes (M) mature subjacent to the endothelium of marrow sinuses.

(Adapted from Tavassoli and Yoffey, 1983).



1.8. Leukaemia and exposure to benzene

Leukaemia occurs at a rate of approximately 9 per 100, 000 people worldwide each year, accounting for 2.5 % of overall cancer incidence and 3.5 % of cancer mortality in the USA (Smith and Zhang, 1998). Leukaemia is the most common form of childhood cancer, (with cancer being the second biggest killer of children) accounting for 12 % of all leukaemias (Smith and Zhang, 1998).

Leukaemia and lymphomas are malignant tumours of the haematopoietic cells of the bone marrow. Leukaemias proliferate as single cells whereas lymphomas tend to grow as tumour masses within a lymphoid tissue such as the bone marrow, lymph nodes or thymus (Kuby, 1994). Leukaemias are monoclonal diseases arising from cells in the haematopoietic stem and progenitor cell compartments, which have lost the capacity to differentiate normally to mature blood cells (Irons and Stillman, 1996a, Smith and Zhang, 1998 and Snyder and Kalf, 1994). The disease is detected by an increase in cell numbers (normally one cell type) in the blood or lymph. As with other cancers, leukaemia is a progressive, multistep disease that can be characterised depending on which cell type is affected, for example, lymphoid or myeloid and whether the disease is acute or chronic (Tavassoli and Yoffey, 1983). Leukaemias are quite unique in that the differences between normal and malignant cells are quite subtle i.e. normal progenitor cells also possess the ability to proliferate, survive intravascular transit and transmigrate into tissues (Tavassoli and Yoffey, 1983).

Acute leukaemias are characterised by aggressively proliferating cells that rapidly colonize the bone marrow and prevent normal blood cell maturation. Chronic leukaemia in comparison, progresses much more slowly. Acute leukaemias include acute lymphocytic leukaemia (ALL) and acute myeloid leukaemias (AML) and these leukaemias can be seen at any age. The chronic leukaemias include chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemias (CML), as these leukaemias progress much more slowly than the acute forms they are only observed in adults (Tavassoli and Yoffey, 1983). AML is the most common leukaemia in adults, whereas in children ALL is most common (this cancer rarely occurs among adults) (Smith and Zhang, 1998).

Leukaemia incidence increased rapidly between 1900 and 1940 and over the last 25 years the incidence of AML has increased significantly in men over 40 years of age (Smith and Zhang, 1998). This increase in incidence, particularly in older males, suggests the importance of occupational and environmental risk factors. An assessment of recent time trends (1984-1993) of various leukaemias and lymphomas however, reported an overall decrease in AML (McNally *et al.*, 1999). As benzene exposure has been associated with AML this may be a reflection on the decrease in the occupational exposure limit to benzene (see section 1.9.2.1. for further details), although it's most likely to be due to improved methods of diagnosis.

The risk factors thought to be involved in the development of leukaemia include genetic factors, for example, Eastern European Jews, individuals with a family history, parents of Down syndrome children and individuals with Down syndrome all have an increased risk of leukaemia whereas Asians have a decreased risk. Other risk factors include ionising radiation, for example, x-rays (Marie Curie and her daughter probably died from leukaemia), chemical exposure, which includes treatment with chemotherapeutic agents (secondary myeloid diseases account for up to 20 % of all AML and MDS cases) and viral infection (risks factors reviewed by Smith and Zhang, 1998). Risk factors in childhood leukaemia include paternal smoking habits prior to conception, the possibility of genetic damage in female and /or male germ cells prior to conception and the geographical location of the child's home with respect to industries involved in benzene and petroleum product manufacture (Smith and Zhang, 1998).

Benzene exposure has been associated with non-Hodgkin's lymphoma, lymphocytic leukaemia, lung cancer, nasopharyngeal cancer (Smith and Zhang, 1998) and an increased incidence of AML (Rinsky *et al.*, 1981). Benzene associated AML is historically preceded by a period of preleukaemia and accompanied with clonal cytogenetic abnormalities involving loss of all or part of chromosomes 5 and 7. In *de novo* AML these abnormalities occur at a much less frequency (approximately 4 % compared to approximately 90 % of cases with benzene associated AML) (Irons and Stillman,1996a). A cluster of genes involved in the regulation of haematopoiesis are located on q31 on chromosome 5, including GM-CSF, IL-3, IL-4, IL-5 CD14 (a myeloid specific surface molecule which has structural characteristics of a receptor)

and early growth response 1 (EGR1) and hence loss of part or whole of chromosome 5 could lead to production of a non functional protein or reduction in level of gene products (Irons and Stillman, 1996a).

The most common translocation found in AML is a reciprocal translocation between chromosomes 8 and 21, resulting in fusion of the AML-1 (a potential leukaemia gene at 21q22) and the ETO (eight twenty one) genes producing a highly active transcription factor which promotes both excessive growth and differentiation paralysis in myeloid progenitor cells (Smith, 1996). Another translocation commonly occurs at 11q23, the site of the MLL (myeloid/lymphoid or mixed lineage leukaemia) gene, a rearrangement commonly seen in infants with leukaemia (Smith, 1996 and Smith and Zhang, 1998).

1.9. Exposure to benzene

Of all the organic chemicals known to be human carcinogens benzene is produced in the greatest volume with the widest number of possibilities for human exposure both in the workplace and in the general environment. Occupational exposure generally gives the greatest exposure potential but due to benzene's presence in such sources as petrol and cigarettes, albeit present as a constituent of a more complex chemical mixture, which may exacerbate or inhibit the effects of benzene, the general public is at risk of exposure to benzene.

1.9.1. Environmental exposure to benzene

Much information regarding non-occupational exposure to benzene was based on studies carried out by the U.S. Environmental Protection Agency (EPA) in the 1980's (Wallace, 1996). Their study concluded that the main sources of human exposure outside the workplace are due to personal activities, which include smoking and the use of petrol driven cars.

1.9.1.1. Smoking related benzene exposure

It has been estimated that smokers (non-occupationally exposed) receive 90 % of their benzene exposure from mainstream smoke (smoke drawn from the mouth end of a cigarette) with an average smoker in the U.S. inhaling approximately 1.8mg of benzene

per day (Wallace, 1989 and Wallace, 1996). Benzene is present in both mainstream and side stream cigarette smoke (material released into the air from the burning tip of the cigarette plus that which diffuses through the cigarette paper) at approximate concentrations of 45 μg/cigarette and 450 μg/cigarette, respectively (Korte *et al.*, 2000). The amount of benzene inhaled depends on the number and type of cigarettes smoked on a daily basis, with a light smoker (20 cigarettes/day) inhaling approximately 0.45 g benzene/year whereas a heavy smoker (40 cigarettes/day) will inhale up to 0.9 g benzene/year (Korte *et al.*, 2000). Ultimately, levels of exposure will depend on individual differences in regard to absorption, metabolism and excretion. Mainstream smoke also contains various benzene metabolites, which include both HQ and catechol (110-300 μg and 100-360 μg, respectively) (IARC, 1986).

Cigarette smoking has been associated with an increased risk of leukaemia, with the relative risk being estimated as 1.5 to 2.0 times that expected for non-smokers (Korte *et al.*, 2000). Other components present in cigarette smoke, which are known or suspected to be leukemogens include styrene, 1,3-butadiene and urethane and therefore benzene exposure is unlikely to be independently responsible for all smoking induced leukaemia. A study carried out by Korte *et al.*, 2000, suggested that benzene is responsible for approximately 10-50 % of smoking induced total leukaemia and up to 60 % of smoking induced AML.

1.9.1.2. Non-smoking related exposure to benzene

The daily inhalation of benzene for non-occupationally exposed, non-smoking individuals in the general urban atmosphere has been estimated to be 0.6 mg (Arfellini *et al.*, 1985). Non-smokers receive their benzene exposure from passive smoking (as detailed above), vehicle exhaust fumes (benzene is a constituent of high octane gasoline used in aeroplanes and accounts for 1-2 % of the petrol used in automobiles) and living close to major sources of benzene such as petrochemical plants or refineries.

Exposure to benzene from vehicle exhaust fumes accounts for both outdoor and indoor exposure due to the intrusion of fumes from attached garages and from such activities as driving and refuelling of vehicles (Gilli *et al.*, 1996). Refuelling of vehicles accounts for an average benzene exposure of 1 ppm per refuel (Wallace, 1989). A

recent study in a number of European cities has determined that benzene pollution is approximately 1.5 times higher in the home than at outside street level (Cocheo *et al.*, 2000), possibly due to adsorbent surfaces in the home attracting pollutants from the outside environment.

Benzene has been shown to be present in a range of consumer products, including adhesives, paints and rubber products (Wallace, 1989). Initial worries that benzene was also present in food was quelled by studies carried out by the U.S. Food and Drug Administration (FDA) who analysed more than 50 foods for benzene content and concluded that negligible amounts were present (Wallace, 1996).

1.9.2. Occupational exposure to benzene

Industrially, benzene is produced mainly from petroleum and coal sources with production in 1980 in the USA totalling more than 5 million tonnes with approximately 2 million workers potentially exposed to benzene (Parodi *et al.*, 1989). In 1987, the number of exposed workers had decreased to nearly a quarter of a million people in the USA, but of these workers, 10,000 were exposed to benzene at levels between 1 and 10 ppm and 370 were exposed to levels greater than 10 ppm (Nicholson and Landrigan, 1989).

Benzene has been used in a number of industries including the leather industry, in the production of artificial rubber, gilding, bronzing, silvering, varnish, in the printing industry and in dry cleaning (Cohen *et al.*, 1978). Benzene has been used in the chemical industry as a starting material in the production of explosives, pesticides, plastics, detergents, antioxidants and medicinals. Benzene has also been used as a solvent in the manufacture of a number of compounds including rubber gums, resins, celluloid, fats and alkaloids (Cohen *et al.*, 1978 and Snyder 1984).

1.9.2.1. Regulation of benzene use in the workplace

Due to early reports of benzene toxicity in occupationally exposed individuals a recommended exposure level to benzene was first introduced in 1941 and was set at 100 ppm /8 h time weighted average (TWA). Over the years this level has decreased to 50 ppm / 8 h TWA (1947), 35 ppm/ 8 h TWA (1948), 25 ppm (1957) and 10 ppm

(1969) (Rinsky *et al.*, 1981). Benzene was declared a carcinogen by NIOSH (National Institute for Occupational Safety and Health) in 1976, by the U.S. EPA in 1979 and by the International Agency for Research on Cancer (IARC) in 1982 (Nicholson and Landrigan, 1989). As a consequence of these declarations, the Occupational Safety and Health Administration (OSHA) reduced the permissible work place concentrations of benzene to the new 8 h TWA of 1 ppm (Nicholson and Landrigan, 1989 and Rinsky *et al.*, 1987). This decision however was overruled in 1980 by the U.S. Supreme court who claimed lack of substantial evidence, and for a further 7 years, until 1987 when the OSHA 1 ppm standard was reintroduced, workers could be exposed to 10 ppm benzene in the workplace. It has been estimated that due to delays in regulation, between 30 and 490 excess leukaemia deaths will occur due to occupational exposure to benzene greater than 1 ppm between 1978 and 1987 (Nicholson and Landrigan, 1989).

Estimates of risk have predicted that 44 to 152 excess leukaemia deaths will result from exposure to benzene at 10 ppm over a 45 year working lifetime. In comparison, exposure to 1 ppm over the same 45 year working lifetime will result in 4-15 leukaemia deaths per 1000 people exposed (Nicholson and Landrigan, 1989), a level estimated by the US OSHA to double the lifetime risk of dying from myelogenous leukaemia (Goldstein, 1989).

In the UK, AML incidence has shown a decline between 1984 and 1993. Although this has not been linked to the lowering of the exposure limit to benzene, it may be related (McNally *et al.*, 1999).

1.9.2.2. Epidemiological studies of benzene exposed workers

Epidemiological studies of benzene-exposed workers are broadly classified as individuals who work with benzene based solvents and those involved in the manufacture of petroleum products. A significant number of leukaemia cases of occupationally exposed individuals have been reported. Such individuals include workers in Ohio, USA, involved in the synthesis of rubber hydrochloride (Pliofilm) (Rinsky *et al.*, 1981, Rinsky *et al.*, 2002 and Silver *et al.*, 2002), vehicle mechanics in the District of Columbia (Hunting *et al.*, 1995) and shoe workers in Istanbul, Turkey (Aksoy, 1989; Aksoy *et al.*, 1974 and Tunca and Egeli, 1996).

The first epidemiological study reporting the role of benzene in the development of leukaemia was carried out in chronically exposed shoe workers in Istanbul between 1967 and 1973. The concentration of benzene was found to reach between 210-650 ppm during working hours and the content of benzene in the adhesives and thinners used was between 9 and 88 % (Aksoy, 1989). Of 28,500 workers, 26 presented with leukaemia or pre-leukaemia between 1967 and 1973 and in 1974 this number rose to 31. The incidence of leukaemia in this select population was 13.59 per 100,000, a significant increase over the incidence of leukaemia in the general population (6 per 100,000) (Aksoy, 1989). This number started to decrease after the discontinuation of the use of benzene, which was introduced in 1969.

Workers in the petroleum industry represent one of the largest populations exposed to benzene in the workplace and numerous studies have been carried out both in the USA and in the UK, where historically the benzene content of petroleum is twice that of petroleum in the USA (Raabe and Wong, 1996). The benzene content of petroleum has always varied but rose significantly with the introduction of catalytic cracking in the 1930s, with the typical benzene content of petroleum ranging between 1-5 % (Schnatter, 2000). As can be seen in Table 1.3, which has combined data from 19 cohorts of petroleum workers (adapted from Raabe and Wong, 1996), the distribution of leukaemia deaths amongst workers was comparable to the figures reported for the general public. This lack of increase, in particular in AML cases, is probably due to a threshold of exposure, which is required to produce a significant increase in AML. The data therefore suggests that petroleum workers would not have accumulated sufficient benzene exposure in excess of the AML threshold. An increase observed in low exposed individuals can be more likely accounted for due to cigarette smoking or exposure to other risk factors (Schnatter, 2000). It has been estimated that only 2 % of workers at refineries were exposed to between 25 and 50 ppm, with 60 % being exposed to less than 1 ppm.

In later studies, which occurred after strict regulations were placed on benzene in the workplace, little correlation between leukaemia risk and exposure was observed suggesting that there is no risk at the present set limits.

Table 1.3: Comparison of leukaemia cell types between petroleum workers in the USA and UK with comparison to cell types observed in the general population

Cell Type	No. of Petroleum Workers (%)		General Population, %
	USA (%)	UK (%)	
Acute myeloid leukaemia	103 (46)	45 (45)	43
Chronic myeloid leukaemia	43 (19)	19 (19)	19
Acute lymphocytic leukaemia	25 (11)	9 (9)	11
Chronic lymphocytic leukaemia	55 (24)	28 (28)	28

Table adapted from Raabe and Wong, 1996

1.10. Routes of exposure to benzene

Exposure to benzene can occur by a number of routes, the main one being inhalation. Other, more minor routes include ingestion and dermal contact (Cohen *et al.*, 1978 and Medeiros *et al.*, 1997). On inhalation, benzene rapidly reaches equilibrium with the alveolar air and saturation is thought to occur within minutes (Hunter and Blair, 1972). Only 40-50 % of the inhaled benzene is retained (Aksoy, 1985). See section 1.12, for further details in regard to benzene elimination.

1.11. Benzene metabolism

Benzene is the smallest, most stable aromatic hydrocarbon and must undergo metabolism to exert its toxic effects (Snyder *et al.*, 1993b). It is the general consideration that more than one benzene metabolite, acting through a number of mechanisms produces the observed biological effects (Goldstein, 1989). It is not known however, which metabolites are responsible for benzene toxicity, but the main metabolites considered to date include benzene oxide, *trans, trans*-muconaldehyde, the polyphenolics and quinones. An overview of benzene metabolism can be seen in Figure 1.5.

Partial hepatectomy (Sammett *et al.*, 1979) or co-administration of benzene with toluene (a competitive inhibitor of benzene metabolism) reduces both benzene

metabolism and toxicity in mice (Andrews *et al.*, 1977) although at low doses realistic to human exposure there is no evidence of such an inhibition (Melikian *et al.*, 2002). Pre-treatment with inducers of metabolism i.e. ethanol, a known inducer of cytochrome P450 2E1, increases benzene metabolism and toxicity (Gad-el-Karim *et al.*, 1986 and Johansson and Ingelman-Sundberg, 1988).

Cytochrome P450 2E1 is involved in the primary step of benzene oxidation as demonstrated in CYP2E1 knockout mice (Valentine *et al.*, 1996). Following benzene administration, the excretion of benzene metabolites in the urine of these animals was reduced by up to 90 % and a complete lack of benzene myelotoxicity and cytotoxicity was reported. Cytochrome P450s are not confined solely to the liver but are ubiquitous to all tissues and with relevance to benzene toxicity are found in the endoplasmic reticulum of leukocytes (Parke, 1989) and in cells of the lung (Ross, 1996).

In all species studied, benzene metabolism has been shown to occur primarily in the liver by two metabolic pathways, one which leads to the production of ring hydroxylated compounds and one which involves the ring opening of benzene (Ross, 1996). Initial metabolism of benzene involves the synthesis of the electrophilic intermediate benzene oxide, mainly via cytochrome P450 2E1 oxidation but there are reports that cytochrome P450 2B1 may also be involved. (Gut *et al.*, 1996b; Ross, 1996 and Snyder *et al.*, 1993a).

Benzene oxide can react with glutathione to form a pre-phenylmercapturic acid, which on transportation to the kidney can undergo further metabolism to the water-soluble *S*-phenylmercapturic acid (*S*-PMA). Approximately 1 % of absorbed benzene is converted to *S*-PMA, which has an elimination half-life of approximately 9 h (Aston *et al.*, 2002) and can be detected as a urinary metabolite within 24-48 h of benzene exposure (Snyder and Kalf, 1994). *S*-PMA is thought to be derived only from benzene metabolism, hence making *S*-PMA an ideal biomarker of choice for monitoring occupational uptake of benzene.

The metabolic pathway leading to the ring opening of benzene can give rise to *trans-trans* muconaldehyde a precursor to *trans-trans* muconic acid, which can also be

detected as a urinary metabolite. Urinary *trans-trans* muconic acid however is not specific for benzene exposure being identified as a metabolite of sorbic acid, a known food additive (Medeiros *et al.*, 1997).

The alternative pathway for benzene metabolism involves the formation of hydroxylated compounds. Benzene oxide is considered to be not very stable and hence undergoes spontaneous rearrangement to form phenol, a reaction that is accelerated in protic media (i.e. water) with a higher rate of conversion at lower pHs (Golding and Watson, 1999). Work carried out by Lindstrom *et al.*, 1997 and 1998, demonstrated that benzene oxide is more stable than previously thought with a half life in blood of 6.6, 7.9 and 7.2 min for mice, rats and humans, respectively, enough time to allow for transport from the liver to the bone marrow, the target organ for benzene toxicity. Phenol can also be formed from the acid catalysed ring opening of benzene oxide followed by aromatisation via loss of a proton (Snyder *et al.*, 1993b). Phenol can undergo conjugation to form phenyl sulphate or further oxidation to form various hydroxylated intermediates, which include HQ, catechol and 1,2,4-benzenetriol (Rangan and Snyder, 1997; Snyder *et al.*, 1993b and Snyder and Hedli, 1996).

An alternative pathway for the formation of catechol involves the reaction of benzene oxide with water in the presence of epoxide hydrolase, to form benzene dihydrodiol, which in turn may be oxidised by dihydrodiol dehydrogenase to form catechol (Snyder *et al.*, 1993b).

In the liver, HQ and catechol can undergo further oxidation to form the reactive quinones, *p*-BQ (an α, β-unsaturated carbonyl compound) and *ortho*-benzoquinone (*o*-BQ), respectively. There is no report that the quinone compounds cause liver toxicity and this is probably because the liver contains NAD(P)H: quinone oxidoreductase (NQO1), an enzyme that detoxifies the quinones by a two electron reduction step (Moran *et al.*, 1999). Siegel and Ross, 2000, however, associate the lack of liver toxicity to carbonyl reductase as they have found little NQO1 activity in the liver.

The liver is structurally divided into three zones (I, II and III). The primary site of conjugating enzymes is in zone I whereas that of cytochrome P450 2E1 is in zone III. Blood entering the liver passes through each region in turn, hence phenol and hydroxylated benzene metabolites entering the liver from a direct administration, would immediately be conjugated in zone I. Benzene however, would escape conjugation going directly to zone III where it would undergo metabolism to phenol and other hydroxylated compounds, which would escape the liver unconjugated (Smith, 1996).

The hydroxylated metabolites can accumulate in the bone marrow (Rickert *et al.*, 1979), the main target organ of benzene toxicity. In rats, low levels of cytochrome P450 2E1 have been observed in bone marrow, but to date there is no evidence of this enzyme being present in human bone marrow (Irons *et al.*, 1980). The bone marrow is rich in peroxidases and low in the detoxifying enzyme, NQO1 (Ross, 1996). Peroxidases reported to be present in the bone marrow include myeloperoxidase (MPO), prostaglandin synthase and eosinophil peroxidase; of these, MPO is known to be present in the highest concentrations (Ross, 1996). Peroxidases activate most substrates by one electron oxidation via radical intermediates (the zymbal gland, an additional target organ in the rat is also rich in peroxidase activity (Ross, 1996)). The hydroxylated compounds, after transportation to the bone marrow, can undergo peroxidase metabolism giving rise to reactive quinones and semiquinone radicals, with the additional generation of reactive oxygen species (Greenlee *et al.*, 1981). The main quinone formed is *p*-BQ, a strong alkylating agent, which can modify both DNA and protein (Snyder *et al.*, 1993b).

Alternatively, the hydroxylated compounds can be further metabolised by sulphation or glucuronidation to give the conjugated end products that are excreted in urine (Snyder and Hedli, 1996). Glutathione (GSH) and quinone reductase (QR) in the bone marrow can act to detoxify these metabolites, for example, 2-(*S*-glutathionyl)hydroquinone is thought to be formed from the reaction of glutathione with *p*-BQ (Snyder *et al.*, 1993b).

A minor route of exposure to benzene is via the skin. Benzene metabolism in human dermal fibroblasts leads to the production of the non-phenolic metabolites, toluene, benzaldehyde, benzoic acid and aniline (Philips *et al.*, 2001).

The metabolic pathways for benzene are similar in all species studied, however differences are seen in the amounts of benzene metabolised and the metabolites produced. Mice metabolise more benzene and produce more HQ metabolites than rats or primates (Golding and Watson, 1999 and Henderson, 1996). In general, a higher proportion of benzene is metabolised at lower doses than higher doses (Rothman *et al.*, 1998), probably due to the saturation of the metabolism of phenol, which is inhibited by high levels of benzene (Schlosser *et al.*, 1993). In humans for the same level of benzene exposure, women have been shown to excrete more metabolites than men (Melikian *et al.*, 2002).

1.12. Benzene excretion

Excretion of benzene metabolites is similar in all species studied, each showing a ready absorption and elimination of benzene in both urine and breath, with almost the entire dose being excreted by these routes within 48 h of exposure (Snyder and Hedli, 1996). However, the amount of benzene excreted was dependent on dose, with the majority being excreted unchanged in expired breath at high benzene doses (Mathews *et al.*, 1998) and excreted in urine at lower doses (Henderson, 1996). The percentage of benzene excreted also reflected the cytochrome P450 2E1 activity in individual species with 50 % and 22 % being eliminated for rats and mice, respectively (Mathews *et al.*, 1998).

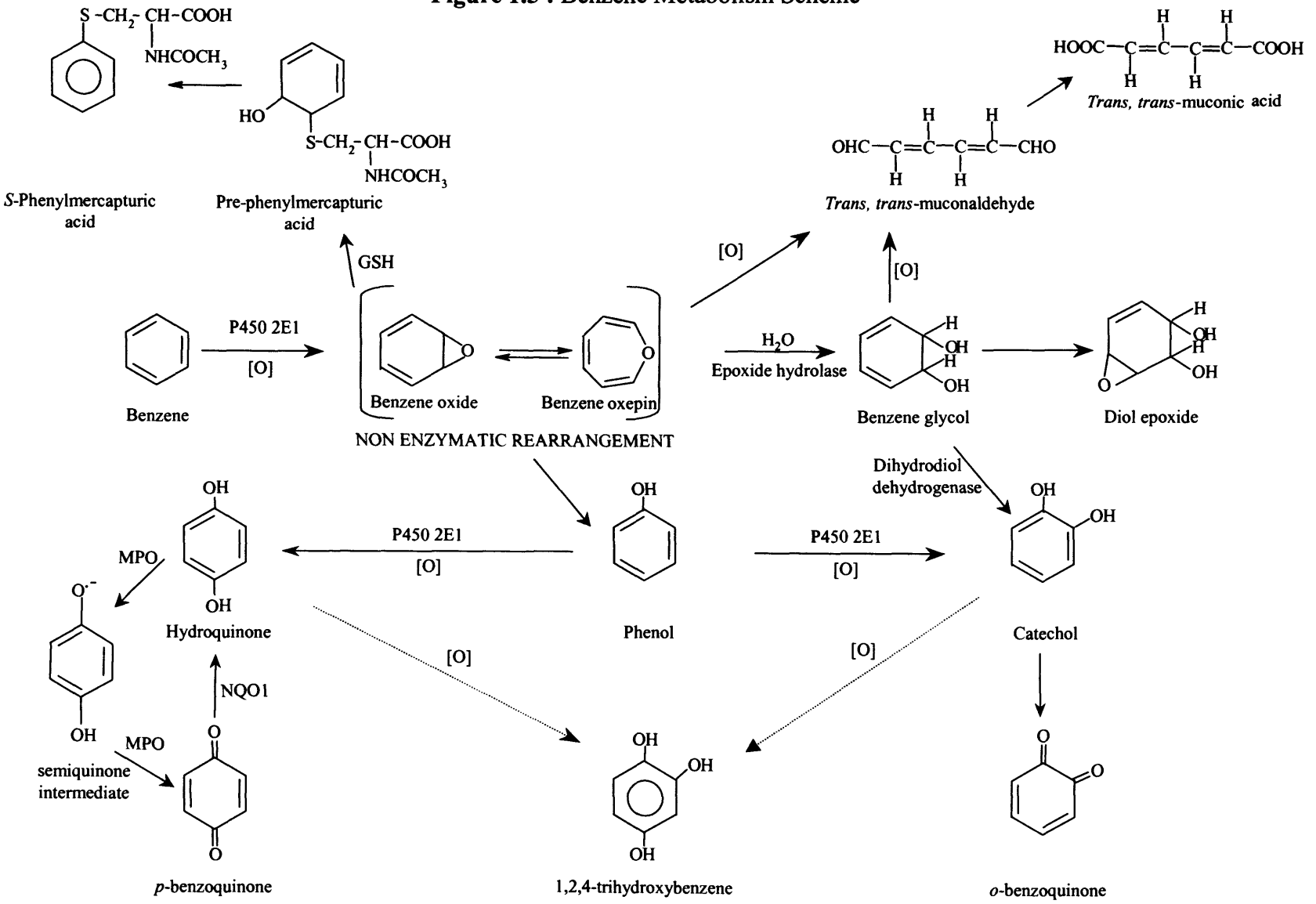
Work carried out on benzene-exposed rats demonstrated a biphasic elimination of benzene from expired air (0.7 and 13.1 h) with benzene reaching steady state concentrations within 4 h in blood, 6 h in fat and less than 2 h in bone marrow. Phenol present in the blood and bone marrow quickly disappeared unlike catechol and HQ, which persisted in both tissues for at least 9 h after cessation of the exposure. Benzene is considered to be rapidly lost from circulating blood, next from tissues including muscle and last from fat, which slowly accumulates and releases benzene (Cohen *et al.*, 1978 and Medeiros *et al.*, 1997). Due to the lipid solubility of benzene it has been

estimated to be 20 times more abundant in the bone marrow than in the blood (Aksoy, 1985) and overweight individuals have a higher incidence of haematological disorders compared to people of an average weight (Cohen *et al.*, 1978 and Medeiros *et al.*, 1997). The biological half-life of the clearance of benzene from fat has been estimated to be 24 h (Medeiros *et al.*, 1997).

Interspecies differences in regard to urinary metabolites have been observed. Mice produce predominantly phenyl sulphate and hydroquinone glucuronide, rats produce mainly phenyl sulphate and hamsters, hydroquinone glucuronide and muconic acid (Mathews *et al.*, 1998). Mice excrete more muconic acid than rats, and hamsters produce detectable amounts of 1,2,4-benzenetriol and catechol sulphate, which is not observed in the urine of rats or mice. Rabbits gave similar results to rats, excreting 23 % of the dose in urine as phenyl sulphate (Parke, 1996).

The low levels of benzene metabolites not excreted can be transported round the body to the site of their toxic effects (Medeiros *et al.*, 1997).

Figure 1.5 : Benzene Metabolism Scheme



adapted from Snyder *et al.*, 1993b.

NQO1 : NAD(P)H: quinone oxidoreductase
MPO : myeloperoxidase

1.13. Biomarkers of benzene exposure

1.13.1. Biomarkers of exposure

Biomarkers of benzene exposure include benzene itself and its metabolites and adducts with macromolecules, which include DNA, RNA and protein. Benzene exposure can be monitored by the analysis of exhaled breath, blood or urine for the presence of benzene or a metabolite of benzene.

1.13.1.1. Exhaled breath

Exhaled breath can be monitored for the presence of benzene itself with approximately 70 % of inhaled benzene being exhaled; benzene which is not absorbed or retained accounts for 50 % of this figure, the remainder accounts for 40 % of the absorbed dose being exhaled (Medeiros *et al.*, 1997). Exhalation of benzene only accounts for 12 % of the total dose during the first 6 h of exposure (Cohen *et al.*, 1978). Elimination of benzene in the breath occurs in two phases, the first phase having a half-life of 2.6 h and the second slower phase having a half-life of 24 h hence monitoring of exhaled breath needs to be carried out shortly after exposure as the concentration in breath decreases rapidly. Sampling of exhaled breath is advantageous as no invasive technique is required, however monitoring of exhaled breath is unable to detect environmentally relevant, low doses of benzene (less than 1 ppm) (Medeiros *et al.*, 1997).

1.13.1.2. Urinary metabolites

Absorbed benzene is metabolised to ring hydroxylated metabolites as mentioned earlier (see section 1.11), which include phenol, HQ, catechol and 1,2,4-trihydroxybenzene these metabolites can be excreted in the form of sulphate or glucuronide conjugates in the urine within 24-48 h of exposure. Monitoring of urine for these metabolites to assess for extent of benzene exposure has disadvantages as these metabolites are not specific for benzene exposure, being derived from various foodstuffs. For example, significant background levels of phenol derived from exogenous and endogenous sources are always present in urine and hence its use as a biomarker is limited as specificity is lacking at exposures less than 5 ppm (Bechtold and Henderson, 1993;

Medeiros *et al.*, 1997 and Smith, 1996). Phenol is currently used as a benzene biomarker only after accidental high level exposure (Smith, 1996).

Other urinary metabolites of benzene include the ring-opened metabolite, *trans, trans*-muconic acid. *Trans, trans*-muconic acid is a minor metabolite of benzene and has been shown to be a sensitive and specific biomarker for exposures as low as 0.5 ppm. Again its presence in urine may not solely be due to benzene exposure as *trans, trans*-muconic acid has been reported to be a minor metabolite of the food additives sorbic acid and potassium sorbate. Sorbic acid based preservatives are used in several food categories including processed cheese slices and spreads, refrigerated flavoured drinks, sweet baked goods, mayonnaise, margarine and salad dressing (Weaver *et al.*, 2000). Despite this, methods utilising HPLC coupled to tandem mass spectrometry or GC-MS have been employed to distinguish heavy smokers from non smokers (Melikian *et al.*, 1999), glue and shoe workers or rubber and glue workers in China from unexposed individuals (Bechtold and Henderson, 1993 and Melikian *et al.*, 2002) respectively, and smokers from occupationally exposed individuals (Lin *et al.*, 2002).

The reaction of benzene oxide with glutathione results in the production of pre-phenylmercapturic acid, which, under acidic conditions, can undergo aromatisation by dehydrogenation to yield *S*-phenylmercapturic acid (*S*-PMA). *S*-PMA is excreted in the urine 24-48 h following benzene exposure. Its use as a biomarker has demonstrated high sensitivity and specificity, with levels detectable after exposures to benzene as low as 0.3 ppm, although saturation of the assay used occurs at higher exposures (Medeiros *et al.*, 1997). Recently, simple HPLC methods, HPLC coupled to tandem mass spectrometry (MS), and a competitive ELISA have been developed specific for *S*-PMA analysis. The ELISA method allows for large scale, routine monitoring of individuals and has been applied to individuals working in the petroleum, steel and chemical industries, the assay has a working range of 40-1200 nmol *S*-PMA/litre urine (Pople *et al.*, 2002). The HPLC methods with and without tandem MS, have been applied to the analysis of urine from a number of occupationally exposed groups. These groups include chemical plant workers exposed to between 0.01- 21.1 ppm benzene (Ghittori *et al.*, 1999), glue and shoe workers in China exposed to between 0.06 and 122 ppm (Melikian *et al.*, 2002) or gasoline station attendants exposed in some cases to less than

1 ppm benzene (Inoue *et al.*, 2001). The analysis of urine from the gasoline station attendants involved the *in vitro* conversion of pre-PMA to *S*-PMA, the limit of detection of this assay being 0.2μg/litre (Inoue *et al.*, 2001). All the methods employed were capable of determining exposed from non-exposed individuals. The studies carried out by Melikian *et al.*, 1999 (LC-tandem MS) and Ghittori *et al.*, 1999 (HPLC) were also able to determine a difference between smokers and non-smokers in the control subjects. In the Ghittori study, the control subject smokers had a similar *S*-PMA level to occupationally exposed individuals.

N-acetyl-*S*-(2,5-dihydroxyphenyl)-L-cysteine is formed from the reaction of *p*-BQ with glutathione followed by further metabolism, and has been detected in the urine of animals but not humans. As *p*-BQ is formed originally from phenol, this biomarker also shares the same limitations in regard to exposure from other sources (Medeiros *et al.*, 1997).

When monitoring individuals in an occupational setting, there is a significant risk that exposure to toluene has also taken place, which results in a mutual suppression of metabolism. This effect will therefore have a confounding effect on biomarker quantitation of benzene exposure (Medeiros *et al.*, 1997).

1.13.2. Biomarkers of effective dose

The presence of DNA and protein adducts can be used as a measure of effective dose, a measure that can assess an exposure which occurred over a more distant time frame as opposed to monitoring of benzene and benzene metabolite excretion.

1.13.2.1. DNA adducts

Benzene DNA adducts will be discussed in greater detail in Chapters 2 and 5, but will be briefly mentioned here.

Through the use of rodents the covalent binding of benzene metabolites to DNA in various tissues has been demonstrated. Scintillation counting of DNA following the administration of radiolabelled benzene detected binding to rat liver DNA (Lutz and Schlatter, 1977 and Mazzullo *et al.*, 1989), rat spleen, kidney, lung and stomach DNA

(and RNA) (Mazzullo *et al.*, 1989) and mitochondrial DNA from mouse liver and bone marrow (Gill and Ahmed, 1981). Specific DNA adducts have also been determined using the sensitive assay of ^{32}P-postlabelling. This assay was applied to the analysis of DNA from various tissues, which included both bone marrow and white blood cells of male B6C3F1 mice treated with 440mg/kg benzene, twice a day for up to 7 days (Bauer *et al.*, 1989; Bodell *et al.*, 1996; Levay *et al.*, 1996 and Pathak *et al.*, 1995).

The main DNA adduct from treatment of rodents with benzene was identified as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate. However, to date, benzene-DNA adducts have not been determined in human DNA following *in vivo* exposure.

Other DNA adducts include the unstable adduct, N-7-phenylguanine, whose presence has yet to be determined in animal or human urine (Medeiros *et al.*, 1997; Norpoth *et al.*, 1996 and Schell *et al.*, 1993), and 8-hydroxy 2'-deoxyguanosine, a marker of oxidative damage. Oxidative damage has been measured in both benzene-exposed mice and Italian gas station attendants (Smith, 1996) although this marker of DNA damage is not specific for benzene exposure.

1.13.2.2. Protein adducts

Proteins routinely used in biomarker analysis include albumin (plasma) and haemoglobin (red blood cells), and in the case of benzene exposure bone marrow proteins are also utilised. Due to the lifetimes of albumin (20-25 days for human albumin) and haemoglobin (human haemoglobin has a lifetime of approximately 120 days, haemoglobin from F344 rats has approximately a 66 day lifetime), protein adducts represent a longer term or more distant exposure (1-4 months) compared to, for example, metabolites in urine which reflect a more recent exposure i.e. hours (Yeowell-O' Connell *et al.*, 1998).

Various benzene metabolites, which include benzene oxide (BO), *o*-BQ and *p*-BQ are capable of binding to the nucleophilic sites of proteins, i.e. the thiol moiety of cysteine and the protein adducts formed are considered to be specific biomarkers for benzene exposure. Haemoglobin, albumin or bone marrow protein adducts of the three

metabolites given above have successfully been detected *in vivo* in rats (F344) and mice ($B6C3F_1$), from a range of benzene doses and from various exposure routes (Bechtold *et al.*, 1992a; Bechtold *et al.*, 1992b; Bechtold and Henderson, 1993; Bechtold and Strunk, 1996; McDonald *et al.*, 1994; Melikian *et al.*, 1992; Yeowell-O'Connell *et al.*, 1996) with rats giving a generally higher adduct level compared to mice (Bechtold *et al.*, 1992a and Rappaport *et al.*, 1996). Adducts have also been detected in human protein following occupational exposure to benzene (Bechtold *et al.*, 1992a; Bechtold *et al.*, 1992b; Bechtold and Henderson, 1993).

Binding of metabolites to proteins as assessed by i*n vitro* studies however, has shown differential binding i.e. benzene oxide is more reactive to haemoglobin of rats than mice or humans, whereas for albumin, binding was greatest for human and rat protein compared to that of the mouse. Reactivity of benzene oxide towards human albumin was thirty times greater than with human haemoglobin although this may be influenced by the turnover rate, haemoglobin being slower than albumin and the level of glutathione in human red blood cells being 2000 times higher than in plasma (Lindstrom *et al.*, 1998). Differential binding has also been observed *in vivo*, with different metabolites; in rat blood, BO formed the majority of the adducts whereas in bone marrow, adducts of *o*-BQ predominated (McDonald *et al.*, 1994). In mice however, *p*-BQ gave the majority of adducts observed in both blood and bone marrow (McDonald *et al.*, 1994).

Benzene-protein adduct detection has proved to be dependent on both method sensitivity and specificity. In early studies, high levels of background adducts were detected for *o*- and *p*-BQ after reaction *in vitro* with rat and human haemoglobin and albumin (McDonald, 1993). A high background level was also observed *in vivo* in haemoglobin and bone marrow proteins of unexposed humans and animals (McDonald *et al.*, 1993). These high background values were thought to be due to environmental, dietary or endogenous sources, which were independent of benzene exposure. Due to this finding it was considered that monitoring of samples for *o*- and *p*-BQ adducts would only be useful in highly exposed individuals (Rappaport *et al.*, 1996). Benzene oxide-protein adducts (*S*-phenylcysteine) which are thought to originate solely from cytochrome P450 metabolism of benzene, were considered more specific for benzene

exposure. Early attempts to detect such adducts in occupationally exposed individuals were not particularly successful due to the limit of detection of the assay at that time being approximately 500 pmol adduct / g haemoglobin (Bechtold *et al.*, 1992a). Since then the limit of detection has been improved upon and is now approximately 20 pmol adduct / g haemoglobin (Waidyanatha *et al.*, 1998 and Yeowell-O' Connell *et al.*, 1996). This improved method has been used for the analysis of both BO-albumin and BO-haemoglobin adducts in mice, rats (Lindstrom *et al.*, 1998) and humans (Lindstrom *et al.*, 1998 and Yeowell-O' Connell *et al.*, 1998).

Although there is 6 times more haemoglobin than albumin in human blood, albumin adducts have proved to be a better biomarker than haemoglobin adducts due to albumin's increased reactivity (McDonald, 1993) as reflected in the results of Yeowell-O' Connell *et al.*, 1998, who observed a high relative abundance of BO-albumin adducts compared to BO-haemoglobin adducts.

Protein adducts may also be used as an indirect biomarker of effect as benzene metabolites have been shown to bind selectively to histone proteins (Williams *et al.*, 2002). Such an interaction could affect DNA folding and packaging assisting in the production of strand breaks as demonstrated with catechol (Oikawa *et al.*, 2001).

1.13.3. Biomarkers of susceptibility

Inherited polymorphisms in enzymes involved in benzene metabolism and detoxification, which include cytochrome P450 2E1, glutathione *S*-transferase (GST) (involved in the detoxification of epoxides including benzene oxide), NAD(P)H quinone oxidoreductase I (NQO1), myeloperoxidase (MPO) and epoxide hydrolase can all be used as biomarkers of susceptibility (Smith and Zhang, 1998).

1.13.3.1. Cytochrome P450 2E1

Cytochrome P450 2E1 is involved in the initial metabolism of benzene to benzene oxide and further metabolism to phenol. A specific mutation in the 5'-regulatory region of the CYP2E1 gene causes over expression of the gene (Marsh *et al.*, 1999) and

hence results in an increase in benzene metabolism. Individuals carrying this mutation have an increased risk of benzene toxicity (Marsh *et al.*, 1999).

1.13.3.2. NQO1

NAD(P)H quinone oxidoreducatase I (NQO1), also known as DT-diaphorase after its discovery as a cytosolic diaphorase in 1958, is a flavoprotein involved in the detoxification of quinones to HQs via a two electron reduction mechanism.

NQO1 activity has been detected in human bone marrow mononuclear cells, $CD34^+$ progenitor cells (Moran *et al.*, 1999) and murine fibroblasts (Thomas *et al.*, 1989a) and to a lesser extent in murine bone marrow macrophages (Ganousis *et al.*, 1992 and Trush *et al.*, 1996).

A polymorphism in NQO1 has been identified which involves a C to T point mutation at position 609 of the NQO1 cDNA, which confers a proline to serine substitution at position 187 of the NQO1 protein. Individuals homozygous (T/T) for this change have no detectable NQO1 activity and hence are at an increased risk of benzene toxicity (Moran *et al.*, 1999). The frequency of homozygosity among Caucasians and African Americans is 4-5 % (Larson *et al.*, 1999), whilst Asians have a much higher prevalence, with a frequency ranging from 4-20 % (Moran *et al.*, 1999). In a group of leukaemia patients, the incidence of the mutant allele was 1.4 fold higher in patients who had myeloid leukaemia secondary to chemotherapy (t-AML) and was 1.6 fold higher in patients with abnormalities in chromosomes 5 and/or 7 compared to what would be expected in the general population (Larson *et al.*, 1999 and Smith, 1999).

1.13.3.3. Myeloperoxidase

Myeloperoxidase (MPO) is a phase I metabolic enzyme found in monocytes/macrophages and in the primary granules of neutrophils. MPO is involved in the oxidation of procarcinogens. MPO has a polymorphic region located in a hormone response element region 463 bp upstream of the MPO gene, which appears to reduce transcriptional activity (Schabath *et al.*, 2000). The polymorphism is a G→A nucleotide base shift which negates the binding region for the general transcription factor Sp1 (Schabath *et al.*, 2000). In normal mature cells, MPO occurs exclusively as

a dimer, whilst in immature leukaemia cells there are both monomeric and dimeric species (Taylor *et al.*, 1990).

1.13.3.4. Epoxide hydrolase

Microsomal epoxide hydrolase (*HYL1*), occurs with the highest activity in the liver and is involved in the metabolism of benzene oxide to benzene dihydrodiol, which can subsequently be metabolised to catechol and *o*-BQ (Snyder and Hedli, 1996). A number of polymorphisms in *HYL1* exist. The *HYL1*2* allele polymorphism in exon 3 involves substitution of histidine for tyrosine at amino acid position 113, this polymorphism is associated with a 40 % decrease of enzyme activity *in vitro*. The *HYL1*3* allele involves substitution of an arginine for histidine at amino acid position 139, and is associated with a 25 % increase in activity (reviewed in Lebailly *et al.*, 2002). In a study of AML cases the presence of *HYL1*3* was associated with an increased risk of AML in males with t(8:21) or –7/del(7q) which was not observed with people of a normal karyotype. Males who were heterozygotes for *HYL1*2* with these chromosomal aberrations were associated with a decreased risk of AML (homozygotes showed a similar level to controls) (Lebailly *et al.*, 2002).

1.13.4. Biomarkers of effect

Metabolites of benzene have been shown to cause a number of effects in cells, both after *in vitro* and *in vivo* exposure. These effects include micronuclei formation, sister chromatid exchange, unscheduled DNA synthesis, gene mutations and various chromosomal aberrations, which include breaks and loss or gain of whole or part of chromosomes.

1.13.4.1. Micronuclei formation

Metabolites, which include phenol, catechol, HQ and *p*-BQ have been shown to cause chromosomal loss (aneuploidy) and breakage (clastogenicity) as measured by the formation of micronuclei (MN). Micronuclei are formed when either an entire chromosome or a chromosome fragment fails to segregate into either of the two main daughter nuclei during cell division. Micronuclei have been observed in human lymphocytes in culture and in various cells of the mouse which include bone marrow

cells, spleen cells, lung fibroblasts and erythrocytes, following exposure to benzene *in vivo* (Chen *et al.*, 1994; Ciranni *et al.*, 1988; Gad-El Karim *et al.*, 1986; Healy *et al.*, 2001; Ranaldi *et al.*, 1998; Tunek *et al.*, 1982 and Yager *et al.*, 1990) and in the lymphocytes of gasoline station workers (Bukvic *et al.*, 1998).

1.13.4.2. Sister chromatid exchange

Benzene and its metabolites have also induced sister chromatid exchange (SCE), which arise from the reciprocal exchange of DNA between two sister chromatids of a duplicated chromosome. Sister chromatid exchanges have been observed both *in vivo* in benzene exposed or smoking individuals (Bukvic *et al.*, 1998; Popp *et al.*, 1992 and Xu *et al.*, 1998) and *in vitro,* in human lymphocytes (Erexson *et al.*, 1985), Syrian hamster embryo cells and human T lymphocytes (Tsutsai *et al.*, 1997) after dosing with benzene or one of its metabolites.

1.13.4.3. Gene mutations

Benzene and its metabolites have been shown to be weak or non-mutagenic in simple gene mutation assays in bacteria and mammalian cells (Glatt *et al.*, 1989). *In vivo* however, benzene has been shown to be mutagenic both in animal systems and in humans, and this is reviewed in greater detail in Chapter 6.

1.13.4.4. Strand breaks

Single strand breaks have been detected following exposure to benzene *in vivo,* in mouse liver and blood cells, (Loft *et al.*, 1998) in human lymphocytes (Andreoli *et al.*, 1997 and Sul *et al.*, 2002) and *in vitro* after exposure to benzene and its metabolites in human lymphocytes (Andreoli *et al.*, 1999) and peripheral blood mononuclear cells (Fabiani *et al.*, 2001). The comet assay was utilised for this detection, which can also be used in the detection of alkali labile sites, DNA crosslinking and incomplete excision repair sites.

The comet assay has successfully been utilised to determine single strand breaks in individuals exposed to levels of benzene as low as 0.3 mg/m^3 (Andreoli *et al.*, 1997). It has also been applied to the analysis of T and B-lymphocytes and granulocytes of

workers in a printing company in South Korea who were exposed to low levels of benzene from between 4 months and 25 years (level not reported). The cells analysed have a life span of 4-10 years, 3-4 days and 5 days, respectively. Damage was observed in all cells compared to controls, with B cells being more sensitive to benzene than granulocytes, which in turn were more sensitive than T cells (Sul *et al.*, 2002). This damage may be linked to the 80 % of lymphoblastic leukaemia and 90 % of non-Hodgkin's lymphoma, which are of B cell derivation (Sul *et al.*, 2002).

1.13.4.5. Chromosomal aberrations

The relevance of chromosomal alterations is of great importance as both structural and numerical alterations have been observed in the peripheral blood cells of occupationally exposed individuals (Rinsky *et al.*, 1981) and chromosomal aberrations, compared to MN and SCE may be predictive of cancer risk (Bonassi and Au, 2002).

Patients suffering from benzene induced bone marrow dysfunction (aplastic anaemia and preleukaemia) often exhibit chromosome loss or gain i.e. chromosomes 5, 7 and 8, which may be a lasting, direct consequence of benzene exposure rather than a secondary effect due to induction of disease. Chromosomal abnormalities associated with benzene exposure in humans include ring chromosomes and dicentric or tricentric chromosomes (Cohen *et al.*, 1978). In a follow up study of Turkish shoe workers previously exposed to benzene, a significant increase in chromosomal aberrations, mainly gaps and breaks, was observed (Tunca and Egeli, 1996). These findings however were only observed in long term exposed individuals (up to 50 years in one case).

Shipyard workers acutely exposed to benzene (>60 ppm) over several days showed haematological abnormalities which persisted for up to one year in a number of the subjects. Exposure was marked mainly by the presence of large granular lymphocytes in peripheral blood (Midzenski *et al.*, 1992). Chromosomal aberrations in lymphocytes have been shown to persist in heavily exposed (hundreds of ppm) individuals up to 20 years after exposure (Forni, 1996), although it's not certain whether these aberrations are due to an age effect or from exposure to other genotoxic agents.

1.14. Application of biomarkers of benzene exposure in humans

Over recent years a number of cohort studies have been carried out which have involved the biological monitoring of occupational exposure to benzene and have been conducted in a number of industries including the petroleum, steel and chemical industries. One early study carried out between 1972 and 1987 in 12 cities in China, assessed approximately 110,000 workers; approximately 75,000 were exposed occupationally to benzene and 35,000 were considered to be control individuals, with women accounting for 47 % and 40 % of the groups, respectively. Exposed individuals were chosen from a number of industries including painting and printing and from rubber, chemical and shoe production. Benzene exposure was estimated for the time period involved by local hygienists, with only 38 % being based on actual monitoring data.

The general findings from this large cohort study were that a mortality increase was observed in exposed individuals, the majority of these deaths being accounted for by cancer, with perhaps a slight (but not significant) increase in deaths due to oesphageal and nasopharynx cancers. Lymphatic and haematopoietic malignancies increased with increasing cumulative exposure to benzene with an excess risk demonstrated at low levels of exposure (<10 ppm), most notably with the subtypes, acute myelogenous leukaemia and aplastic anaemia (Hayes *et al.*, 1996; Hayes *et al.*, 1997 and Yin *et al.*, 1996).

A cross section of these individuals were selected to allow biomarker analysis to be performed. The individuals selected were 44 healthy, benzene exposed individuals with a minimal exposure to toluene and a median 8 h, TWA benzene exposure of 31 ppm (exposure ranged from 1-328 ppm) and 44 unexposed individuals. All individuals were from Shanghai, China. The biomarkers examined are detailed in Table 1.4 and include urinary metabolites, protein adducts, inherited polymorphisms, gene mutations and chromosomal aberrations.

Overall, the findings suggest aberrations in chromosomes 5, 7, 8 and 21 may act as useful biomarkers of early benzene exposure (Smith and Rothman, 2000).

Table 1.4: Biomarker analysis of a sub-population of benzene exposed individuals from the Chinese cohort study

Biomarker Type	Biomarker	Finding	Reference
Biomarker of recent exposure (hours)	Urine metabolites	Benzene metabolites phenol, catechol, HQ and *t,t*-muconic acid in urine showed strong correlation with air benzene levels	Smith and Rothman, 2000; Rothman *et al.*, 1998 and Waidyanatha *et al.*, 2001
Biomarker of longer term exposure (months)	Albumin Adducts	Adducts of 1,4-BQ : median levels were 2110 (n=44), 5850 (n=21) and 13,800 (n=22) pmol adduct / g albumin for controls, workers exposed to < 31 ppm benzene and workers exposed to > 31 ppm benzene respectively	Yeowell-O'Connell *et al.*, 2001
		Adducts of BO : median adduct levels were 106 (n =44), 417 (n=21) and 2400 (n=22) pmol adduct/g albumin for controls, workers exposed to < 31 ppm benzene and workers exposed to > 31 ppm benzene respectively.	Yeowell-O'Connell *et al.*, 2001
	Haemoglobin Adducts	Adducts of BO : median adduct levels were 32.0 (n=22), 46.7 (n =21) and 129 (n=22), pmol adduct/g globin for controls, workers exposed to < 31 ppm benzene and workers exposed to > 31 ppm benzene.	Yeowell-O'Connell *et al.*, 1998
Susceptibility	Inherited polymorphisms	20% of the population carried the homozygous mutation for NQO1	Smith, 1999
Early Effect	Haematotoxicity (complete blood cell count)	Benzene poisoning assessed by decreased WBC count, red blood cells and platelet count	Dosemeci *et al.*, 1996 and Rothman *et al.*, 1996
	Gene mutations; (glycophorin A (GPA))	An increased level of gene duplicating mutations in GPA observed in benzene exposed workers which correlated with increased cumulative benzene exposure	Rothman *et al.*, 1995
	Chromosomal abberations	Increased hyperdiploidy of chromosome 9 in lymphocytes of individuals with high benzene exposure (trisomy 9 being most prevalent)	Zhang *et al.*, 1996
		Increased hyperdiploidy of chromosomes 8 and 21 in lymphocytes of individuals with high benzene exposure. Also increase in translocations between chromosomes 8 and 21, increase in monosomy 5 and 7 with increasing exposure, increases of trisomy and tetrasomy of chromosomes 1,5 and 7 and long arm deletion of chromosomes 5 and 7	Smith and Zhang, 1998; Zhang *et al.*, 1998

1.15. Potential mechanisms of benzene carcinogenesis

To date, the mechanism by which benzene causes carcinogenesis is unknown. It is considered however, that more than one metabolite is involved acting through more than one mechanism on critical targets within the bone marrow. Several hypotheses exist, these include the covalent binding of benzene metabolites to critical proteins and DNA, the causation of oxidative damage and the resultant damage, which includes DNA strand breaks, aneuploidy, mitotic recombination and chromosome translocations (Golding and Watson, 1999 and Smith, 1996). The consequence of any of these effects may involve activation of proto-oncogenes or the inactivation of tumour suppressor genes and if occurring in stem or early progenitor cells may lead to the production of a leukaemic clone.

Critical proteins within a cell include the spindle fibre protein tubulin, histone proteins and topoisomerase II. The covalent binding of quinones to tubulin may inhibit cell replication, whereas binding to the histone proteins can affect both DNA folding and packaging assisting in the production of strand breaks (Williams *et al.*, 2002). Binding to topoisomerase II, an enzyme which participates in DNA replication and transcription by breaking and resealing DNA, hence uncoiling DNA, can again cause DNA strand breaks (Chen and Eastmond, 1995). Strand breaks have the potential to lead to aberrant mitotic recombination and ultimately chromosome aberrations, which include chromosome translocations (Smith, 1996).

The formation of a DNA adduct in a critical gene can hinder both transcription and replication and if not repaired correctly during replication may give rise to a mutation which can affect the activity and function of crucial proteins, activate oncogenes and inactivate tumour suppressor genes (La and Swenberg, 1996). Benzene metabolites may also cause oxidative stress, giving rise to reactive oxygen species, again damaging DNA and giving rise to mutations.

1.16. Aim of work

The overall aim of the work described in this thesis was to develop a sensitive ^{32}P-postlabelling system coupled to HPLC for the determination and analysis of benzene-DNA adducts in occupationally exposed individuals. The assay involved initial development of an antibody recognising the benzene-DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the only adduct identified *in vivo* following treatment of rodents with benzene. The antibody would then be used in an immunopurification step, enriching the DNA adduct, prior to ^{32}P-postlabelling. The assay development and validation involved synthesis and characterisation of a number of adducts formed from the direct reaction of certain benzene metabolites with DNA, *in vitro*.

Upon synthesis of all of the respective benzene-DNA adducts, the mutational assay, *supF*, was utilised to determine the mutational consequence of such adducts and elucidate a potential role for benzene-DNA adducts in the mechanism of benzene induced carcinogenesis.

Chapter 2
In Vitro Adduct Synthesis

2.1 Introduction

Benzene is a known rodent carcinogen causing malignancy in a number of organs in rats and mice (Huff *et al.,* 1989) and exposure has been associated with bone marrow toxicity and an increased risk of leukaemia in humans (Goldstein, 1977). The exact mechanism by which benzene exerts these effects is still unknown. It is known however, that due to its stability, benzene must be metabolised in order to produce its toxic effects. As discussed in Chapter 1, benzene is metabolised in the liver to a number of metabolites, which include phenol and hydroquinone (HQ), two metabolites that can be transported to the bone marrow, where further metabolism can take place. It is a general consideration that to cause its genotoxic and carcinogenic effects, more than one metabolite is involved, acting through multiple mechanisms and on more than one cellular target. Binding of benzene metabolites to macromolecules in the bone marrow, including protein, DNA or RNA may be one mechanism by which benzene exposure gives rise to bone marrow toxicity (other potential mechanisms are discussed in Chapter 1, section 1.15). A number of studies have been performed demonstrating the covalent binding of various benzene metabolites to DNA both *in vitro* and *in vivo.* DNA adduct formation *in vivo,* will be discussed in Chapter 4.

2.1.1. Reactive metabolites of benzene

Metabolism of benzene results in a number of metabolites, which include benzene oxide, phenol, HQ, catechol, 1,2,4-benzenetriol, *para*-benzoquinone (*p*-BQ) and the ring open structure *trans, trans*-muconaldehyde. Of these metabolites only a small number are expected to, or have been shown to react with RNA, DNA and protein. These include, benzene oxide, BQs and muconaldehydes (Golding and Watson, 1999).

2.1.1.1. Benzene oxide

Benzene oxide is the primary metabolite of benzene oxidation. *In vivo*, benzene oxide has a half-life of 7.9 min in the blood of rats (Lindstrom *et al.*, 1997), enough time to allow for transport of the electrophilic intermediate to the target organ, the bone marrow. Benzene oxide has been shown to be capable of reaction with glutathione, and various proteins, both *in vitro* and *in vivo* (Aston *et al.*, 2002; Lindstrom *et al.*, 1998; McDonald *et al.*, 1994; Rappaport *et al.*, 2002a; Rappaport *et al.*, 2002b; Snyder

and Kalf, 1994; Troester *et al.*, 2000; Yeowell-O'Connell *et al.*, 1996; Yeowell-O' Connell *et al.*, 1998 and Yeowell-O'Connell *et al.*, 2001) but to date, there is no evidence to suggest that benzene oxide reacts with DNA to form stable DNA adducts or the unstable adduct, N-7-phenylguanine (Krewet *et al.*, 1993; Norpoth *et al.*, 1996 and Schell *et al.*, 1993).

2.1.1.2. *Trans, trans*-muconaldehyde

Trans, trans-muconaldehyde, a ring open metabolite of benzene has been suggested to act as a bifunctional alkylating agent with the potential to act as a crosslinking agent (Witz *et al.*, 1989). This metabolite has been shown to react *in vitro*, with deoxyguanosine and deoxyadenosine to produce a number of adducts (Bleasdale *et al.*, 1996; Latriano *et al.*, 1989 and Witz *et al.*, 1989). The role of *trans, trans*-muconaldehyde in benzene toxicity however is uncertain, particularly as studies have shown that less than 1 % of this metabolite produced in the liver reaches the bone marrow of exposed mice (Zhang *et al.*, 1997) and to date no muconaldehyde related DNA adducts have been identified in intact DNA after reaction *in vitro* (Bleasdale *et al.*, 1996).

2.1.1.3. Catechol

Quinones formed from the oxidation of catechol can react with DNA to form the depurinating adducts at the N-7 and N-3 of guanine and adenine, respectively (Cavalieri *et al.*, 2002). These adducts however have only been observed *in vitro* and no stable DNA adducts of catechol have been observed.

2.1.1.4. HQ and *p*-BQ

The benzene metabolites HQ and *p*-BQ have been shown to induce a number of effects in cells, both *in vitro* and *in vivo,* these effects include sister chromatid exchanges *in vitro* in human lymphocytes (Erexson *et al.*, 1985), micronuclei formation in human lymphocytes *in vitro* and mouse erythrocytes *in vivo* (Tunek *et al.*, 1982 and Yager *et al.*, 1990), mutations in V79 cells (Glatt *et al.*, 1989), the inhibition of DNA replication (Schwartz *et al.*, 1985) and DNA transcription and the ability to induce single strand breaks (Pellack-Walker and Blumer, 1986). In fact, HQ induces chromosomal damage

in lymphocytes *in vitro* similar to that observed in benzene-exposed workers (Zhang *et al.*, 1998).

Like benzene oxide, *p*-BQ has been shown to react with protein both *in vitro* and *in vivo* but unlike benzene oxide, HQ and *p*-BQ have been shown to react with DNA and are thought to be responsible for the main DNA adduct formed in exposed rodents (Bodell *et al.*, 1996 and Pathak *et al.*, 1995). It is the general consideration that *p*-BQ, or the semiquinone derived from HQ is the major toxic metabolite of benzene (Greenlee *et al.*, 1981).

2.1.2. Benzene DNA adducts formed *in vitro*

A number of studies have been carried out *in vitro* that have demonstrated the formation of benzene-DNA adducts. Direct reaction of the benzene metabolites, HQ and *p*-BQ, with individual nucleotides or DNA resulted in the formation of 3 exocyclic adducts which have been identified as (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate (Jowa *et al.*, 1986; Jowa *et al.*, 1990; Levay *et al.*, 1991; Pongracz *et al.*, 1990 and Pongracz and Bodell, 1991), respectively. It has been hypothesised that these adducts are formed from the reaction of *p*-BQ with the nucleotide base at the N^2, N^6 and the N^4 position of guanine, adenine and cytosine, respectively, resulting in the loss of one water molecule followed by stabilisation through enolization (Jowa *et al.*, 1986).

Adducts forming at the N^2 (and O^6) position are considered to be extremely stable molecules which are not as susceptible to depurination as, for example, adducts forming at the N-7 position. Such adducts are thought to be exposed in the minor groove of DNA where the N^2 is exposed, probably resulting in distortion of the helix (Jowa *et al.*, 1986).

Following reaction with the benzene metabolites HQ and *p*-BQ, one adduct for each nucleotide (excluding thymidine 3'-monophosphate (Tp)) has so far been identified. There is however the possibility that other adducts may be formed. Other benzene

DNA adducts have been observed but have not been fully characterised. Work carried out by Jowa *et al.*, 1986, 1990 and Snyder *et al.*, 1987 showed the presence of two adducts in a reaction of dG with HQ or *p*-BQ. A second group (Chenna and Singer, 1997), also identified two adducts in a similar reaction. In both cases one adduct was identified as (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine (see above) and the second, although not fully characterised appeared to be different in both cases. Work by Jowa *et al.*, 1986, 1990 and Snyder *et al.*, 1987, identified the second adduct as having a molecular weight of 339 (18 mass units less than (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine). From NMR data this adduct was thought not to have been formed at the N-7 or N^2 position of the deoxyguanosine and still remains uncharacterised. Chenna and Singer, 1997, identified the second adduct as a possible bicyclic adduct containing two *p*-BQ moieities (N-1, N^2 and C-8, N-7). In both cases, the second adduct was considered to be relatively unstable and possibly an intermediate adduct which slowly decomposed to form the known adduct (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine).

Pre-incubation of rabbit bone marrow mitoplasts and rat liver mitoplasts (mitochondria stripped of their outer membrane) with radiolabelled nucleoside triphosphates followed by dosing with radiolabelled benzene resulted in the formation of a number of benzene-DNA adducts. In rat liver mitoplasts, six guanosine DNA adducts were observed whereas in rabbit bone marrow mitoplasts, seven guanosine and two adenosine DNA adducts were seen (Rushmore *et al.*, 1984). Dosing of bone marrow mitoplasts with individual benzene metabolites identified HQ and 1,2,4-benzenetriol as responsible for the formation of three of the guanosine adducts. Direct reaction of mitoplasts with catechol resulted in one DNA adduct which was not observed in benzene treated cells, a result which suggested that perhaps catechol is not a major metabolite of benzene (Rushmore *et al.*, 1984). The adducts in this study were not characterised.

Unlike most other genotoxic compounds which form the same adducts both *in vitro* and *in vivo*, this may not be the case for benzene, as a different adduct has been identified *in vivo* (see Chapter 5 for more details).

2.2. Aim

The main aim of this part of the work was to synthesise the adducts which are known to form *in vitro* and identify any other adducts formed from the benzene metabolites benzene oxide, HQ and *p*-BQ. These adducts would then be utilised as standards in the development of a ^{32}P-postlabelling assay coupled to HPLC. At present, only one adduct has been identified which forms *in vivo*, albeit in rodent models. There is a possibility that these '*in vitro*' adducts could form *in vivo*, but the methods so far developed have not been adequately sensitive to determine this. ^{32}P-postlabelling with use of these synthesised adduct standards will ultimately be used for the analysis of DNA from tissues of exposed animals and/or humans.

2.3. Materials

Calf thymus DNA (ct-DNA), hydroquinone (HQ), nucleotides (2'-deoxycytidine 3'-monophosphate, 2'-deoxyadenosine 3'-monophosphate, thymidine 3'-monophosphate and 2'-deoxyguanosine 3'-monophosphate), 8-hydroxy 2'-deoxyguanosine, nuclease P1, micrococcal nuclease and cyclohexadiene were purchased from Sigma (Poole, Dorset, UK). 2'-deoxyguanosine, *p*-benzoquinone (*p*-BQ), bromine, dichloromethane, *meta*-chloroperbenzoic acid, sodium bisulfite, sodium bicarbonate, sodium sulphate, sodium chloride and 1,8 diazabicyclo(5.4.0)undec-7-ene were obtained from Fluka (Gillingham, Kent). All other chemicals used were of the highest grade available and were obtained from Fisher Scientific Ltd. (Loughborough, Leicestershire).

2.4. Instrumentation

2.4.1. HPLC with UV detection

Analytical HPLC with UV detection was conducted using a Gilson 715 system (Gilson, Middleton, WI, USA) using a Hypersil, C18, BDS, 5μm, 4.6 x 250 mm reversed-phase column. Semi-preparative HPLC was carried out using the same Gilson 715 system with a Hypersil, C18, 5 μm, 10 x 250 mm reversed-phase column.
UV absorbance was monitored at 254 nm.

2.4.1.1. System A

Separation was carried out using a linear gradient of methanol (solvent B) in 10 mM ammonium acetate, pH 5.1 (0-70 % B, 0-30 min; 70 % B, 30-35 min; 70-0 % B, 35-40 min; 0 % B, 40-45 min), flow rate 1 mL/min.

2.4.1.2. System B

Chromatography was performed using a linear gradient of methanol (solvent B) in 10 mM ammonium acetate, pH 5.1 (10 % B, 0-10 min; 10-70 % B, 10-35 min; 70-10 % B, 35-45 min; 10 % B, 45-50 min), flow rate 1 mL/min.

2.4.1.3. System C

Chromatography was performed using a linear gradient of methanol (solvent B) in 50 mM ammonium acetate, pH 5.4 (0-35 % B, 0-15 min; 35 % B, 15-25 min; 35-70 % B, 25-35 min; 70-0 % B, 35-40 min; 0 %B, 45-50 min). Flow rate was 1 mL/min for analytical analysis, 5 mL/min for semi-preparative analysis.

2.4.1.4. System D

Separation was carried out using a linear gradient of methanol (solvent B) in water (0-70 % B, 0-15 min; 70 % B, 15-20 min; 70-0 % B, 20-25 min; 0 % B, 25-30 min). Flow rate was 1 mL/min for analytical analysis, 5 mL/min for semi-preparative analysis.

2.4.2. UV spectroscopy

UV spectra of the isolated products (HPLC fractions) were recorded with a Uvikon 860 spectrophotometer (Kontron Instruments, Zurich, Germany).

DNA concentration and purity was assessed using a UV spectrophotometer (GeneQuant, Pharmacia Biotech, St Albans, Hertfordshire).

2.4.3. Mass spectrometry

Mass spectra of the isolated nucleotide or nucleoside products were recorded on a Micromass Quattro BioQ tandem quadrupole mass spectrometer with an electrospray interface using the negative ionisation mode (Micromass, Manchester, UK). Solutions were introduced by constant infusion using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Edenbridge, UK) and a Hamilton 1 mL gastight syringe pumped at a flow rate of 10 μL/min. Samples were dissolved in 1:1 methanol/water (v/v). Data was generated by combination of all spectra. A Varian 9012 pump (Varian Analytical Instruments, Surrey, UK) was coupled to the MS to facilitate online LC-MS. The chromatography for LC-MS was as that described for the adduct under investigation (see sections 2.5.2 and 2.5.3, respectively). A tee-piece was attached post column, allowing reduction of the eluant volume entering the source (120 μL/min). Source temperature was maintained at 150 °C to aid desolvation. Ions of interest were

selected post run and data was generated by combination of spectra underneath the peaks.

2.4.4. Proton (^{1}H) NMR

^{1}H NMR spectra of the isolated nucleotide and nucleoside products were recorded at room temperature on a Bruker ARX 250 (250 MHz) instrument. Samples were dissolved in d_4-methanol or d_3-acetonitrile in the case of benzene oxide.

2.5. Methods

MS and NMR analysis were carried out by Dr. D.J.L. Jones and Ms. R. Jukes, respectively.

2.5.1. Reaction of HQ and *p*-BQ with calf thymus DNA.

Calf thymus DNA (ct-DNA; 1 mg), HQ (0.5 mg; 4.5 μmol) and *p*-BQ (0.5 mg; 4.6 μmol) or ct-DNA (1 mg) and *p*-BQ (0.5mg; 4.6 μmol) were reacted together in 30 mM ammonium formate, pH 7.0 (1 mL), at 37°C for 18 h, with shaking (Pongracz *et al.*, 1990 and Levay *et al.*, 1991).

Alternatively ct-DNA (1 mg), HQ (1 mg; 9 μmol) and varying amounts of *p*-BQ (0-2 mg; 0-18 μmol) or ct-DNA (1 mg), *p*-BQ (1 mg; 9.3 μmol) and varying amounts of HQ (0-2 mg; 0-18 μmol) were reacted together as described above.

DNA was precipitated by the addition of 0.7 volumes of isopropanol. DNA was transferred to an eppendorf and washed twice with 70 % ethanol in water (v/v) followed by one wash with ethanol. DNA was allowed to air dry briefly and was redissolved in SSC buffer (1:100 dilution in water; 150 mM NaCl, 15 mM trisodium citrate, pH 7.5) to give an approximate concentration of 1mg/ml.

DNA concentration and purity was assessed using a UV spectrophotometer and DNA concentration was calculated using the following formula:

$$\text{DNA concentration } (\mu g/\mu L) = \frac{\text{Absorbance at 260 nm x dilution factor x 50}}{1000}$$

where 50 represents the amount (μg/ mL) of double stranded DNA which gives one absorbance unit at 260 nm.

The DNA purity was assessed using the absorbance at 260 nm divided by absorbance at 280 nm value (ratio). (For the DNA to be classed as free from contaminating RNA and protein, the 260/280 ratio should be between 1.7 and 1.9).

2.5.2. Reaction of 2'-deoxycytidine 3'-monophosphate with HQ and *p*-BQ

2'-Deoxycytidine 3'-monophosphate (dCp; 1 mg; 3μmol), HQ (1 mg; 9 μmol) and *p*-BQ (1 mg; 9.3 μmol) were dissolved in 50 mM ammonium formate, pH 6.0 (1 mL) and incubated at 37 °C, for 15 h (Pongracz *et al.*, 1990). The main product of the reaction was separated from the starting materials using HPLC conditions A (section 2.4.1.1). The main product was collected for further analysis.

2.5.3. Reaction of 2'-deoxyadenosine 3'-monophosphate with HQ and *p*-BQ

2'-Deoxyadenosine 3'-monophosphate (dAp; 1 mg; 3 μmol), HQ (1 mg; 9 μmol) and *p*-BQ (1 mg; 9.3 μmol) were dissolved in 30 mM ammonium formate, pH 6.0 (1 mL) and incubated at 37 °C for 15 h (Pongracz and Bodell, 1991). The main product of the reaction was separated from the starting materials using HPLC conditions B (section 2.4.1.2), and collected for further analysis.

2.5.4. Reaction of thymidine 3'-monophosphate with HQ and *p*-BQ

Thymidine 3'-monophosphate (Tp; 10 mg; 31 μmol), HQ (10 mg; 91 μmol) and *p*-BQ (10 mg; 93 μmol) were dissolved in 50 mM ammonium formate, pH 6.0 (1 mL). Alternatively, Tp (5 mg; 15.5 μmol), HQ (5 mg; 45 μmol) and *p*-BQ (5 mg; 46 μmol) were dissolved in 30 mM ammonium formate, pH 6.0 (1 mL). Both samples were incubated at 37 °C for 72 or 15 h, respectively. HPLC analysis was carried out using HPLC conditions C (section 2.4.1.3).

2.5.5. Reaction of 2'-deoxyguanosine 3'-monophosphate with HQ and *p*-BQ

2'-Deoxyguanosine 3'-monophosphate (dGp; 5 mg; 14 μmol), HQ (5 mg; 45 μmol) and varying amounts of *p*-BQ (0-10 mg; 0-93 μmol) or alternatively dGp (5 mg; 14 μmol), *p*-BQ (5 mg; 46 μmol) and varying amounts of HQ (0-10 mg; 0-91 μmol) were

dissolved in 10 mM ammonium formate, pH 6.0 (1 mL) and incubated for 15 h at 37 °C (Levay *et al.*, 1991). Following incubation, the reaction mixture was extracted with ethyl acetate to remove unreacted benzene metabolites as detailed in Section 2.5.9. The main products of the reaction were separated from the starting materials using HPLC conditions A (section 2.4.1.1) and were collected for further analysis.

2.5.6. Reaction of 2' deoxyguanosine with HQ and *p*-BQ

2'-Deoxyguanosine (dG; 5 mg; 19 μmol), HQ (5 mg; 45 μmol) and varying amounts of *p*-BQ (0 – 10 mg; 0-93 μmol) or alternatively dG (5 mg; 19 μmol), *p*-BQ (5 mg; 46 μmol) and varying amounts of HQ (0 – 10 mg; 0-91 μmol) were dissolved in 10 mM ammonium formate, pH 6.0 (1 mL), and incubated for 15 h at 37 °C.

The reaction was scaled up (20 fold) to allow for product analysis by NMR. The reaction mixture underwent ethyl acetate extraction to remove excess benzene metabolites as detailed in section 2.5.9. The large scale reaction volume was partially evaporated using a cold finger rotary evaporator at approx. 40 °C and the resultant concentrate was filtered through a cotton wool plugged pasteur pipette.

The main products from each reaction underwent separation using HPLC conditions C (section 2.4.1.3). The main products were collected and any salt contamination was removed by re-injection onto the HPLC using conditions D as detailed in section 2.4.1.4. Product peaks were again collected and dried to completeness using the cold finger rotary evaporator at approx. 40 °C.

2.5.7. Reaction of 8-hydroxy 2'-deoxyguanosine with HQ and *p*-BQ

8-Hydroxy 2'-deoxyguanosine (8-OH dG; 5 mg; 18 μmol), HQ (5 mg; 45 μmol) and *p*-BQ (0.5 mg; 4.6 μmol) were dissolved in 10 mM ammonium formate, pH 6.0 (1 mL) and incubated for 15 h at 37 °C. The reaction mixture underwent ethyl acetate extraction prior to separation of the products using HPLC conditions C (sections 2.5.9. and 2.4.1.3, respectively). The main products were collected for further analysis.

2.5.8. Removal of the 3'-phosphate group

The 3'-phosphate group was removed from the isolated, adducted dGp products (dried HPLC fractions) by incubating the samples with nuclease P1 (2 μg/μL in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5.0) for 24 h at 37 °C. Products of this reaction were co-chromatographed with the products of reaction 2.5.6. using HPLC conditions C as detailed in section 2.4.1.3.

2.5.9. Ethyl acetate extraction

To remove excess (unreacted) benzene metabolites prior to HPLC analysis the reaction mixture underwent ethyl acetate extraction.

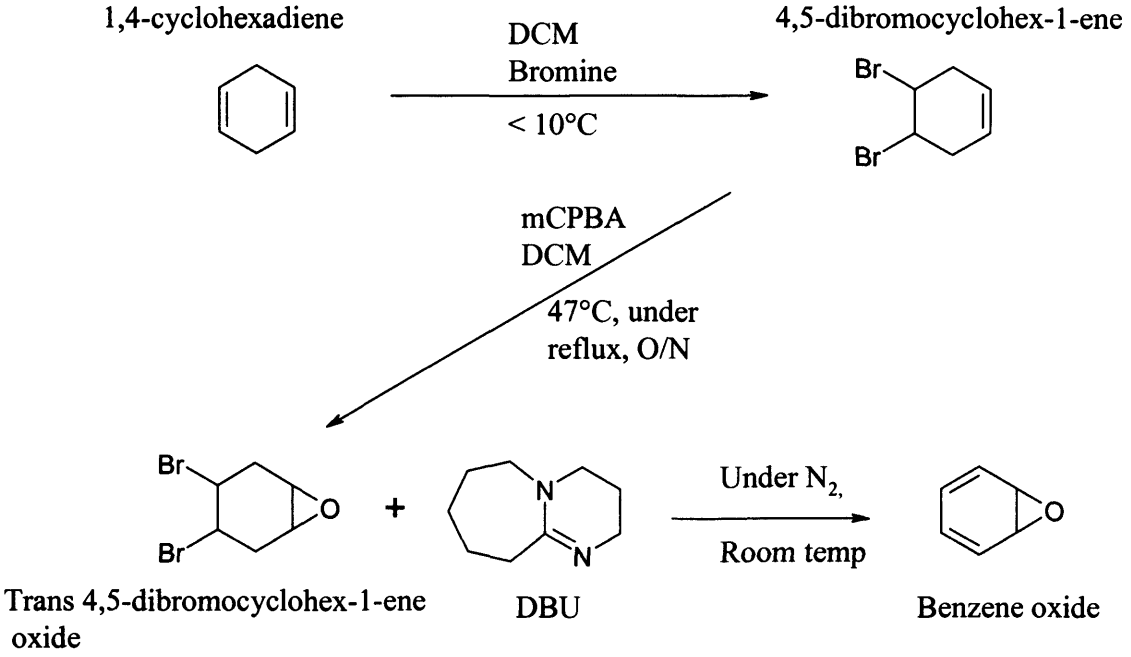
An equal volume of water-saturated ethyl acetate was added to each reaction mixture and samples were mixed by inverting the tubes a number of times, the organic and aqueous layers were allowed to separate. The organic layer containing the excess metabolites was removed and discarded. The washing procedure was repeated until the organic layer remained clear.

2.5.10. Synthesis of benzene oxide

Benzene oxide was synthesised according to the methods of Gillard *et al.*, 1991 and Vogel and Gunther, 1967 by Prof. B. Golding and Dr. A. Henderson of Newcastle University. In brief, benzene oxide was synthesised by a multistep reaction and is outlined in Figure 2.1. The initial product, 4,5-dibromocyclohex-1-ene was synthesised by bromination of the starting material, 1,4-cyclohexadiene (1:1 molar ratio) at less than 10 °C. The resulting solution was dried, and then heated under reflux in the presence of *meta*-chloroperbenzoic acid (*m*-CPBA) in dry dichloromethane (DCM) in a 1:1.2 molar ratio, for 22 h. The resultant product was cooled to 0 °C and the precipitate was filtered and discarded. The filtrate was washed twice with 20 % sodium bisulfite ($NaHSO_3$), and three times with saturated sodium bicarbonate ($NaHCO_3$). The solution was dried by the addition of solid sodium sulphate (Na_2SO_4), which was ultimately filtered and concentrated resulting in a white product (trans-4, 5-dibromocyclohex-1-ene oxide). Trans-4, 5-dibromocyclohex-1-ene oxide underwent dehydrohalogenation in the presence of 1,8 diazabicyclo(5.4.0)undec-7-ene (DBU) in dry ether at a 1:4 molar ratio, and was stirred at room temperature, under nitrogen for 3

days which resulted in the formation of a white precipitate. The solution was washed three times with saturated $NaHCO_3$, once with saturated sodium chloride (NaCl) and again dried by the addition of solid Na_2SO_4. The solution was filtered and the resultant filtrate was concentrated and purified by distillation. The structure and purity of the final product was confirmed by proton NMR.

Figure 2.1. : Schematic diagram showing the synthesis of benzene oxide. Full details of the reaction can be found in the text in section 2.5.10.
(DCM, dichloromethane; DBU, 1,8 diazabicyclo(5.4.0)undec-7-ene; *m*CPBA, *meta*-chloroperbenzoic acid)



2.5.11. Reaction of benzene oxide with 2'-deoxyguanosine and 2'-deoxyguanosine 3'-monophosphate

Due to the instability of benzene oxide in aqueous solution initial reactions were carried out in organic solvents as follows: dG (50 mg; 187 μmol) was added to 37 °C-prewarmed dimethyl formamide (DMF; 1 mL) or dimethyl sulphoxide (DMSO; 1 mL). Benzene oxide (20 μL of stock; approximately 200 μM) was added to each sample and samples were incubated overnight at 37 °C. The reaction mixture was analysed using HPLC conditions C (section 2.4.1.3).

Subsequent reactions were carried out using dGp (4 mg; 11.5 μmol) dissolved in 10 mM ammonium formate, pH 8.0 (100 μL) and benzene oxide (approximately 30 mM diluted in acetone) or acetone alone as the control. Reactions were incubated for 2 h at 37 °C.

2.5.12. Reaction of benzene oxide with DNA

Ct-DNA (0.5-3.0 mg) in 10 mM potassium phosphate buffer, pH 7.4 (1 mL) was reacted with benzene oxide (0-5 mM) or phenol (0-5 mM) at 37 °C for 2 h (reaction based on unpublished information obtained from Prof. W.J. Bodell, University of California).

The DNA was precipitated, washed, redissolved and the concentration and purity determined using the method outlined in section 2.5.1.

2.6. Results

2.6.1. Reaction of HQ and *p*-BQ with calf thymus DNA

Calf thymus DNA (ct-DNA) following reaction with HQ and *p*-BQ was utilised in development of the ^{32}P-postlabelling assay coupled to HPLC or TLC, see Chapter 3 for further details. The adducts formed in intact DNA, which after digestion to its nucleotide components, were not detectable by HPLC-UV detection (results not shown).

2.6.2. Reaction of dCp with HQ and *p*-BQ

Reaction of the nucleotide, dCp with HQ and *p*-BQ resulted in the formation of one main product with t_R (retention time) 17.5 min as determined by HPLC analysis, represented by peak 4 in Figure 2.2, B. Peaks 1, 2 and 3 (Figure 2.2, B) were identified as the starting materials, dCp, HQ and *p*-BQ respectively, by injection of authentic standards onto the HPLC and also by comparison of the UV spectra (results not shown). Peak 5 was identified as quinhydrone, a polymerisation product of HQ and *p*-BQ. Identification of quinhydrone was based on the data obtained from MS (quinhydrone has a molecular weight of 218). The UV absorption spectrum of the main reaction product gave UV_{max} at 323 nm and a UV_{min} at 268 nm (Figure 2.2, A). Further analysis of the product (peak 4) using on-line LC-MS with negative ion electrospray (Figure 2.2, C) resulted in a molecule ion $[M-H]^-$ at *m/z* 396, which is consistent with a product of molecular weight 397. The major insource dissociation product ions, $[M-H]^-$ at *m/z* 195 and 200 were consistent with a deoxyribose plus phosphate and an adducted base moiety, respectively. Comparison of the results to previously published data (Pongracz *et al.*, 1990) allowed identification of this adduct as (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate. The structure of the resultant product is illustrated in Figure 2.2, C.

2.6.3. Reaction of dAp with HQ and *p*-BQ

Reaction of dAp with HQ and *p*-BQ gave one main product, peak 4 as shown in Figure 2.3, B with t_R of 23 min. Peaks 1, 2 and 3 were identified as unreacted nucleotide, HQ and *p*-BQ, respectively by comparison of the UV spectra obtained to authentic standards and injection of the standards onto the HPLC (results not shown). Peak 5 was identified as quinhydrone, a product from the reaction of HQ and *p*-BQ.

Collection of peak 4 and analysis by UV spectrophotometry gave a UV spectrum as shown in Figure 2.3, A, with UV_{max} 274nm and UV_{min} 268nm. LC-MS/MS analysis of the product using negative ion electrospray gave the molecule ion $[M-H]^-$ at *m/z* 420, which is consistent with a product of molecular weight 421 (as shown in Figure 2.3, C). The major product ions, $[M-H]^-$ at *m/z* 195 and 224 were consistent with a deoxyribose plus phosphate and an adducted base moiety, respectively. Comparison of the data collated to published results (Pongracz and Bodell, 1991) showed similar findings and allowed the identification of the product as (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, the structure of which is shown in Figure 2.3, C.

Figure 2.2: Full characterisation of the product from the reaction of dCp with HQ and *p*-BQ. Reaction mixture underwent HPLC (**B**) using a linear gradient of methanol (solvent B) in 10 mM ammonium acetate, pH 5.1 (0-70 % B, 0-30 min; 70 % B, 30-35 min; 70-0 % B, 35-40 min; 0 % B, 40-45 min), flow rate 1 mL/min). Peaks 1-3 represent starting material, peak 4 the product of the reaction and peak 5 a compound formed by reaction of the two metabolites. The main product represented by peak 4, underwent analysis by UV spectrophotometry (**A**) and online LC-MS with negative ion electrospray (**C**), which resulted in a molecule ion $[M-H]^-$ at *m/z* 396, consistent with a product of molecular weight 397 and insource dissociation products $[M-H]^-$ at *m/z* 195 and 200 which are consistent with deoxyribose plus phosphate and the adducted base, respectively. The structure of the main product is shown (**C**).

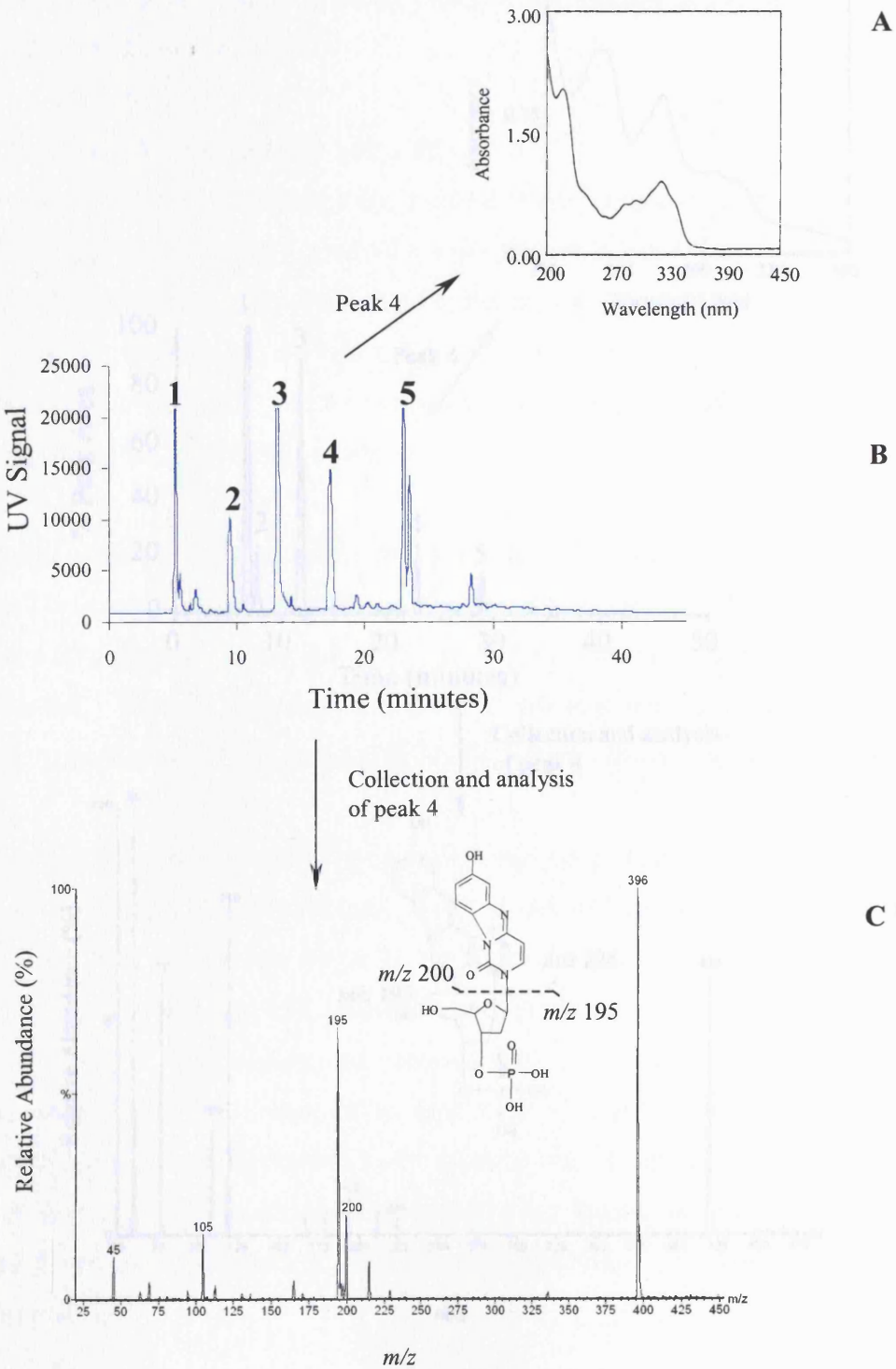
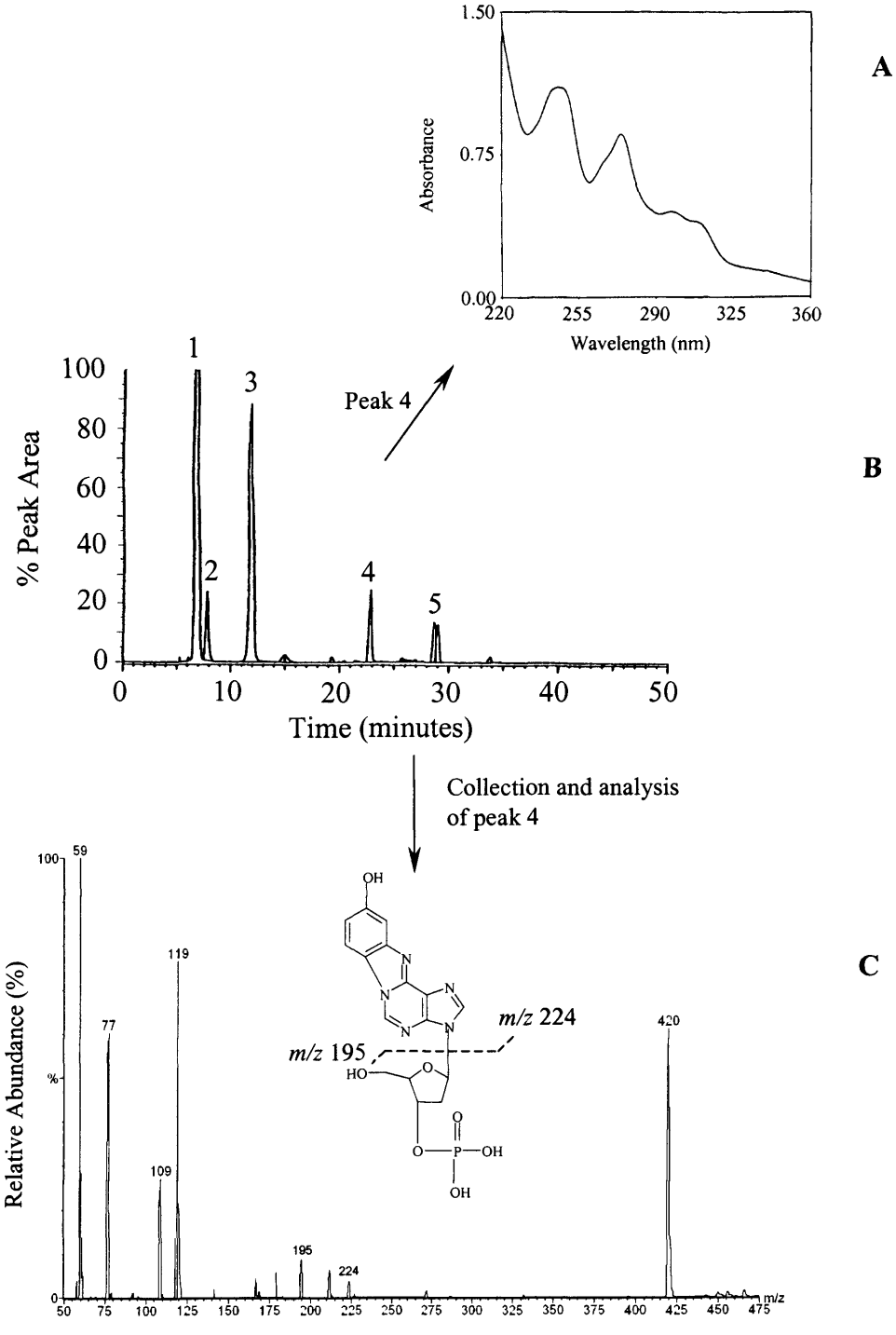


Figure 2.3: Identification of the main product from the reaction of dAp with HQ and *p*-BQ. The product (peak 4) was separated from the starting material (Peaks 1,2 and 3 (**B**)) using HPLC with a linear gradient of methanol (solvent B) in 10 mM ammonium acetate, pH 5.1 (10 % B, 0-10 min; 10-70 % B, 10-35 min; 70-10 % B, 35-45 min; 10 % B, 45-50 min), flow rate 1 mL/min. Peak 4 was identified as the reaction product from UV spectrophotometry (**A**) and LC-MS/MS with negative ion electrospray (**C**), which resulted in a molecule ion $[M-H]^-$ at *m/z* 420, which is consistent with a product of molecular weight 421 and product ions $[M-H]^-$ at *m/z* 195 and 224 which are consistent with a deoxyribose plus phosphate and adducted base, respectively. The structure of the identified product is shown in (**C**).



2.6.4. Reaction of Tp with HQ and *p*-BQ

The reaction of Tp with HQ and *p*-BQ resulted in the production of up to 4 products not identified as starting material when analysed by HPLC (results not shown). Only one product (t_R of 20 min), gave a significant peak when analysed by ^{32}P-postlabelling as discussed further in Chapter 3, Section 3.6.6, which when analysed by MS gave a molecule ion $[M-H]^-$ at *m/z* 217, consistent with a product of molecular weight 218. It is postulated that this product is quinhydrone, a polymerisation product formed from the reaction of HQ with *p*-BQ.

2.6.5. Reaction of dGp with HQ and *p*-BQ

The reaction of dGp with HQ and *p*-BQ resulted in the formation of two major products with t_R of 15.5 min (product 1) and 17.5 min (product 2) when isolated by HPLC. A typical HPLC chromatogram demonstrating the product separation can be observed in Figure 2.4, A. Peaks 1 and 2 (Figure 2.4, A) were identified as unreacted nucleotide and HQ, respectively (*p*-BQ was not present due to removal with ethyl acetate), peaks 3 and 4 represent the two reaction products.

Collection of the two product peaks and analysis by UV spectrophotometry gave two similar UV spectra as demonstrated in Figures 2.4, B and C. The UV absorption spectrum of product 1 gave a peak at 203 nm and shoulders at approximately 235, 270 and 330 nm. The UV spectrum of product 2, although similar to that obtained for product 1, gave more defined peaks at 203, 229, 271 and 330 nm, with no shoulders.

Analysis of each product peak by tandem MS using continuous infusion, negative ion electrospray resulted in molecule ions $[M-H]^-$ at *m/z* 452 and 436 and product ions at *m/z* 256 and 240, respectively (Figure 2.5). This data was consistent with products of molecular weights 453 and 437, respectively, with a mass difference between the two compounds of 16. The product ions observed ($[M-H]^-$ at *m/z* 256 and 240; products 1 and 2, respectively) are consistent in each case with loss of the deoxyribose and phosphate moieties and correspond to the product ions of the adducted base. Again a mass difference of 16 was observed between the two fragments, which confirmed that product 1 was not an adduct of contaminating guanosine 3'-monophosphate in the starting material.

From the data obtained, it was possible to identify product 2 by comparison with data in the published literature (Jowa *et al.*, 1986 and 1990). The adduct was identified as (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. The mass difference between products 1 and 2, suggested the presence of an extra oxygen molecule on the adducted base. Possible locations of this molecule could be at the C-8 position, a common lesion associated with oxidative damage (Figure 2.6, A) or at positons 2, 4 or 5 of the exocyclic ring (Figure 2.6, B, C and D, respectively). To determine the structure of product 1, this compound underwent further investigation as detailed in the following sections.

Figure 2.4: Typical UV-HPLC trace (**A**) of the products from the reaction of dGp with HQ and *p*-BQ. Starting material is represented by peaks 1 and 2 (nucleotide and HQ, respectively) whereas products of the reaction are represented by peaks 3 and 4 (products 1 and 2, respectively). HPLC separation was carried out using a linear gradient of methanol (solvent B) in 10 mM ammonium acetate, pH 5.1 (0-70 % B, 0-30 min; 70 % B, 30-35 min; 70-0 % B, 35-40 min; 0 %B, 40-45 min) flow rate 1 mL/min). Typical UV absorption spectra representing product 1 (**B**) and product 2 (**C**) are shown.

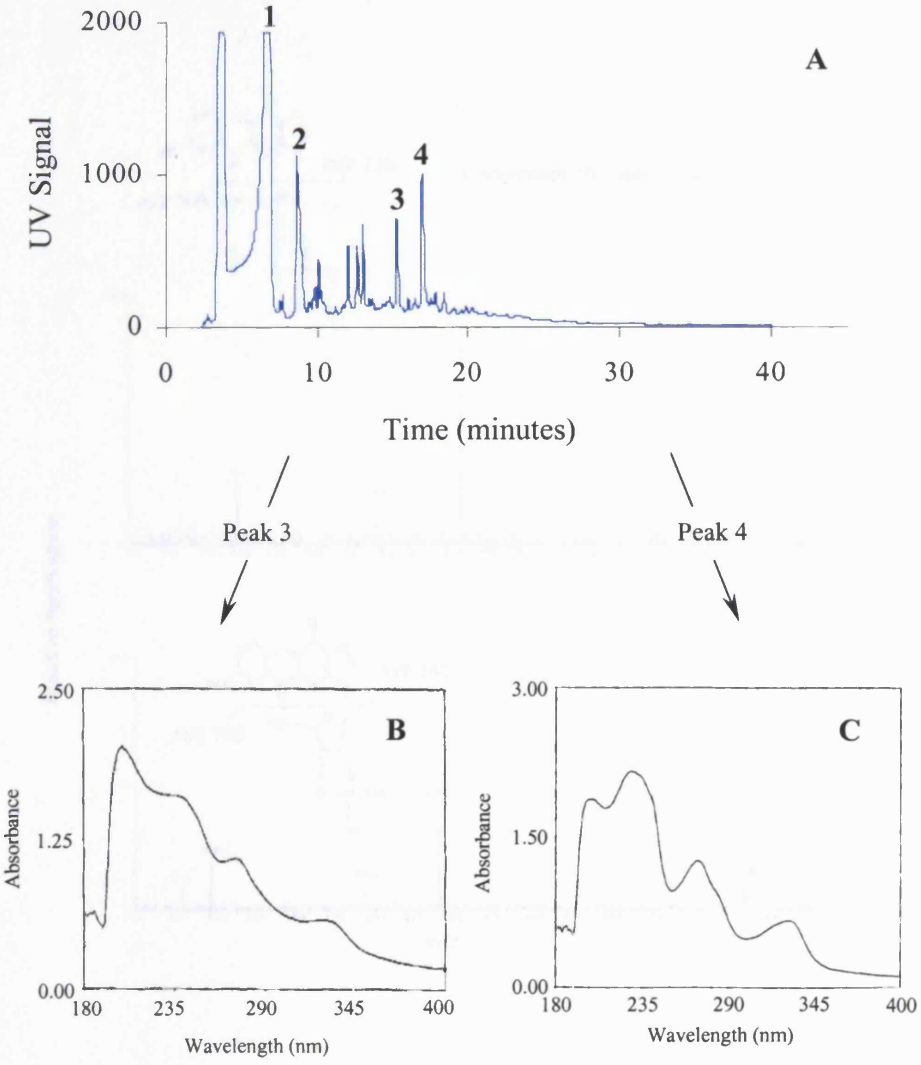


Figure 2.5: Typical MS/MS analyses of products 1 and 2 from the reaction of dGp with HQ and *p*-BQ in 10 mM ammonium formate, pH 6.0 at 37 °C, 15 h. Product 1 (**A**) has a molecule ion $[M-H]^-$ at *m/z* 452, with a product ion at *m/z* 256. Product 2 (**B**) has a molecule ion $[M-H]^-$ at *m/z* 436 with a product ion at *m/z* 240. Product ions at *m/z* 79, 97 and 195 correspond to PO_3^-, phosphate group and deoxyribose plus phosphate moieties, respectively.

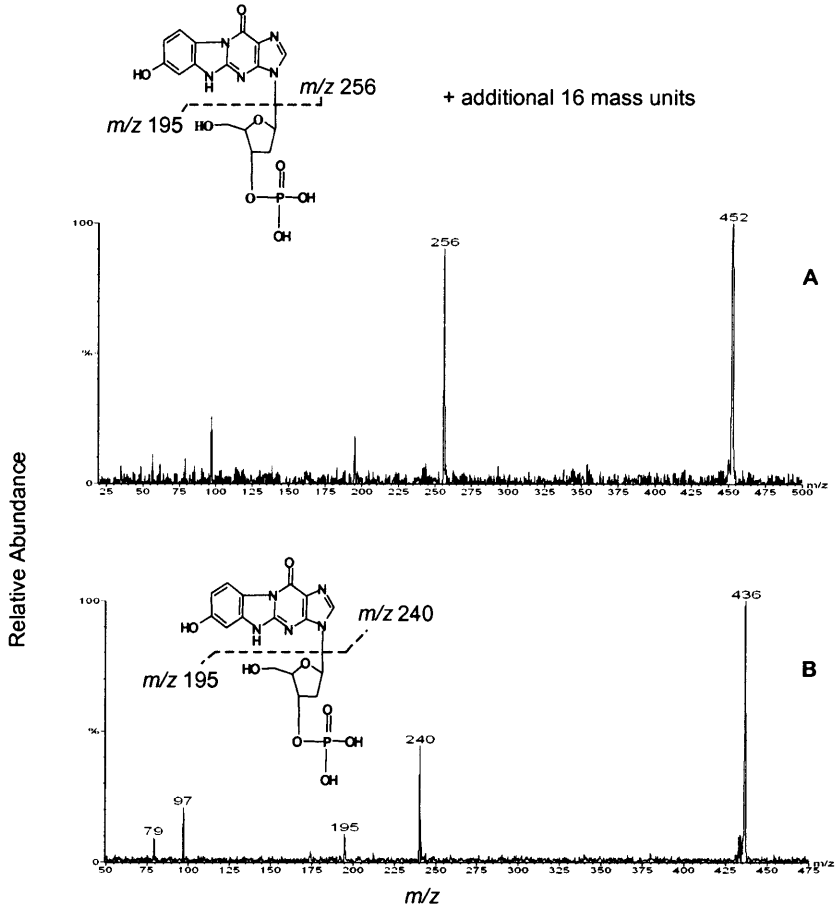
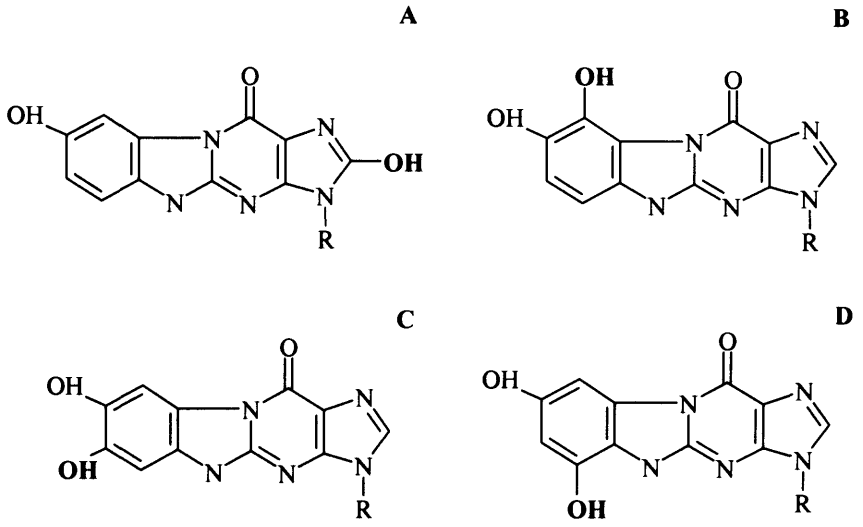


Figure 2.6: Possible structures of product 1 from the reaction of dGp with HQ and *p*-BQ based on the knowledge obtained from MS/MS, UV spectrophotometry and the current literature. The mass difference when compared to product 2, whose structure is known, is 16, suggesting the presence of an oxygen molecule. Mono-oxygenation could have occurred at the C-8 position (**A**), or at positions 2, 4 or 5 on the exocyclic ring (**B**, **C** and **D** respectively) as denoted by the bold typeface.
R denotes the deoxyribose and phosphate moieties.



2.6.6. Reaction of dG with HQ and *p*-BQ

Reaction of dG with HQ and *p*-BQ resulted in two main products with t_R of 19.5 min (product 1) and 22 min (product 2) as determined by HPLC separation (Figure 2.9, A). Tandem MS analysis of the two collected products using continuous infusion negative ion electrospray resulted in molecule ions $[M-H]^-$ at *m/z* 372 and 356 and product ions at *m/z* 256 and 240, respectively (Figure 2.8, A and B). The molecule ions observed were consistent with products of molecular weight 373 and 357, respectively and also, as observed with the nucleotide adducts (section 2.6.5) a mass difference of 16 was demonstrated between the two products. The product ions were comparable to those observed for the nucleotide products (section 2.6.5), again showing the loss of the deoxyribose moiety.

Products 1 and 2 were synthesised on a large scale and analysed by ^{1}H NMR and the results are shown in Figure 2.7. The data obtained for product 1 were as follows: 8.16 (s, 1H, H-8), 8.11 (s, 1H, ring-2"), 6.92 (s, 1H, ring-5"), 6.44 (t, 1H, H-1'), 4.61 (m, 1H, H-3'), 4.07 (m, 1H, H-4'), 3.82 (m, 2H, H-5'), 2.81 (m, 1H, H-2'$_b$), 2.44 (m, 1H, H-2'$_a$).

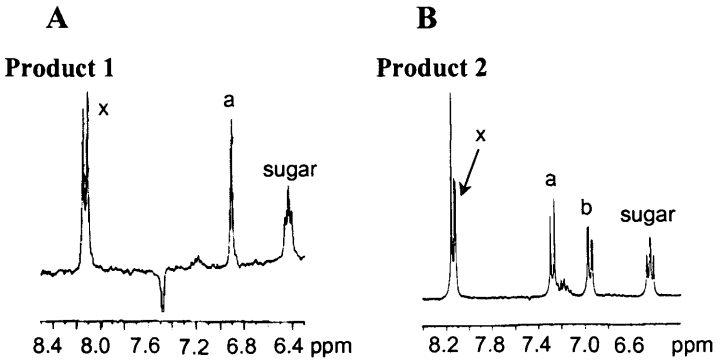
The data for product 2 were as follows: 8.15 (s, 1H, H-8), 8.11 (dd, 1H, ring-2", *J*=2.32 Hz), 7.27 (d, 1H, ring-5", *J*=8.66 Hz), 6.95 (dd, 1H, ring-4", *J*=2.37, 8.62 Hz), 6.43 (t, 1H, H-1'), 4.60 (m, 1H, H-3'), 4.06 (m, 1H, H-4'), 3.83 (m, 2H, H-5'), 2.81 (m, 1H, H-2'$_b$), 2.45 (m, 1H, H-2'$_a$).

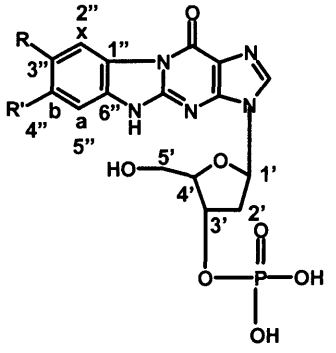
In summary, the spectrum of product 2 shows one doublet at 7.27 ppm and two doublet of doublets at 8.11 and 6.95 ppm. The doublet at 7.27 ppm and the doublet of doublets at 6.95 ppm correspond to the adjacent protons of the aromatic ring, represented by **a** and **b** (Figure 2.7), the splitting pattern confirming this. The third doublet, which is a fine doublet of doublets, at 8.11 ppm corresponds to the proton of the aromatic ring, which is adjacent to the hydroxyl group, represented by **x** on Figure 2.7. The fine splitting pattern is due to the long range coupling effect of proton b.

The ^{1}H NMR spectrum of product 1 shows 2 singlets at 8.11 and 6.92 ppm. This is consistent with 2 protons on the aromatic ring, which are *para-* to each other, with two

hydroxyl groups at the *ortho-* and *meta-* positions. The singlet at 8.2 ppm on both spectra is assigned to the proton at the C-8 position on the purine ring.

Figure 2.7.: Typical ^{1}H NMR spectra recorded in d_4-methanol of products 1 and 2 (**A** and **B**, respectively) from the reaction of dG with HQ and *p*-BQ in 10 mM ammonium formate, pH 6.0 at 37 °C, 15 h. The proposed structure of product 1, and the structure of product 2 are shown.





	R(3")	R'(4")
Product 1	OH	OH
Product 2	OH	H

2.6.7. Reaction of 8-OH dG with HQ and *p*-BQ

Reaction of 8-OH dG with HQ and *p*-BQ produced 2 major products, which had t_R of 20.5 min (product i) and 23 min (product ii) when isolated by HPLC (Figure 2.9, B). Further analysis of these two products by tandem mass spectrometry gave molecule ions $[M-H]^-$ at *m/z* 388 (product i) and 372 (product ii), and product ions at *m/z* 272 and 256, respectively (Figure 2.8, C and D). The molecule ions were consistent with products of molecular weight 389 and 373, with product ii having the same molecular weight and product ion as product 1 from the dG reaction (section 2.6.6).

HPLC co-chromatography studies of products i and ii, with the products from the reaction of dG, showed no product co-elution. This observation suggested that the products from both reactions with identical molecular weights were structurally different (Figure 2.9, C).

Product ii from the 8-OH dG reaction was known to have the extra mono-oxygenation at the C-8 position, the HPLC co-chromatography results therefore suggest that product 1 (dG reaction) was not modified at the C-8 position. Further evidence to support this theory comes from product i of the 8-OH dG reaction. MS/MS analysis of this product gave a molecule ion with $[M-H]^-$ at *m/z* 388 and a product ion with at *m/z* 272 (Figure 2.8, C). This data is consistent with a product of molecular weight 389, which is a further addition of 16 mass units. This data suggests that compound i contains the (3"-hydroxy)-1, N^2-benzetheno adduct, the hydroxylation at the C-8 position plus a further oxygenation somewhere on the molecule. The observed product ion again supports the theory that the additional mono-oxygenation is present on the adducted base.

Figure 2.8: Typical MS/MS analyses of the main products from the reaction of dG or 8-OH dG with HQ and *p*-BQ in 10 mM ammonium formate, pH 6.0, 15 h at 37 °C. Product 1 from the dG reaction (**A**) has a molecule ion $[M-H]^-$ at *m/z* 372, with a product ion at *m/z* 256. Product 2 from the dG reaction (**B**) has a molecule ion $[M-H]^-$ at *m/z* 356, with a product ion at *m/z* 240. Product i from the 8-OH dG reaction (**C**) has a molecule ion $[M-H]^-$ at *m/z* 388, with a product ion at *m/z* 272. Product ii from the 8-OH dG reaction (**D**) has a molecule ion $[M-H]^-$ at *m/z* 372, with a product ion at *m/z* 256.

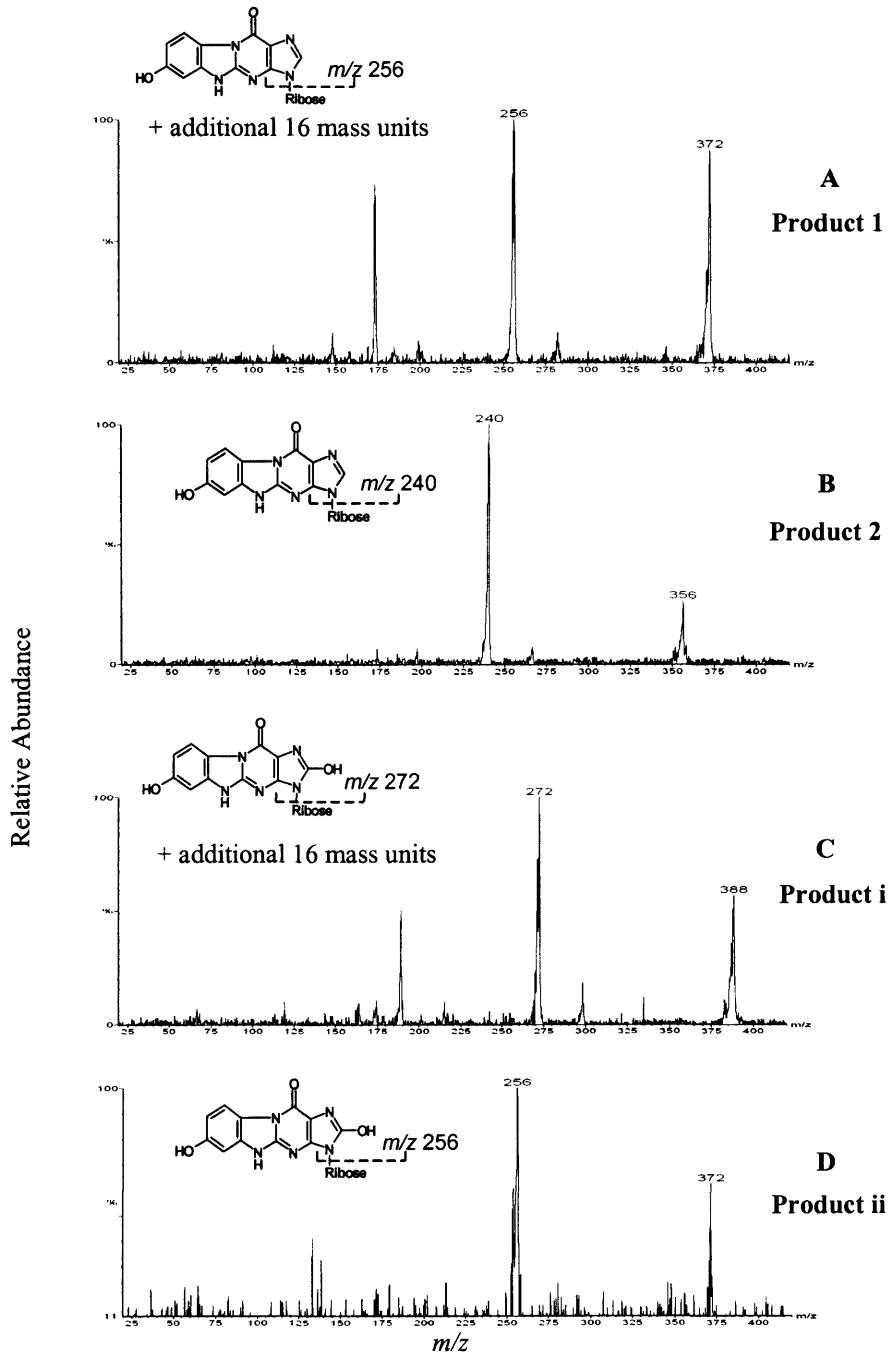
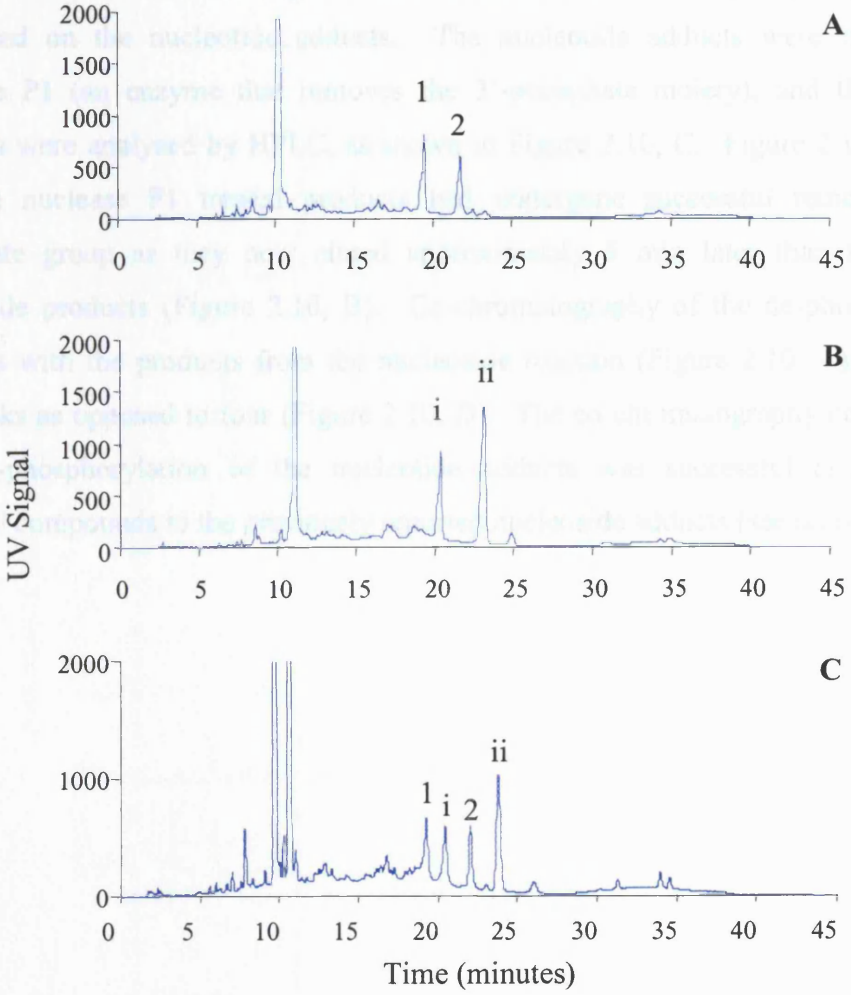


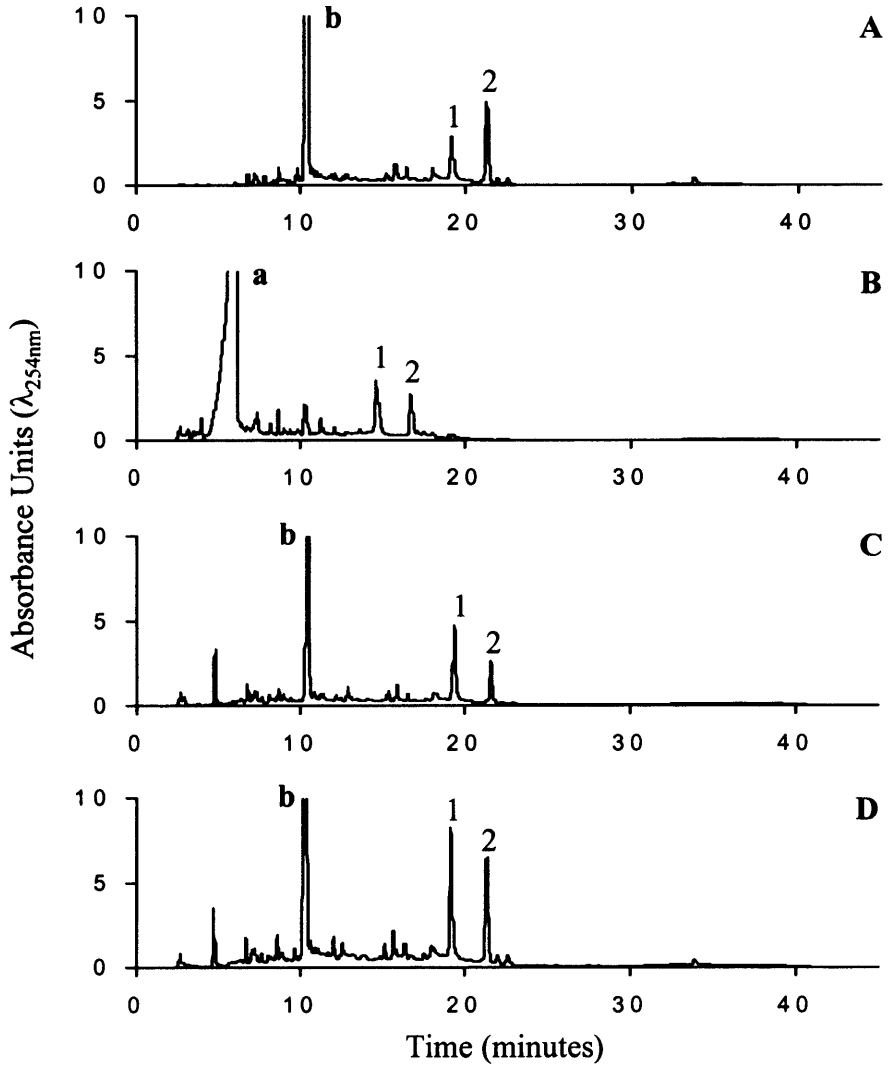
Figure 2.9: Typical UV-HPLC traces of products 1 and 2, from the reaction of dG with HQ and *p*-BQ (**A**), products i and ii from the reaction of 8-OH dG with HQ and *p*-BQ (**B**) and co-elution of all four products (**C**).
Chromatography was carried out using a linear gradient of methanol in 50 mM ammonium acetate, pH 5.1 (0-35 % B, 0-15 min; 35 % B, 15-25 min; 35-70 % B, 25-35 min; 70-0 % B, 35-40 min; 0 % B, 45-50 min), with a flow rate of 1 mL/min.



2.6.8. Removal of the 3'-phosphate group from the nucleotide products

Two products were synthesised in both the dGp reaction with HQ and *p*-BQ (Section 2.6.5) and the dG reaction with the same two metabolites (Section 2.6.6). The nucleoside adducts have undergone full characterisation with the aid of ^{1}H NMR analysis (section 2.6.6). To confirm that adduct formation from the nucleotide reaction was identical to that occurring for the nucleoside reaction, de-phosphorylation was performed on the nucleotide adducts. The nucleotide adducts were treated with nuclease P1 (an enzyme that removes the 3'-phosphate moiety), and the resultant products were analysed by HPLC, as shown in Figure 2.10, C. Figure 2.10, C shows that the nuclease P1 treated products had undergone successful removal of the phosphate group as they now eluted approximately 5 min later than the original nucleotide products (Figure 2.10, B). Co-chromatography of the de-phosphorylated products with the products from the nucleoside reaction (Figure 2.10, A) resulted in two peaks as opposed to four (Figure 2.10, D). The co-chromatography demonstrated that de-phosphorylation of the nucleotide adducts was successful and produced identical compounds to the previously reported nucleoside adducts (see section 2.6.6).

Figure 2.10: Typical UV-HPLC traces of the products (designated as peaks 1 and 2) from the reaction of dG (**A**) or dGp (**B**) with HQ and *p*-BQ. Chromatography of the products from the nucleotide reaction after nuclease P1 treatment (**C**) and co-elution of the nuclease P1 treated products with the nucleoside products (**D**). Identical chromatography conditions were used for both nucleotide and nucleoside reaction products: linear gradient of methanol in 50 mM ammonium acetate, pH 5.1 (0-35 % B, 0-15 min; 35 % B, 15-25 min; 35-70 % B, 25-35 min; 70-0 % B, 35-40 min; 0 % B, 45-50 min), with a flow rate of 1 mL/min.
Peaks a and b represent unreacted nucleotide and nucleoside, respectively.



2.6.9. Investigation of the formation of product 1

When dGp was reacted with equal amounts (w/w) of HQ and *p*-BQ then the (3"-hydroxy)-1, N^2-benzetheno adduct (product 2) was the major product formed (Figure 2.11, A). When the amount of *p*-BQ in the reaction was decreased relative to HQ, an increase in the yield of product 1 was observed (Figure 2.11, A). The highest yield of product 1 was obtained when no *p*-BQ was present. If the amount of HQ in the reaction was decreased in relation to *p*-BQ no overall effect on adduct formation was observed (Figure 2.11, B). Similar findings were also observed when dGp was substituted for dG (results not shown). The formation of product 2 ((3"-hydroxy)-1, N^2-benzetheno adduct) does not appear to be influenced by the ratio of the two metabolites, maintaining a steady rate of production independent of the ratio of HQ to *p*-BQ.

2.6.9.1. Auto-oxidation of HQ

A solution of HQ in 50 mM ammonium formate, pH 6.0 was prepared and at set time intervals a sample of the solution was analysed by UV-HPLC. The metabolite, *p*-BQ, is present in the solution at time 0 min, and the presence of this metabolite increased significantly over time as shown in Figure 2.12. These results are indicative of the slow auto-oxidation of HQ over time.

Figure 2.11: Comparison of adduct formation from the reaction of dGp with varying amounts of the two benzene metabolites HQ and *p*-BQ. Analysis of the samples was carried out using UV-HPLC (A_{254}) by comparison of peak areas. Figure **A** is the graphical representation of the pattern of adduct formation when the amount of *p*-BQ in the reaction is varied and the amount of HQ is constant. Figure **B** shows the pattern of adduct formation when the amount of HQ in the reaction is varied and the amount of *p*-BQ is constant. Data shown are mean ± SD (n=3). An asterisk indicates significant differences from *a* ($p < 0.05$), and x indicates a significant difference from *b* ($p < 0.05$) as determined by oneway ANOVA followed by Tukey's post hoc test.

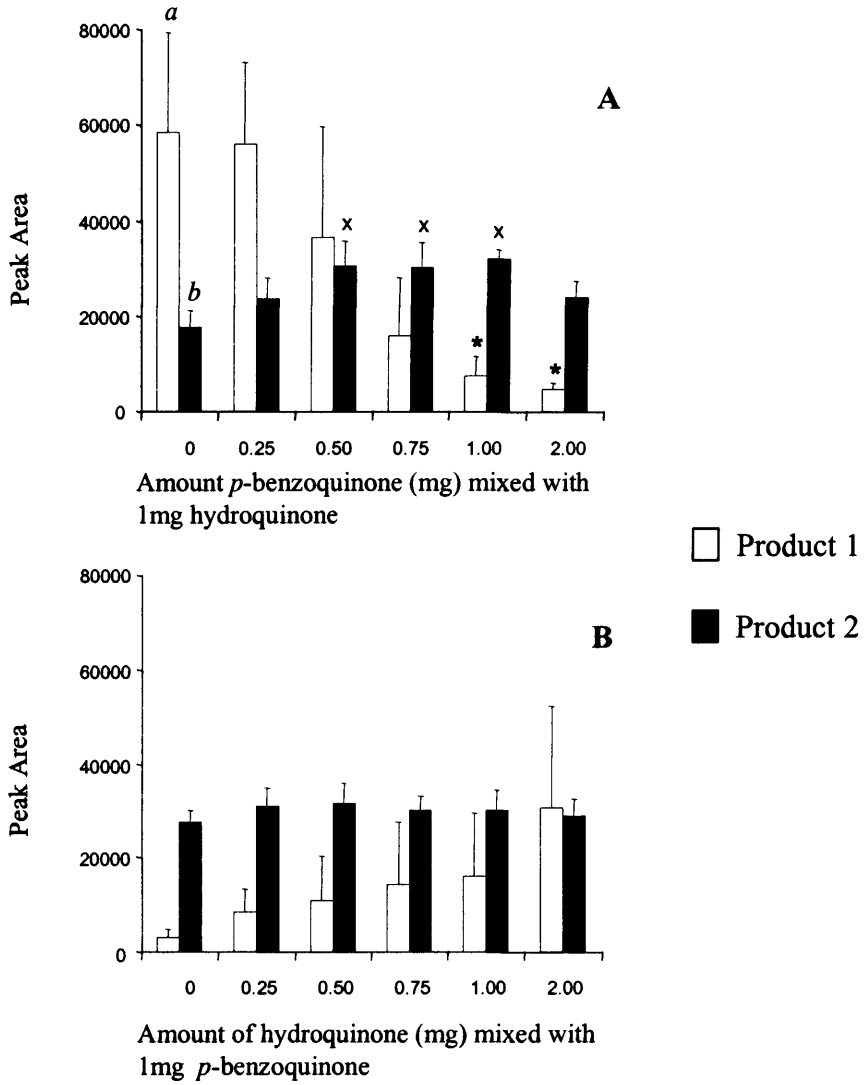
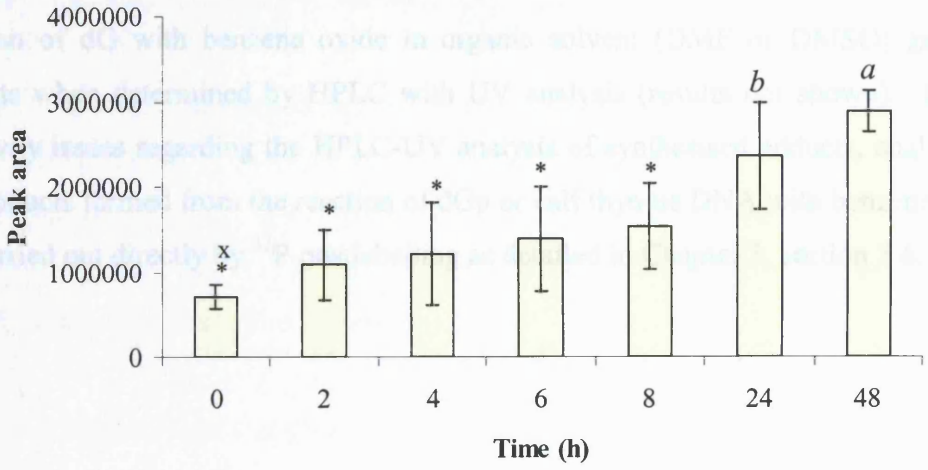


Figure 2.12: A graphical representation showing the auto-oxidation of HQ to *p*-BQ over time. Bars represent the peak area of *p*-BQ formed over time (0-48 h) as determined by HPLC. Data shown are mean ± SD (n=3).
* indicates significant difference from *a* ($p < 0.05$), ^{x} indicates significant difference from *b* ($p < 0.05$), as determined by oneway ANOVA followed by Tukey's post hoc test.



2.6.10. Benzene oxide reaction

The synthesis and purity of the benzene metabolite, benzene oxide was confirmed by ^{1}H NMR (results not shown) which showed that the resulting product was only 66 % pure (contaminated with ether).

Reaction of dG with benzene oxide in organic solvent (DMF or DMSO) gave no products when determined by HPLC with UV analysis (results not shown). Due to sensitivity issues regarding the HPLC-UV analysis of synthesised adducts, analysis of any products formed from the reaction of dGp or calf thymus DNA with benzene oxide was carried out directly by ^{32}P-postlabelling as detailed in Chapter 3, section 3.6.7.

2.7. Discussion

The reaction of individual nucleotides with the benzene metabolites HQ and *p*-BQ has shown the formation of at least one major adduct per nucleotide (excluding Tp) which have been characterised and identified by a number of analytical techniques, including UV spectrophotometry, negative ion electrospray mass spectrometry and ^{1}H NMR.

The adducts were identified as (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and a novel, not previously characterised adduct, whose identification will be explained below.

The novel adduct was identified by using a number of analytical techniques and different reactions. MS determined a mass difference of 16 between (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and the novel adduct suggesting an extra mono-oxygenation present on the novel adduct. MS/MS showed this to be present on the base and not due to contamination from guanosine 3'-monophosphate in the starting material. Reaction with 8-OH dG ruled out the possibility of a hydroxyl substitution at the C-8 position. Using mass spectral and ^{1}H NMR analysis we have confirmed that the extra oxygenation had occurred on the adducted part of the base and from this information we were able to identify the product as a new benzetheno adduct, identified as (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate.

This adduct was shown, by dephosphorylation and HPLC co-chromatography, to be identical to the unknown adduct formed from a similar reaction with dG. The yield of this new adduct was shown to be influenced by the amount of *p*-BQ in the reaction in comparison to HQ (w/w), with the yield increasing with decreasing levels of *p*-BQ. At physiological pH, HQ, albeit slow, can undergo auto-oxidation to *p*-BQ, a reaction, which is thought to occur via the semiquinone intermediate (Greenlee *et al.*, 1981), an observation also made in our laboratory at pH 6.0 (Figure 2.12).

In a 1:1 reaction (w/w) of HQ and *p*-BQ with dGp, the main product formed is (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Perhaps, in this reaction the adduct is formed directly from reaction of *p*-BQ with the nucleotide rather than the semi-quinone. Work carried out by Smart and Zannoni, 1984, investigating the influence of DT-diaphorase and peroxidase on the covalent binding of benzene metabolites confirmed that it was *p*-BQ and not its semi-quinone intermediate that was responsible for adduct formation. With a decreasing amount of *p*-BQ in the reaction mixture, formation of the second adduct, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate occurred maybe due to a shift in equilibrium in favour of the formation of reactive oxygen radicals. These radicals may react directly with HQ forming a 1,2,4-benzenetriol like compound which could react directly with dGp, or the radicals could react with (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate resulting in the new adduct.

Reaction of DNA and nucleotides with the electrophilic reactive compound, benzene oxide, resulted in no observable product when analysed by HPLC. A result also observed by Tunek *et al.*, 1978, who saw negligible binding of benzene oxide to macromolecules when incubated in a microsomal system. These results were probably due to benzene oxide's instability in aqueous medium, which has a half-life in water at pH 7.0, of 2 min (Tunek *et al.*, 1978). Golding and Watson, 1999 suggested that reaction of benzene oxide with a nucleophile, which includes DNA bases, results in elimination of the DNA base and the formation of phenol.

The synthesised adducts were utilised in the development of a ^{32}P-postlabelling method coupled to HPLC or TLC designed specifically for the analysis of benzene DNA adducts (see Chapter 3 for further details).

Chapter 3
^{32}P-Postlabelling Method Development

3.1. Introduction

As previously described in Chapter 1, the presence of DNA adducts in various tissues can be used as reliable biomarkers in human biomonitoring and help assess the extent of an individual's exposure to a genotoxic carcinogen. In the past, adduct analysis was reliant on the administration of the carcinogen/mutagen of interest as a radiolabelled compound, the covalent binding of which, to DNA (and other biomolecules) could be determined by scintillation counting. This procedure has a number of disadvantages; radiolabelled compounds cannot be easily administered to humans due to ethical reasons and the technique is also not suitable for monitoring human exposure to environmental or occupational carcinogens.

Over the years a number of procedures have been developed for the analysis of DNA adducts which do not require administration of radiolabelled compounds, the sensitivity and selectivity of which have improved greatly over time. Such procedures include MS, immunoassays, fluorescence detection, HPLC with electrochemical detection, and ^{32}P-postlabelling. These techniques have been discussed in brief in Chapter 1. The ^{32}P-postlabelling method will be discussed in more detail in this chapter.

3.1.1. ^{32}P-postlabelling

^{32}P-postlabelling is a sensitive technique for detecting DNA adducts induced by structurally diverse endogenous or exogenous mutagens or carcinogens, and has proved a suitable approach for the detection of DNA lesions formed from unidentified carcinogens in experimental animals and humans (Randerath and Randerath, 1994). ^{32}P-postlabelling can be used, not only to assess the extent of initial DNA binding of a carcinogen but also the persistence of the resultant adducts over time.

A procedure for the ^{32}P-postlabelling of unmodified bases was first described in 1979 (Davies *et al.*, 1979) and its application to the analysis of carcinogen-modified deoxynucleotides was described in 1981 by Reddy *et al.*

The basic method of ^{32}P-postlabelling involves an enzymatic hydrolysis of DNA to deoxynucleoside 3'-monophosphates using an endonuclease (micrococcal nuclease)

and an exonuclease (spleen phosphodiesterase). The hydrolysis products are subsequently converted to 5'-^{32}P labelled 3', 5'-bisphosphate derivatives with excess [γ-^{32}P] ATP as the [^{32}P] phosphate donor and T4 polynucleotide kinase as the catalyst. The labelled deoxynucleotides are traditionally separated by multi-directional polyethyleneimine (PEI) cellulose anion exchange thin layer chromatography (TLC) followed by autoradiography and scintillation counting (Gupta *et al.*, 1982; Gupta, 1993 and Randerath and Randerath, 1994). Adduct levels are determined as relative adduct labelling (RAL), which is a ratio of labelled adducts compared to labelled unmodified deoxynucleotides (Gupta *et al.*, 1982). A RAL value of 10^{-7} corresponds to a modification level of about 0.3 pmol of adduct/mg of DNA (Randerath *et al.*, 1985).

The original ^{32}P-postlabelling method as described above, can detect one adduct in 10^{6}-10^{7} deoxynucleotides. This method radiolabels both adducted and unmodified deoxynucleotides (Scheme 1, Figure 3.1) and separation of unmodified from adducted deoxynucleotides is dependent on the TLC conditions employed. Unmodified deoxynucleotides are removed from TLC plates using non-denaturing, high salt solvents, whilst the adducts, which have remained at, or close to the origin are resolved using high salt, high urea, denaturing solvents (Gupta *et al.*, 1982 and Randerath and Randerath, 1994).

A slight modification of the basic method employs a limiting amount of radiolabel. This procedure leads to a preferential labelling of bulky and aromatic adducts over the unmodified deoxynucleotides (Randerath and Randerath, 1994) but this method is not quantitative and further improvements to the method are required (reviewed in Gorelick, 1993).

3.1.2. Improvements to the ^{32}P-postlabelling method

Since its introduction, the technique of ^{32}P-postlabelling has undergone a number of modifications to increase its sensitivity. One such modification involves the inclusion of an adduct enrichment step which is routinely used in many laboratories and will be discussed in detail below.

Adaptations of the basic method, as can be seen in Schemes 2-4 of Figure 3.1, incorporates a number of different enrichment procedures which involve the removal of unmodified deoxynucleotides, either by a physical method or enzymatically. The physical enrichment procedures include butanol extraction (Gupta, 1985), C_{18} reversed-phase TLC (Reddy, 1993b), HPLC (Dunn and San, 1988), ion exchange or C_{18} reversed phase cartridge chromatography (reviewed in Gorelick, 1993) or immunoaffinity chromatography (see Chapter 4 for a review of the technique). Enzymatic enrichment procedures involve the digestion of unmodified deoxynucleotides with nuclease P1 (Reddy and Randerath, 1986), or nuclease S1 (Reddy, 1991). The application of adduct enrichment has improved the sensitivity of the assay over a 1000 fold and is currently reported as being capable of detecting one adduct per 10^{10}-10^{11} deoxynucleotides i.e. at least one adduct per cell (Reddy and Randerath, 1987).

The two main enrichment techniques are butanol extraction and nuclease P1 digestion. Butanol extraction relies on the hydrophobicity of adducts, which allows selective extraction of nucleotide adducts into butanol in the presence of the phase transfer agent, tetrabutylammonium chloride. Butanol extraction allows successful recovery of most aromatic adducts but a less effective recovery for some small adducts containing one aromatic ring or non-aromatic bulky adducts (Reddy, 1993a).

Nuclease P1 (or S1) digestion relies on the ability of the enzyme to dephosphorylate unmodified deoxynucleotides, resulting in the formation of deoxynucleosides, which are not substrates for postlabelling. Certain modified deoxynucleotides are resistant to the action of the enzyme, a characteristic which appears to be influenced by the adduct position on the deoxynucleotide (Gorelick, 1993). Nuclease P1 digestion has proved effective for the analysis of most aromatic adducts, however, arylamine adducts are not resistant to nuclease P1 and are almost entirely lost (Reddy and Randerath, 1987). Unlike butanol extraction, nuclease P1 digestion can be used for the successful analysis of small aromatic and bulky adducts.

In addition to the ^{32}P-postlabelling bisphosphate method, an alternative method, shown in Scheme 5 of Figure 3.1, has been employed in several laboratories (Randerath *et al.*,

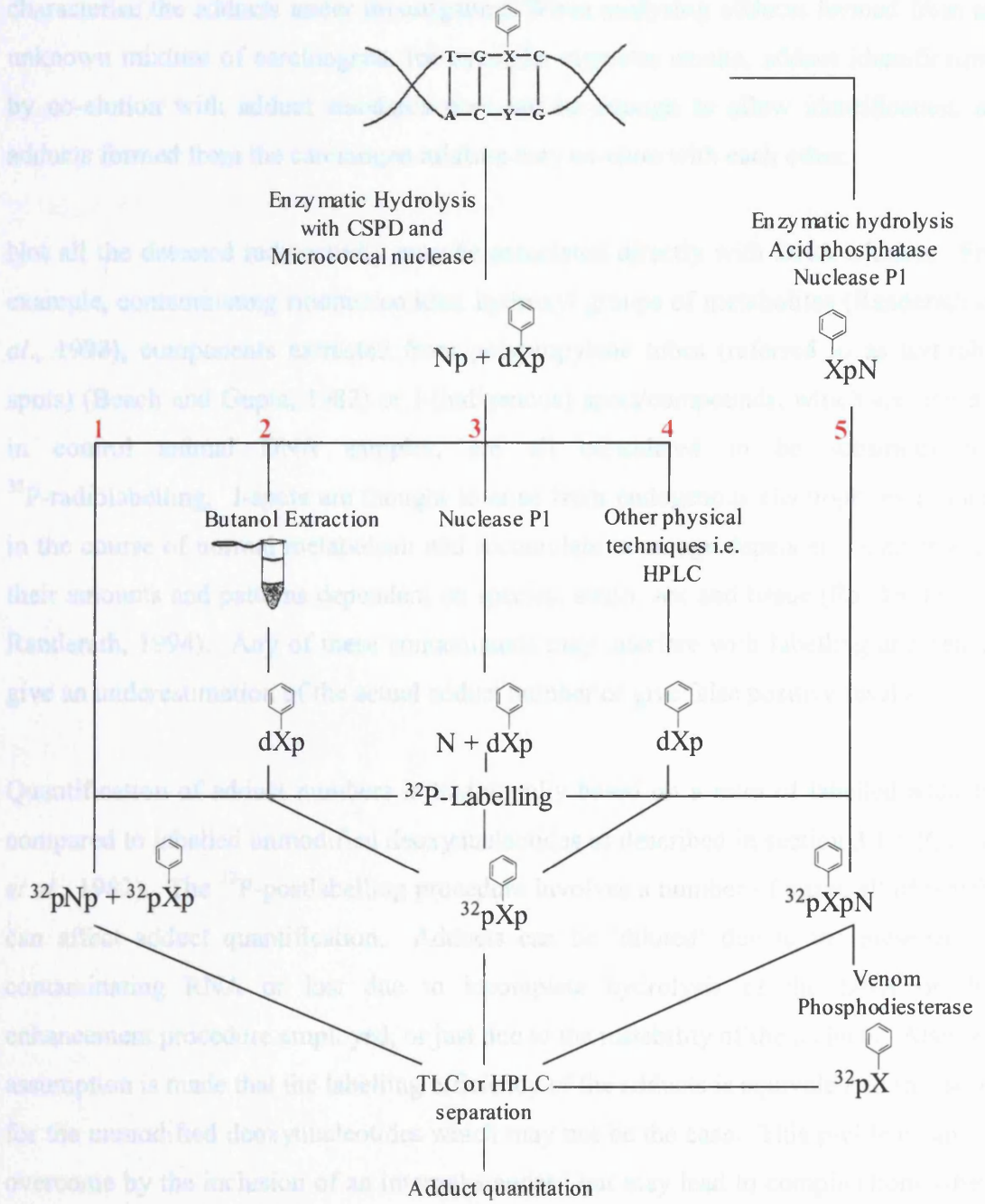
1989). In this method, adducts are excised as 5'-phosphorylated dinucleotides whereas unmodified deoxynucleotides are released from DNA as 5'-monophosphates. The adducted dinucleotides are substrates for the labelling reaction, and can either be analysed as radiolabelled dinucleotides or can be further digested to ^{32}P-labelled deoxynucleoside 5'-monophosphates. These products have an improved mobility on TLC (and HPLC) compared to their bisphosphate counterparts, which may help in the analysis of large, bulky adducts or aid in the analysis of adducts which have a similar mobility/retention time, when analysed as bisphosphates.

A further improvement to the ^{32}P-postlabelling method involves the analysis of the radiolabelled adducts by HPLC coupled to a radiochemical detector as opposed to TLC, the traditional method for adduct analysis. HPLC affords an improved reproducibility and reduced analysis time, although sensitivity of detection by HPLC is reduced compared to TLC, a problem highlighted in Chapter 5.

Figure 3.1: Schematic representation of the ^{32}P-postlabelling assay highlighting the different procedures which may be employed. The basic methodology involves digestion of DNA to deoxynucleoside 3'-monophosphates, which can be ^{32}P-labelled directly (1), adduct enhanced by organic partitioning (2), enzyme digestion (3) or other physical methods (4). Alternatively, a different procedure involves digestion of DNA to dinucleotides and after labelling, subsequent digestion to deoxynucleoside 5'- monophosphates (5). Analysis of samples is typically by 2D-TLC or HPLC followed by adduct quantitation.

represents an adducted base, N and Y represent any of the 4 possible bases.

Adapted from Beach and Gupta, 1992 and Randerath and Randerath, 1994.



3.1.3. Advantages and limitations of the ^{32}P-postlabelling assay

The main advantages of the ^{32}P-postlabelling assay over other techniques are that it requires only microgram quantities of DNA for each analysis and no prior detailed knowledge of adduct structure is required. No knowledge of adduct structure however, could be classed as a disadvantage of the technique as co-chromatography with chemically synthesised standards or further techniques, such as MS are required to characterise the adducts under investigation. When analysing adducts formed from an unknown mixture of carcinogens, for example, cigarette smoke, adduct identification by co-elution with adduct standards may not be enough to allow identification, as adducts formed from the carcinogen mixture may co-elute with each other.

Not all the detected radioactivity may be associated directly with DNA adducts. For example, contaminating ribonucleotides, hydroxyl groups of metabolites (Randerath *et al.*, 1998), components extracted from polypropylene tubes (referred to as test-tube spots) (Beach and Gupta, 1982) or I-(indigenous) spots/compounds, which are present in control animal DNA samples, are all considered to be substrates for ^{32}P-radiolabelling. I-spots are thought to arise from endogenous electrophiles formed in the course of normal metabolism and accumulate in an age dependent manner with their amounts and patterns dependent on species, strain, sex and tissue (Randerath and Randerath, 1994). Any of these contaminants may interfere with labelling and hence give an underestimation of the actual adduct number or give false positive results.

Quantification of adduct numbers is traditionally based on a ratio of labelled adducts compared to labelled unmodified deoxynucleotides as described in section 3.1.1 (Gupta *et al.*, 1982). The ^{32}P-postlabelling procedure involves a number of steps, all of which can affect adduct quantification. Adducts can be 'diluted' due to the presence of contaminating RNA or lost due to incomplete hydrolysis of the DNA or the enhancement procedure employed, or just due to the instability of the adducts. Also, an assumption is made that the labelling efficiency of the adducts is equivalent to that seen for the unmodified deoxynucleotides which may not be the case. This problem can be overcome by the inclusion of an internal standard but may lead to complications when more than one adduct is under analysis.

3.1.4. Application of the ^{32}P-postlabelling assay

The ^{32}P-postlabelling assay has proved to be highly sensitive and has been applied to the detection of DNA adducts in human and rodent tissues from a wide range of carcinogens and mutagens which include alkylating agents, polycyclic aromatic hydrocarbons (PAHs), heterocyclic PAHs, nitro-PAHs, aromatic amines, quinones, dyes, chemotherapeutic agents and pesticides to name but a few examples (Beach and Gupta, 1992; Randerath *et al.*, 1985 and Reddy *et al.*, 1984).

In humans, DNA from a wide range of tissues has been investigated for the presence of DNA adducts using the ^{32}P-postlabelling technique. Such tissues include blood (white blood cells), skin, placenta, tissues taken at autopsy, biopsy samples, buccal smears and sperm. Human tissue samples have been used to assess an individual's exposure to chemicals in cigarette smoke, chemicals used in a clinical setting or from an occupational exposure, for example, iron foundry workers, coke oven workers and roofers have been assessed for their exposure to PAHs (Beach and Gupta, 1992).

3.2. Aim

The main aim of the work described in this chapter was to develop a reliable ^{32}P-postlabelling assay coupled to either TLC or HPLC for the analysis of benzene-DNA adducts which had been synthesised *in vitro,* as detailed in Chapter 2. This work involved an investigation of all aspects of the general ^{32}P-postlabelling procedure in order to optimise adduct recovery and analysis.

Upon development of an optimised ^{32}P-postlabelling method for the analysis of the *in vitro* benzene-DNA adducts it was hoped that the same method in combination with an immunoaffinity purification step (detailed in Chapter 4), could be employed for the sensitive and specific analysis of the adduct formed *in vivo,* (N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate), which will be discussed in Chapter 5.

3.3. Materials

Micrococcal nuclease, apyrase, tetrabutylammonium chloride and nuclease P1 were purchased from Sigma (Poole, Dorset). [γ-^{32}P]ATP (>185 TBq / mmol, > 5000 Ci /mmol) was obtained from Amersham (Amersham, Buckinghamshire). T4 polynucleotide kinase (3'-phosphatase free) and calf spleen phosphodiesterase (CSPD) were purchased from Boehringer Mannheim (Lewes, East Sussex). TLC plates were obtained from VWR (Lutterworth, Leicestershire) and Camlab (Over, Cambridge). All other chemicals were of the highest grade available and were obtained from Fisher (Loughborough, Leicestershire).

3.4. Instruments

HPLC of ^{32}P-radiolabelled samples was performed using a Varian system with a Star 9012 pump (Varian Analytical Instruments, Surrey) and a Hypersil, C18, BDS, 5 μm, 4.6 x 250 mm column. Eluate from the HPLC passed through a radiochemical detector (β-ram, LabLogic, Sheffield) fitted with a solid phase cell (500 μL). Data collection was performed using Laura, a Microsoft Windows package (LabLogic, Sheffield).

Visualisation of TLC plates was carried out using phosphorimager cassettes, which were scanned using a phosphorimager (Molecular Dynamics, part of Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Buckinghamshire). Analysis of the TLC plates was carried out using ImageQuant software (Molecular Dynamics, part of Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Buckinghamshire).

3.5. Methods

3.5.1. Adduct synthesis

Reaction of calf thymus DNA or the individual deoxynucleotides with the benzene metabolites, HQ, *p*-BQ or benzene oxide has been detailed previously (see Chapter 2, section 2.5).

3.5.2. ^{32}P-postlabelling

3.5.2.1. DNA digestion

DNA (5 μg), dried using vacuum centrifugation, was enzymatically digested to deoxyribonucleoside 3'-monophosphates using micrococcal nuclease (0.175 U), calf spleen phosphodiesterase (0.003 U) and SSCC (100 mM sodium succinate, 50 mM calcium chloride pH 6.0; 1 μL) in a total volume of 6.25 μL, at 37 °C, for 15 h.

3.5.2.2. Adduct enrichment

3.5.2.2.1. Nuclease P1 digestion

The digested DNA underwent adduct enrichment based on the nuclease P1 enhancement method originally described by Reddy and Randerath, 1986.

The digested DNA (4 μg; the remaining 1μg was used for nucleotide dilution, see section 3.5.2.4) underwent enrichment with nuclease P1 (9 μg; 2 μg/μL in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5.0) at 37 °C for 60 min (this incubation time changed during the optimisation of the technique, see section 3.6.4.2 for further details). Tris HCl, (2.4 μL; 1 M, pH 7.6) was added to each sample in order to stop the digestion.

3.5.2.2.2. Butanol extraction

The digested DNA underwent adduct enrichment using the modified butanol extraction procedure as described by Phillips and Castegnaro, 1999.

The digested DNA (4 μg; the remaining 1 μg was used for nucleotide dilution, see section 3.5.2.4) was diluted to 120 μL with water and a mixture of tetrabutylammonium chloride (TBAC; 10 mM; 15 μL), ammonium formate (100 mM; 15 μL) and water saturated butan-1-ol (150 μL) was added to each sample. Samples were vortexed for 30 s and centrifuged at 14,000 rpm for 1 min. The organic layer from each sample was removed and the extraction was repeated on the remaining aqueous layer. Organic layers for each extraction were combined. Butan-1-ol saturated water (250 μL) was added to the pooled organic layers and mixing and centrifugation was carried out as detailed above. The organic layer was again removed and the aqueous layer was discarded. The butan-1-ol saturated water wash was

repeated. Tris HCl, pH 7.6, (200 mM; 3 μL) was added to each sample and the samples were dried to completeness using vacuum centrifugation. Each sample was resuspended in water (9.5 μL) and 1 M tris base, pH 7.6 (2.4 μL) in preparation for radiolabelling.

3.5.2.3. Radiolabelling of enriched adducted deoxynucleotides

Radiolabelling of the enriched adducts and the synthesised adducted deoxynucleotides (see Chapter 2 for further details of adduct synthesis) was performed using 3' phosphatase free, T4 polynucleotide kinase (5 U) and [γ-^{32}P]ATP (50μCi; specific activity > 5000Ci/mmol; specific activity was corrected by calculating the extent of decay) in the presence of labelling buffer (2 μL; 200 mM Tris HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 10 mM spermidine, pH 7.6, filter sterilised) in a total volume of 20 μL (adjusted with water). Samples were incubated at 37 °C for 1 h.

Samples were either frozen at –20°C (for HPLC analysis) or spotted onto TLC plates as shown in Figure 3.2. (The plates had been pre-developed overnight with water).

3.5.2.4. Nucleotide dilution

The remaining digested DNA (1 μg), which did not undergo adduct enrichment, (see sections 3.5.2.2.1 and 3.5.2.2.2 for further details), underwent dilution with water to a final concentration of 0.1 ng/μL. An aliquot of this dilution (1.2 ng DNA) was radiolabelled as described above (section 3.5.2.3), except 20 μCi [γ-^{32}P] ATP was used. After incubation at 37 °C for 1 h, the sample was divided into 2 x 10 μL aliquots. To one aliquot, apyrase (0.08 U) was added and these samples were incubated at 37 °C for a further 30 min. Both aliquots were diluted to 100 μL with tris HCl (10 mM): EDTA (5 mM, pH 9.5).

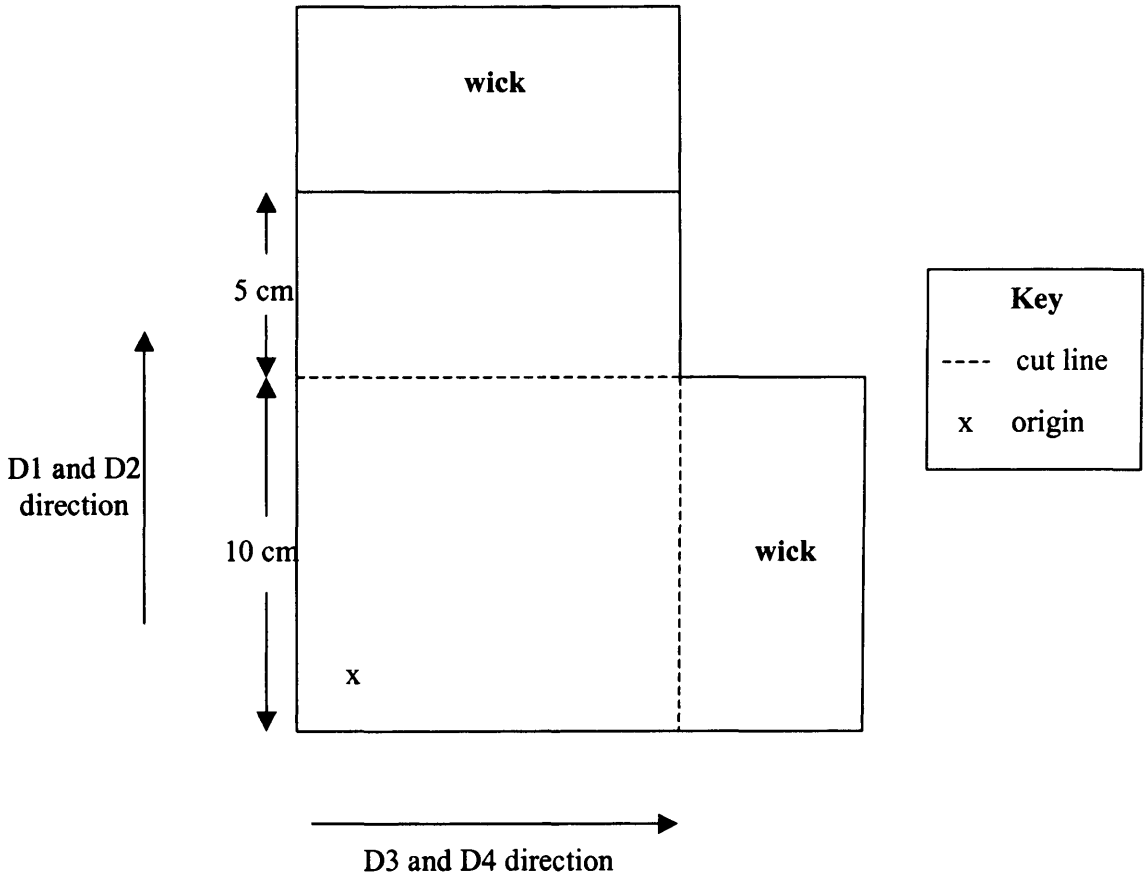
Aliquots of each sample (2 μL) were spotted onto TLC plates, the apyrase treated samples were spotted in duplicate. The TLC plates were developed in one direction using sodium phosphate buffer (0.12 M; pH 6.8). These plates were used for quantifying adduct number as detailed in section 3.5.2.7.

3.5.2.5. TLC methodology

The published method for the TLC development for benzene-DNA adducts involved four chromatography steps, as detailed in Pongracz *et al.*, 1990. The direction of each chromatography step can be seen in Figure 3.2. The plates (15 x 10 cm) were developed overnight onto a wick using 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction, this step was optional). The top 5 cm of each plate plus the wick were removed and discarded and the remaining 10 x 10 cm plates were washed (and also for all subsequent chromatography steps), for 5 min in water (washing after D2 chromatography included an additional 5 min wash with 13 mM tris base). Plates were developed in the same direction, to the end of each plate, using 1.8 M lithium formate, 4.5 M urea, pH 3.5 (D2). A wick was attached to the right edge of each plate (spotting origin in bottom left hand corner) and plates were developed using 0.36 M lithium chloride, 0.22 M tris, 3.8 M urea, pH 8.0 (D3). The final chromatography step was carried out in the same direction as D3, 5-6 cm onto a wick using 1.7 M sodium phosphate buffer, pH 6.0.

TLC conditions were improved upon using the above-mentioned buffers in various combinations and dilutions for varying lengths of time as detailed in the results, section 3.6.1.

Figure 3.2: Plate preparation for the multi-directional separation of benzene-DNA adducts. D1- D4 refers to the chromatography conditions employed and the direction of adduct development as detailed in the main text.



3.5.2.6. HPLC methodology

Samples were injected manually (see Section 3.3 for further details) and a method for DNA adduct separation was developed using a range of concentrations of aqueous mobile phase (0.1 – 2.0 M ammonium formate, pH 4.0 containing 0.27 mM EDTA), varying between methanol or acetonitrile as the organic phase. See results section 3.6.2 for full details of method development.

3.5.2.7. Adduct quantitation from TLC

TLC plates, including those for the adducted samples (section 3.5.2.5), the diluted nucleotides (section 3.5.2.4) and an ATP calibration line ($7.2x10^{-5}$ - 0.2 μCi equivalent to 1.6×10^2 - 5×10^5 disintegrations per min (dpm), as based on theoretical values) were exposed in a phosphorimager cassette for a maximum of 2 h and then scanned using a phosphorimager as detailed in Section 3.4. The phosphorimager software

(ImageQuant) reports radioactivity as a 'volume', therefore the ATP calibration line was used to convert 'volume' data to a dpm value.

Using the dpm values calculated, adducts were quantified as numbers of adducts / 10^8 nucleotides using the following equations (Gupta *et al.*, 1982 and Gupta, 1993):

$$RAL = \frac{1}{\text{dilution factor}} \times \frac{\text{(dpm for adduct spot – background)}}{\text{dpm for diluted nucleotide - background}}$$

Where RAL is relative adduct labelling

dpm for diluted nucleotides is the average radioactivity for the apyrase treated samples, which were spotted in duplicate (see section 3.5.2.4).

$$\text{Dilution factor} = \frac{\text{Amount of DNA spotted onto adduct plate}}{\text{Amount of DNA spotted onto diluted nucleotide plate}}$$

RAL is converted to number of adducts / 10^8 nucleotides by multiplying RAL by a factor of 10^8.

3.5.2.8. Adduct quantitation from HPLC

Known amounts of radioactivity were injected onto the HPLC. Eluate corresponding to the ATP peak was collected and radioactivity was determined by scintillation counting (measured as dpm) and compared to theoretical dpm values. A calibration line based on peak areas from HPLC and corresponding values from scintillation counting (or theoretical values) were plotted. dpm values for the actual samples were read from the calibration line and relative adduct labelling was calculated based on the method described by Reddy and Randerath, 1986 using the following equation:

$$RAL = \frac{\text{dpm in adduct peak}}{\text{Specific activity of ATP x pmol dNp used for analysis}}$$

Specific activity of [γ-^{32}P] ATP is expressed as dpm/pmol

1μg DNA corresponds to 3240 pmol deoxyribonucleoside 3'-monophosphates (dNp) (Reddy and Randerath, 1986).

3.5.3. Spot elution

Radioactive spots were eluted from TLC plates using 4 M pyridinium formate, pH 4.5. Spots were traced, excised from TLC plates and placed into 20 mL vials with enough pyridinium formate to cover each piece of TLC plate. Vials were placed on a shaking table and incubated for 15 h at room temperature.

The resultant, radioactive solution was filtered using 0.2 μm sterile filters and the eluate was centrifuged at 10,000 rpm for 10 min. The supernatant was dried to completeness using vacuum centrifugation and reconstituted in water (100 μL) prior to injection onto the HPLC column.

3.6. Results

Benzene-DNA adducts were initially synthesised by reaction of calf thymus DNA with *p*-BQ, with and without HQ and these DNA samples was used for the development of both TLC and HPLC methods for the detection and separation of ^{32}P-postlabelled benzene-DNA adducts.

3.6.1. TLC method development

DNA treated with *p*-BQ was postlabelled using the standard nuclease P1 enhanced postlabelling method of Reddy and Randerath, 1986 as detailed in the Materials and Methods, section 3.5.2.2.1. To check that the reaction resulted in adduct synthesis, multidirectional, multisolvent thin layer chromatography was carried out using the published method of Pongracz *et al.*, 1990, which has also been detailed in the Materials and Methods section. The resultant TLC maps can be seen in Figure 3.3, plates were developed without the initial D1 chromatography step and are shown before and after the final chromatography step (D4). As shown in Figure 3.3 D, comparing it to Figure 3.3 C, the final chromatography step, which was intended as a 'wash' to reduce the background radioactivity on the plates, has resolved a number of ^{32}P-labelled products which, by comparison to TLC of control DNA (Figure 3.3, A and B) are radiolabelled DNA adducts. This adduct migration would normally be expected in the D3 chromatography step, which was not achieved (shown in Figure 3.3, C).

Subsequent modification of the TLC conditions resulted in more defined adduct elution as shown in Figure 3.4. Inclusion of all 4 chromatography steps (D2 and D3 were carried out without wicks) resulted in 3 major and several minor adduct spots as observed in Figure 3.4, C. The length of time allowed for D4 chromatography appears to be critical, with a loss of adduct spots occurring when D4 was carried out for 15 h as opposed to 8 h (Figure 3.4, B and C, respectively). Exclusion of the D1 step resulted in only 2 adduct spots, which had different retention times to the adducts achieved when D1 was included (Figure 3.4, D and C, respectively). This finding led to the conclusion that the two 'wash' steps, D1 and D4 were actually resolving the adducts as highlighted in Figure 3.3 (described above). Elution of radioactive spots from the TLC plates and injection onto HPLC further clarified the fact that the TLC conditions were not

appropriate for the separation of the benzene-DNA adducts as highlighted in Figure 3.8 and explained in more detail in section 3.6.2.

Further modification of the TLC conditions which included various dilutions of the D2 and D3 chromatography solvents, eventually led to the exclusion of the D2 and D3 chromatography steps, with adduct spot separation reliant only on the two wash steps, D1 and D4. As observed in Figure 3.5, migration of the spots was dependent on the length of time that each chromatography step was carried out for, with adducts eluting approximately two thirds of the way up the plate after a 4 h development in D1 buffer and four fifths of the way across the plate after a 4 h development with D4 buffer. These results led to the modification of the TLC conditions from those published (Pongracz *et al.*, 1990), with chromatography now being carried out using only two steps which involved adduct elution in the D1 direction for 3 h (adduct mobility was similar to that observed after 4 h) using 0.4 M sodium phosphate buffer, pH 6.8 and the D4 direction using 1.7 M sodium phosphate buffer, pH 6.0 for 4 h. Using these conditions, adducts were resolved as 4 major spots and several minor ones as observed in Figure 3.5.

TLC analysis of control calf thymus DNA using the modified conditions resulted in no observable radioactive spots as can be seen in Figure 3.5, E. This chromatogram clearly demonstrates successful removal of excess ATP, inorganic phosphate and any radiolabelled unmodified deoxynucleotides, therefore suggesting that the radiolabelled spots observed on the TLC plates shown in Figure 3.5, A-D are benzene-modified (adducted) deoxynucleotides.

3.6.1.1. Identification of adducts on TLC

Identification of the main adduct spots was carried out using the synthesised deoxynucleotide adduct standards (see Chapter 2 for full details). ^{32}P-postlabelled deoxynucleotide adduct standards were co-chromatographed with the HQ and *p*-BQ treated calf thymus DNA and an example of this is shown in Figure 3.6, A-C for the benzene adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate.

TLC spots 1, 2, 3 and 4 corresponded to (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, respectively. The chemical structure of each adduct is shown in Figure 3.6, D.

Figure 3.3: Thin layer chromatography of ^{32}P-postlabelled calf thymus DNA (**A** and **B**) and *p*-BQ treated calf thymus DNA (**C** and **D**) using the published standard chromatography conditions (Pongracz *et al.*, 1990) except the initial chromatography step (D1) was omitted for all 4 plates, a wick was included in the D3 chromatography and the final chromatography step (1.7 M sodium phosphate, pH 6.0) was included for plates **B** and **D**.

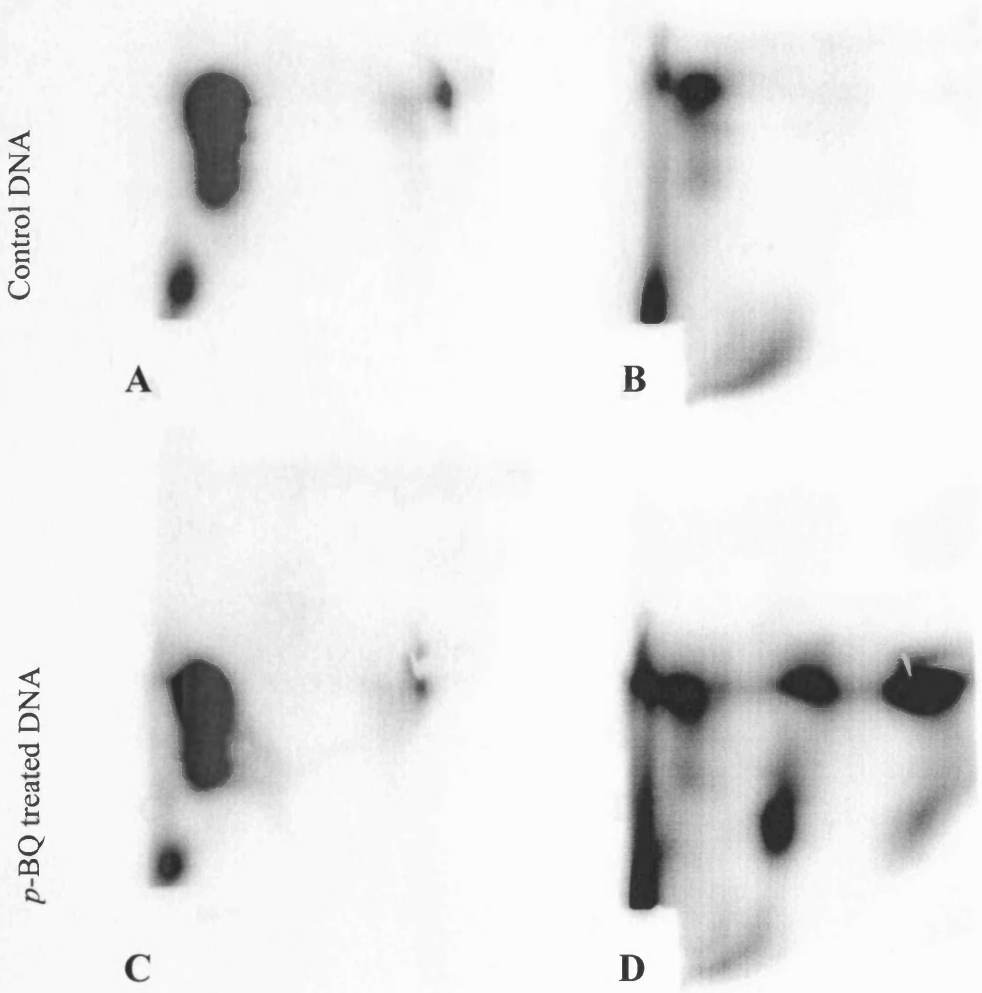


Figure 3.4: TLC separation of ^{32}P-postlabelled control calf thymus DNA (**A**), and ^{32}P-postlabelled calf thymus DNA following treatment with *p*-BQ (**B**, **C** and **D**). Plates **A**, **B** and **C** were initially developed for 15 h in the D1 direction. All 4 plates were developed using the D2 and D3 (without wicks) chromatography. D4 chromatography was carried out either for 8 h (**A**, **C** and **D**) or 15 h (**B**).

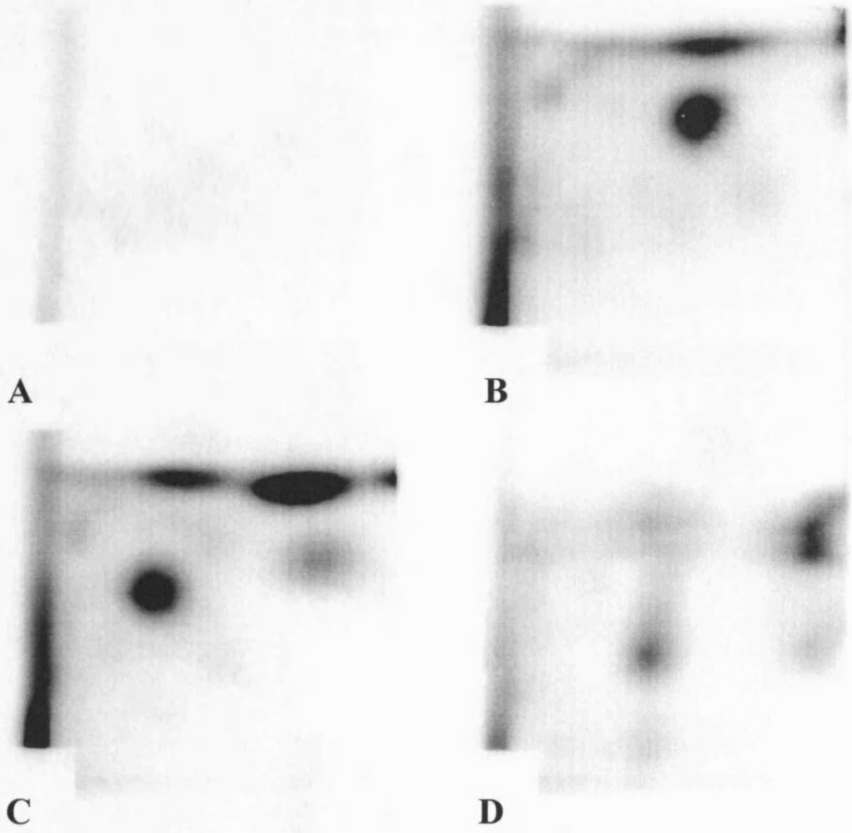


Figure 3.5: TLC chromatograms of ^{32}P-postlabelled DNA adducts from the reaction of calf thymus DNA with *p*-BQ (**A-D**) or control calf thymus DNA (**E**). Plates were developed in the D1 direction using 0.4 M sodium phosphate, pH 6.8 for either 1 h (plates **A** and **B**) or 4 h (plates **C, D** and **E**) and the D4 direction using 1.7M sodium phosphate, pH 6.0 for either 3 h (plates **A** and **C**) or 4 h (plates **B, D** and **E**).

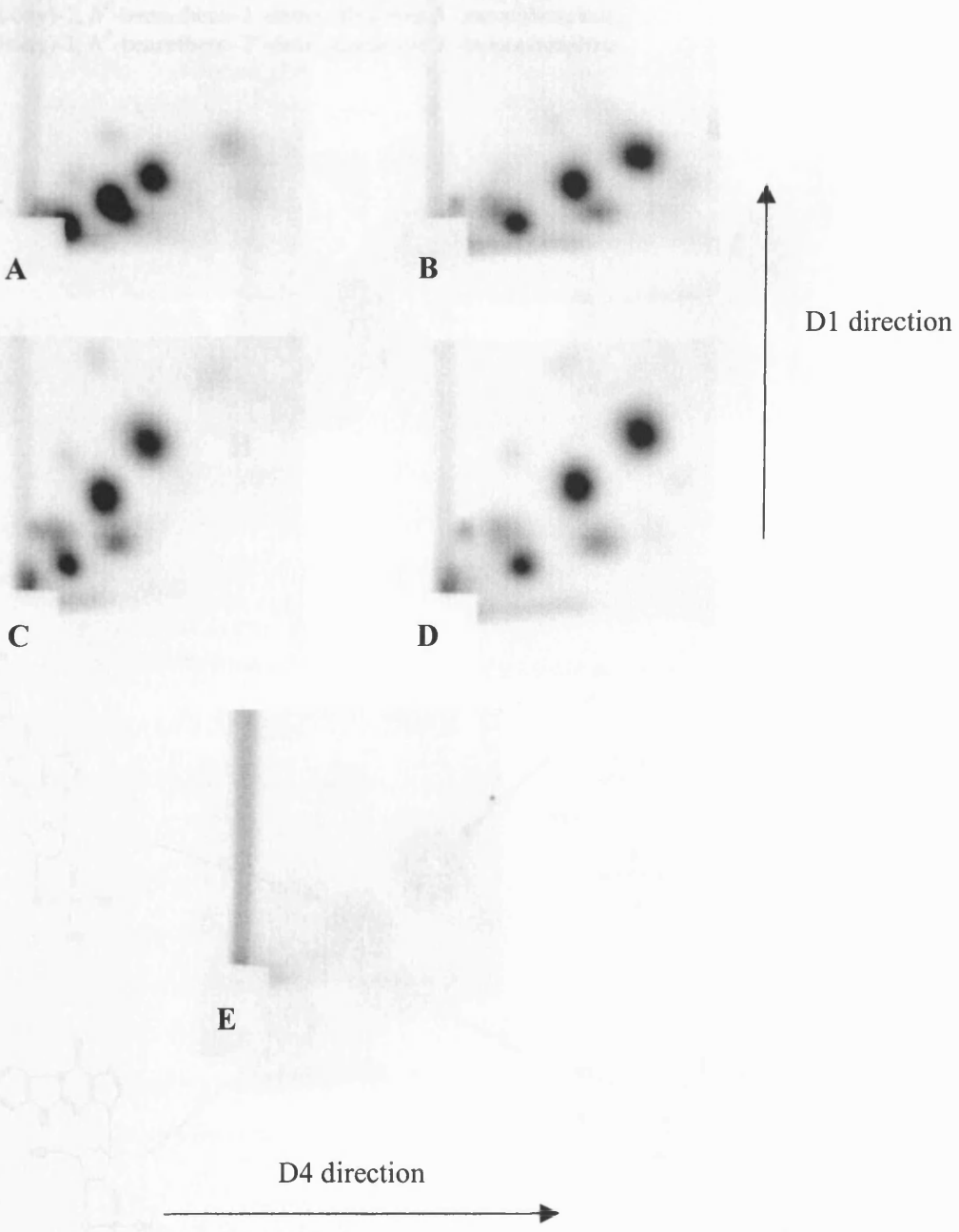
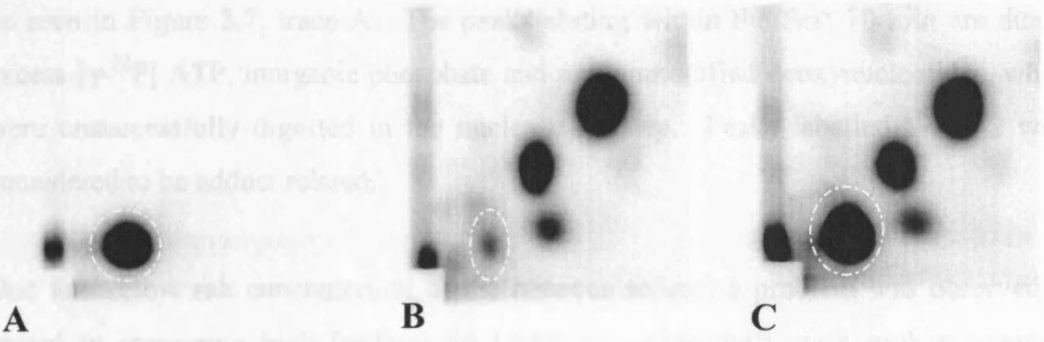
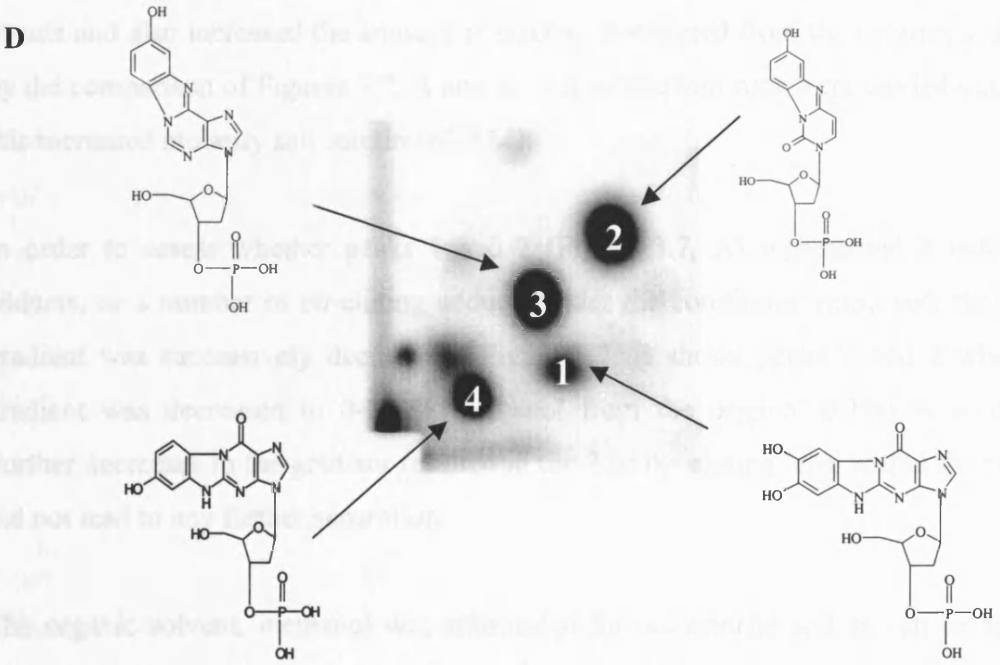


Figure 3.6: Typical TLC maps demonstrating the retention of ^{32}P-postlabelled (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (**A**), adducts formed from HQ and *p*-BQ treated calf thymus DNA (**B**) and co-elution of the deoxynucleotide adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate with HQ and *p*-BQ treated calf thymus DNA (**C**). The adduct spot of interest is highlighted by the white dotted line.
Adducts formed after treatment of calf thymus with HQ and *p*-BQ were identified by co-elution with the individual synthesised nucleotide adducts (**D**). TLC adduct spots were identified as follows:
Spot 1: (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate.
Spot 2: (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate,
Spot 3: (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate,
Spot 4: (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate





3.6.2. HPLC method development

Calf thymus DNA treated with *p*-BQ, with and without HQ, was nuclease P1 treated and ^{32}P-postlabelled as detailed in the Methods section (Section 3.5.2). The resulting radiolabelled mixture was used to develop a HPLC system capable of resolving benzene-DNA adducts.

Initially, a linear gradient of methanol in 0.1 M ammonium formate, pH 4.0, containing 0.27 mM EDTA, (0-100 %; 65 min) was employed and the resulting elution profile can be seen in Figure 3.7, trace A. The peaks eluting within the first 10 min are due to excess [γ-^{32}P] ATP, inorganic phosphate and any unmodified deoxynucleotides, which were unsuccessfully digested in the nuclease P1 step. Peaks labelled 1 and 2 were considered to be adduct related.

Due to the low salt concentration of the aqueous solvent a problem was observed in regard to increasing high background levels of radioactivity with each subsequent HPLC run. To counter this problem HPLC runs were carried out with increasing concentrations of 0.5, 1.0 and 2.0 M ammonium formate in the aqueous solvent. It was found that 2.0 M ammonium formate successfully reduced the background radioactive counts and also increased the amount of product that eluted from the column as shown by the comparison of Figures 3.7, A and B. All subsequent runs were carried out using this increased molarity salt solution (2.0 M).

In order to assess whether peaks 1 and 2 (Figure 3.7, A) represented 2 individual adducts, or a number of co-eluting adducts under the conditions employed, the linear gradient was successively decreased. Figure 3.7, B shows peaks 1 and 2 when the gradient was decreased to 0-80 % methanol from the original 0-100 % methanol. Further decreases in the gradient resulted in the 2 peaks eluting later within the run but did not lead to any further separation.

The organic solvent, methanol was substituted for acetonitrile and as can be seen in Figure 3.7, C, a 0-30 % gradient gave a similar peak elution pattern to that observed when a gradient of 0-80 % methanol was used.

Data from the TLC analysis of the *p*-BQ (and HQ) treated DNA suggested that at least 3 major and several minor adducts were present in the sample. Under the HPLC conditions employed here only two adduct peaks were observed, suggesting that adducts were either co-eluting or eluting with the ATP related peaks etc at the start of the run. An attempt was made to try and determine if peak 1 represented one adduct or two or more adducts eluting together. To achieve this, the gradient was decreased to 0-15 % over 40 min followed by an increase over 25 min to 30 % acetonitrile in order to elute peak 2. The resultant trace is shown in Figure 3.7, D. Peak 1 appears to be resolved into 2 peaks. These two peaks were not observed in control calf thymus DNA as shown in Figure 3.7, E.

In order to clarify the situation further, and help determine which peaks were significant in regard to whether they were adduct related, the adduct spots from the TLC plates (during the early method development) were excised, the radioactivity eluted and injected onto the HPLC. The TLC map used and the resultant HPLC traces can be seen in Figure 3.8. Comparison of the results to the trace showing separation of HQ and *p*-BQ treated DNA (Figure 3.8, F) demonstrated that the HPLC conditions (and most probably the TLC conditions at this time) were not optimal for adduct separation.

Based on the HPLC conditions developed so far, with peak 1 eluting between 5 and 10 % organic mobile phase, an isocratic system of 6 % acetonitrile over 45 min was employed, followed by an increase to 50 % over the next 20 min. The increase to 50 % was used as a wash step to elute any remaining radioactivity from the column. The resultant trace from this chromatography can be seen in Figure 3.9, A. The peaks now eluted within the first 20 min. Reduction of the isocratic system to 3 % organic mobile phase resulted in 4 main peaks eluting between 15 and 50 min and a further reduction to 2 % organic mobile phase gave the same peak profile with the peaks eluting approximately 3-4 min earlier than previously seen. The resultant traces can be seen in Figure 3.9, B and C, respectively, with comparison to untreated calf thymus DNA (Figure 3.9, D). Radiolabelled untreated calf thymus DNA showed no peaks eluting during the 15-50 min window, suggesting that the four peaks observed in the treated DNA sample represented the benzene-DNA adducts.

Further elution of the radioactivity from the adduct spots on the TLC plates (in the early stages of method development) as shown in Figure 3.8 and subsequent injection onto the newly developed HPLC system confirmed that the TLC conditions at this time were not appropriate for adduct elution. The main peak on the HPLC, peak 2, was not identifiable from the TLC spots, suggesting that this adduct was lost from the TLC plate which was confirmed by chromatography of the individually synthesised adduct (results not shown).

Further development of the TLC conditions as outlined in Section 3.6.1, led to the separation of 3 major and several minor adduct spots. Elution of the radioactivity from the adduct spots on the TLC plates, with subsequent injection onto HPLC and co-elution with *p*-BQ treated calf thymus DNA confirmed that 4 of the radiolabelled spots on TLC corresponded to the 4 main peaks on HPLC (Figure 3.10). TLC spots 1, 2, 3 and 5 as shown in Figure 3.10, corresponded to HPLC peaks 4, 1, 3 and 2, respectively (see Figure 3.10, A) and were hence renumbered as shown in Figure 3.6, to avoid confusion.

3.6.2.1. Identification of adducts by HPLC

The synthesised benzene DNA adducts; (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate (see Chapter 2) were ^{32}P-postlabelled and chromatography was carried out using the newly developed HPLC system. Co-chromatography of the individual adducts with radiolabelled, *p*-BQ treated DNA led to the identification of the main HPLC peaks observed. An example of the HPLC chromatogram observed can be seen in Figure 3.11, A, with peak identification shown in Figure 3.12.

HPLC peaks 1, 2, 3 and 4 corresponded to (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1,

N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, respectively. This result was confirmed by comparison to the chromatography observed when the same adducts were analysed by TLC, thus proving that the TLC spots 1, 2, 3 and 5 corresponded to HPLC peaks 4, 1, 3 and 2, respectively.

Figure 3.7: HPLC traces of ^{32}P-radiolabelled *p*-BQ treated calf thymus DNA (2 μg, **A** or 1 μg **B-D**) or 1 μg untreated calf thymus DNA (**E**). Separation was carried out using a linear gradient of methanol in 0.1 M ammonium formate, pH 4.0 (0-100 %, 65 min) (**A**). Separation in **B** is carried out using a linear gradient of methanol in 2.0 M ammonium formate, pH 4.0, 0-80 %, 65 min. Separation in **C** was performed using a gradient of acetonitrile in 2.0 M ammonium formate, pH 4.0 0-30 %, 65 min. Separation in **D** and **E** was performed using a gradient of 0-15 % acetonitrile in 2.0 M ammonium formate, pH 4.0, 0-40 min, then 15-30 % B, 40-65 min.
The main two adduct peaks are numbered 1 and 2.

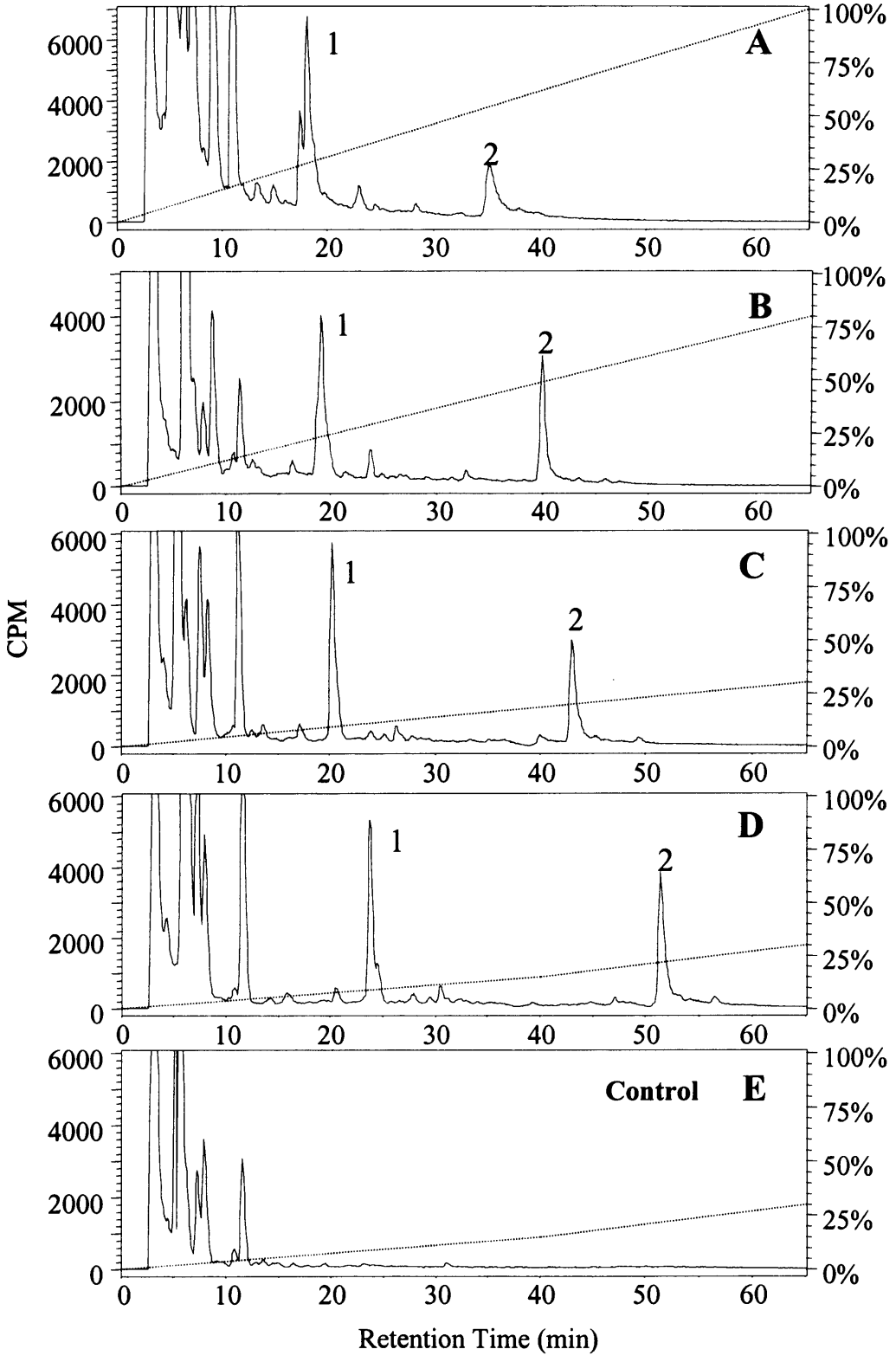


Figure 3.8: Elution of radioactive spots 1-4 from TLC (**A**) (suspected benzene-DNA adducts) with subsequent injection onto HPLC (traces **B-E** correspond to spots 1-4 respectively). Trace **F** shows the separation of 1μg ^{32}P-radiolabelled calf thymus DNA after treatment with *p*-BQ and is shown for comparison. Chromatography was performed using a gradient of acetonitrile in 2.0 M ammonium formate, pH 4.0, 0-15 % B, 0-40 min; 15-30 % B, 40-65 min.

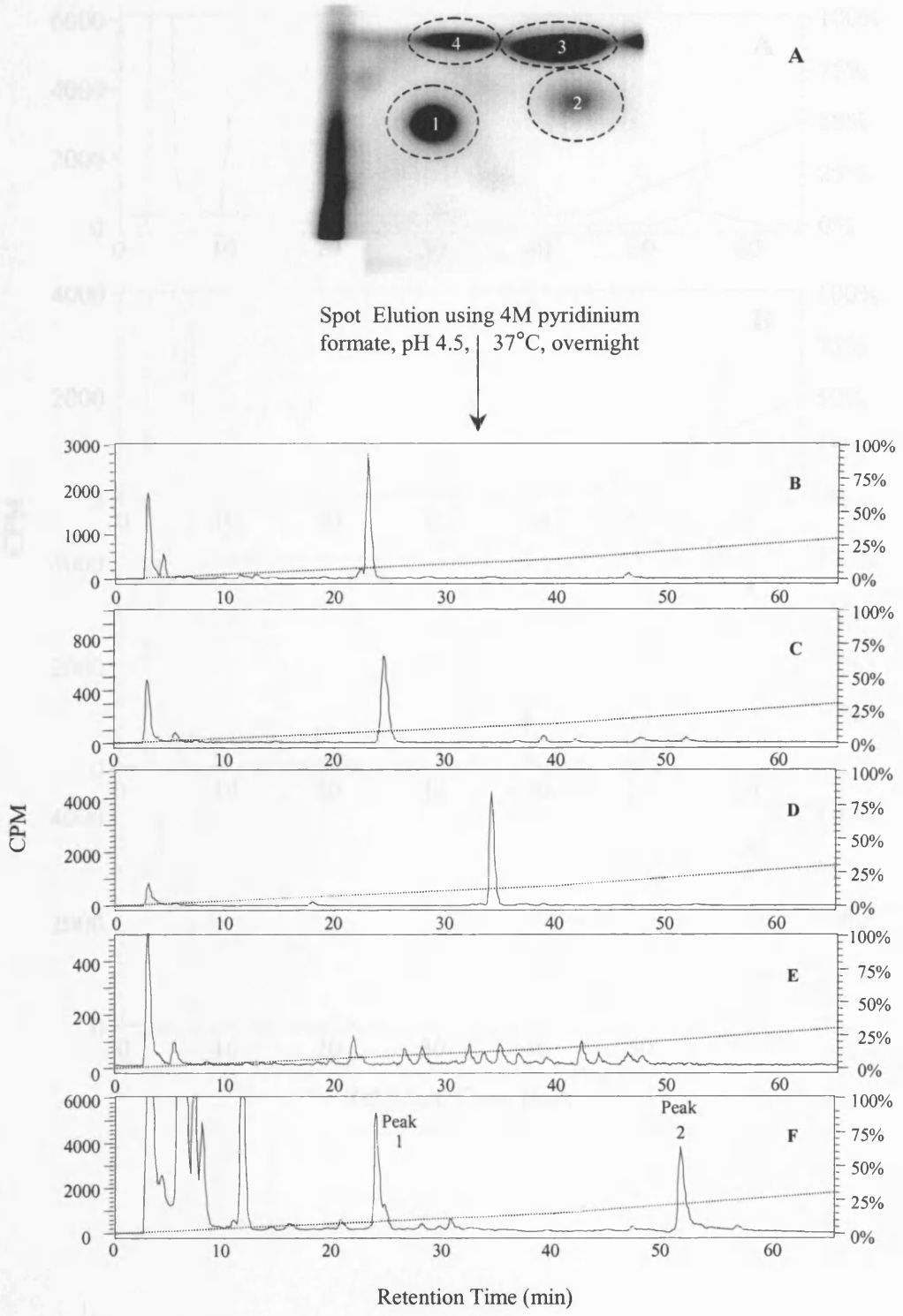


Figure 3.9: HPLC traces of ^{32}P-radiolabelled calf thymus DNA after treatment with *p*-BQ (1 μg; **A-C**) or untreated calf thymus DNA (1 μg; **D**). Separation was carried out using 6 % (**A**), 3 % (**B**) or 2 % (**C** and **D**) acetonitrile in 2.0 M ammonium formate, pH 4.0 containing 0.27 mM EDTA. Peaks labelled 1-4 were suspected adduct peaks when compared to control DNA (**D**).

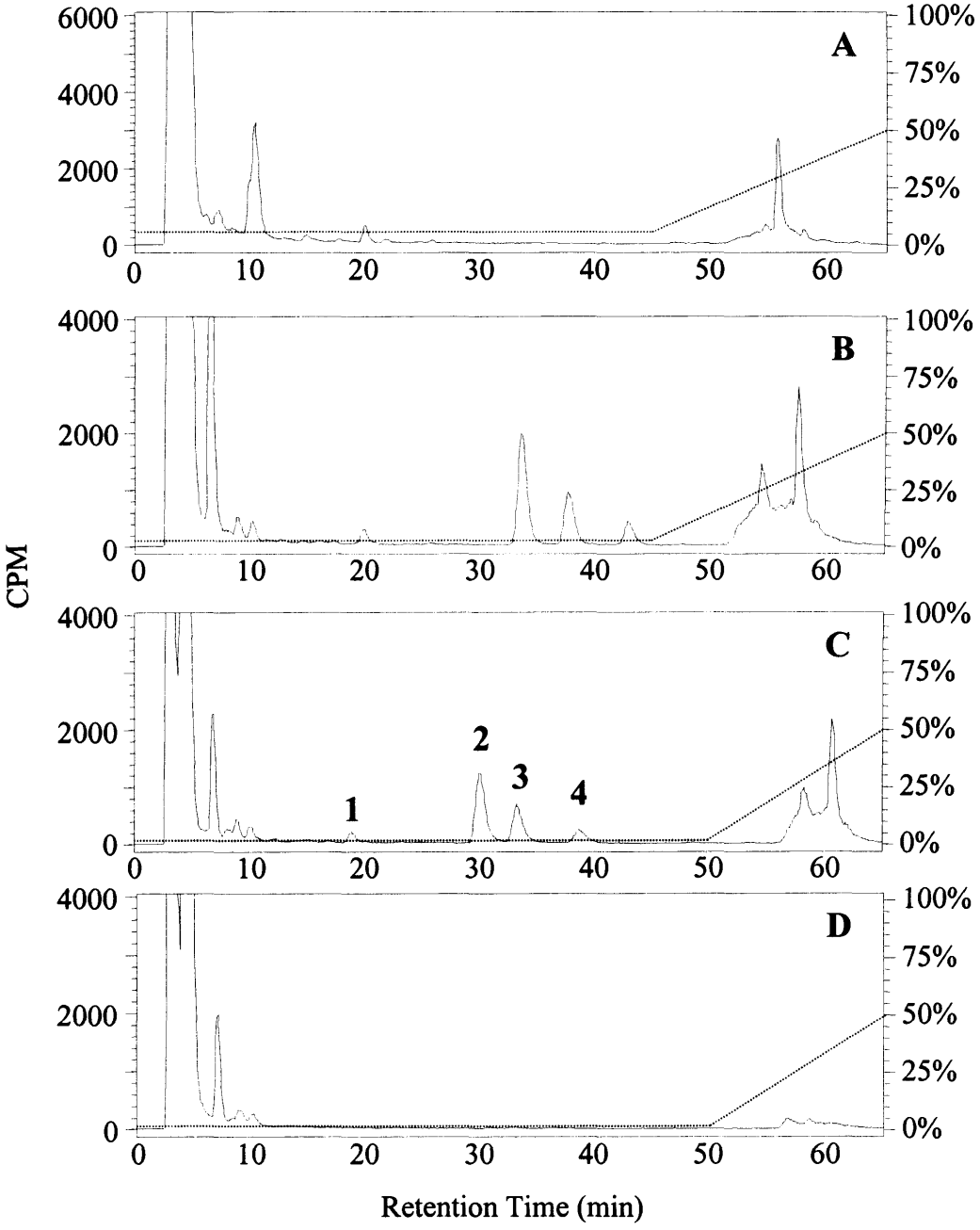


Figure 3.10: TLC map of 5 adduct spots from the separation of radiolabelled DNA after treatment with *p*-BQ (**A**). Radioactivity from each spot was eluted with 4.0 M pyridinium formate, pH 4.5 and mixed with radiolabelled *p*-BQ treated DNA. Co-chromatography was carried out on the HPLC system using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA). Traces **B-F** correspond to spots 1-5, respectively. Trace **G** shows the separation of adducts from radiolabelled DNA after treatment with *p*-BQ and is shown for comparison purposes. * indicates co-elution of peaks.

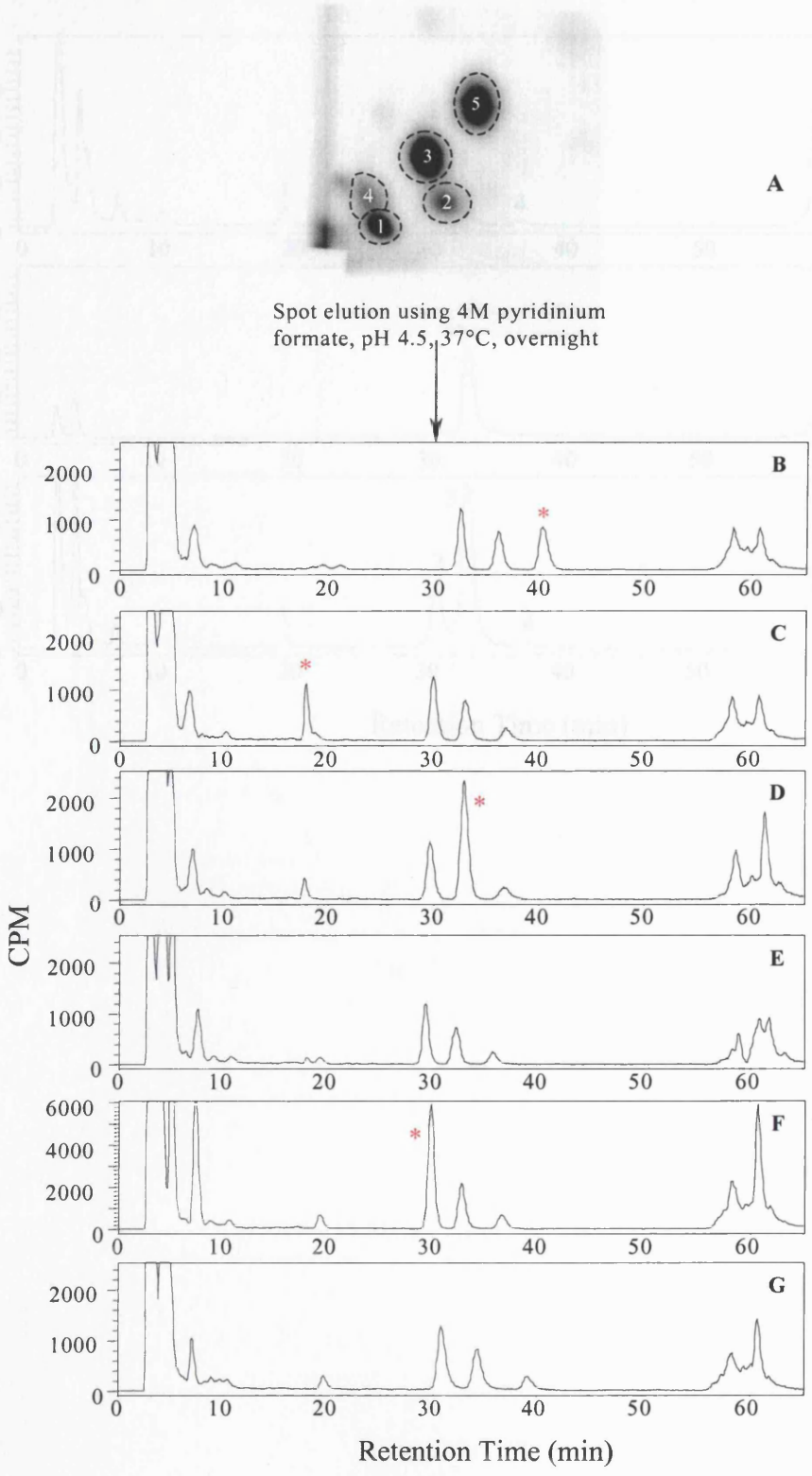


Figure 3.11: HPLC of ^{32}P-radiolabelled *p*-BQ treated DNA (**A**) with the four main peaks highlighted (numbered 1-4), the synthesised nucleotide adduct, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate (**B**) and co-elution of both samples (**C**) demonstrating that the synthesised adduct co-elutes with the peak identified as 3 in the treated DNA.
HPLC was carried out using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0

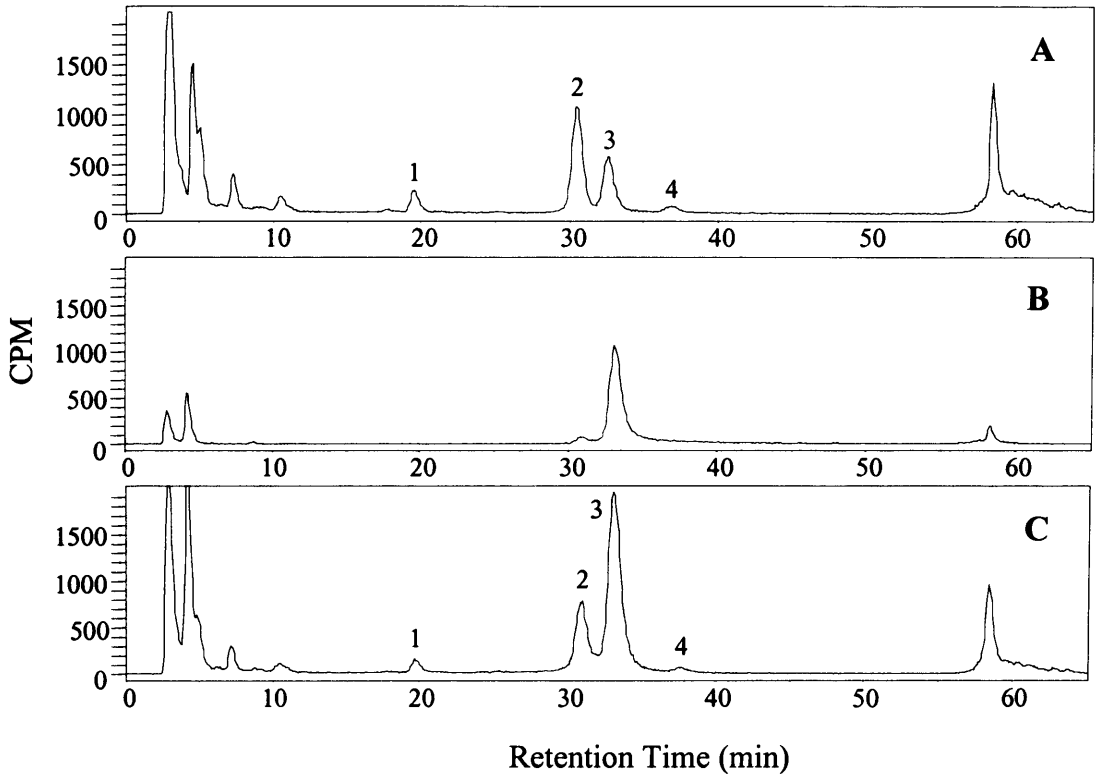
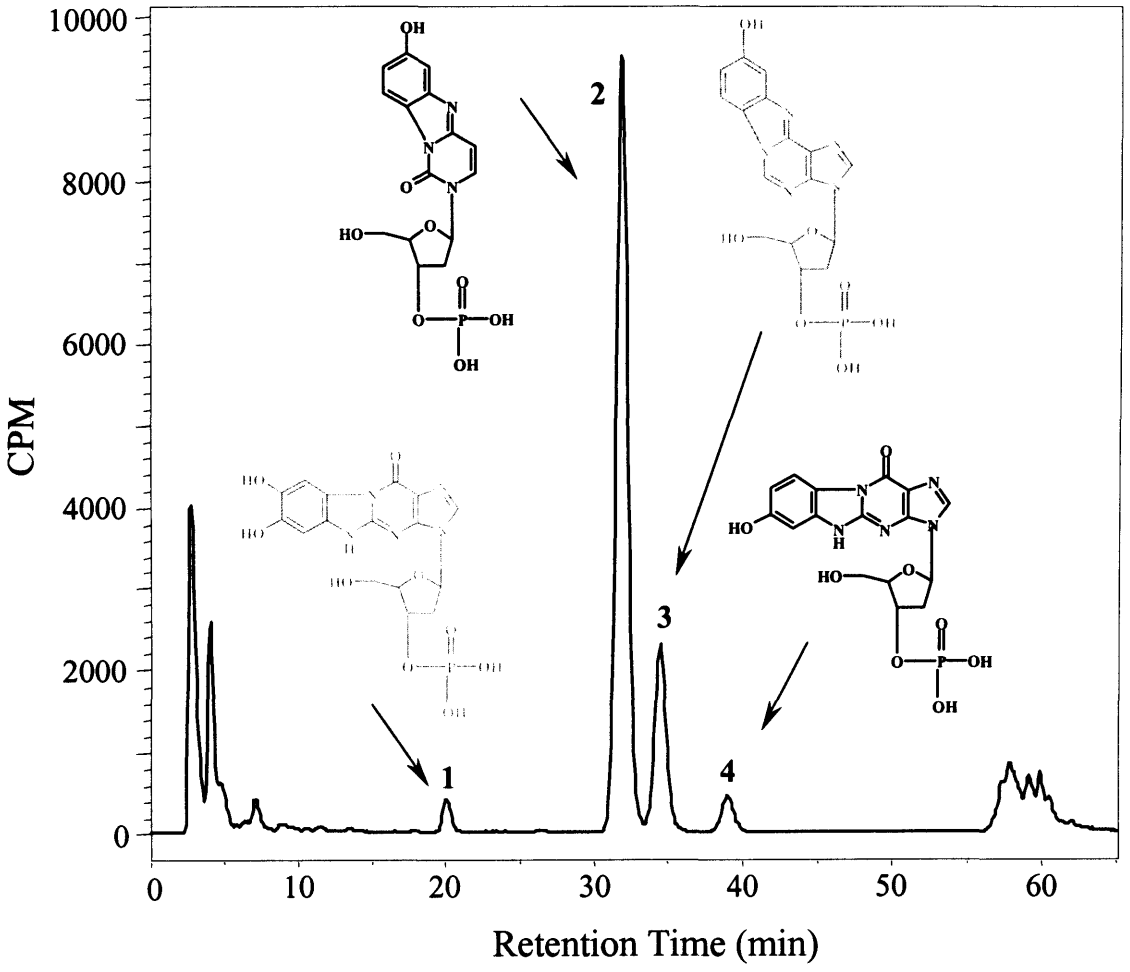


Figure 3.12: HPLC separation of ^{32}P-postlabelled *p*-BQ treated DNA with the four main peaks identified previously by co-chromatography as (3",4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (**peak 1**), (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate (**peak 2**), (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate (**peak 3**) and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (**peak 4**).
HPLC was carried out using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).



3.6.3. Comparison of TLC with HPLC

Reaction of calf thymus DNA with HQ and *p*-BQ with subsequent ^{32}P-postlabelling and analysis by TLC and HPLC gave a similar adduct recovery as shown in Table 3.1. The average total adduct number (n = 5) when the treated DNA was analysed by TLC was 24.64 ± 2.87 / 10^5 deoxynucleotides compared to 23.16 ± 1.07 / 10^5 deoxynucleotides when analysed by HPLC. The adduct ratio was 9.60 ± 0.78 %, 54.40 ± 3.87 %, 30.77 ± 2.38 % and 5.23 ± 0.76 % for TLC analysis compared to 6.93 ± 2.34 %, 64.24 ± 6.49 %, 25.67 ± 3.67 % and 3.16 ± 0.80 % for HPLC analysis for (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, respectively. The adduct ratios obtained for each method were similar, except for the ratios achieved for (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate which were shown to be significantly different ($p < 0.05$) as determined by Oneway ANOVA followed by Tukey's post hoc test.

Table 3.1: Average adduct number (n = 5) ± SD, obtained for each individual adduct when ^{32}P-postlabelled calf thymus DNA after treatment with HQ and *p*-BQ was analysed by TLC or HPLC. Adduct number is expressed as number / 10^5 deoxynucleotides. Values in brackets are percentage of total adduct number.
[a] is significantly different from [b] ($p < 0.05$) as determined by Oneway ANOVA followed by Tukey's post hoc test.

Adduct	Average adduct number from TLC (adducts / 10^5 deoxynucleotides) (n=5) ± SD	Average adduct number from HPLC (adducts / 10^5 deoxynucleotides) (n=5) ± SD
(3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate	2.38 ± 0.39	1.61 ± 0.54
(3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate	13.38 ± 1.56[a]	14.88 ± 1.54[b]
(3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate	7.60 ± 1.12	5.95 ± 0.83
(3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate	1.30 ± 0.26	0.73 ± 0.18
Total Adduct number ± SD	**24.64 ± 2.87**	**23.16 ± 1.07**

3.6.4. Optimisation of the DNA digestion and labelling procedure

For the TLC and HPLC method development described in sections 3.6.1 and 3.6.2, the digestion of DNA, adduct enhancement by nuclease P1 digestion and ^{32}P-postlabelling was carried out using the standard procedure employed in our laboratory, outlined in the Methods Section 3.5. When the analytical methods for the analysis of benzene-DNA adducts had been developed, the actual sample preparation procedure required some refinement to optimise adduct yield and allow for quantitative adduct analysis.

3.6.4.1. Optimisation of the DNA digestion procedure

The general procedure for the digestion of DNA to deoxynucleoside 3'-monophosphates is outlined in Section 3.5.2.1. To determine whether the standard procedure was adequate to give full digestion of the HQ and *p*-BQ treated DNA, the digestion enzymes were employed in varying concentrations ranging from 0.25 - 2 times the standard concentration. The data obtained from the ^{32}P-postlabelling of the resultant total, adducted deoxynucleotides, based on three determinations, can be seen in Figure 3.13. These results indicated that the standard enzyme concentration of 175 mU micrococcal nuclease and 3 mU calf spleen phosphodiesterase for 5 μg DNA, which was routinely used in our laboratory as detailed in section 3.5.2.1, gave the best adduct recovery (1 times normal concentration). Adduct recovery for the normal concentration of hydrolysis enzymes gave an adduct level which was significantly different from the other concentrations employed, as determined by oneway ANOVA followed by Tukey's post hoc test.

3.6.4.2. Optimisation of the adduct enhancement procedure

Previously, nuclease P1 has been employed for benzene DNA adduct enhancement (Pongracz *et al.*, 1990 and Reddy *et al.*, 1990) and as described by Reddy, 1993a and Reddy and Randerath, 1986 this is the most appropriate enrichment procedure for adducts of this size. To confirm that this method was the most suitable, both nuclease P1 and butanol enrichment were carried out and the samples were analysed alongside a sample which had undergone no adduct enrichment. The sample used was HQ and *p*-BQ treated calf thymus DNA and the resultant HPLC traces can be seen in Figure 3.14. Total adduct numbers were very similar comparing butanol extraction with

nuclease P1 digestion, however, when looking at individual adducts, large adduct losses were observed when comparing butanol extraction with nuclease P1 digestion, with losses in the region of 65 %, 94 % and 80 % for (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, respectively. A 70 % increase in adduct number was observed for (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, a result which may be due to this adducts sensitivity towards nuclease P1 (see below for further details). Without adduct enhancement no adduct peaks were observed, as shown in Figure 3.14, C.

The optimum enrichment procedure was therefore deemed to be nuclease P1 digestion, which gave the highest overall adduct recovery. Nuclease P1 digestion successfully removed greater than 99 % (n=5) of unmodified deoxynucleotides as determined by comparison of one directional TLC of samples which had undergone nuclease P1 digestion with samples that had received no such enrichment (data not shown). Other laboratories utilising nuclease P1 digestion have reported greater than 97 % removal of unmodified deoxynucleotides when analysing safrole DNA adducts (Reddy and Randerath, 1986).

Nuclease P1 digestion of the individually synthesised adducts (Chapter 2) for 1 h followed by ^{32}P-postlabelling resulted in complete loss of (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate and (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate. The two 2'-deoxyguanosine 3'-monophosphate adducts appeared resistant to nuclease P1 digestion (results not shown). Analysis of the HQ and *p*-BQ treated calf thymus DNA following nuclease P1 digestion for varying lengths of time demonstrated that adduct losses were also occurring with this sample (Figure 3.15). Again, loss of (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate and (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate predominated, with losses correlating with the increasing incubation time. After a 1 h incubation with nuclease P1 only 13.5 % and 45.6 % remained of these adducts respectively, with this decreasing

to 2.7 % and 14.0 % after a 2 h incubation (values calculated on the assumption that the adduct level after a 15 min nuclease P1 digestion reflected 100 % adduct recovery).

The two 2'-deoxyguanosine 3'-monophosphate adducts were stable in the presence of nuclease P1 for up to 1 h. After this time, as shown in Figure 3.15, the adduct yield started to decline.

As previously shown in Figure 3.14, C, lack of adduct enhancement resulted in no observable adducts, therefore some adduct enrichment procedure was required and as previously stated, nuclease P1 proved to be the best method. Reduction of the nuclease P1 digestion to 15 min gave the best adduct recovery.

3.6.4.3. Optimisation of the ^{32}P-labelling procedure

^{32}P labelling of samples (DNA treated with HQ and *p*-BQ) with high specific activity [γ-^{32}P]ATP (>5000 Ci/mmol); 12.5 μCi/1μg DNA, resulted in a 48 % ± 9 % (n=5) surplus of ATP. To analyse DNA adducts quantitatively a molar excess (30-80 %) of ATP is required (Gupta, 1993), therefore it was considered that the amount of ATP used here was adequate for this purpose.

Figure 3.13: Graphical representation showing the comparison of adducts recovered after DNA hydrolysis using differing concentrations of enzymes. Concentrations ranged from 0.25 – 2 times standard conditions (standard concentrations are 0.175 U micrococcal nuclease and 0.003 U calf spleen phosphodiesterase for 5µg DNA).
Data shown are mean ± SD (n=3), * indicates significant difference from *a* ($p < 0.05$), x indicates a significant difference from *b* ($p < 0.05$) and + indicates a significant difference from *c* ($p < 0.05$) as determined by oneway ANOVA followed by Tukey's post hoc test.

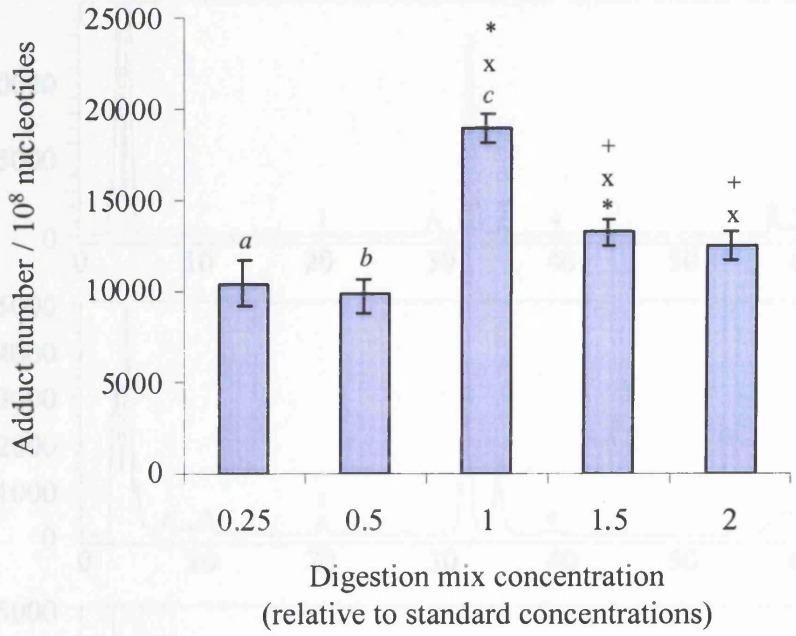


Figure 3.14: Typical HPLC traces showing the differences between adduct enrichment by butanol extraction (**A**), nuclease P1 digestion (**B**) and no enhancement (**C**). DNA analysed was previously treated with HQ and *p*-BQ and the DNA concentrations varied for each analysis; 0.5μg (**A**), 0.25μg (**B**), 2μg (**C**).
Peaks 1-4 represent (3",4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, respectively.

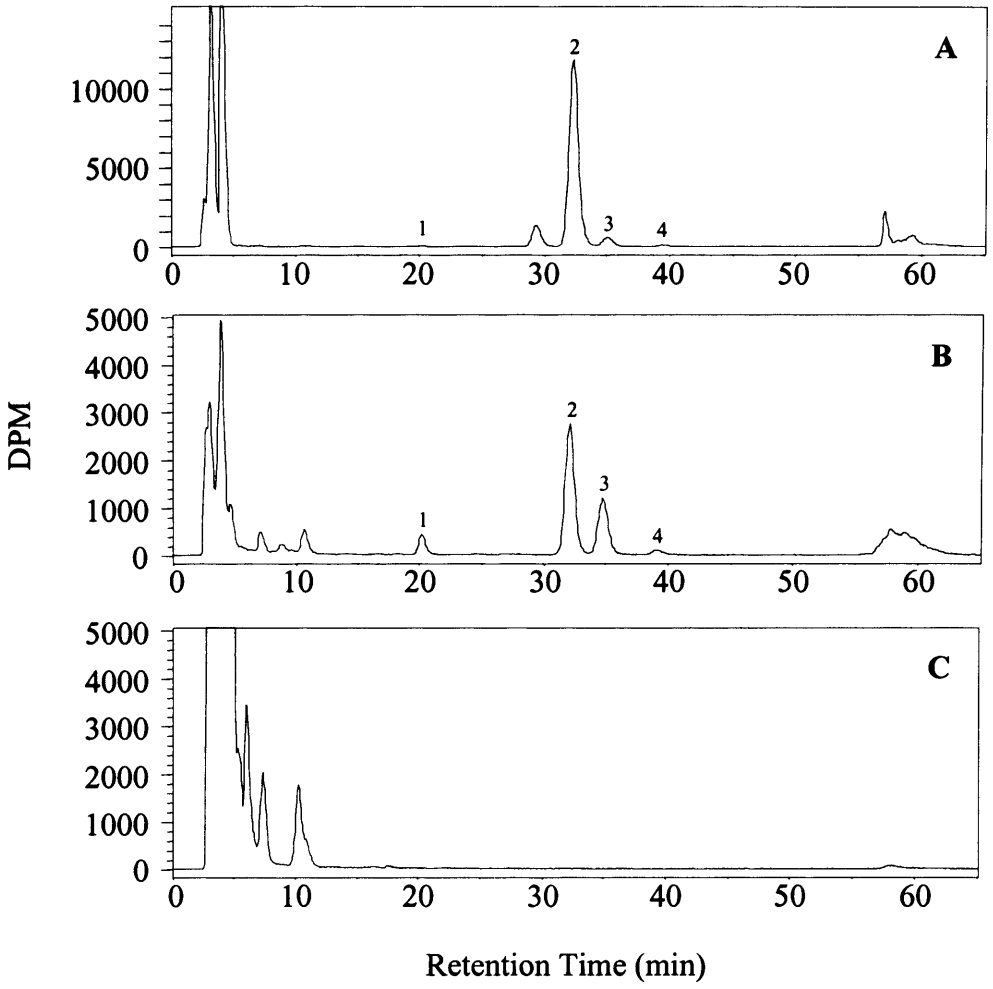
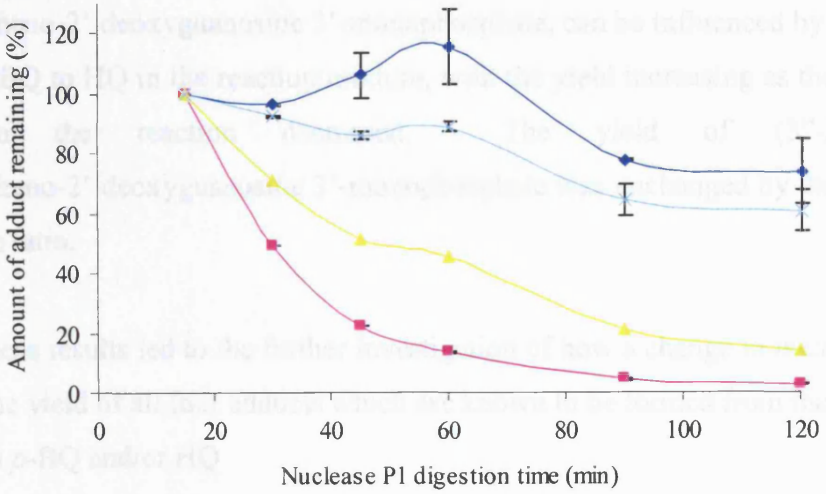


Figure 3.15: Graphical representation highlighting the stability of the adducts following nuclease P1 digestion for 15 -120 min as assessed by ^{32}P-postlabelling followed by HPLC analysis. Values are an average of 3 determinations and are all normalised for the adduct number achieved at the 15 min time point.
Key :
(3",4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate
(3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate
(3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate
(3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate



3.6.5. Effect of metabolite ratio on adduct synthesis

During the initial development of the ^{32}P-postlabelling procedure coupled to either TLC or HPLC, two reactions were carried out to provide a 'positive' control (a sample containing a high adduct number). These reactions involved the synthesis of benzene DNA adducts from the incubation of calf thymus DNA with *p*-BQ and/or HQ. The presence of HQ in the reaction appeared to influence the adduct yield, with a five fold increase in adduct number observed (data not shown). As previously described in Chapter 2, the yield of the benzene DNA adduct, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, can be influenced by altering the ratio of *p*-BQ to HQ in the reaction mixture, with the yield increasing as the amount of *p*-BQ in the reaction decreased. The yield of (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate was unchanged by this change in metabolite ratio.

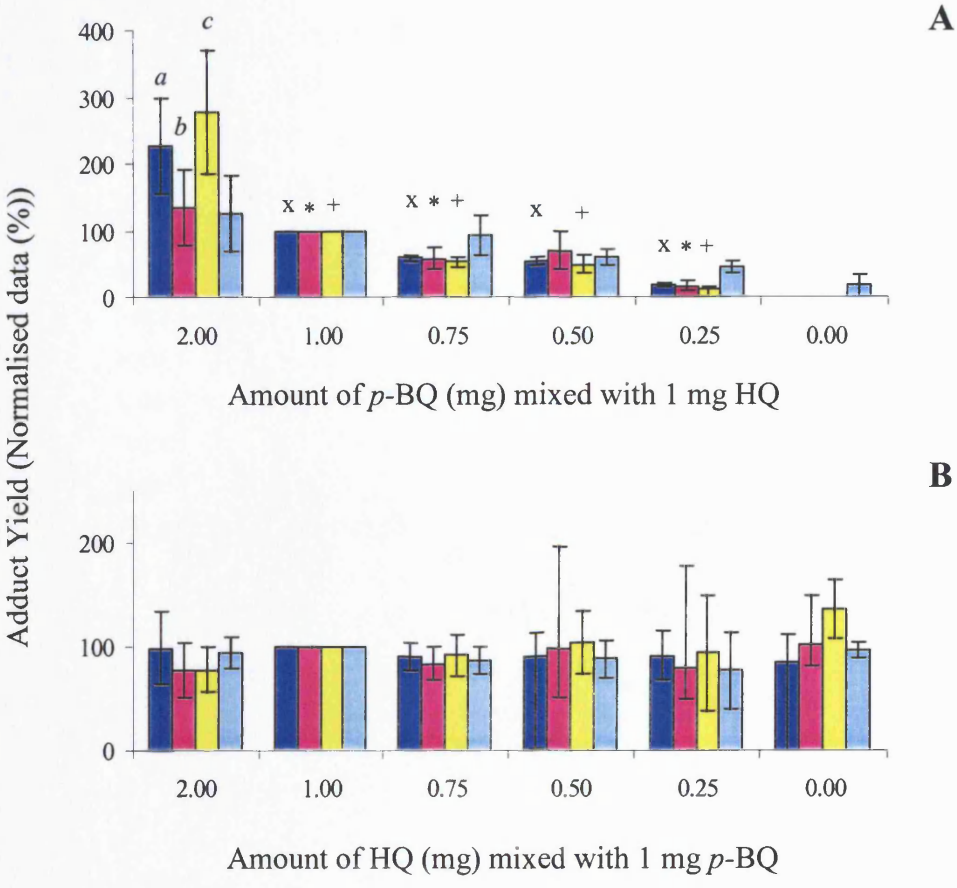
Both of these results led to the further investigation of how a change in metabolite ratio affected the yield of all four adducts which are known to be formed from the reaction of DNA with *p*-BQ and/or HQ

The results obtained are shown in Figure 3.16, A, indicating that as the amount of *p*-BQ in the reaction was increased (HQ remains constant) then the overall adduct yield also increased. On an individual basis, when *p*-BQ was present at twice the amount of HQ, the adduct number for (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate was significantly different from the yield achieved at the lower *p*-BQ concentrations. For the adduct, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, a significant difference was only observed when 0.75 mg *p*-BQ or less was used in the reaction. No significant difference was observed for any of the *p*-BQ concentrations for (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, an observation also determined when the adduct was investigated on an individual basis (see Chapter 2).

When the amount of *p*-BQ in the reaction was kept constant and the amount of HQ varied, no difference in adduct yields for any of the four adducts was observed (Figure

3.16, B). This result was similar to that previously observed when the two 2'-deoxyguanosine 3'-monophosphate adducts were investigated (Chapter 2).

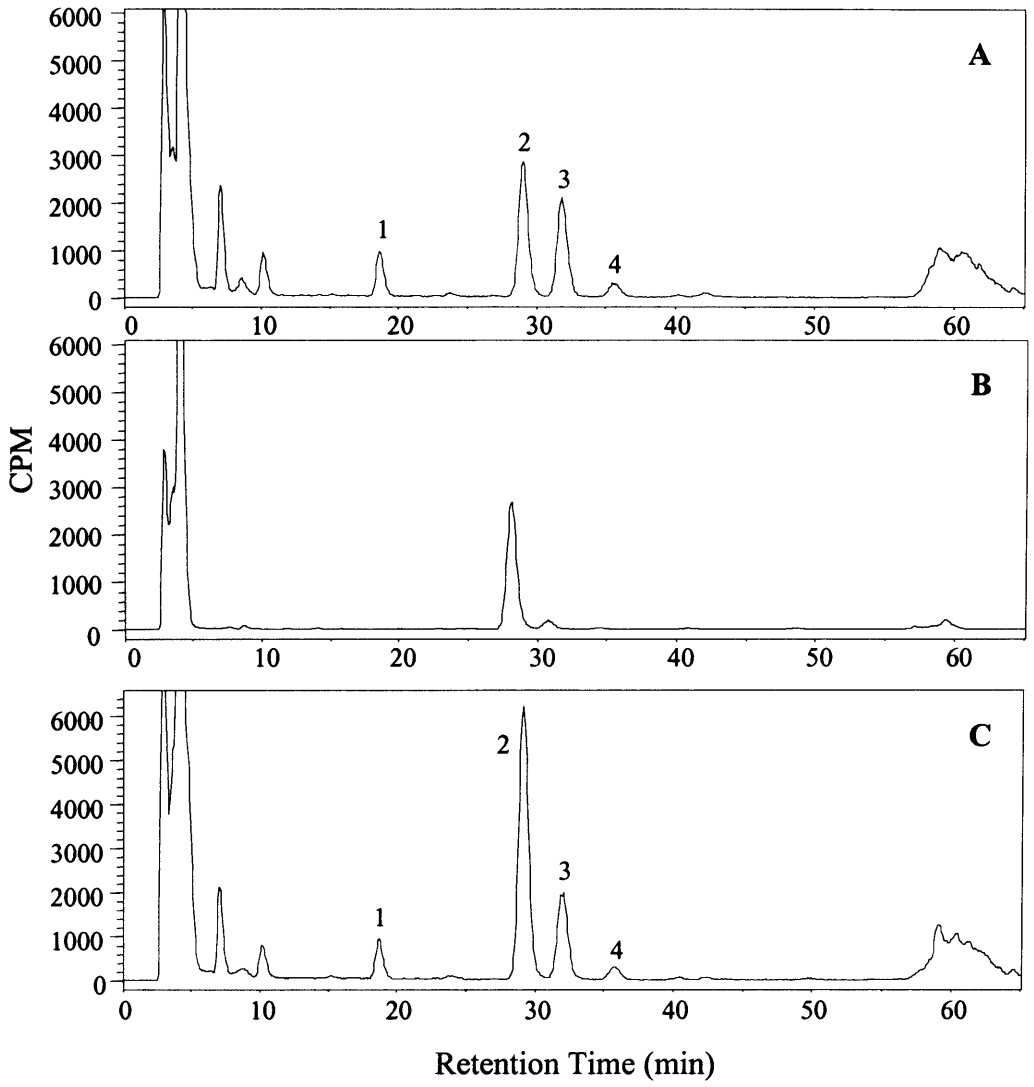
Figure 3.16: Comparison of adduct formation from the reaction of calf thymus DNA with varying amounts of the two metabolites hydroquinone (HQ) and *p*-benzoquinone (*p*-BQ). Analysis of samples was carried out using ^{32}P-postlabelling coupled to HPLC with an online radiochemical detector.
Graphical representation of the adduct pattern observed when the amount of *p*-BQ in the reaction is varied and the amount of HQ is constant (**A**) and that observed when the amount of HQ is varied and *p*-BQ is kept constant (**B**).
Data shown are mean ± SD (n = 3), x indicates significant difference from *a* ($p < 0.05$), * indicates a significant difference from *b* ($p < 0.05$) and + indicates a significant difference from *c* ($p < 0.05$) as determined by oneway ANOVA followed by Tukey's post hoc test.
Key:
(3",4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate
(3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate
(3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate
(3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate



3.6.6. Reaction of thymidine 3'-monophosphate with HQ and *p*-BQ

Reaction of Tp with HQ and *p*-BQ gave one significant product as discussed in Chapter 2, section 2.6.4. ^{32}P-postlabelling and HPLC analysis of this product using the method developed for benzene-DNA adducts described in section 3.5.2, gave a radiolabelled peak that eluted at approximately 27 min (Figure 3.17, B). Co-chromatography of this sample with radiolabelled calf thymus DNA treated with HQ and *p*-BQ showed co-elution with the adduct peak previously identified as representing (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate (Figure 3.17, C). MS analysis of this sample however indicated that this product was not a true Tp related adduct and was in fact quinhydrone, a polymerisation product formed from the reaction of HQ with *p*-BQ.

Figure 3.17: Typical HPLC chromatograms of HQ and *p*-BQ treated DNA (**A**), the main product from the reaction of Tp with HQ and *p*-BQ (**B**) and co-elution of HQ and *p*-BQ treated DNA with the main product from the reaction of Tp with HQ and *p*-BQ (**C**).
HPLC was carried out using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).



3.6.7. Reaction with benzene oxide

Benzene oxide is considered to be a major electrophilic metabolite of benzene and hence highly reactive. Reaction of benzene oxide with dGp or directly with DNA, as described in Chapter 2, resulted in no observable peaks following ^{32}P-postlabelling and analysis by HPLC when compared to control samples. Treated DNA underwent three methods of analysis which included no adduct enhancement or both adduct enrichment procedures (nuclease P1 digestion or butanol extraction) to confirm that no adducts were observable (results not shown). The lack of adduct production has been discussed in Chapter 2, section 2.6.10.

3.7. Discussion

The overall aim of this part of the work was to develop and optimise a ^{32}P-postlabelling method coupled to HPLC for the analysis of benzene-DNA adducts, which, coupled to immunoaffinity purification for adduct enrichment, could then be employed for the analysis of DNA samples obtained from individuals who had been exposed to benzene in an occupational setting (see Chapters 4 and 5 for further details). For the initial development and optimisation of the method as discussed in this chapter, adducts synthesised *in vitro* were utilised. The ^{32}P-postlabelling assay had been used previously by other groups (Pongracz *et al.*, 1990 and Reddy *et al.*, 1990) for the analysis of such adducts but the procedure employed by both groups used the standard ^{32}P-postlabelling assay coupled to TLC for adduct visualisation and quantification.

HPLC was the preferred method used here for adduct analysis since TLC has a number of disadvantages. TLC can often be quite subjective with adducts having the potential of being lost from TLC plates (polar adducts) or remaining at, or close to the origin (bulky adducts). Development of TLC conditions to retain the more polar adducts would normally affect the resolution of adduct spots with several adducts potentially eluting together (Moller *et al.*, 1993), although this would probably not be a factor here. HPLC gives better resolution of adducts as gradient elution can be employed. TLC also suffers problems in regard to reproducibility and is more time consuming compared to HPLC analysis. The main drawback of HPLC however, is the loss of sensitivity when compared to TLC, a problem highlighted in Chapter 5.

Quite interestingly, Pongracz *et al.*, 1990 and Reddy *et al.*, 1990 both came to different conclusions regarding the synthesis of benzene-DNA adducts *in vitro,* based on their observations from TLC analysis, which highlights the issues regarding adduct losses etc when using multi-directional TLC for adduct separation. Reddy *et al.*, 1990, concluded that from the reaction of DNA with the benzene metabolite, *p*-BQ (9 mM), one major and several minor adducts are formed, with a total adduct number of 1.6 adducts / 10^5 deoxynucleotides, the major adduct they concluded as being on dGp. Pongracz *et al.*, 1990, however, observed 3 major and several minor adducts from a similar reaction (4.6 mM *p*-BQ), the major adduct they concluded was (3"-hydroxy)-3, N^4-benzetheno-2-deoxycytidine 3'-monophosphate and the total adduct number was 3.1

adducts / 10^5 deoxynucleotides, almost double the number observed by Reddy *et al*, 1990 and they concluded that the dGp adduct was in fact a minor product of the reaction.

The published TLC method employed by Pongracz *et al.*, 1990, was used here initially, to confirm that benzene-DNA adducts had been formed in the calf thymus DNA reaction. There was no intention at this stage to utilise TLC in the analysis of benzene-DNA adducts. When using this method, adduct separation was found to be sub-optimal with the discovery that the adducts were migrating in the sodium phosphate buffers. These buffers are usually employed to remove excess ATP, inorganic phosphate and any labelled unmodified deoxynucleotides, whilst the adducts remain at or close to the origin. These buffers were adapted for successful separation of adducts from *p*-BQ treated DNA, with four major and several minor adduct spots clearly resolved. The total adduct number reported was 4.6 ± 0.3 / 10^5 deoxynucleotides, findings which were similar to that observed by Pongracz *et al.*, 1990.

Reaction of DNA with both *p*-BQ and HQ, resulted in an approximate 5-fold increase in adduct yield. Adduct recovery was on average 24.6 ± 2.9/ 10^5 deoxynucleotides, compared to 4.6 ± 0.3 / 10^5 deoxynucleotides as previously seen when the reaction was carried out with *p*-BQ alone. For a similar reaction involving both metabolites, Pongracz *et al.*, 1990, only observed an adduct yield of 5.5 / 10^5 deoxynucleotides, less than double the yield observed when DNA was treated with *p*-BQ alone, and approximately 4.5 times less than the yield observed here. This result may imply that the method employed in the laboratory of Pongracz *et al.*, 1990 whilst suitable for low adduct numbers results in adduct losses at higher yields due to overloading of TLC plates. The new, improved TLC method developed here which involved only two chromatography steps retained the adducts more efficiently and cut down the processing time by approximately 1 day.

Chromatography and co-elution studies of the synthesised adducts (synthesis detailed in Chapter 2), confirmed that the major adduct observed here from the reaction of calf thymus DNA with *p*-BQ (and HQ) was (3"-hydroxy)-3, N^4-benzetheno-2-

deoxycytidine 3'-monophosphate, a result also reported by Pongracz *et al.*, 1990. The other major adducts were confirmed as being (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and the novel adduct not previously identified, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (see Figure 3.6 for further information).

Following confirmation from the TLC data, that *in vitro* benzene DNA adducts had been successfully prepared, a HPLC method for their separation was developed, based on the method previously described by Moller *et al.*, 1993. This method employed a high molarity ammonium formate buffer to act as the aqueous solvent. After many attempts at adduct separation using differing concentrations of the aqueous buffer, different organic solvents and gradients, it was found that an isocratic system of acetonitrile (2 %) in ammonium formate (2.0 M, pH 4.0 containing 0.27 mM EDTA) was the most successful at separating the 4 products from the DNA reaction. These four products were identified by co-elution studies to correspond to (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, a result which was similar to that seen by TLC analysis.

Analysis of the adducts from HQ and *p*-BQ treated calf thymus DNA by the newly developed TLC or HPLC conditions gave comparable adduct numbers. The ratio of the recovered adducts however did vary, with the major adduct, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, accounting for 54.40 ± 3.87 % of the adducts when analysed by TLC and 64.24 ± 6.49 % when analysed by HPLC, these ratios were shown to be significantly different as determined by oneway ANOVA followed by Tukey's post hoc test. This difference could be due to an underestimation of the adduct numbers when analysed by TLC due to the saturation of the phosphorimager screen utilised for adduct analysis.

The ^{32}P-postlabelling procedure involved a number of steps which included the digestion of DNA to its deoxynucleotide components, adduct enhancement and the radiolabelling procedure itself, all of which can play a role in the underestimation of adduct number. Each step was taken in turn and optimised for adduct recovery.

DNA digestion to its deoxynucleotide components was found to be optimal using the normal conditions employed for ^{32}P-postlabelling (Figure 3.13). An increase in enzyme concentration actually reduced the numbers of adducts recovered, an observation seen by other groups when hydrolysis has been carried out for long periods (Phillips and Castegnaro, 1999). At two times the normal concentration, digestion would be expected to give the same nucleotide recovery when carried out for 7.5 h as digestion with one times the standard concentration, carried out for 15 h. Incubation for an extended period was therefore causing depletion in the actual enzyme substrates and hence the adducted deoxynucleotides could be acting as a surrogate substrate.

Nuclease P1 digestion was employed as the enrichment method of choice as this method is substantially more effective for the enrichment of small aromatic and bulky non-aromatic adducts when compared to other enrichment procedures i.e. butanol extraction (Reddy, 1993a). Comparison of both nuclease P1 digestion and butanol extraction gave 23.1 and 28.3 adducts / 10^5 deoxynucleotides respectively, but on an individual adduct basis, the recovery was extremely poor for butanol extraction. Adduct losses ranged from 65-94 %, when compared to the yields achieved for nuclease P1 digestion, with the greatest loss occurring for (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Using butanol extraction, the adduct, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate accounted for 85 % of the adducts recovered, compared to 61 % when nuclease P1 was employed, with adduct numbers ranging from 24.1 and 14.1 adducts / 10^5 deoxynucleotides, respectively. Nuclease P1 was therefore considered the most efficient adduct enhancement procedure, although the major adduct, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, may be underestimated, which required further investigation.

Incubation of the individually synthesised adducts with nuclease P1 for the normal time of 1 h, resulted in total loss of the two adducts, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate and (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate. Incubation of *p*-BQ and HQ treated DNA with nuclease P1 for varying lengths of time showed adduct losses for these two adducts after 15 min of incubation and losses of the two dGp adducts after incubations longer than 1 h. Total adduct numbers following a 15 min nuclease P1 digestion were now 47.5 adducts / 10^5 deoxynucleotides, approximately double that seen previously, when incubation was carried out for 1 h. From this study, it was apparent that the benzene-DNA adducts were, like the unmodified deoxynucleotides, substrates for nuclease P1 digestion which could explain why, for (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, a 70 % increase in yield was observed when butanol extraction was employed. Recovery of this adduct following digestion with nuclease P1 for 15 min exceeded the yield observed when butanol extraction was used.

The optimal conditions selected for benzene DNA adduct analysis, involved an initial digestion to its nucleotide components using, (for 5 μg DNA), micrococcal nuclease (175 mU) and calf spleen phosphodiesterase (3 mU) in SSCC (100 mM sodium succinate, 50 mM calcium chloride pH 6.0; 1 μL) for 15 h, at 37 °C followed by an incubation with nuclease P1 (9 μg; 2 μg/μL in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5.0) for 15 min. Actual ^{32}P-labelling was carried out with [γ-^{32}P] ATP (50 μCi; specific activity > 5000 Ci/mmol) in the presence of 3'-phosphatase free, T4 polynucleotide kinase (5 units) and labelling buffer (2 μL; 200 mM Tris HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM Spermidine, pH 7.6), for 1h at 37 °C. DNA adducts were analysed with 2D-TLC using sodium phosphate at two different concentrations; 0.4 M, pH 6.8 and 1.7 M, pH 6.0, or by HPLC using an isocratic system of 2 % acetonitrile in ammonium formate (2.0 M, pH 4.0 containing 0.27 mM EDTA).

To optimise the procedure fully and allow accurate adduct quantitation, internal standards should be employed, which will allow errors in sample preparation to be taken into account. The synthesised adducts, detailed in Chapter 2, could be employed for this purpose, but as the labelling efficiency for each individual adduct may vary, an

adduct standard would need to be employed for each adduct under analysis. At present, the adduct standards have not been accurately quantitated and therefore cannot be used for this purpose. Re-synthesis of the adducts perhaps using tritiated deoxynucleotide or metabolites could overcome this problem.

Radiolabelling of the main product from the Tp reaction gave a product which co-eluted with (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate when analysed by HPLC. Based on the data obtained from MS it was concluded that this product was quinhydrone, a polymerisation product from the reaction of HQ with *p*-BQ. Other workers have also observed the ^{32}P-labelling of hydroxyl groups on metabolites under the conditions employed (Randerath *et al.*, 1998) which is what may be occurring here, although attempts to produce quinhydrone independently and ^{32}P-postlabel the product has to date failed. This work requires further investigation to determine whether or not benzene adducts are formed on Tp.

Previously, as detailed in Chapter 2, adducts synthesised from the reaction of dGp with varying amounts of HQ and *p*-BQ were investigated. This study showed the increased production of (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate as levels of *p*-BQ in the reaction decreased in relation to HQ. The yield of the adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, remained unchanged by the decreasing ratio of *p*-BQ relative to HQ. This study was applied to the investigation of the adducts produced in the reaction of DNA with varying amounts of HQ and *p*-BQ. Adduct levels for (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate increased as the amount of *p*-BQ in the reaction increased in relation to HQ. If *p*-BQ was the major reactive metabolite then this would be expected. Previously, we speculated that the shift in equilibrium of the two metabolites favoured the formation of reactive oxygen species which resulted in the increased formation of the (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate adduct. Perhaps the difference in reaction pH, 7.0 as opposed to pH 6.0, or the presence of a more complex structure,

double stranded DNA as opposed to dNp's, which has many more sites for reaction could explain the difference in adduct production.

Adduct yield for (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate was unaffected by the changing metabolite ratios, a finding which was similar to that seen when the individual adduct was synthesised (see Chapter 2, section 2.6.9, for further details), this result may imply that a different reaction mechanism, compared to the other adducts may be involved. When the amount of HQ in the reaction was varied and the level of *p*-BQ remained the same, no overall differences in any of the adduct yields was observed, again a finding observed for the individual dGp adducts (Chapter 2).

This chapter describes the successful development of an optimised ^{32}P-postlabelling method coupled to either TLC or HPLC for the separation, identification and quantification of benzene DNA adducts formed *in vitro*. This method was applied to the analysis of DNA adducts from benzene exposed rodents and humans, as detailed in Chapter 5.

Chapter 4
Antibody Production

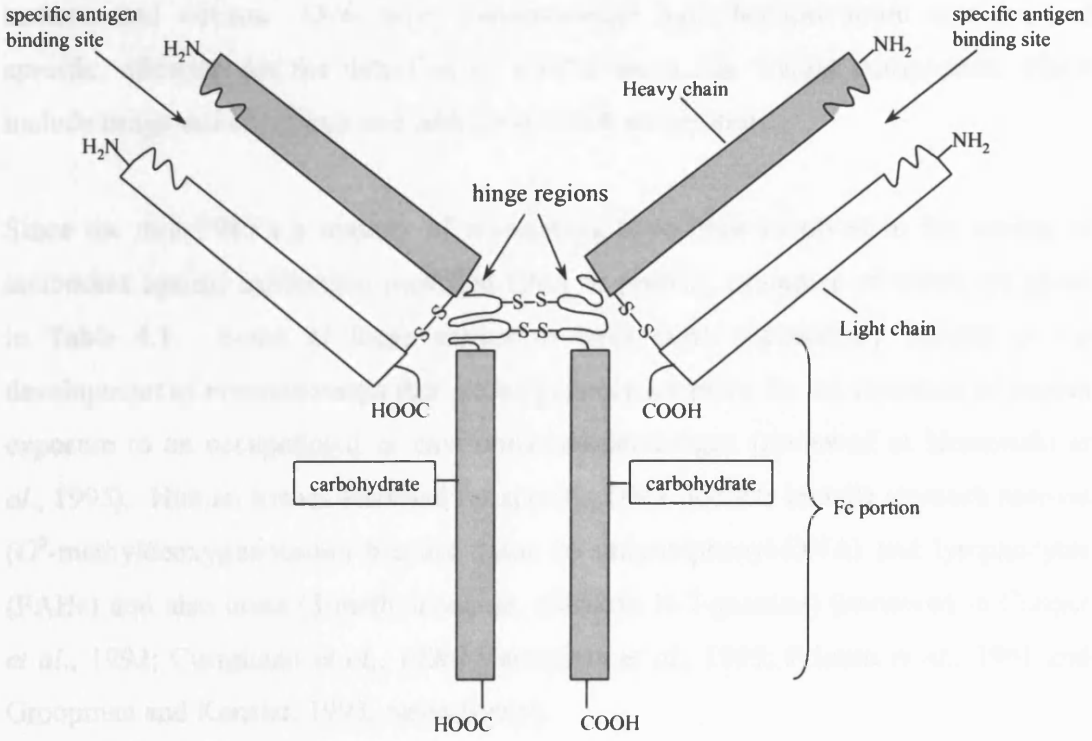
4.1. Background

Antibodies belong to the family of proteins known as immunoglobulins (Ig) and are produced in response to a molecule or an organism, which is recognised by the immune system as foreign to the host; such an agent is known as an antigen. Antibodies are either present on the surface membrane of B cells (a sub population of lymphocytes) or are secreted by these cells into the serum or lymph where they can bind to antigens. Antibody-antigen complexes are removed from the circulatory system mainly by macrophages through the process of phagacytosis.

There are five main classes of antibody which are known as IgG, IgM, IgA, IgD and IgE and each is composed of four interlinked polypeptide chains (heavy and light chains), as highlighted in Figure 4.1, which form a Y shaped unit. The difference between the antibody classes is determined by the amino acid sequences in the lower half of the two heavy chains (the Fc portion), this sequence being identical for all antibodies of the same class. The number of Y-like units that join to form the complete protein also differs between classes; for example, IgG antibodies have only one unit, whereas IgM contains 5 such units. Within antibody classes the amino acid sequence of the antigen binding site varies from one antibody to the next giving rise to many thousands of unique antibodies, each capable of combining with only one specific antigen or several antigens of a closely related structure.

During an immune response IgM is the primary antibody produced, the secondary response is carried out mostly by IgG, which is the most abundant antibody in serum, due to this abundance most immunoassays use IgG antibodies (Rosner *et al.*, 1991).

Figure 4.1: A typical antibody molecule. (adapted from Alberts *et al.*, 1989)



4.1.1. Antibodies in immunoassay development

Immunoassays are analytical methods, which detect an interaction between an antibody and an antigenic analyte, and have been used in biological monitoring since the mid-20th century (Rosner *et al.*, 1991). In the early days of immunoassay development, antibodies were used to detect the presence of large biological molecules, such as bacteria and viruses. Over time, immunoassays have become more sensitive and specific, allowing for the detection of smaller molecular weight compounds, which include drugs and chemicals and adducts of DNA and protein.

Since the mid 1980's a number of researchers have been involved in the raising of antibodies against carcinogen modified DNA or protein, examples of which are given in Table 4.1. Some of these antibodies have been successfully utilised in the development of immunoassays that are sufficiently sensitive for the detection of human exposure to an occupational or environmental carcinogen (reviewed in Hemminki *et al.*, 1995). Human tissues screened for specific DNA adducts include stomach mucosa (O^6-methyldeoxyguanosine) bladder tissue (4-aminobiphenyl-DNA) and lymphocytes (PAHs) and also urine (3-methyladenine, aflatoxin N-7-guanine) (reviewed in Cooper *et al.*, 1992; Curigliano *et al.*, 1996; Hemminki *et al.*, 1995; Friesen *et al.*, 1991 and Groopman and Kensler, 1993, respectively).

Table 4.1: Examples of some of the carcinogen modified DNA or protein adducts used for successful antibody production.

AdductType/Description	Reference
Benzo[*a*]pyrene modified guanosine and modified DNA	Santella *et al.*, 1984
The etheno dA and dC adducts formed after exposure to vinyl chloride or urethane	Guichard *et al.*, 1993 and Nair *et al.*, 1995
Malondialdehyde dG adduct	Sevilla *et al.*, 1997
The food mutagens, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,4,8-trimethylimidazo{4,5-*f*]quinoxaline (4,8-DiMeIQx). Antibodies raised can detect adducts in DNA.	Dragsted *et al.*, 1995
O^6-alkylguanine and O^4-alkylthymine adducts from exposure to *N*-nitroso compounds for example nitrosomethylurea which forms O^6-methyldeoxyguanosine adducts	Cooper *et al.*, 1992
aflatoxin-N-7-guanine	Reviewed in Hemminki *et al.*, 1995 and Groopman and Kensler, 1993
4-aminobiphenyl adducted DNA	Curigliano *et al.*, 1996
3-methyladenine formed after exposure to methylating carcinogens	Friesen *et al.*, 1991

4.1.2. Immunoassays

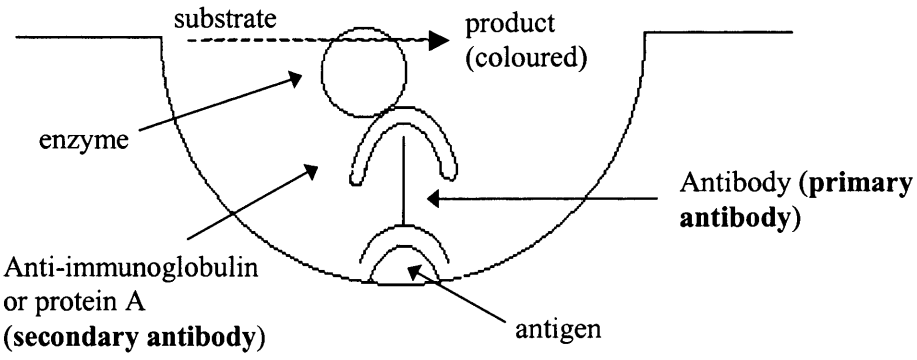
Antibodies raised against specific DNA or protein adducts can be used for analysis of samples in a number of different methods. Immunoaffinity or immunopurification chromatography is generally utilised as a clean-up step prior to analytical quantitation or determination i.e. via ^{32}P-postlabelling or mass spectrometry whereas immunoassays can directly quantitate adduct levels. Other techniques include immunohistochemistry, which involves immunofluorescence or immunoperoxidase analysis of tissue sections.

A number of immunoassays exist which include radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), ultrasensitive enzyme radioimmunoassay (USERIA), immunoslot blot and a new highly sensitive method, immuno-PCR. The basic principles of each immunoassay are similar, with each method reliant on the isolation and quantitation of an antigen-antibody complex detectable by a number of different methods, which include spectrophotometry, fluorometry, chemiluminescence or radioactivity (Poirier, 1991). In ELISA the antigen-antibody complex is bound by a secondary antibody conjugated to an enzyme. Upon incubation with a substrate the enzyme cleaves the substrate resulting in an observable colour change, the intensity of

which can be measured by spectrophotometry. In USERIA, the substrate is radiolabelled and upon enzymatic cleavage the product can be monitored by scintillation counting. A diagrammatical outline of the basic ELISA procedure can be seen in Figure 4.2. RIA relies on a radioactively labelled antigen for its sensitivity as the entire antigen-antibody complex is monitored by scintillation counting. The new procedure of immuno-PCR is similar to ELISA except the secondary antibody is conjugated to a DNA label, which is amplified by PCR (Case *et al.*, 1999). Immuno-slot blot involves the immobilization of adducted single stranded DNA to a nitrocellulose filter. Detection can be based on a colour change or detection of a radiolabelled antibody, as described for ELISA and RIA, respectively or chemiluminescence (Nehls *et al.*, 1984).

Figure 4.2: Representation of the enzyme-linked immunosorbent assay (ELISA)

Adapted from Goding, 1986.



In general, immunoassays are quantitative, highly sensitive and selective for the adduct of choice, albeit with some exceptions. For example, the possible lack of selectivity caused by cross reactivity of the antibody with adducts containing chemically related

moieties, which can give rise to a number of false results. An example of this is the antibody made against a benzo[*a*]pyrene DNA adduct (anti-BPDE-N^2-dG) which demonstrates a cross reactivity with other aromatic hydrocarbons of a similar chemical structure, which include chrysene and benz[*a*]anthracene (Booth *et al.*, 1994). This cross reactivity has also been seen as an advantage in certain situations particularly when an exposure to an unknown mixture of PAHs has occurred. The antibody has been used to determine adduct levels in the lymphocytes of coke-oven workers, foundry workers, roofers, firefighters and individuals living in a highly polluted area close to a coke oven plant (reviewed in Hemminki *et al.*, 1995). The antibody has also been utilised in an immunoaffinity step prior to ^{32}P-postlabelling to allow for detection of a range of DNA adducts formed by PAH compounds in mouse skin (Randerath *et al.*, 1998).

4.1.3. Antibody production

Antibodies can be raised to specific adducts of nucleosides, nucleotides, deoxynucleosides, deoxynucleotides or an adduct in an oligonucleotide. Upon injection into an animal, these adducts although capable of binding to a region of the antibody binding site would be unable to elicit an antibody response, mainly due to their small size. To overcome this problem and hence achieving a sufficient immunogenic stimulus, the adducted molecule would routinely be coupled to a larger carrier molecule, normally a protein, producing a synthetic antigen (Stollar, 1980).

The coupling procedure takes advantage of a number of reaction sites on the protein, which include the ε-amino group of lysine, sulphydryl groups, phenolic hydroxyl groups, the imidazole group of histidine and the α-amino groups (Poirier, 1991). The disadvantage of this coupling however, is that the antibodies produced may have a strong binding affinity for the adduct (hapten)-protein conjugate but relatively poor binding affinity for the adduct (hapten) itself, a problem reported by a number of investigators (Aston *et al.*, 2002; Rosner *et al.*, 1991 and Seedhouse *et al.*, 2001) or immunisation may give rise to anti-DNA antibodies (Poirier, 1991).

Another disadvantage may arise due to the nature of the original hapten, that is whether the adduct was on a single (deoxy)nucleoside or (deoxy)nucleotide or whether it was

incorporated into an oligonucleotide. A number of groups report that antibodies raised to an adduct in an oligonucleotide have no or a low specificity towards individual adducts and vice versa. For example, antibodies raised to α-acetoxytamoxifen modified DNA or BPDE modified DNA, are useful at detecting adducts in intact DNA but not at the individual adduct level (Divi *et al.*, 1999 and Santella *et al.*, 1984, respectively).

There are two main methods of producing antibodies, both of which involve immunisation of animals with the target antigen or hapten carrier molecule. The first method produces what is known as polyclonal antibodies, which are harvested from the sera of the animal. Polyclonal sera contains many different antibodies, however the antibody for the desired analyte will normally predominate. The main disadvantages of this method are that the quantity of sera is finite and may be difficult to reproduce on subsequent immunisations and the antibodies produced are less specific than monoclonal antibodies (discussed below).

The second method gives rise to monoclonal antibodies and is outlined in Figure 4.4 (reviewed by Goding,1986). This procedure is more involved and time consuming than polyclonal antibody production and involves the isolation of the B cells from the spleen of the immunised animal. The B cells, which are mortal are fused with immortal cells (normally an established myeloma cell line), resulting in hybrid cells (hybridomas). The hybridomas are isolated as individual cells and grown in cell culture medium, the monoclonal antibodies can then be harvested from the medium (Rosner *et al.*, 1991). This method as well as giving antibodies of a single, high specificity can be grown indefinitely in culture.

4.2. Aim

The main aim of this work was to raise an antibody to the DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate either as part of a polyclonal sera or as a monoclonal antibody. This DNA adduct is considered by other researchers to be the only DNA adduct formed *in vivo* following exposure to benzene (Bodell *et al.*, 1996 and Pongracz and Bodell, 1996). To date however, detection of this adduct in human samples has failed even after using the sensitive method of ^{32}P-postlabelling with nuclease P1 adduct enhancement.

It was hypothesised that use of an antibody against N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate in an immunoaffinity clean up step prior to ^{32}P-postlabelling may improve both the sensitivity and selectivity of the assay allowing for specific adduct determination in human samples, perhaps even in subjects exposed to low levels of benzene. Immunoaffinity chromatography prior to postlabelling has been used successfully by other investigators (Guichard *et al.*, 1993 and Widlak *et al.*, 1996) and in one case (Guichard *et al.*, 1993), an improvement in adduct recovery up to 80 % was determined.

If the antibody production from either the polyclonal or monoclonal methods proved successful, the antibody would be used not only as an enhancement step in postlabelling but also in the development of an immunoassay. For example the immuno-slot blot assay, which has a high sample throughput and uses less DNA than ^{32}P-postlabelling, which would be advantageous when dealing with human samples where the amount of DNA can be limited.

4.3. Materials

2-Thioxanthine was obtained from Lancaster (Morecambe, Lancashire). Hydrobromic acid, bromine, sodium cyanoborohydride and sodium periodate were purchased from Aldrich (Gillingham, Kent). Thymidine, purine nucleoside phosphorylase, thymidine phosphorylase, ribose 1-phosphate, guanosine, ovalbumin, bovine serum albumin, *p*-hydroxyaniline, *o*-phenylenediamine dihydrochloride tablets, foetal calf serum (FCS), HAT medium, complete/incomplete Freunds adjuvant and anti-rabbit and anti-mouse IgG peroxidase conjugates were all purchased from Sigma (Poole, Dorset). RPMI 1640, Glutamax, penicillin and streptomycin were obtained from Invitrogen (Paisley, UK). NSO cells were purchased from the European Collection of Cell Cultures (ECACC; Centre for Applied Microbiology and Research, Salisbury, Wiltshire) and polyethylene glycol was obtained from Boehringer Mannheim (Lewes, East Sussex). Silica was purchased from Fluka (Gillingham, Kent) and ELISA plates (Nunc 96 well microtitre plates with maxisorp surface) were purchased from Life Technologies Ltd (Paisley, UK). Vivaspin 20 concentrators (MWCO 10,000) were obtained from Sartorius (Epsom, Surrey) and dialysis cassettes were purchased from Pierce (Tattenhall, Cheshire).

All other chemicals were of the highest grade possible and were purchased from either Fisher (Loughborough, Leicestershire) or Sigma (Poole, Dorset).

4.4. Instrumentation

Analysis of ELISA plates was carried out using an automatic plate reader (Labsystems Multiskan Plus utilising Genesis software; Labsystems Ltd, Basingstoke, Hampshire).

Mass spectra (MS) of the products from the stepwise antigen synthesis were recorded on an Autospec-UltimaQ mass spectrometer with an electrospray interface using the positive ionisation mode (Micromass, Manchester). Solutions were introduced by constant infusion using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Edenbridge, UK) and a Hamilton 1 mL gastight syringe pumped at a flow rate of 10 μL/minute. Samples were dissolved in 1:1 methanol/water (v/v). Data was generated by combination of all spectra. MS was carried out by Dr. G. Lord.

4.5. Methods

Antigen synthesis was carried out using the method of Girault *et al.*, (1996) by Ms Rebekah Jukes. Animal dosing was carried out either at Biomedical Services, University of Leicester or the Leicester Royal Infirmary facilities. Hybridoma production was carried out in the CMHT cell culture facility by Mrs Julie Chamberlain and the initial testing of animal blood for the presence of antibodies was carried out by Mr James Fisher.

4.5.1. Antigen synthesis

A schematic representation of the following reactions can be seen in Figure 4.3.

1. Synthesis of 2-bromo-6-hydroxypurine. Hydrobromic acid (48 % aq., 50 mL) and methanol (20 mL) were placed in a sealed conical flask and cooled on an ice bath. 2-Thioxanthine (5.02 g; 30 mmol) was added to the solution with constant stirring. Bromine (10 mL; 195 mmol) was added slowly to the reaction at a rate of 0.2 mL / min followed by further stirring for 4 h. The reaction mixture was filtered and the solid was washed twice with cold acetone, three times with water followed by a final wash with cold acetone. The solid was dried in a vacuum dessicator. To further purify, the solid was dissolved in water (40 mL) and enough sodium hydroxide (1 M) to aid dissolution. The solution was again filtered and acidified with hydrochloric acid (5 M). The resulting yellow precipitate was filtered and dried.

2. Synthesis of bromoinosine. 2-Bromo-6-hydroxypurine (**1**) (214 mg; 1 mmol) and ribose 1-phosphate (242 mg; 1 mmol) were incubated at 37 °C for 3-7 days in potassium phosphate buffer (100 mL, 20 mM, pH 7.4) with purine nucleoside phosphorylase (120 units). The reaction mixture was concentrated to dryness on silica (60 particle). Flash column chromatography (20 cm x 3 cm) was carried out using dichloromethane: methanol: triethylamine (80:15:5) with fraction collection (10-20 mL). Fractions were evaporated to dryness prior to molecular weight determination by MS to allow identification of the fraction containing the desired product, bromoinosine.

3. Synthesis of N^2-(4-hydroxyphenyl)-guanosine. Bromoinosine (**2**) (20 mg; 58 μmol) and *p*-hydroxyaniline (50 mg; 460 μmol) were mixed together and refluxed for 2

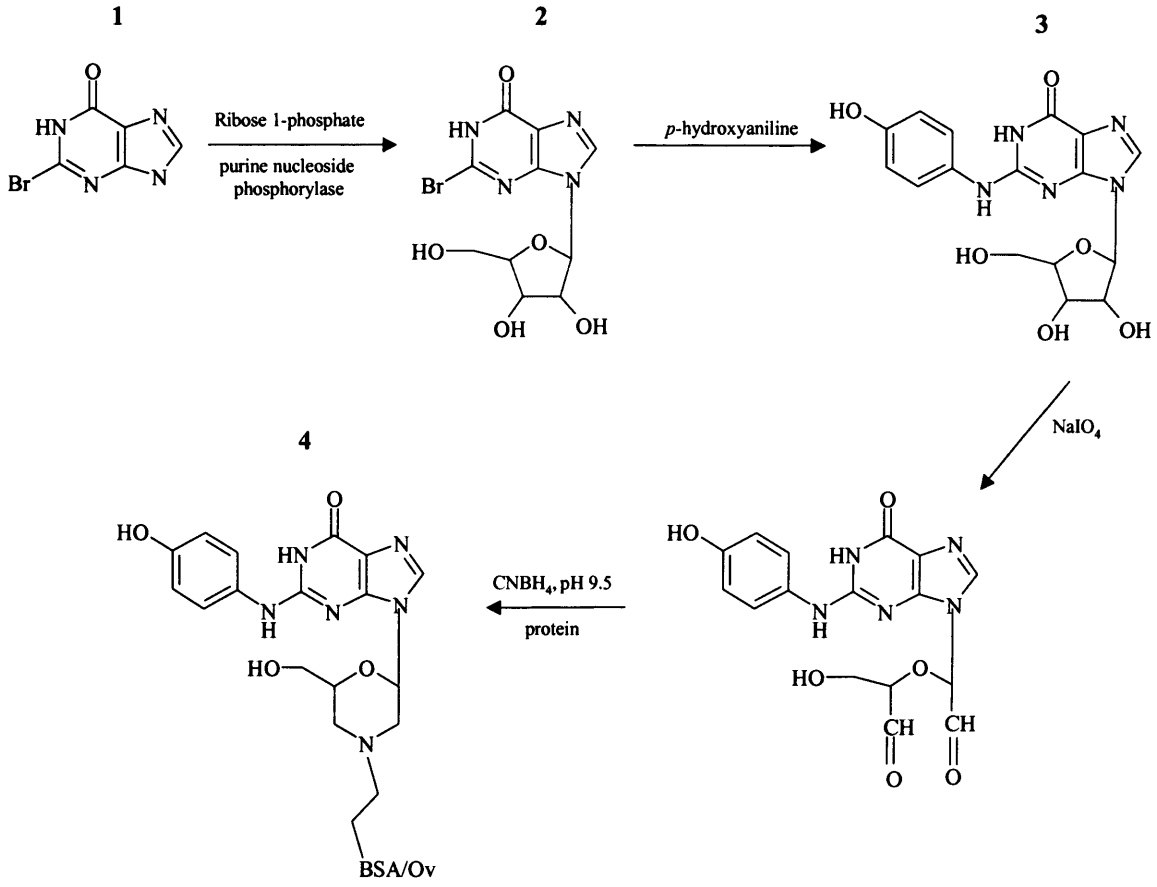
h in methoxyethanol:water (4:1; 5 mL). The reaction mixture was cooled to room temperature and centrifuged at 3000 rpm. The resultant pellet was washed with cold methanol and the sample was repelleted by centrifugation at 3000 rpm. Washing was repeated until the supernatant remained clear.

4. Synthesis of N^2-(4-hydroxyphenyl)-guanosine or guanosine coupled to protein. N^2-(4-hydroxyphenyl)-guanosine (**3**) or guanosine (10 mg in 0.5 mL water; 27 μmol and 35 μmol, respectively) underwent reaction with sodium periodate ($NaIO_4$; 21.4 mg in 0.5 mL water; 100 μmol) for 15 min at room temperature. Ethylene glycol (5 μL; 90 μmol) was added to stop the reaction.

The product from this reaction was added to either ovalbumin or bovine serum albumin (BSA) (10 mg in 0.5 mL water, pH 9.5, adjusted using sodium bicarbonate (Na_2CO_3; 0.2 M). The reaction was carried out for 45 min at room temperature with constant stirring (pH was maintained throughout at 9.5). Sodium cyanoborohydride (15 mg in 0.5 mL water; 366 μmol) was added to the solution and the reaction was carried out for 18 h at 4 °C. The product was dialysed against PBS for 18 h using MWCO 2000 dialysis cassettes. The resultant product was freeze dried. (Method originally described by Erlanger and Beiser, 1964 and a modified version by Stollar, 1980)

Figure 4.3 : Schematic representation for the synthesis of the antigen N^2-(4-hydroxyphenyl)-guanosine coupled to carrier protein

Numbers in bold refer to the synthesis steps described in the main text



4.5.2. Quantification of bound hapten

The amount of hapten bound to protein was determined by measuring the absorbance of a standard solution of the appropriate protein and the modified protein at 270 and 300 nm. Various amounts of N^2-(4-hydroxyphenyl)-guanosine were added to a known amount of protein (1 mg), and a calibration line was constructed from the absorbance values. The absorbance reading of the actual conjugated sample was read from the calibration line in order to determine the average number of residues per molecule of protein.

4.5.3. Polyclonal antibody production

Two New Zealand white (NZW) rabbits (1.5 – 2 kg) were initially inoculated subcutaneously (s.c.) with 200 μg antigen in 800 μL of 50:50 water: complete Freunds adjuvant (v/v) over 4 sites. Each animal was initially inoculated on day 0 and then boosted every 14 days thereafter for the first 3 boosts. Boosts were carried out using incomplete Freunds adjuvant. Timing of subsequent boosts varied as shown in Table 4.2. After the fourth boost, the dose was increased to 1000 μg of antigen in 800 μl of 50:50 water: incomplete Freunds adjuvant.

A pre-bleed was taken on day 0 and the first test bleed was taken 35 days after the initial inoculation. Subsequent test bleeds were taken at different time intervals as detailed in Table 4.2. Up to a maximum of 6 test bleeds were taken. Animals were culled and blood was removed. Blood was centrifuged and plasma removed and stored at –20 °C.

Table 4.2: Protocol for the production of polyclonal antibodies in New Zealand White Rabbits (Animals 775 and 776) detailing the dosing regime, taking of test bleeds and exsanguination of animals (** dose of antigen increased to 1000 μg)

Day Number	Boost Details	Test Bleed Taken?
0	initial inoculation	yes (T0)
14	1st boost	
28	2nd boost	
35		yes (T1)
42	3rd boost	
55		yes (T2)
62	4th boost	
77		yes (T3)
98	5th boost **	
112		yes (T4)
131	6th boost **	
159		yes (T5)
169	7th boost **	
181		Animal 776 exsanguinated
263	8th boost ** (775 only)	
270		yes (T6)
286		Animal 775 exsanguinated

4.5.4. Dosing of mice for monoclonal antibody production (method 1)

Three BALB/C mice (6-8 week old) were dosed (s.c.) with 50 μg antigen in 200 μL of 50:50 water:Freunds complete adjuvant over 4 sites.

Each animal was initially inoculated on day 0 and then boosted every 7 days up to 21 days after the initial boost and then every 14 days thereafter for a maximum of 9 boosts. Boosts were carried out using incomplete Freunds adjuvant. A pre-bleed was taken at day 0, and test bleeds were taken at days 21, 28 and then every 14 days thereafter. A maximum of 5 test bleeds were taken. Each animal was culled in turn, with removal of blood and spleen. The spleen was used in the hybridoma production assay as detailed in section 4.5.6.

4.5.5. Dosing of mice for monoclonal antibody production (method 2)

Three female, BALB/C mice (6-8 week old) were immunised subcutaneously in the flank with 25 μg antigen in 100 μL of diluted adjuvant (1:3; water: Titremax Gold adjuvant) at one site only. Animals were boosted 4 weeks later, with 10 μg antigen in 100 μL of diluted adjuvant at one site (i.p., flank). A second boost was given 15 days after the first boost at a dose of 20 μg antigen in 100 μl diluted adjuvant plus 1 μg choleratoxin. Test bleeds were taken 7 days after each boost. One month after the second boost all animals were reboosted as for boost 2 (choleratoxin excluded) and mouse two was selected for fusion. Four weeks later the remaining animals were reboosted and a second animal was selected for fusion.

4.5.6. Monoclonal antibody production

The procedure used for the production of monoclonal antibodies is outlined in Figure 4.4. and is based on a method reviewed by Goding, 1986. Each mouse was sacrificed and the spleen was immediately removed and transported to the cell culture facility in supplemented medium (5 mL; RPMI 1640 medium containing 2 % 1 M HEPES buffer, 1 % 200 mM glutamax, 1 % 0.1 M sodium pyruvate, 5000 i.u. (international unit) penicillin and 5000 μg streptavidin). The spleen was homogenised by forcing the tissue through a supplemented medium pre-washed, 125 μm mesh into a sterile beaker. Cells were washed with supplemented medium and counted with a haemacytometer.

Spleen cells were mixed with NSO cells, a mouse myeloma cell line, in a ratio of 4:1 (Spleen: NSO), and cells were centrifuged at 300 g. Medium was discarded and the cell pellet was placed in a waterbath at 41 °C. Pre-warmed polyethylene glycol (PEG) 1500 (800 µL), was slowly added to the cell pellet and cells were continuously mixed for 1 min. Supplemented medium was added slowly to the cell suspension, 1 mL/min for the first 2 min, 2 mL/min for 1 min and 3 mL/min for 2 min.

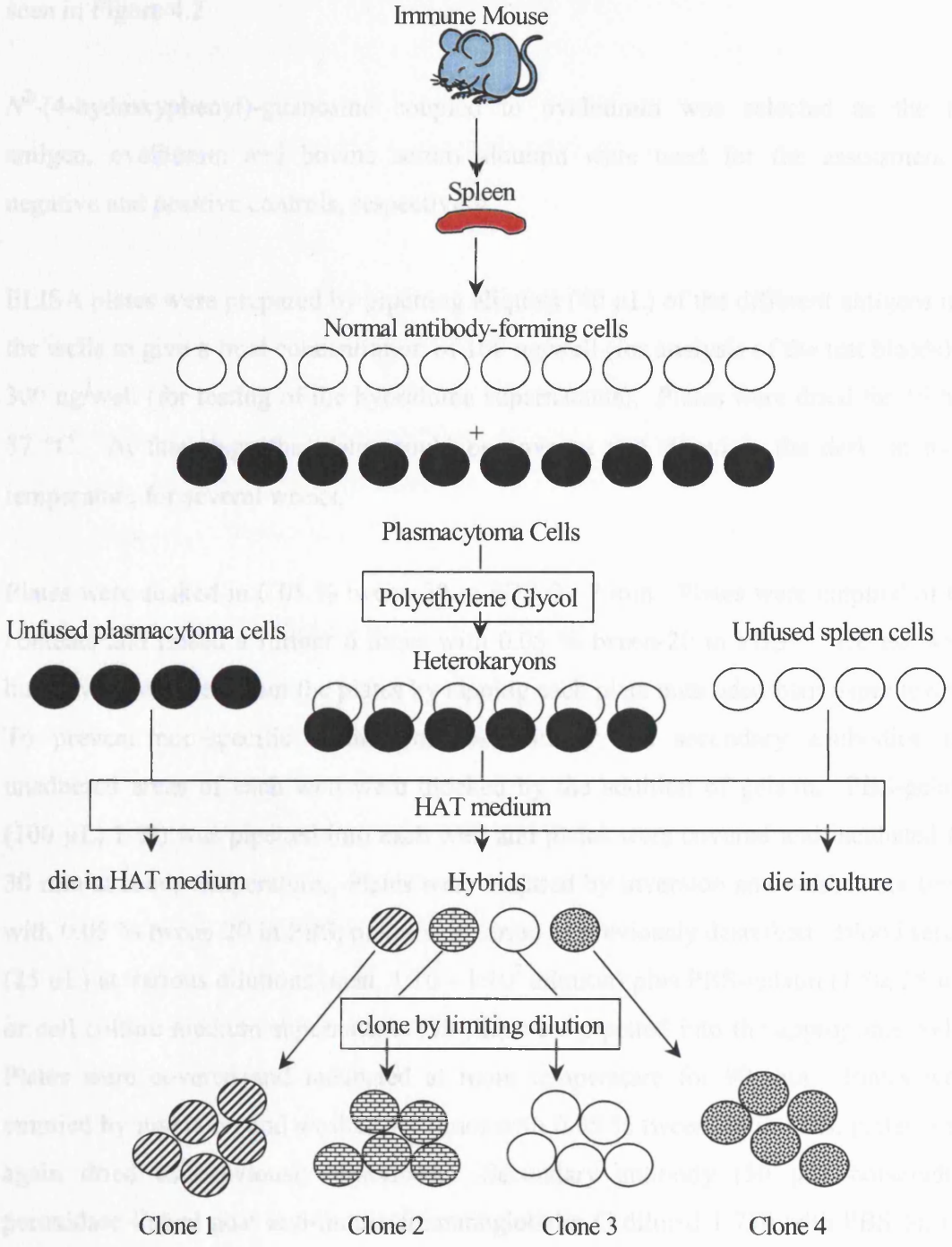
Cells were pelleted by centrifugation and resuspended at a cell density of 2 x 10^6 spleen cells/mL in supplemented medium, which now contained 15 % foetal calf serum (FCS). Cells were plated into 24 well plates, 1 mL of cell suspension/ well. Cells were incubated for 15 h at 37 °C, 5 % CO_2.

Following incubation a further 1 mL of supplemented medium plus 15 % FCS and 1x HAT (0.1 mM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine) was added to each well.

Cells were maintained with the fully supplemented medium (FCS plus HAT) until the wells were almost confluent. The medium was removed from each well for antibody testing using the ELISA procedure.

Cell aliquots which gave a positive response for the antibody were harvested and diluted to 10 cells /mL. Cells were plated into 96 well flat-bottomed plates at 100 µL/well i.e. 1 cell/well. Cells were incubated at 37 °C, 5 % CO_2 for approximately 6 days when wells were assessed for clone numbers. Supernatants were tested at around day 9. The supernantant from wells that gave a positive response for the antigen were again subjected to the cloning procedure as described above to ensure single clones.

Figure.4.4: Schematic representation for the production of monoclonal antibodies using the hybridoma technique (Figure adapted from Goding, 1986)



4.5.7. ELISA (enzyme -linked immunosorbent assay) procedure

4.5.7.1. Non-competitive ELISA

A diagrammatical representation of the enzyme-linked immunosorbent assay can be seen in Figure 4.2.

N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin was selected as the test antigen, ovalbumin and bovine serum albumin were used for the assessment of negative and positive controls, respectively.

ELISA plates were prepared by pipetting aliquots (40 μL) of the different antigens into the wells to give a final concentration of 100 ng/well (for analysis of the test bleeds) or 300 ng/well (for testing of the hybridoma supernatants). Plates were dried for 15 h at 37 °C. At this stage the plates could be covered and stored in the dark, at room temperature for several weeks.

Plates were soaked in 0.05 % tween-20 in PBS for 2 min. Plates were emptied of the contents and rinsed a further 6 times with 0.05 % tween-20 in PBS. Excess wash buffer was removed from the plates by tapping each plate onto adsorbent paper towels. To prevent non-specific binding of the primary and secondary antibodies any unadhered areas of each well were blocked by the addition of gelatin. PBS-gelatin (100 μL, 1 %) was pipetted into each well and plates were covered and incubated for 30 min at room temperature. Plates were emptied by inversion and washed six times with 0.05 % tween-20 in PBS, plates were dried as previously described. Blood serum (25 μL) at various dilutions (neat, 1:10 - $1:10^7$ dilution) plus PBS-gelatin (1 %; 25 μL) or cell culture medium supernatants (50 μL) were pipetted into the appropriate wells. Plates were covered and incubated at room temperature for 90 min. Plates were emptied by inversion and washed six times with 0.05 % tween-20 in PBS, plates were again dried as previously described. Secondary antibody (50 μL; horseradish peroxidase-linked goat anti-mouse immunoglobulin G diluted 1:750 with PBS for the mouse samples and horseradish peroxidase-linked goat anti-rabbit immunoglobulin G diluted 1:10,000 with PBS for the rabbit samples) was pipetted into each well, plates were covered and incubated for a further 90 min at room temperature. Plates were

emptied by inversion and washed six times with 0.05 % tween-20 in PBS followed by a final wash with distilled water. The enzyme substrate was prepared by dissolving *o*-phenylenediamine dihydrochloride (OPD) peroxidase substrate tablets in 20 mL distilled water. The enzyme substrate (200 μL) was pipetted into each well. Plates were covered with an adhesive plastic cover and wrapped in foil to protect from light. The plates were then placed on a shaking platform for 15 min to allow for colour development. HCl (50 μL; 1 M) was added to each well to stop the reaction and absorbance was monitored at 492nm using an automatic microplate reader.

4.5.7.2. Competitive ELISA

For a competitive ELISA, the procedure was identical to that previously described (section 4.5.7.1) with the following variations:

Known concentrations of the competing antigen were pipetted into wells prior to the addition of the primary antibody (only one dilution of primary antibody was used). In some cases pre-incubation of the primary antibody with the competing antigen was carried out for 1-15 h prior to addition of the sample to the ELISA plate.

4.5.8. Antibody identification

Antibody from the cloning assay was characterised using an ISO1 kit from Sigma according to the manufacturer's instructions.

An isotyping strip and hybridoma supernatant (3 mL) were placed into a test tube and incubated for 30 min at room temperature. Supernatant was removed and the isotyping strip was washed by addition of PBS-T-BSA (3 mL; phosphate buffered saline containing 0.05 % Tween 20 and 1 % BSA). Diluted biotinylated secondary antibody (1:50 dilution in PBS-T-BSA) was added to the tube and incubated for 30 min at room temperature. Secondary antibody was removed and the strip was washed with PBS-T-BSA. ExtrAvidin-peroxidase (diluted 1:50 with PBS-T-BSA) was added to the tube and the incubation was carried out for 15 min. ExtrAvidin-peroxidase was removed from the tube and the strip was washed with PBS-T-BSA followed by a wash with PBS. Substrate solution (acetate buffer, pH 5.0 (2.5 M), chromogen; 3-amino-9-ethyl-carbazole in N, N-dimethyl formamide (AEC-DMF) and hydrogen peroxide (2 %)) was added to the tube and incubation was carried out until an isotype

signal became evident. The strip was immediately immersed in NaOH (0.1 M; 2 min). The strip was washed in distilled water and dried.

4.5.9. Antibody concentration

4.5.9.1. Method 1: Ammonium sulphate precipitation

Hybridoma supernatant was centrifuged at 3000 rpm for 30 min. The supernatant was transferred to a beaker and an equal volume of saturated ammonium sulphate solution was added, with continuous stirring (4°C, 60 min). The sample was transferred to 50 mL tubes and centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in 0.25 volumes of the original starting volume. The sample was dialysed for 15 h against PBS. The PBS was changed twice and dialysis was continued for a further 24 h.

4.5.9.2. Method 2: vivaspin concentrators

Vivaspin 20 concentrators (MWCO 10,000) were loaded with hybridoma supernatant and centrifuged for 30 min at 3000 rpm or until the remaining volume was approximately ¼ the original starting volume.

4.5.10. Antibody purification

4.5.10.1. Method 1

Purification was carried out using a Protein A Purification Kit (Sigma) according to the manufacturer's instructions.

Filtered supernatant (0.45 μm filter) was mixed with binding buffer (proprietary formulation) at a concentration of 1 mL/10 mL supernatant. The Protein A cartridge was regenerated by passing regeneration buffer (5 mL; proprietary formulation) through the cartridge (1 mL/min). Binding buffer (4 mL; proprietary formulation) was passed through the protein A cartridge and the sample (previously mixed with binding buffer) was loaded onto the protein A column and eluted at a flow rate no greater than 0.5 mL/min. Once the sample had eluted, the Protein A cartridge was washed with binding buffer (6 mL, 1 mL/min) and the cartridge was attached to the desalting column (previously regenerated by washing with HEPES buffer (10 mL) at a flow rate of 1 mL/min). The antibody was eluted onto the second column using elution buffer (5

mL, 0.5 mL/min; proprietary formulation). Both cartridges were washed with phosphate buffered saline (PBS; 10 mL) containing 0.02 % sodium azide.

4.5.10.2. Method 2

Purification was carried out using the Affi-gel protein A MAPS II kit (Biorad) according to the manufacturer's instructions.

The supplied column was packed with affi-gel protein A (1 mL) and the column was equilibrated with binding buffer (5 mL; proprietary formulation). The sample (supernatant diluted with an equal volume of binding buffer) was applied to the column and the column was washed with binding buffer (15 mL). The antibody was eluted (elution buffer; 5 mL; proprietary formulation) into a collecting vial containing 1 M tris, pH 9.0 (1.6 mL). The column was regenerated by the addition of regeneration buffer (5 mL; proprietary formulation). (This method gave no product).

4.5.11. Antibody quantitation

The concentration of the purified antibody was determined using the following equation (Harlow and Lane, 1999):

1.25 Absorbance units at 280nm is equivalent to 1.0 mg/mL

4.6. Results

4.6.1. Antigen synthesis

The antigen N^2-(4-hydroxyphenyl)-guanosine coupled to BSA, was synthesised via ribosylation of 2-bromo-6-hydroxypurine followed by reaction with *p*-hydroxyaniline and subsequent conjugation to protein. The product from each reaction was confirmed by MS using continuous infusion, positive ion electrospray which resulted in molecule ions $[M+H]^+$ at *m/z* 347, 349 and 376. This data was consistent with products of molecular weight (MW) 346, 348 and 375 which corresponded to the products 2-bromoinosine (MW 346 (^{79}Br) and 348 (^{81}Br)) and N^2-(4-hydroxyphenyl)-guanosine (MW 375). Conjugation of the antigen to protein gave an average of 3.5 residues of N^2-(4-hydroxyphenyl)-guanosine per molecule of protein linked through lysine.

4.6.2. Polyclonal antibodies

Two New Zealand White rabbits (animals 775 and 776) were immunised and boosted up to 8 times with N^2-(4-hydroxyphenyl)-guanosine coupled to BSA (test antigen). The initial inoculation and the following 4 boosts involved injection of the animals with 200 μg of the test antigen. At appropriate time points after the initial inoculation and subsequent boosts, three test bleeds (T1-T3) were taken from each animal (see Table 4.2 in the methods section for more details). The sera was used in the enzyme-linked immunosorbent assay (ELISA) to assess for the presence of antibodies to BSA (the carrier protein used in immunisation), ovalbumin (the carrier protein for the antigen synthesised specifically for ELISA) and N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (the adduct of interest coupled to a carrier protein not used in the immunisation procedure). The results are shown in Figure 4.5. Each animal produced antibodies for the carrier protein BSA, with maximum yield being achieved by the first time point (T1). However, for these three time points, no antibody response was observed for either animal, for the antigen, N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin, as compared to the negative control, ovalbumin.

Further boosts with the test antigen were increased 5-fold, to 1000 μg. This increase in dose was reflected in the antibody titre observed for BSA at T4, as shown in Figure 4.5. Again, after this initial increase in dose the antibody response for BSA increased for the first time point and then plateaued for subsequent time points. An antibody response

was now observed for the test antigen, N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin, a response which increased at each time point. Animal 776 demonstrated a higher antibody titre than animal 775, therefore this animal was exsanguinated at day 181. Animal 776 received a further boost and was culled and bled at day 286.

Final animal bleeds were assayed for antibody selectivity and sensitivity using competitive ELISA. The compounds tested included, the metabolite HQ, the purine base guanine, the deoxynucleoside, deoxyguanosine (dG), the adducted base, N^2-(4-hydroxyphenyl)-guanine and the nucleoside, guanosine coupled to ovalbumin. The results for both animals were very similar as demonstrated in Figure 4.6. HQ, guanine, dG and N^2-(4-hydroxyphenyl)-guanosine showed no competition with N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (the coating antigen) whereas guanosine attached to ovalbumin did, a result observed for both animals. Incubation of the competing antigen with the primary antibody for 15 h prior to addition to the ELISA plate again resulted in no observable competition except for guanosine coupled to ovalbumin (results not shown).

Figure 4.5: Graphical representation of the polyclonal antibody production in the sera of 2 New Zealand White Rabbits (animal 775 (**A**) and animal 776 (**B**)) after inoculation and boosting with N^2-(4-hydroxyphenyl)-guanosine coupled to BSA. Sera was assessed by ELISA at 5 time points (T1-T5) for the presence of antibodies to BSA (■), ovalbumin (●) or N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (▲).
(All values were corrected for the T0 (background) reading, sera was diluted 1:10).

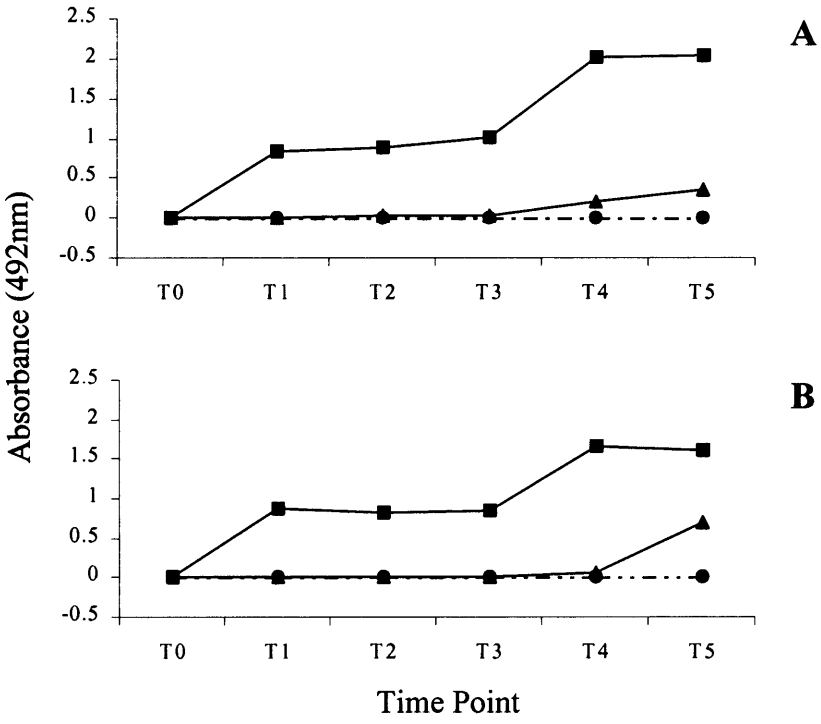
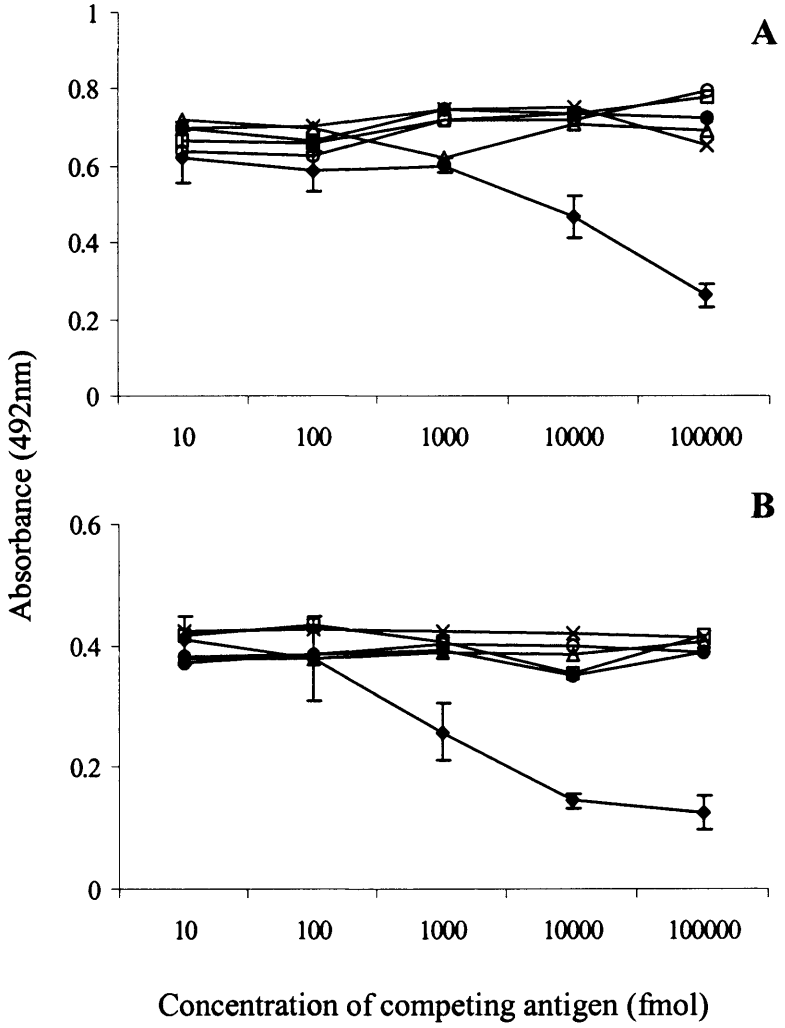


Figure 4.6: Graphical representation demonstrating the antibody response observed when competing agents were included in the ELISA. The coating antigen, N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (300 ng) was competed against HQ (x), guanine (o), dG (□), guanosine coupled to ovalbumin (◆), N^2-(4-hydroxyphenyl)-guanosine (△) and PBS (control,●) for the antibodies present in the final bleeds (1 in 10 dilution) of both animals 775 (**A**) and 776 (**B**).
(n=3 for guanosine coupled to ovalbumin, n=2 for all other competing antigens)



4.6.3. Monoclonal antibody production (method 1)

Using monoclonal production method 1 (see section 4.5.4), 3 mice were inoculated and boosted a maximum of 9 times with the antigen N^2-(4-hydroxyphenyl)-guanosine coupled to BSA. A test bleed was taken on day 0 (T0), prior to inoculation and a maximum of 5 test bleeds were taken thereafter (T1-T5). Sera from each time point were utilised in the ELISA assay, testing for an antibody response to N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin, the adduct of interest coupled to a different protein not utilised in the dosing programme. Unlike with the antibody production in the rabbits (section 4.6.2) an antibody response towards N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin, albeit weak, was observed after the initial inoculation and three subsequent boosts, at day 28 (T2). This response increased at each subsequent time point, although the general response for animal 2 was overall poor as shown in Figure 4.7, B. The antibody response recorded for each time point (T0-T5) is shown in Figure 4.7.

BSA, the carrier protein used in the dosing protocol, was utilised in the ELISA as a positive control (an antigen which would give a positive response and be indicative of a problem with the procedure). A strong response was observed for BSA at the first time point (T1, day 21), which increased with subsequent time points (T2-T5) as observed in Figure 4.7.

Ovalbumin, which is coupled to the adduct of interest and used as the test antigen in the ELISA (see above) was used alone to clarify that any response observed to N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin was directed towards the adduct and not the carrier protein. As deduced from Figure 4.7, ovalbumin elicited no antibody response.

4.6.3.1. Hybridoma production

Each animal in turn was sacrificed and each spleen was used in the hybridoma assay as detailed in section 4.5.6. All three mice proved successful in the growing of clones but only mouse 2, produced clones which elicited a positive antibody response for the adduct N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin. From 60 clones,

showing varying degrees of antibody response, 6 clones were selected for re-cloning to ensure a pure cell line. The supernatant from the resultant 18 clones all elicited a positive response for antibodies towards N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin and a negative response for antibodies towards BSA and guanosine coupled to ovalbumin as shown in Figure 4.8. The cells underwent a further round of purification, which resulted in the selection of one clone (sample 8, Figure 4.8) for a final re-cloning exercise. From this, 7 clones were selected and all 7 clones were isotyped and identified as IgG. One clone was selected for expansion and the remaining 6 were archived at -80°C.

Supernatant from the selected clone underwent concentration and purification using several different methods as outlined in sections 4.5.9 and 4.5.10. Use of the resultant purified antibody with and without prior concentration gave very similar results to the unconcentrated/unpurified cell culture supernatant when tested for antibody response to N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (results not shown).

Supernatant, which was concentrated to approximately ¼ the starting volume, when compared using ELISA to the unconcentrated sample, also gave a similar response, suggesting that the assay was saturated at the diluted concentration of antibody. Checkerboard ELISA (comparison of varying amounts of coating antigen with varying dilutions of antibody) was carried out to determine a coating antigen concentration and a primary antibody dilution to be utilised in competition ELISA, the results of which can be seen in Figure 4.9, A. The antibody dilution chosen should lie on the linear portion of the dilution curve and give antibody-antigen binding levels at least fourfold above background (Poirier, 1991). A series of coating antigen concentrations (300, 500 and 1000 ng) and dilutions of primary antibody (1:1000, 1:2000, 1:5000 and 1:10,000) were selected for competition with the antigen N^2-(4-hydroxyphenyl)-guanosine. No competition was observed, compared to control (PBS), for any antibody dilution or coating antigen concentration utilised, as shown in Figure 4.9, B, C and D. Incubation of the competing antigen (N^2-(4-hydroxyphenyl)-guanosine) with primary antibody for 4 h prior to incubation with the coating antigen (N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin) also resulted in no observable competition (results not shown).

Testing of the previously archived clones for competition towards the adduct, N^2-(4-hydroxyphenyl)-guanosine also resulted in no observable competition even with prior incubation for 15 h of the competing antigen with the primary antibody.

Figure 4.7: Graphical representation of the antibody production in the sera of 3 BALB/C mice (animal 1 (**A**), animal 2 (**B**) and animal 3 (**C**)) after inoculation and boosting with N^2-(4-hydroxyphenyl)-guanosine coupled to BSA. Sera was assessed by ELISA for a maximum of 5 time points (T1-T5) for the presence of antibodies to BSA (▲), ovalbumin (■) or N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (●).
(All values were corrected for the T0 (background) reading, sera was diluted 1:100).

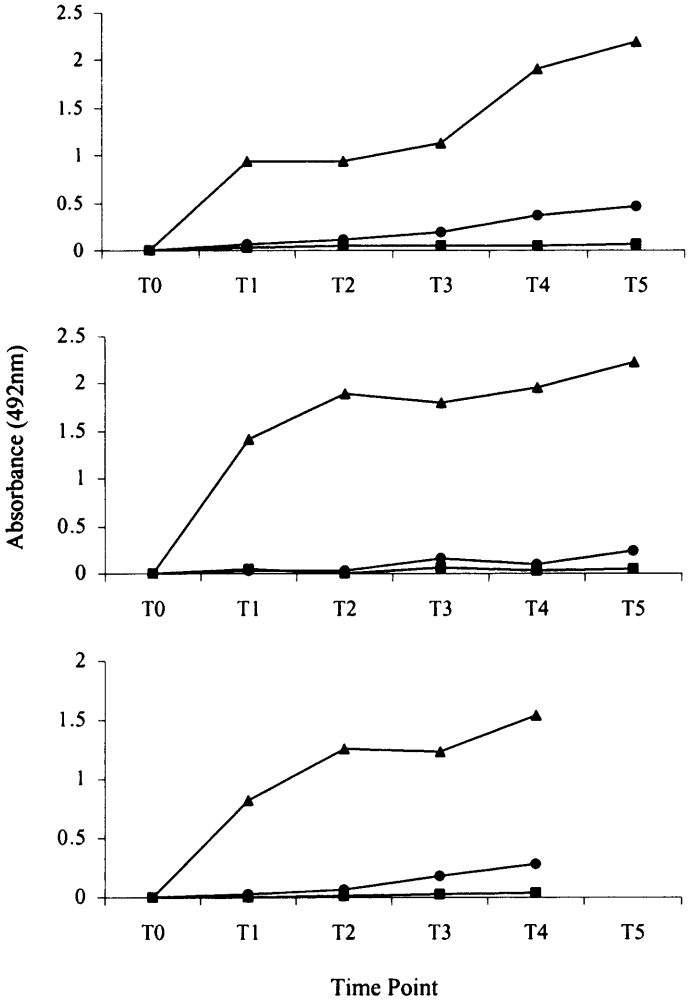


Figure 4.8: Graphical representation of the antibody response observed from the supernatant of 18 hybridoma clones (clone numbers 1-18) and sera from the final test bleed of mouse 2 (clone number 19) when tested using ELISA against BSA (■), guanosine coupled to ovalbumin (■), ovalbumin (■) and N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (■)

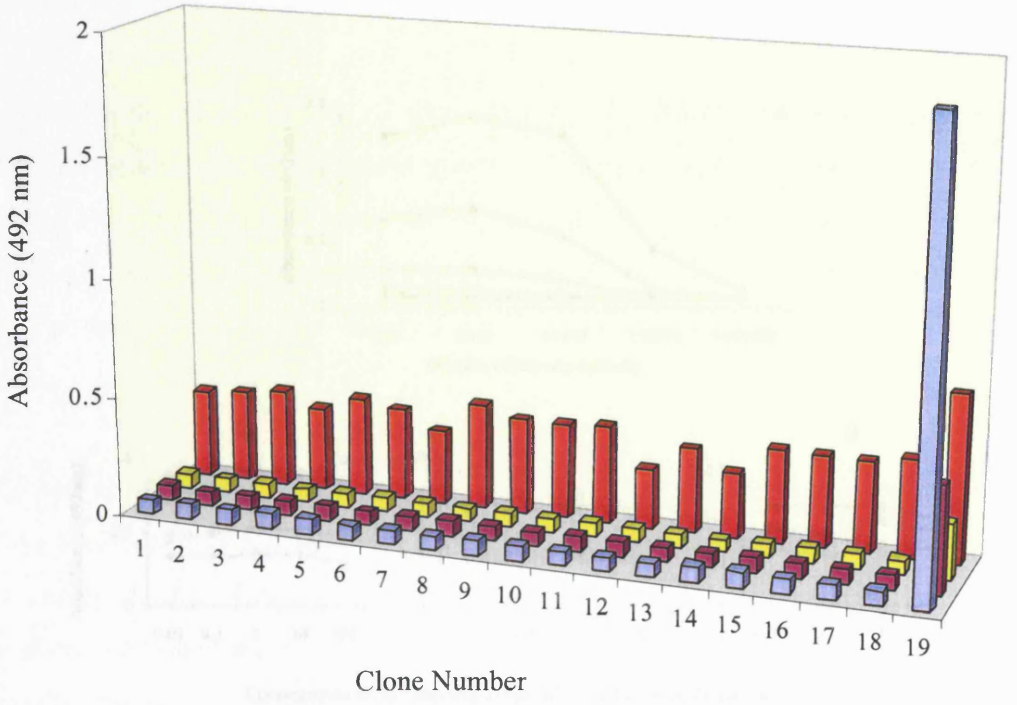
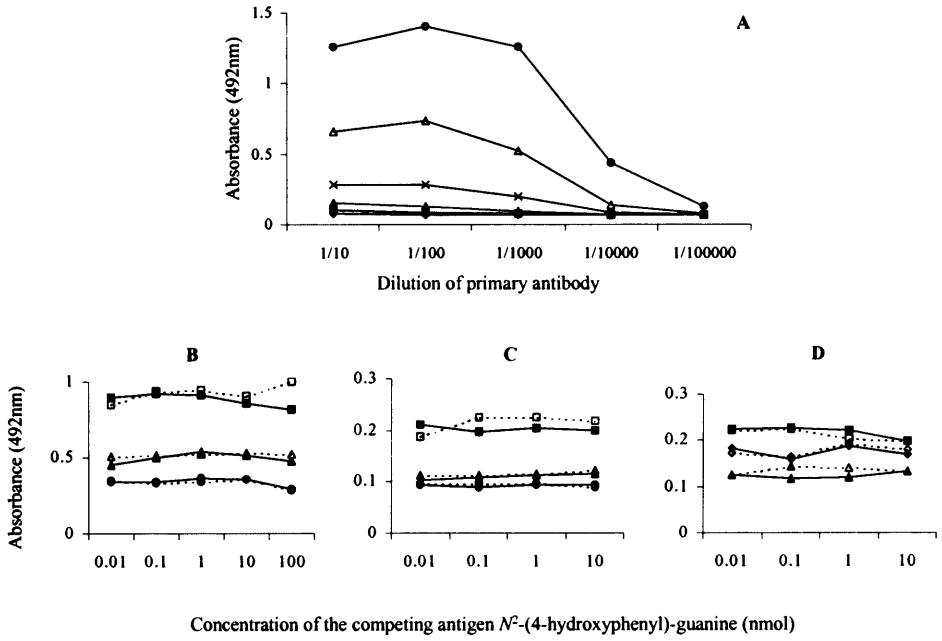


Figure 4.9: Graphical representation of the checkerboard ELISA (**A**) carried out for known dilutions of the supernatant of the selected hybridoma clone against known concentrations of the coating antigen, N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (1000ng (●), 300ng (△), 100ng (x), 30ng (▲), 10ng (■) and 0ng (◆).
Competition ELISA was carried out with 1000ng (**B**), 500ng (**C**) and 300ng (**D**) of the coating antigen, N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin against a range of concentrations (0.01-100fmol) of N^2-(4-hydroxyphenyl)-guanosine with a number of different dilutions of the primary antibody (1:1000 diln (■), 1:2000 diln (◆), 1:5000 diln (▲) and 1:10000 diln (●). For control samples PBS was substituted for the competing antigen and are represented by the open symbols and dashed lines.



4.6.4. Monoclonal antibody production (method 2)

Using monoclonal production method 2 (see section 4.5.5), 3 mice were inoculated and boosted a maximum of 4 times with the antigen N^2-(4-hydroxyphenyl)-guanosine coupled to BSA. A different procedure was utilised as opposed to method 1.

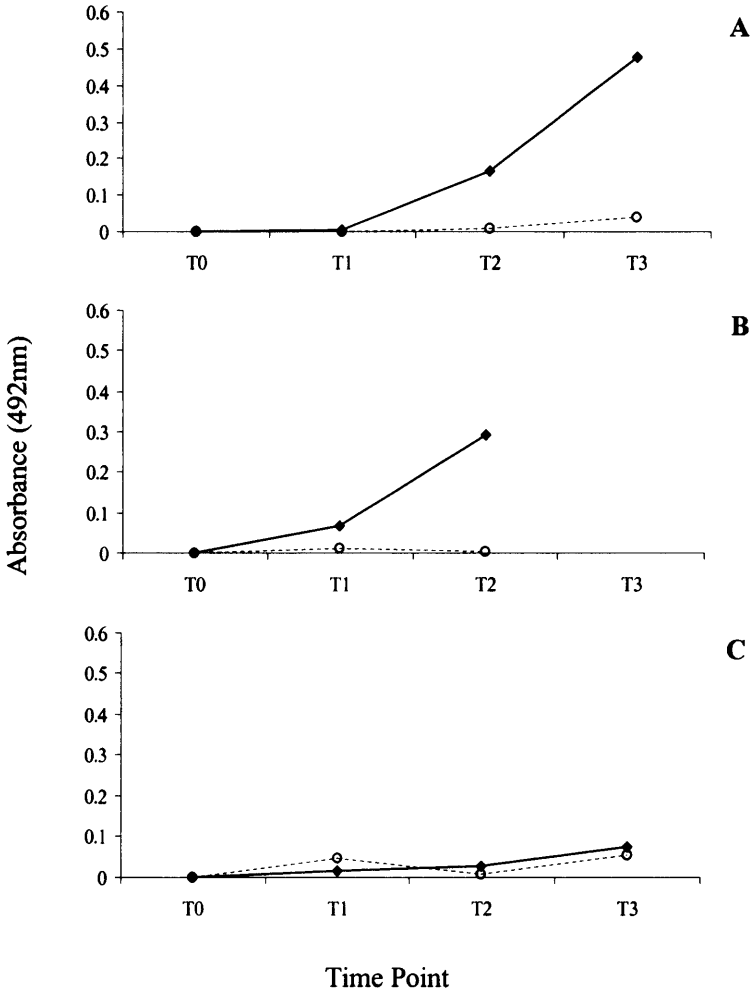
Due to the limiting amounts of blood received at each test bleed, bloods were tested using ELISA against N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin and ovalbumin only.

After the first test bleed (taken 7 days after the first boost), mouse 2 (Figure 4.10, B) demonstrated an antibody response against N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin, a response not observed for animals 1 or 3. After a subsequent boost and test bleed, the response in animal 2 had increased. Based on the results from these two time points, animal 2 was selected for hybridoma production, which subsequently failed.

Animal 1, as shown in Figure 4.10, A, produced an antibody response against N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin as compared to ovalbumin after the second boost. This response also increased with a subsequent boost. Animal 3, produced no noticeable antibody response to the test antigen throughout the lifetime of the experiment, as compared to the negative control, ovalbumin.

Culling of animal 1 and use of its spleen in the hybridoma assay resulted in 64 successful hybridomas. On testing the supernatant from these hybridomas by ELISA, no supernatant tested positive for N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin when compared to ovalbumin. One hybridoma tested positive for antibodies against BSA (results not shown). Recloning of the hybridomas was not pursued.

Figure 4.10: Graphical representation of the antibody production in sera of three BALB/C mice (Mouse 1 (**A**), mouse 2 (**B**) and mouse 3 (**C**) after dosing and subsequent boosting with N^2-(4-hydroxyphenyl)-guanosine coupled to BSA. Sera was assessed by ELISA for a maximum of 3 time points (T1-T3) for the presence of antibodies to ovalbumin (o) or N^2-(4-hydroxyphenyl)-guanosine coupled to ovalbumin (◆).
(All values were corrected for T0 (background) reading, sera was diluted 1:100, 300 ng coating antigen was used in ELISA for animals 1 and 2 whereas 100 ng was used in the ELISA for animal 3)



4.7. Discussion

The production of an antibody against the DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was considered advantageous in regard to the analysis of benzene exposed human DNA samples. The main use of the antibody would have been in an immunopurification step prior to ^{32}P-postlabelling which would offer both selectivity and sensitivity for the adduct of choice. On analysing human samples using the ^{32}P-postlabelling assay, I-spots (endogenous adducts found in control animals) are often observed which have similar chromatographic properties to the adduct of choice. Immunoaffinity utilisation prior to ^{32}P-postlabelling has demonstrated the ability to remove these I-compounds as observed with PAH adduct analysis (Randerath *et al.*, 1998).

A number of antibodies have been raised to benzene metabolites and benzene-adducts. These include antibodies to the benzene metabolite *S*-phenylmercapturic acid (*S*-PMA), the protein adducts HQ-haemoglobin and *S*-(2,5-dihydroxyphenyl)-protein (an adduct formed from the metabolite *p*-benzoquinone) and the synthesised DNA adduct, N-7-phenylguanine (Aston *et al.*, 2002; Grassman and Haas, 1993; Rombach and Hanzlik, 1997; Schell *et al.*, 1993 and Norpoth *et al.*, 1996, respectively). To date however, antibodies to stable benzene DNA adducts have not been raised.

Polyclonal antibody production using New Zealand White rabbits proved to be unsuccessful. An antibody response was elicited towards the adduct of choice, N^2-(4-hydroxyphenyl)-guanosine coupled to a different carrier protein to that used in the dosing programme, only after a five fold increase in the concentration of the test antigen. This lack of response may be due to the number of haptens per molecule of carrier protein, which was on average 3.5 haptens per molecule of carrier protein. As reported by Stollar, 1980 a concentration of between 8 and 25 haptens per protein molecule is required to elicit a good antisera response.

On testing the polyclonal sera for antibody specificity it was determined that the antibody eliciting the positive response recognised the linking group between the adduct and the carrier protein, a result supported by the lack of competition observed

with guanine, deoxyguanosine and N^2-(4-hydroxyphenyl)-guanosine and confirmed by the competition seen using guanosine linked to ovalbumin.

Monoclonal antibody production (method 1) proved to be more successful with an antibody titre observed after only 3 boosts of the test antigen, a response which increased with subsequent boosts. Removal of the spleen from each animal for hybridoma production was successful for only one animal (mouse 2). However, this animal gave rise to a number of clones which elicted a positive response for the adduct coupled to ovalbumin. Due to the response observed for the polyclonal antibodies raised in the rabbits, supernatant from each hybridoma clone was tested for an antibody response against guanosine coupled to ovalbumin, which all proved negative. At this stage, supernatant also gave a negative antibody response for ovalbumin and BSA and a positive antibody response for the modified guanosine attached to ovalbumin. Competition assays however with the modified nucleoside, N^2-(4-hydroxyphenyl)-guanosine, demonstrated no competition suggesting that both the modified adduct plus the conjugated protein were required to elicit an antibody response, a problem which has often been encountered by other researchers. It is assumed that a DNA lesion conjugated to a protein may not be presented to the immune system in the same context as if it were present in DNA. Antibodies raised against such conjugates often have recognition sites directed towards the protein rather than the DNA adduct (Seedhouse *et al.*, 2001) or alternatively the antibody may recognise part of the protein as well as the hapten (Rosner *et al.*, 1991).

Monoclonal antibody production using method 2 resulted in a greater antibody titre which was demonstrated as early as the first test bleed when compared to method 1 (method 1 involved boosting of animals with twice as much antigen when compared to method 2). This methodology however failed to produce any hybridoma cells which tested positive for the adduct.

For future work, a number of different methodologies could be utilised. To carry out a procedure similar to that described (see methods section, 4.5) it would be advisable to use a different conjugating protein, for example KLH (keyhole limpet haemocyanin) and a different conjugation method for the antigen used in the ELISA i.e. succinylation

of a ribose 2'-hydroxyl group or electrostatic coupling between the phosphate of the nucleotide and the methyl group of the protein (Poirier, 1991 and Stollar, 1980).

A different approach entirely, which would overcome the problem of hapten-carrier protein recognition, would be the use of recombinant antibody display technology (phage display libraries), which eliminates the requirement for conjugation of the DNA adduct with a protein. This technology is exploited to produce antigen-binding fragments of an antibody from a number of species which include humans, mice and rabbits (Daly *et al.*, 2001). One such phage library contains the genes for over 100 billion distinct antibodies (Cambridge Antibody Technology, Cambridge). Seedhouse *et al.*, 2001, have utilised one such library to isolate an antibody against the DNA adduct 8-oxo-2'-deoxyguanosine (8-oxo-dG) which has successfully been utilised for the determination of this lesion in the DNA of systemic lupus erythematosus sufferers.

Chapter 5
Synthesis and Analysis of the *In Vivo* Adduct

5.1. Introduction

Benzene has been associated with an increased risk of aplastic anaemia and acute myeloid leukaemia (AML) in humans (reviewed in Snyder and Kalf, 1994) and has been shown to be carcinogenic in a number of rodent tissues, which include the zymbal gland, forestomach and adrenal gland (Huff *et al.*, 1989). The mechanism of benzene's carcinogenicity however is as yet unknown. One possible mechanism is via the formation of DNA adducts which, if not repaired can cause mutations in critical genes. DNA adducts have been identified from the *in vitro* treatment of DNA with the benzene metabolites, hydroquinone (HQ) and *para*-benzoquinone (*p*-BQ) (Jowa *et al.*, 1986; Jowa *et al.*, 1990; Pongracz *et al.*, 1990 and Pongracz and Bodell, 1991), as detailed in Chapter 2, from treatment of cells in culture (Levay *et al.*, 1991; Levay and Bodell, 1992 and Pongracz and Bodell, 1996) and from exposure of animals to benzene or benzene metabolites (Bauer *et al.*, 1989; Bodell *et al.*, 1996; Hedli *et al.*, 1990; Levay *et al.*, 1996 and Pathak *et al.*, 1995).

The formation and detection of DNA adducts in tissues of benzene-exposed humans however, has to date, proved elusive.

5.1.1. Adduct formation in cellular models

Human promyelocytic cells (HL60 cells), a myeloid cell line with the capacity to differentiate in response to a chemical stimulus into any of the four classes of haematopoietic cells (macrophages, monocytes, granulocytes or eosinophils) have been used to demonstrate benzene-DNA adduct formation in a cellular model. HL60 cells contain myeloperoxidase, an enzyme capable of oxidising HQ to *p*-BQ, via a semiquinone intermediate (Snyder *et al.*, 1993a). Adducts formed in these cells have been shown by ^{32}P-postlabelling to be chromatographically different to the adducts formed from direct reaction of benzene metabolites with DNA (Bodell *et al.*, 1993 and Levay *et al.*, 1991). Treatment of HL60 cells with either HQ or *p*-BQ produced the same major DNA adduct as determined by co-chromatography (Bodell *et al.*, 1993 and Levay *et al.*, 1991) and this adduct was identified as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (Pongracz and Bodell, 1996), whose structure is shown in Figure 5.1.

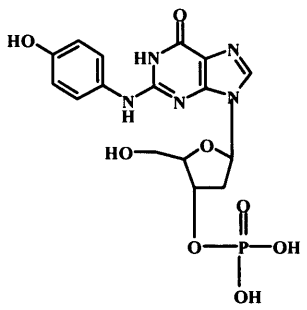


Figure 5.1: Structure of the main adduct identified in cells treated with the benzene metabolites HQ or *p*-BQ or in bone marrow DNA of mice exposed to benzene. The adduct has been characterised as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate.

Treatment of HL60 cells with other benzene metabolites, including catechol and 1,2,4-benzenetriol gave a different pattern of adducts to that observed following treatment with HQ or *p*-BQ. Catechol gave 4 major adducts whilst 1,2,4-benzenetriol gave a single adduct however, none were chromatographically similar. Treatment of cells with a combination of metabolites increased adduct formation synergistically and also gave rise to new adducts not observed with the individual treatments. This finding is an important factor to be aware of when analysing human samples where exposure to benzene would normally occur as part of a chemical mixture (Levay and Bodell, 1992).

Other cell systems utilised include human bone marrow cells, mouse bone marrow macrophages, and two lymphoblastoid cell lines; U-937 and Raji, which are both deficient in peroxidase enzymes (Levay *et al.*, 1993). Treatment of the cells with the benzene metabolite, *p*-BQ, gave an adduct which was common to all and was identified as that formed in HL60 cells (Bodell *et al.*, 1993 and Levay *et al.*, 1993). Treatment with HQ again gave one adduct which was identical chromatographically to that formed with *p*-BQ, but was only observed in HL60 cells, human bone marrow cells and mouse bone marrow macrophages, demonstrating the role of peroxidases in the toxicity of benzene (Levay *et al.*, 1993).

5.1.2. Adduct formation in animal models

Prior to the advent of such sensitive assays as ^{32}P-postlabelling, DNA binding studies relied on the administration of radiolabelled compounds to animals. The administration of radiolabelled benzene demonstrated covalent binding to liver (Lutz and Schlatter, 1977) bone marrow, spleen, kidney, stomach and lung DNA in rats (Arfellini *et al.*, 1985 and Mazzullo *et al.*, 1989) and liver, spleen and bone marrow DNA of mice (Gill and Ahmed, 1981). More recently, accelerator mass spectrometry, a nuclear physics

technique that measures isotope ratios, has been used to determine the tissue distribution and macromolecular binding of radiolabelled benzene in mice and rats at human relevant doses (Mani *et al.*, 1999 and Robertson Creek *et al.*, 1997). The study determined that the highest level of DNA adduct formation in mouse liver occurred 30 min post exposure and adduct levels in mouse bone marrow peaked 12 – 24 h after exposure (Robertson Creek *et al.*, 1997).

However, none of the studies were able to determine which metabolite or metabolites were responsible for such binding.

The sensitive method of ^{32}P-postlabelling has been used to determine DNA adduct formation in a number of benzene exposed animal models. DNA adducts have been observed in both mitochondrial and liver DNA of benzene exposed rabbits (Bauer *et al.*, 1989), bone marrow and white blood cells of benzene exposed mice (Bodell *et al.*, 1996; Levay *et al.*, 1996 and Pathak *et al.*, 1995) and bone marrow DNA of phenol and HQ treated rats (Hedli *et al.*, 1990). Several groups have determined that mice are more sensitive to the toxic effects of benzene than rats but DNA adduct formation in benzene-exposed mice was dependent on the frequency of dosing as opposed to the actual amount of the dose. Treatment of B6C3F1 mice (i.p.) with single daily doses of benzene up to 880 mg/kg for up to seven days, resulted in no observable DNA adducts, (Bodell *et al.*, 1996; Levay *et al.*, 1996; Pathak *et al.*, 1995 and Reddy *et al.*, 1994), whereas twice daily doses of only 100 or 400 mg/kg benzene, gave adducts in both bone marrow and white blood cells, which increased linearly over time (Bodell *et al.*, 1996; Levay *et al.*, 1996 and Pathak *et al.*, 1995). Similar adduct levels were observed in both cell types for the doses and duration investigated (Levay *et al.*, 1996). After dosing with 440 mg/kg twice a day, one adduct was observed after day 1, and then after 3 days a further two minor adducts were observed (Bodell *et al.*, 1996; Levay *et al.*, 1996 and Pathak *et al.*, 1995).

Co-chromatography experiments confirmed that the main adduct formed in *in vivo* studies in rodents corresponded to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the major adduct formed in mouse bone marrow cells (Bodell *et al.*, 1996) or HL60 cells (Pathak *et al.*, 1995) after dosing *in vitro* with HQ. One of the

minor adducts observed corresponded to the DNA adduct formed when HL60 cells were dosed *in vitro* with 1,2,4-benzenetriol (Pathak *et al.*, 1995).

5.1.3. Oxidative DNA damage

Another form of DNA damage occurs through oxidative processes. Exposure of HL60 cells to the benzene metabolites; phenol, HQ, or 1,2,4-benzenetriol resulted in an increased level of oxidative damage, reflected in the production of 8-hydroxy-2'-deoxyguanosine lesions. The greatest level of damage was produced by 1,2,4-benzenetriol. This observation was also seen *in vivo* in the bone marrow of $B6C3F_1$ mice exposed to benzene or a combination of metabolites. This effect was thought to be due to the production of reactive oxygen species (i.e. superoxide anion radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen) during peroxidase metabolism or the auto-oxidation of phenolic metabolites (Kolachana *et al.*, 1993).

Oxidative DNA damage is however not exclusive to benzene exposure, and is caused by many endogenous or exogenous compounds.

5.1.4. Depurination products

Benzene metabolites, in particular benzene oxide, may form unstable DNA adducts which can either be eliminated from DNA during excision repair processes or spontaneously lost due to depurination (loss of guanine or adenine) or depyrimidation (loss of thymine or cytosine). Such excision products can be detected in urine.

N-7-phenylguanine, a theoretical depurination product following reaction of the benzene metabolite, benzene oxide with deoxyguanosine has been synthesised (Krewet *et al.*, 1993; Norpoth *et al.*, 1996 and Schell *et al.*, 1993) and a monoclonal antibody for its detection has been produced (Schell *et al.*, 1993). To date however, analysis of the urine from benzene-exposed rats has failed to detect any such depurination product, however there were indications suggesting that hydroxylated benzene adducts were present in the urine (Krewet *et al.*, 1993 and Norpoth *et al.*, 1996).

5.1.5. DNA adducts in human samples

To date, no benzene DNA adducts have been determined in human DNA following occupational or environmental exposure, and analysis of rodents following dosing at low environmentally relevant doses revealed no genotoxicity or immunotoxicity (Daiker *et al.*, 2000). The main route of human exposure to benzene is via inhalation and inhalation studies carried out in mice revealed a higher level of benzene metabolites in blood and tissues following exposure when compared to oral or i.p. administration (Henderson *et al.*, 1989). A greater toxic and genotoxic effect was observed in mouse bone marrow following inhalation as opposed to oral treatment (Tice *et al.*, 1989).

5.2. Aim

The main aim of this part of the work was to synthesise N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the major benzene-DNA adduct formed *in vivo,* and use this as a standard in the ^{32}P-postlabelling assay coupled to the newly developed TLC or HPLC conditions, as described in Chapter 3. It was envisaged that this assay coupled to an immunoaffinity purification step (detailed in Chapter 4) could be used to improve both the sensitivity and specificity of the ^{32}P-postlabelling assay and hence determine whether benzene-DNA adducts are formed in human tissues.

5.3. Materials

Copper sulphate and sodium carbonate were purchased from BDH. *p*-BQ was obtained from Fluka (Gillingham, Kent). Cell culture medium, glutamax and foetal calf serum (FCS) were purchased from Invitrogen (Paisley, UK). 5'-Dimethoxytrityl-5-fluoro-2'-deoxyinosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (2-F-dI-CE phosphoramidite) and 2-[2-(4,4'-dimethoxytrityoxy)ethylsulfonyl]ethyl-2-succinoyl)-long chain alkylamino-controlled pore glass (3'-phosphate-CPG) were obtained from Glen Research (Sterling, Virginia, USA). Qiagen genomic DNA extraction kits were purchased from Qiagen (Crawley, West Sussex). The Nucleon DNA extraction kit was purchased from Scotlab Biosciences (Coatbridge, Lanarkshire). All other chemicals or enzymes were of the highest grade possible and obtained from Sigma (Poole, Dorset) or Fisher Scientific Ltd (Loughborough, Leicestershire).

HL60 cells were purchased from the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology and Research, Salisbury, Wiltshire). Human embryonic adenovirus-transformed kidney cells (Ad293 cells) were a kind gift from the late Dr. A. Dipple, National Cancer Institute, Frederick, MD, USA.

Liver samples from p53W and 129svWT mice were a kind gift from Dr. Leslie Recio, CIIT, NC, USA. p53W mice were dosed by inhalation with either 0 or 100 ppm benzene for 6 h/day, 5 days/week, for a total of 15 weeks. Each animal received a cumulative dose of 3000 ppm/week as described in Healey *et al.*, 2001 and animals were sacrificed at 15 weeks. 129svWT mice were given a single oral dose (by gavage) of 0, 0.2 or 1.0 mg/g benzene, animals were sacrificed 24 h later.

Human lymphocyte samples were obtained from volunteers working in a petroleum refinery in Oporto, Portugal and were supplied by Joao Paulo Teixeira of the National Institute of Health, Porto, Portugal. Workers were exposed to benzene as part of a chemical mixture. Benzene exposure was estimated to be less than 2 ppm.

The synthesised benzene DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was a kind gift from Dr. William Bodell, University of California, San Francisco, USA.

5.4. Instruments

Protein concentration at 660 nm was recorded using a Uvikon 860 spectrophotometer (Kontron Instruments, Zurich, Germany). DNA concentration and purity was assessed using a different UV spectrophotometer (GeneQuant, Pharmacia Biotech, St Albans, Hertfordshire, UK).

5.5. Methods

5.5.1. Growth and treatment of cells

HL60 cells were grown in RPMI medium supplemented with 10 % FCS and 1 % glutamax. Ad293 cells were grown in Dulbecco's MEM supplemented with 10 % FCS. All cells were grown at 37 °C, 5 % CO_2.

HL60 cells (2 x 10^5 cells /mL) were treated with HQ (0-1000 μM) for 2- 48 h. Viability was assessed at each time point using the trypan blue exclusion assay and the remaining cells underwent genomic DNA extraction.

Ad293 cells were dosed with HQ or *p*-BQ or both metabolites together (0, 250 and 500 μM) for 4, 8, 15, 24 and 48 h. At each time point cells underwent genomic DNA extraction.

5.5.1.1. Cell viability

Cells from each flask were transferred to 50 mL tubes and pelleted by centrifugation at 1000 g for 5 min. The cell pellet was resuspended in 10 mL of supplemented medium. Two 50 μL aliquots were removed from each tube and each were mixed with 50 μL trypan blue. The remaining cells were repelleted for genomic DNA extraction (see section 5.5.1.2. for further details). Cells were scored and assessed for viability using a haemacytometer. Cells, which took up the blue dye were considered dead.

5.5.1.2. Genomic DNA extraction from cells

Each cell pellet was washed twice with PBS and resuspended in PBS to an approximate cell concentration of 10^7 cells / mL. DNA was extracted from cells using Qiagen columns as described below:

An equal volume of ice cold Qiagen buffer C1 (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris/HCl, 1 % Triton X-100, pH 7.5) and 3 volumes of ice cold water were added to each sample. The samples were mixed by inverting the tubes several times and then incubated on ice for 10 min. The samples were repelleted by centrifugation at 3000 rpm, for 15 min at 4 °C. The supernatant was discarded and each pellet was resuspended in 0.5 volumes of ice-cold Qiagen buffer C1 and 1.5 volumes of ice cold water. Samples were re-centrifuged immediately, using the conditions described above. For cell concentrations between 5 x 10^6 cells and 2 x 10^7 cells, cells were resuspended in Qiagen buffer G2 (5 mL; 800 mM GuHCl, 30 mM EDTA, 30 mM Tris/HCl, 5 % Tween-20, 0.5 % Triton X-100, pH 8.0). Proteinase K (2 mg) and ribonuclease A (30 μg) were added to each sample prior to incubation in a shaking waterbath at 37 °C for 2-15 h. The samples were centrifuged at 3000 rpm, 4 °C for 15 min in order to remove any undigested material.

Qiagen columns (100/G) were equilibrated with Qiagen buffer QBT (10 mL; 750 mM NaCl, 50 mM MOPS, 15 % isopropanol, 0.15 % Triton X-100, pH 7.0) and allowed to empty by gravity flow prior to loading of the samples. Each column was washed with Qiagen buffer QC (4 x 7.5 mL; 1.0 M NaCl, 50 mM MOPS, 15 % isopropanol, pH 7.0), and the DNA was eluted from each column using Qiagen buffer QF (5 mL; 1.25 M NaCl, 50 mM Tris/HCl, 15 % isopropanol, pH 8.5). The DNA was precipitated by the addition of 3.5 mL isopropanol, which was washed twice with 70 % ethanol in water (v/v), followed by one wash with ethanol. The DNA for each sample was allowed to air dry briefly before resuspension in an appropriate volume of SSC buffer (1:100 dilution in water; 150 mM NaCl, 15 mM trisodium citrate).

The DNA concentration and purity was determined using UV spectrophotometry as described in Chapter 2, section 2.5.1.

5.5.1.3. Genomic DNA extraction from benzene-exposed mouse liver

The procedure used was similar to that described above (section 5.5.1.2) with a few variations. Liver tissue from benzene exposed mice were homogenised in Qiagen buffer G2 (19 mL) and incubated in a shaking waterbath at 37 °C for 4 h in the presence of the enzymes; proteinase K (20 mg) and ribonuclease A (6 mg). A larger Qiagen column was utilised (500/G), and hence the volume of Qiagen buffers QBT and QC were twice that stated above (section 5.5.1.2). Volumes of Qiagen buffer QF and isopropanol, were 15 mL and 10.5 mL, respectively.

5.5.2. Microsome preparation

Microsomes were prepared from the livers of C57Bl6 mice. Livers were excised and immediately placed into a beaker of ice cold KCl (1.15 %). Liver (3 g) was homogenised in the presence of buffered sucrose (27 mL; 0.25 % sucrose, 10 mM tris, 1 mM EDTA, pH 7.4) and the resultant suspension was centrifuged at 10,000 g for 20 min at 4 °C. The lipid layer was removed and discarded and the supernatant was removed to fresh tubes and re-centrifuged at 100,000 g for 1 h. The resultant supernatant, which contained the cytosolic fraction was frozen as 1 mL aliquots. The pellet was resuspended in KCl (1.15 %, 5 mL) and re-centrifuged at 100,000 g for a further 1 h. The resultant microsome-containing pellet was resuspended in 30 % glycerol in phosphate buffer (v/v) (2 mL /1 g of original liver), aliquoted (1 mL) and frozen at -80 °C. Protein concentration was assessed using the method of Lowry *et al.*, 1951.

Briefly, a series of dilutions of protein (BSA) ranging from 0-200 μg in a total volume of 500 μL (volume adjusted with water) were prepared. An aliquot of microsomal protein was also diluted to 500 μL (in water). Solution A (5 mL; 2 % sodium carbonate: 0.1 M NaOH solution, 1 % copper sulphate, 2 % sodium tartrate) and solution B (0.5 mL; 1:1 dilution of Folin's reagent with water) were added to each sample, samples were mixed and allowed to stand for 10 min prior to analysis by spectrophotometry at 660 nm. A calibration line of known amounts of protein was constructed from which the protein concentration of the microsomal samples was calculated.

5.5.2.1. Microsomal reactions

To calf thymus DNA (0.5 mg), HQ (100 μM) or benzene (100 μM), microsomes (0.5 mg) and HEPES (20 mM) in potassium chloride buffer (1.15 %; pH 7.4) were added, to give a final volume of 1.9 mL. Samples were incubated at 37 °C for 10 min. NADPH generating system (100 μL; NADP+ (5 mM), glucose-6-phosphate (87 mM), glucose-6-phosphate dehydrogenase (5.25 U) in HEPES buffer (700 μL)) was added to each sample and reactions were carried out for 10 - 180 min. The reactions were arrested by the addition of ice-cold chloroform (3 mL) and samples were vortexed and centrifuged at 3000 rpm for 15 min. The aqueous layer was removed and this was washed four times with an equal volume of chloroform: isoamyl alcohol (24:1, v/v), with vortexing and centrifugation at 3000 rpm for 15 min between each wash. The DNA was precipitated by the addition of ethanol (2 times volume) and samples were briefly incubated at –20 °C for 1 h prior to centrifugation at 10,000 g for 10 min. The DNA was washed twice with 70 % ethanol in water (v/v), followed by one wash with ethanol. The DNA was allowed to air dry briefly prior to resuspension in SSC buffer (1:100 dilution in water; 150 mM NaCl, 15 mM trisodium citrate). The DNA concentration and purity was determined using UV spectrophotometry as described in Chapter 2, section 2.5.1.

5.5.3. Peroxidase experiments

Reactions with peroxidase were carried out as described by Davies *et al.*, 1995. Briefly, EDTA, (100 μL, 2 mM), hydrogen peroxide (H_2O_2; 100 μL, 250 μM), 0.3 % tween-20 (100 μL) and horse radish peroxidase (12,500 units) were added to calf thymus DNA (300 μg) followed by incubation for 10 min at 37 °C. To commence the reaction, benzene, phenol or HQ (500 μM) were added to the reaction mixture. The volume was adjusted to 1 mL with HEPES/NaOH buffer (0.05 M) and incubation was carried out for 60 min, at 37 °C. Alternatively, DNA (600 μg), H_2O_2 (100 μL; 2 mM), HQ (1 mM), and/or phenol (2 or 5 mM), *p*-BQ (1 mM) or HQ plus *p*-BQ (500 μM of each) and horse radish peroxidase (130 mU-2,500 U) were incubated in sodium phosphate buffer, (1 mL; 67 mM, pH 7.0) for 70 - 180 min.

The reaction was arrested by the addition of chloroform: isoamyl alcohol (2 mL; 25:1, v/v), followed by vortexing and centrifugation for 5 min at 14,000 rpm. The aqueous layer was removed and the chloroform: isoamyl alcohol (25:1, v/v) wash was repeated a further 3 times. Samples were subsequently washed 6 times with water-saturated ethyl acetate (2 mL) followed by a final clean up step using the Nucleon extraction kit.

Briefly, sodium perchlorate solution (100 µL) was added to the sample, which was inverted seven times prior to the addition of chloroform: isoamyl alcohol (600 µL; 25:1, v/v). Again, the sample was mixed by inversion seven times. Nucleon resin (150 µL) was added, followed by immediate centrifugation at 350 g for 1 min. The aqueous layer was removed and the DNA was precipitated by the addition of ethanol (2 mL). The DNA was washed twice with 70 % ethanol in water (v/v) followed by one wash with ethanol. The DNA was allowed to air dry briefly before resuspension in an appropriate volume of SSC buffer (1:100 dilution in water; 150 mM NaCl, 15 mM trisodium citrate).

The DNA concentration and purity was determined using a UV spectrophotometer as described in Chapter 2, section 2.5.1.

5.5.4. Synthesis of N^2-(4-hydroxyphenyl)-guanosine 3'-monophosphate

The following reaction was based on that previously described by Pongracz and Bodell, 1996.

Guanosine 3'-monophosphate (Gp; 100 mg) and *p*-BQ (100 mg), were dissolved in 200 mM HCl-KCl, pH 1.0 (20 mL) and incubated for 24 -72 h at 37 °C. The reaction was arrested by adjustment of the pH to 6.0 with NaOH. Bond elute cation exchange columns (500 mg) were activated with methanol (3 mL), and equilibrated with sodium phosphate buffer (3 mL; 0.4 M, pH 6.0). The reaction mixture (20 mL) was loaded onto the columns and the product(s) were eluted with sodium phosphate buffer (12 mL, 0.4 M, pH 6.0).

To desalt the sample, C18 sep pak columns were activated with methanol (5 mL) and equilibrated with HPLC grade water (5 mL). Eluate from the cation exchange columns

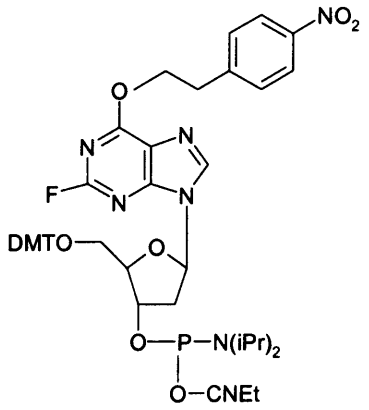
was loaded onto the C18 sep pak column. Columns were washed with 20 % methanol in water (2 mL; v/v) followed by one wash with methanol (5 mL). The methanol washes were dried down under nitrogen.

The products were separated using HPLC System C as outlined in Chapter 2, section 2.4.

5.5.5. Synthesis of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate using 2-F-dI phosphoramidite

5'-Dimethoxytrityl-5-fluoro-2'-deoxyinosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (2F-dI-CE phosphoramidite) (Figure 5.2) can be converted to 2'-modified dG derivatives by reaction with a primary amine which displaces the fluorine atom. Attempts were made to synthesise the N^2-(4-hydroxyphenyl) derivative by displacement of the fluorine with 4-hydroxyaniline.

Figure 5.2: Structure of 5'-Dimethoxytrityl-5-fluoro-2'-deoxyinosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (2F-dI-CE phosphoramidite)



The 2F-dI-CE phosphoramidite was incorporated into several oligonucleotides (prepared by PNACL, University of Leicester), which included:

T T I T T (oligo 1)

T T T I T T T I T T T (oligo 2)

where I represents the 2F-dI-CE phosphoramidite

Using 3'-controlled pore glass (CPG) columns, a single 2F-dI-CE phosphoramidite was attached directly to the support matrix. Following reaction with 4-hydroxyaniline and cleavage from the column it was hoped that the adducted nucleotide, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate would be the major synthesised product.

Because concentrated ammonium hydroxide was used in the oligonucleotide synthesiser, which deprotects and cleaves the oligonucleotides from the support matrix, deprotection and cleavage had to be performed manually (ammonia may displace the fluorine atom hence producing deoxyguanosine).

Several methods were utilised to produce the adduct, the standard method was as follows:
Beads from a synthesis column were transferred into a glass vial. The beads were washed with acetonitrile and dried under argon. 4-Hydroxyaniline (1 mL; 0.5 M in dimethyl sulphoxide (DMSO)) was added to the vial and the beads were incubated in a shaking waterbath at 37 °C for up to 3 days. The beads were washed twice with DMSO and three times with acetonitrile. The beads were incubated twice with diazabicyclo(5.4.0)undec-7-ene (DBU; 1 mL; 1 M in acetonitrile) for 1 h at room temperature. The beads were washed a further two times with methanol and three times with acetonitrile. Deprotection and cleavage was carried out with either concentrated ammonium hydroxide (29.5 %), potassium carbonate in methanol (0.05 M) or 10 %, DBU in methanol (v/v) (Chenna and Singer, 1997) for up to 72 h at room temperature.

C18 sep pak columns conditioned with methanol (5 mL) and equilibrated with HPLC grade water (5 mL) were loaded with supernatant from the reaction or from the washing of the beads. The columns were washed with 20 % methanol in water (1 mL; v/v) followed by one wash with methanol (1mL). The eluate was dried down and analysed by HPLC using the conditions outlined in Chapter 2, Section 2.4.1.3 (system C).

5.5.6. ^{32}P-postlabelling of DNA samples

DNA samples were analysed by ^{32}P-postlabelling as described in Chapter 3.

5.6. Results

5.6.1. Treatment of HL60 cells

Treatment of HL60 cells with the benzene metabolite, HQ (0-1000 μM) for 2-48 h, resulted in one major adduct as detected by ^{32}P-postlabelling coupled with TLC, as shown in Figure 5.3, B. No adduct of similar chromatographic properties was observed in control samples (Figure 5.3, A). Co-elution of ^{32}P-postlabelled DNA from HQ treated HL60 cells (500 μM, 48 h) with the postlabelling adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (obtained from Dr William Bodell, see section 5.6.7 for further details), showed that the major adduct from HL60 treated cells had the same mobility on TLC as the adduct standard (results not shown). For the 250 and 500 μM HQ doses, adduct levels increased linearly for approximately the first 10 h. After this time adduct levels started to plateau, with adduct levels ranging from 3.49 ± 3.46 to 73.15 ± 36.68 and 12.16 ± 6.26 to 65.79 ± 24.12 / 10^8 nucleotides for the 250 μM and 500 μM doses, respectively for the 2- 48 h time period. Overall adduct levels can be seen in Figure 5.5, A.

Cytotoxicity, as assessed by trypan blue exclusion, also increased with both dose and time, as shown in Figure 5.4, with 50 % cell death occurring within the first 24 h for all, except the control and 50 μM HQ dose. Cytotoxicity for the 250 and 500 μM HQ doses can be seen separately in Figure 5.5, B.

Analysis of ^{32}P-postlabelled DNA from HQ treated HL60 cells using the newly developed HPLC conditions as detailed in Chapter 3, gave no adduct peaks when compared to control samples. Elution of the radioactivity from the major adduct spot on the TLC plates gave a non-reproducible pattern of peaks, none of which corresponded to the adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (see Figures 5.11 and 5.12 for elution of this adduct standard).

Figure 5.3: Typical TLC maps of ^{32}P-postlabelled DNA from control HL60 cells (**A**) and HL60 cells following treatment with HQ (500 μM) for 15 h (**B**). The additional adduct spot in the treated sample is highlighted by the dotted line.
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).

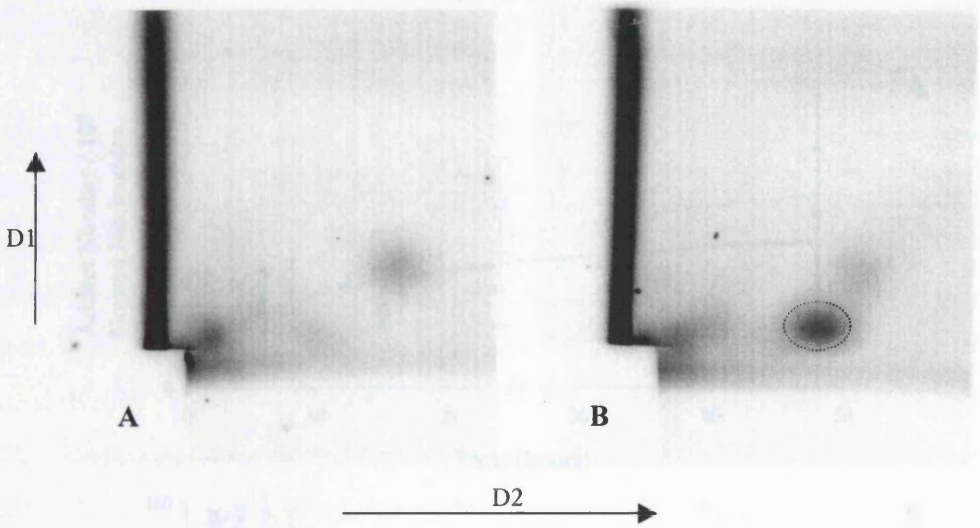


Figure 5.4: Percentage cell survival as determined by trypan blue exclusion after treatement with 0 (◆), 50μM (■), 100μM (▲), 250μM ( ), 500μM (x), 1000μM (•) HQ (**A**). Or percentage cell survival over the range of HQ doses at 2 (◆), 4 (■), 6 (▲), 8 ( ), 24 (x), or 48 h (•) (**B**).

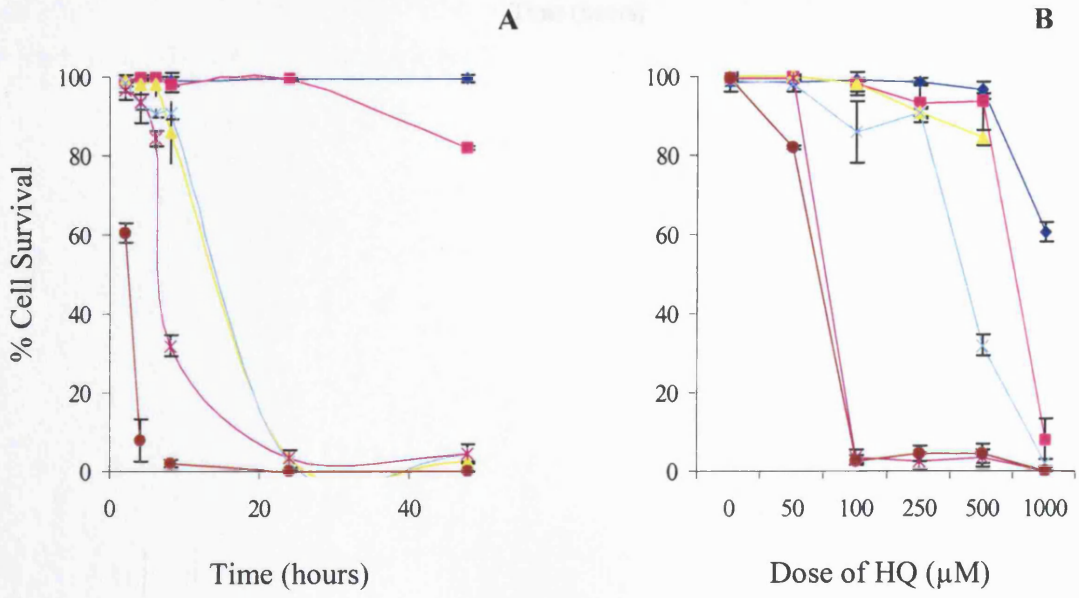
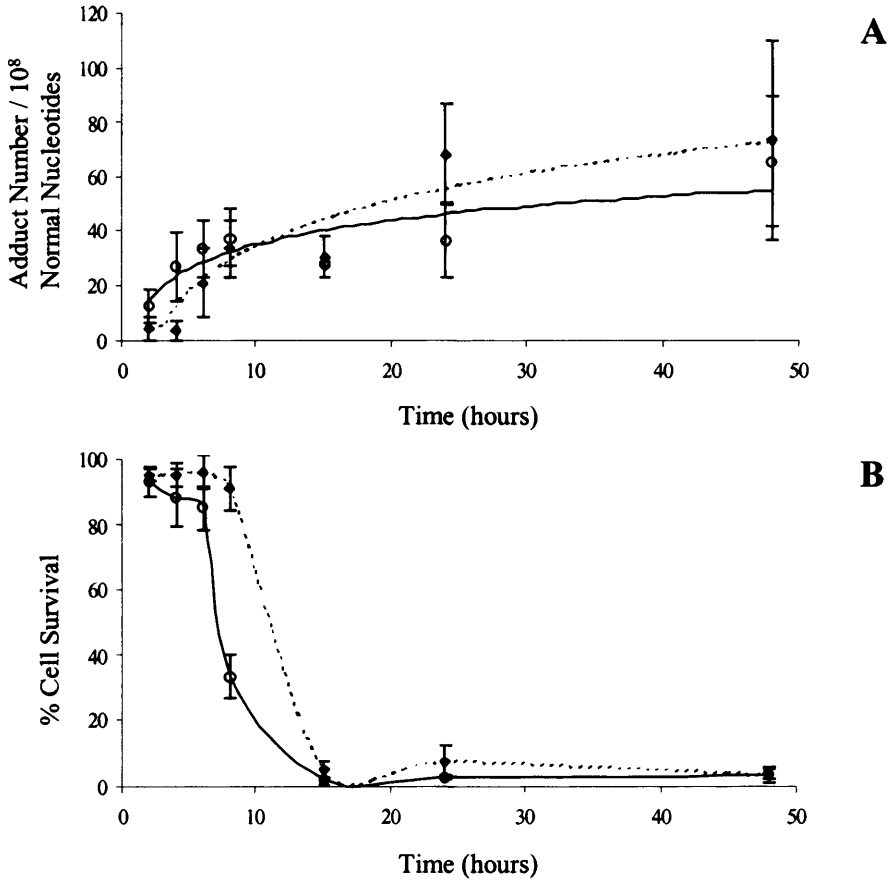


Figure 5.5: Induction of DNA adducts in HL60 cells dosed with 250 (◆, broken line) or 500 μM HQ (o, solid line) (**A**). Percentage of viable cells as determined by trypan blue exclusion for the 250 μM and 500 μM HQ doses (**B**). Cell treatments were repeated independently three times. Data shown are mean ± SD (n=3 for adduct number and n=3-13 for % cell survival).



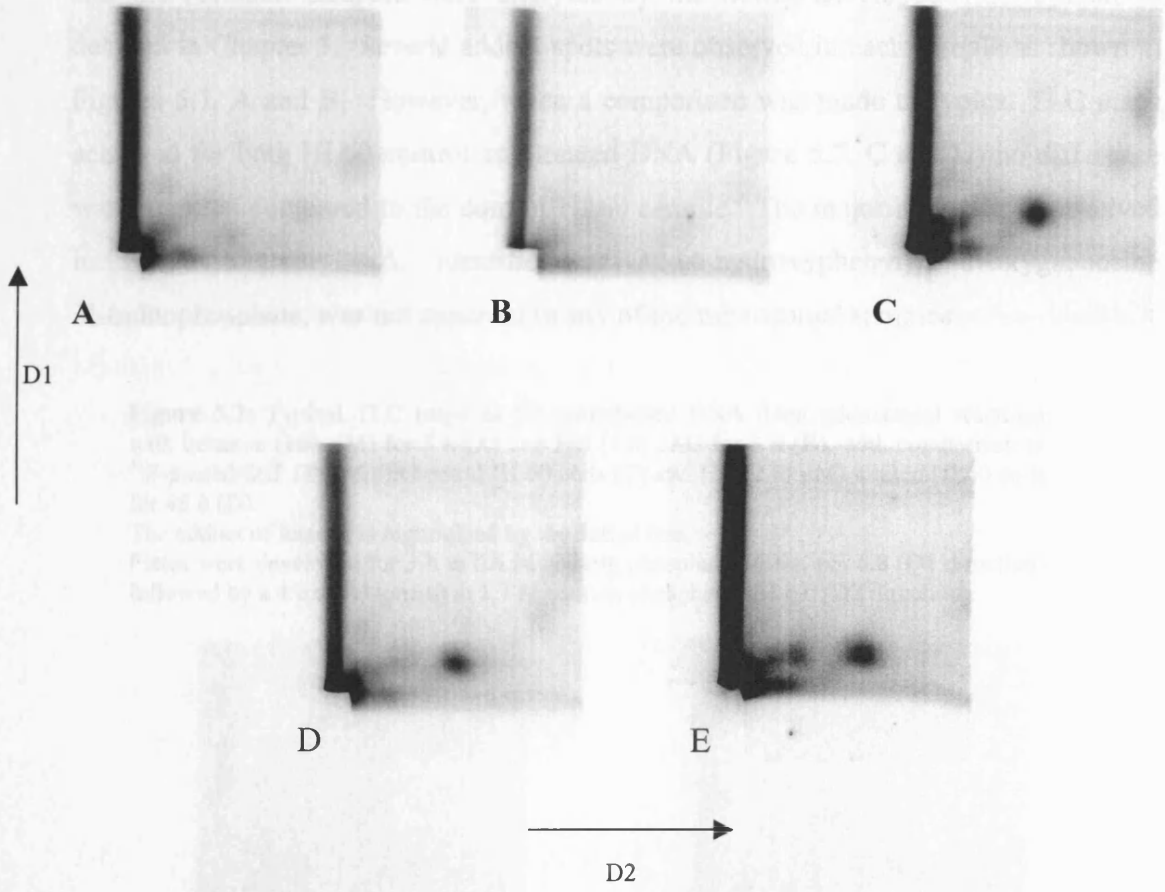
5.6.2. Treatment of Ad293 cells

Ad293 cells, a human embryonic adenovirus-transformed kidney cell line, were utilised in the mutation assay, *supF*, as detailed in Chapter 6. It was proposed that transfection of these cells with the pSP189 plasmid followed by treatment with the benzene metabolites HQ or *p*-BQ or both metabolites together could induce the adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate on the plasmid.

These cells were dosed with either 250 or 500 μM HQ, *p*-BQ or both metabolites together for up to 48 h. HQ failed to induce any such adduct, giving a TLC map similar to that observed for control DNA (Figures 5.6, A and B). However, *p*-BQ and both metabolites together induced one major adduct with similar chromatographical properties as that observed in HL60 cells (see Figures 5.6, C, D and E). Co-elution of ^{32}P-postlabelled DNA from HQ and *p*-BQ treated Ad293 cells (500 μM, 24 h) with the postlabelling standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate confirmed that the major adduct spot observed in Ad293 cells had the same mobility on TLC as the adduct standard (results not shown).

Adduct levels were similar for *p*-BQ treatment and the combined treatment, suggesting that HQ had no influence on adduct levels. Adduct levels for the *p*-BQ and combined treatment for the 4 – 48 h time period ranged from approximately 5 to 150 adducts / 10^8 nucleotides. Adduct levels increased rapidly over the initial 8 h of treatment and then decreased, which may be associated with repair of the adduct in this cell line. Further work would be required to determine if this is the case.

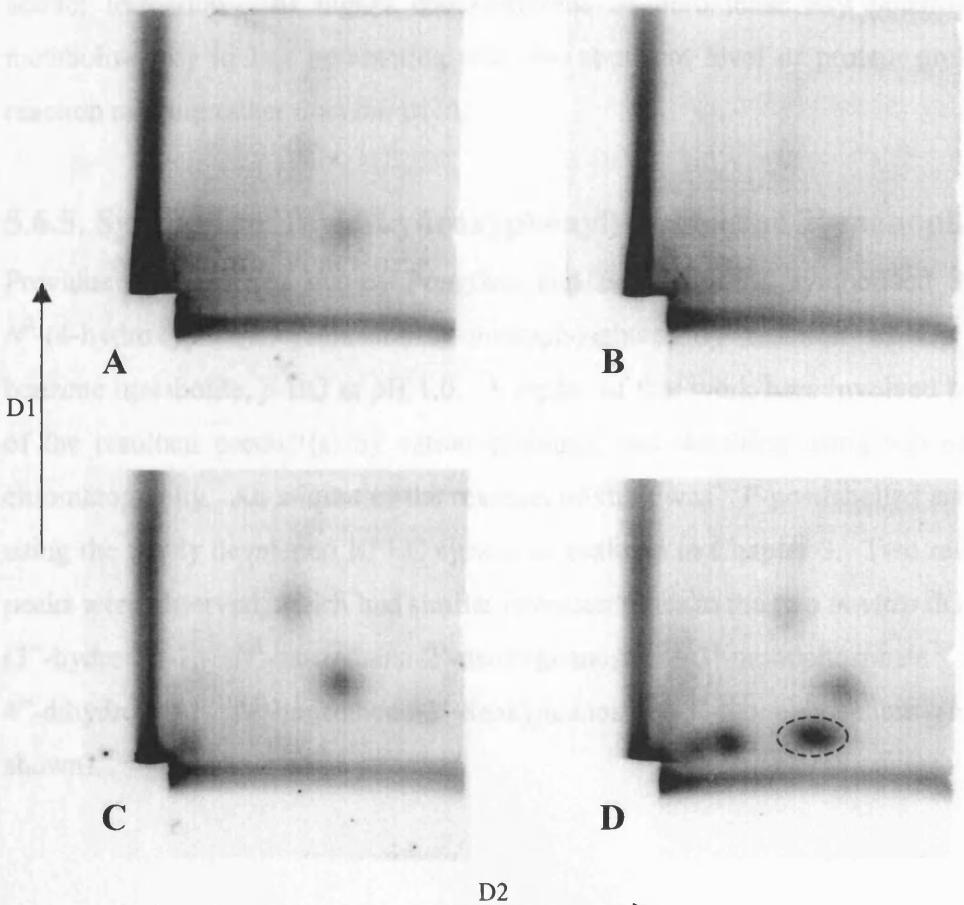
Figure 5.6: Typical TLC maps of ^{32}P-postlabelled DNA from control Ad293 cells (**A**), Ad293 cells following treatment with 500 μM HQ (**B**), 500 μM *p*-BQ (**C**) or 500 μM of both metabolites together (**D**) for 24 h. A TLC map of ^{32}P-postlabelled DNA from HL60 cells treated with 500 μM HQ for 24 h is shown for comparison (**E**).
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).



5.6.3. Microsomal reactions

Reactions were carried out with microsomes prepared from the livers of C57Bl6 mice in the presence of either benzene or the benzene metabolite, HQ. Reaction duration ranged from 10 min up to 180 min. The repurified DNA underwent ^{32}P-postlabelling and the resultant samples were analysed by the newly developed TLC system as detailed in Chapter 3. Several adduct spots were observed in each sample as shown in Figures 5.7, A and B. However, when a comparison was made to typical TLC maps achieved for both HL60 control and treated DNA (Figure 5.7, C and D) no difference was observed compared to the control HL60 sample. The major adduct spot observed in HL60 treated DNA, identified as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, was not apparent in any of the microsomal samples.

Figure 5.7: Typical TLC maps of ^{32}P-postlabelled DNA from microsomal reactions with benzene (100 μM) for 3 h (**A**) and HQ (100 μM) for 3 h (**B**), with comparison to ^{32}P-postlablled DNA from control HL60 cells (**C**) and HQ (250 μM) treated HL60 cells for 48 h (**D**).
The adduct of interest is highlighted by the dotted line.
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).



5.6.4. Peroxidase reactions

Reactions with peroxidase were carried out with a number of individual benzene metabolites and metabolites in combination, which included phenol, HQ and *p*-BQ, with doses ranging from 1 mM to 5 mM for 1 to 3 h. The repurified DNA was ^{32}P-postlabelled and analysed by TLC using the conditions described in Chapter 3. No difference in adduct pattern was observed between treated and control samples for any of the doses, metabolites and combination of metabolites used (results not shown).

An additional adduct spot however, was observed when the amount of peroxidase in the reaction was reduced to 100 ng and the reaction involved dosing with both HQ (1 mM) and phenol (5 mM). This same adduct spot was also observed following a similar treatment except that peroxidase was excluded from the reaction. This adduct had similar chromatographic properties to the benzene DNA adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, the major adduct formed from direct reaction of DNA with the benzene metabolite, HQ (see Chapter 3, section 3.6.5 for further details). This result suggested that the peroxidase was having no effect on adduct formation. At higher concentrations of peroxidase any potential reactive metabolite may in fact be reacting with the abundant level of protein present in the reaction mixture rather than the DNA.

5.6.5. Synthesis of N^2-(4-hydroxyphenyl)-guanosine 3'-monophosphate

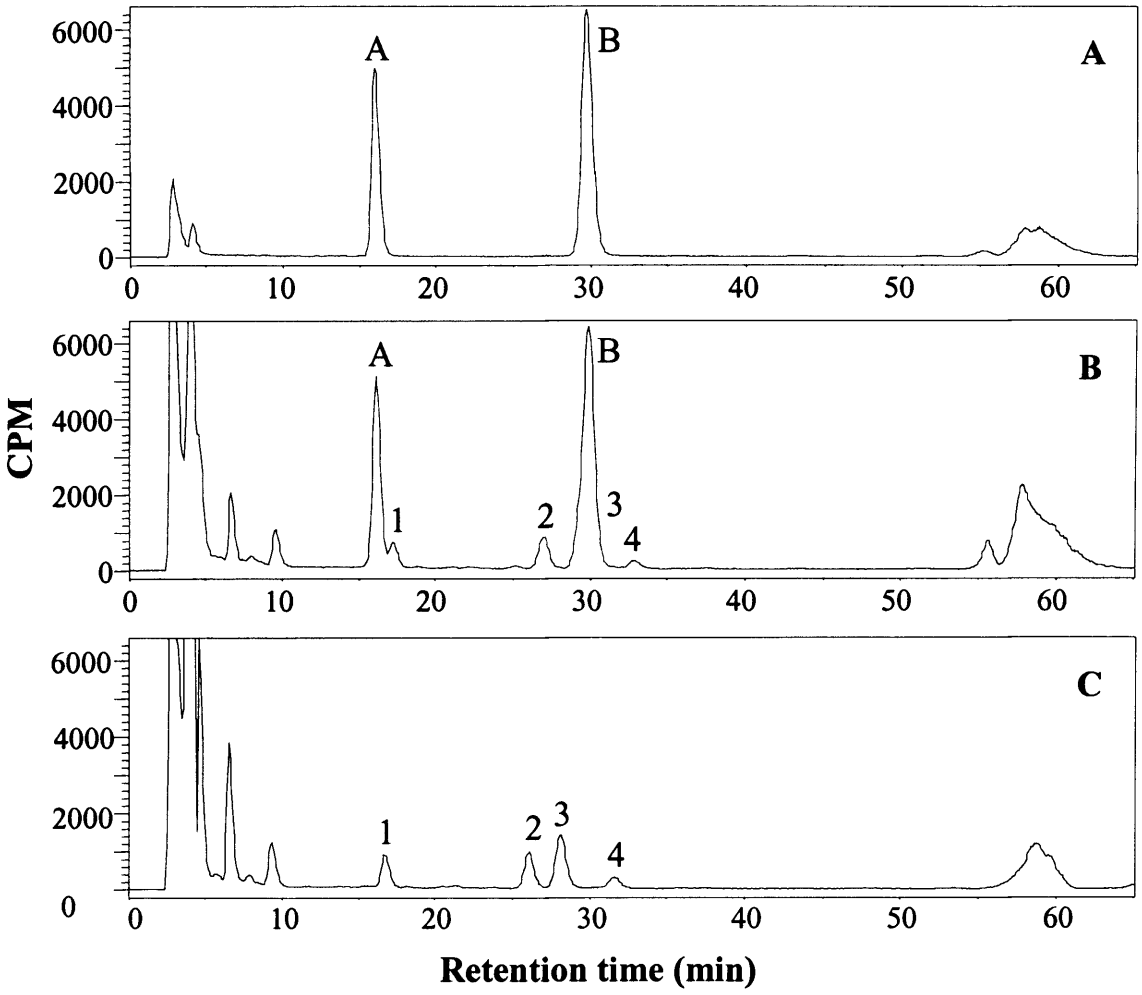
Previous work carried out by Pongracz and Bodell, 1996, synthesised the adduct, N^2-(4-hydroxyphenyl)-guanosine 3'-monophosphate, by reaction of Gp with the benzene metabolite, *p*-BQ at pH 1.0. A repeat of that work here involved purification of the resultant product(s) by cation exchange and desalting using sep pak column chromatography. An aliquot of the reaction mixture was ^{32}P-postlabelled and analysed using the newly developed HPLC system as outlined in Chapter 3. Two radiolabelled peaks were observed, which had similar retention times to the two *in vitro* dGp adducts; (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (results not shown).

UV-HPLC analysis of the 20 % methanol in water (v/v) wash from the sep pak cartridge gave a number of peaks eluting between 10 and 25 min (results not shown). One peak, eluting at approximately 19 min, when collected, dried down, ^{32}P-postlabelled and analysed by HPLC gave two adduct peaks as shown in Figure 5.8, A. These two adduct peaks corresponded to those observed previously when the reaction mixture was analysed by ^{32}P-postlabelling directly, prior to any purification/desalting step. Peaks eluting later in the UV-HPLC run (20 – 25 min) when subjected to the same treatment, all resulted in the adduct peak labelled B in Figure 5.8, A. No peak for product A alone could be identified.

Co-elution of the Gp reaction products with HQ and *p*-BQ treated calf thymus DNA showed the Gp products to elute 2-3 min prior to the dGp adducts; (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. This is probably due to the lack of retention afforded by the extra hydroxyl group found on the Gp adducts (see Figure 5.8, B and C).

Product B, when analysed by tandem MS using continuous infusion, negative ion electrospray resulted in a molecule ion $[M-H]^-$ at *m/z* 452 and a product ion at *m/z* 240 (results not shown). These data are consistent with a product of molecular weight 453, which could correspond to the adduct, (3"-hydroxy)-1, N^2-benzetheno-guanosine 3'-monophosphate. The product ion is consistent with loss of the ribose and phosphate moieties, corresponding to the adducted base. Although no MS data was achieved for product A, based on the information obtained for product B, this product could potentially correspond to (3", 4"-dihydroxy)-1, N^2-benzetheno-guanosine 3'-monophosphate. Further work would be required to determine this.

Figure 5.8: HPLC chromatograms of ^{32}P-postlabelled HPLC purified products from the reaction of Gp with *p*-BQ at pH 1.0 (**A,** peaks A and B), co-elution of these products with the products formed from the reaction of calf thymus DNA with HQ and *p*-BQ (**B,** peaks 1-4) with comparison to the products of the calf thymus DNA reaction (**C**).
HPLC separation was performed using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).



5.6.6. Adduct synthesis using 2F-dI-CE phosphoramidite

The phosphoramidite, 5'-Dimethoxytrityl-5-fluoro-2'-deoxyinosine, 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (2F-dI-CE phosphoramidite) was incorporated into a number of polythymidine oligonucleotides and was also attached directly to 2-[2-(4,4'-dimethoxytrityoxy)ethylsulfonyl]ethyl-2-succinoyl)-long chain alkylamino-controlled pore glass (3'-phosphate CPG), which upon deprotection and cleavage would give 2-fluoro-deoxyinosine 3'-monophosphate. Deprotection and cleavage from the support matrix would normally be carried out in the synthesiser using concentrated ammonia. Due to the potential displacement of the fluorine atom by ammonia, producing 2'-deoxyguanosine 3'-monophosphate, the synthesised oligonucleotides and nucleotide were supplied still coupled to the support matrix. Deprotection and cleavage were performed manually following reaction with 4-hydroxyaniline.

The reactions carried out had to produce the N^2-(4-hydroxyphenyl) adduct then remove the deprotecting groups and cleave the nucleotide or oligonucleotide from the support matrix. A number of different conditions were employed, which incorporated different reaction times and temperatures to synthesise the actual adduct. Different chemical methods were also employed to deprotect and cleave the products from the support matrix.

Initial reactions of 2F-dI-CE phosphoramidite attached to 3'-CPG or oligonucleotide 2 (T T T T T T I T T T-CPG) with 4-hydroxyaniline for 4 – 48 h at both room temperature and 37 °C followed by cleavage and deprotection using concentrated ammonium hydroxide (29.5 %), potassium carbonate (K_2CO_3) in methanol (0.05 M) or 10 % DBU in methanol (v/v), resulted in no observable products when analysed by HPLC with UV detection. However, direct analysis of the K_2CO_3 cleaved product(s) by ^{32}P-postlabelling gave a number of ^{32}P-postlabelled product peaks when analysed by HPLC, compared to the control sample (see Figure 5.9, A and B).

The reaction supernatant and bead washes from a further reaction of 2F-dI-CE phosphoramidite attached to 3'-CPG with 4-hydroxyaniline for 72 h at 55 °C underwent sep pak clean up. An aliquot of the eluate (100 % methanol) was analysed directly by ^{32}P-postlabelling and was shown to give two major product peaks when

analysed by HPLC. The two main products had similar mobility to the two dGp adducts; (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate as shown in Figure 5.10. These results indicate that some deprotection and cleavage of the nucleotide is occurring during the reaction.

Analysis of the sep pak eluate by UV-HPLC gave a number of peaks eluting between 10 and 30 min (results not shown). Collection of the individual peaks followed by analysis using ^{32}P-postlabelling confirmed that the two major peaks previously reported (Figure 5.10) could be separated with retention times on UV-HPLC of 22 and 26 min, respectively. Inadequate sample quantities meant that no conclusive MS data could be obtained in regard to these products.

Similar reactions with oligonucleotide 1 (T T I T T-coupled to the support matrix) with UV-HPLC analysis of the reaction supernatant also gave a number of peaks eluting between 10 and 25 min. Collection of the peaks, digestion to the nucleotide components followed by ^{32}P-postlabelling and analysis by HPLC gave similar results to that achieved for the individual 2F-dI-CE phosphoramidite reaction. Other peaks were also observed suggesting that full deprotection had not been successful.

Without enough product for successful MS analysis it was difficult to determine at this stage whether (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate or the desired product, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate had successfully been synthesised. Both products co-eluted when analysed as ^{32}P-postlabelled products either by TLC or HPLC (see section 5.6.7. for further details). The second product had a similar retention to the adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, which suggested that perhaps the first product is more likely to be (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Further work improving the reaction conditions and product yield should help corroborate this hypothesis.

Figure 5.9: HPLC chromatograms of ^{32}P-postlabelled products from the untreated 2F-dI-CE phosphoramidite followed by cleavage with K_2CO_3 in methanol (0.05 M) (**A**), products from the reaction of 2F-dI CE-phosphoramidite with 4-hydroxyaniline at 37 °C for 48 h, followed by cleavage with K_2CO_3 in methanol (0.05 M) (**B**) and products from the reaction of calf thymus DNA with HQ and *p*-BQ (**C**). Co-elution of the ^{32}P-postlabelled products from the 4-hydroxyaniline treatment of 2F-dI CE-phosphoramidite cleaved with K_2CO_3 in methanol (0.05 M), with the products from the treatment of calf thymus DNA with HQ and *p*-BQ (**D**).
HPLC separation was performed using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).
Peaks 1, 2, 3 and 4 represent (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, respectively.

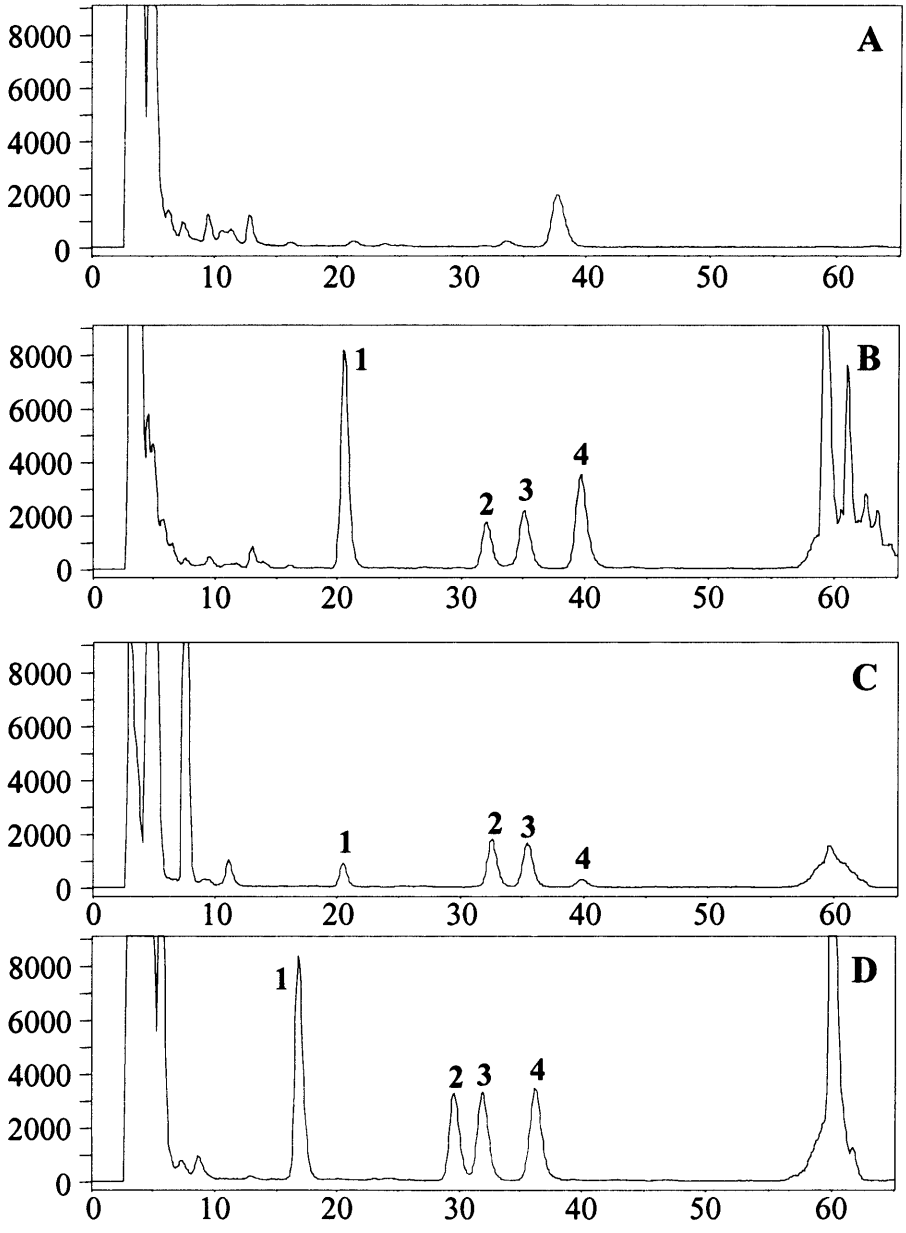
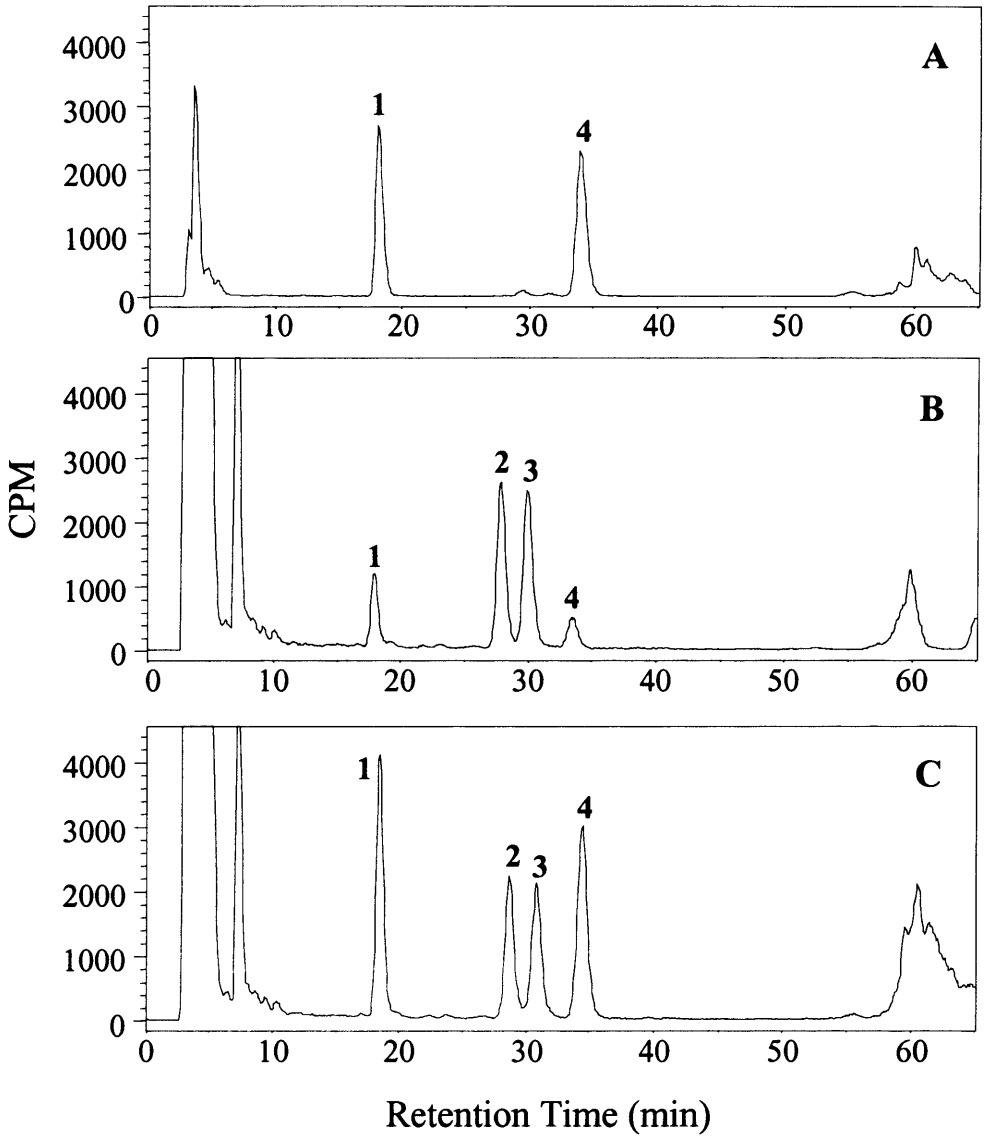


Figure 5.10: Typical HPLC chromatograms of the products extracted using sep pak column chromatography from the reaction supernatant of 2F-dI-CE phosphoramidite with 4-hydroxy aniline for 72 h at 55 °C, with subsequent ^{32}P-postlabelling (**A**), the ^{32}P-postlabelled products from the reaction of calf thymus DNA with HQ and *p*-BQ (**B**) and co-elution of both samples (**C**).
HPLC separation was performed using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).



5.6.7. ^{32}P-postlabelling of the synthesised adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate

The DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was kindly provided by Dr. William Bodell of the University of California, San Francisco, USA. To confirm the stability of the adduct during transit, the adduct underwent MS analysis using negative ion electrospray which resulted in a molecule ion $[M-H]^-$ at *m/z* 438 (results not shown). This data was consistent with a product of molecular weight 439, which corresponded to the adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the structure of which can be seen in Figure 5.1.

The adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, was analysed by ^{32}P-postlabelling and comparison was made to the products from the reaction of calf thymus DNA with the benzene metabolites HQ and *p*-BQ. Initial analysis was carried out using the TLC conditions outlined in Chapter 3, and the resultant TLC maps are shown in Figure 5.11. The adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was shown to co-elute with the adduct previously identified as (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (see Figure 5.11, C).

Further analysis using the HPLC conditions outlined in Chapter 3, also confirmed this finding as shown in Figure 5.12. Further modification of the HPLC gradient failed to separate the two adducts.

Figure 5.11: TLC maps demonstrating the retention of ^{32}P-postlabelled N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (0.25 pmol) (**A**), the adducts formed from the treatment of calf thymus DNA with HQ and *p*-BQ (**B**) and co-elution of the two samples (**C**), the adduct of interest is highlighted by the dotted circle.
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).

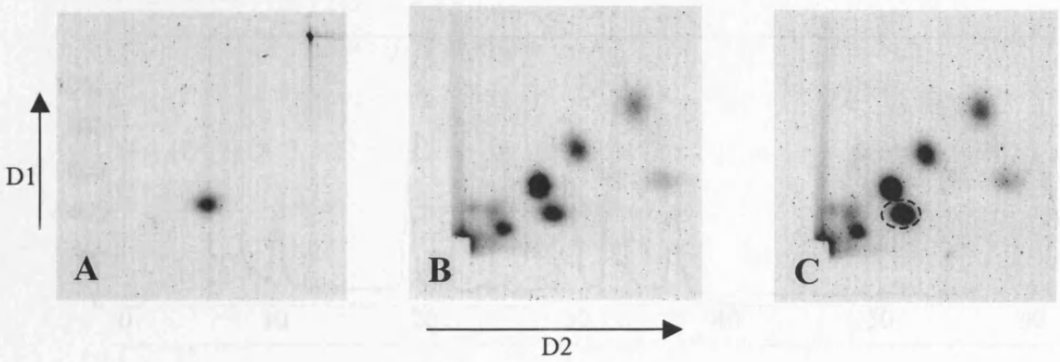
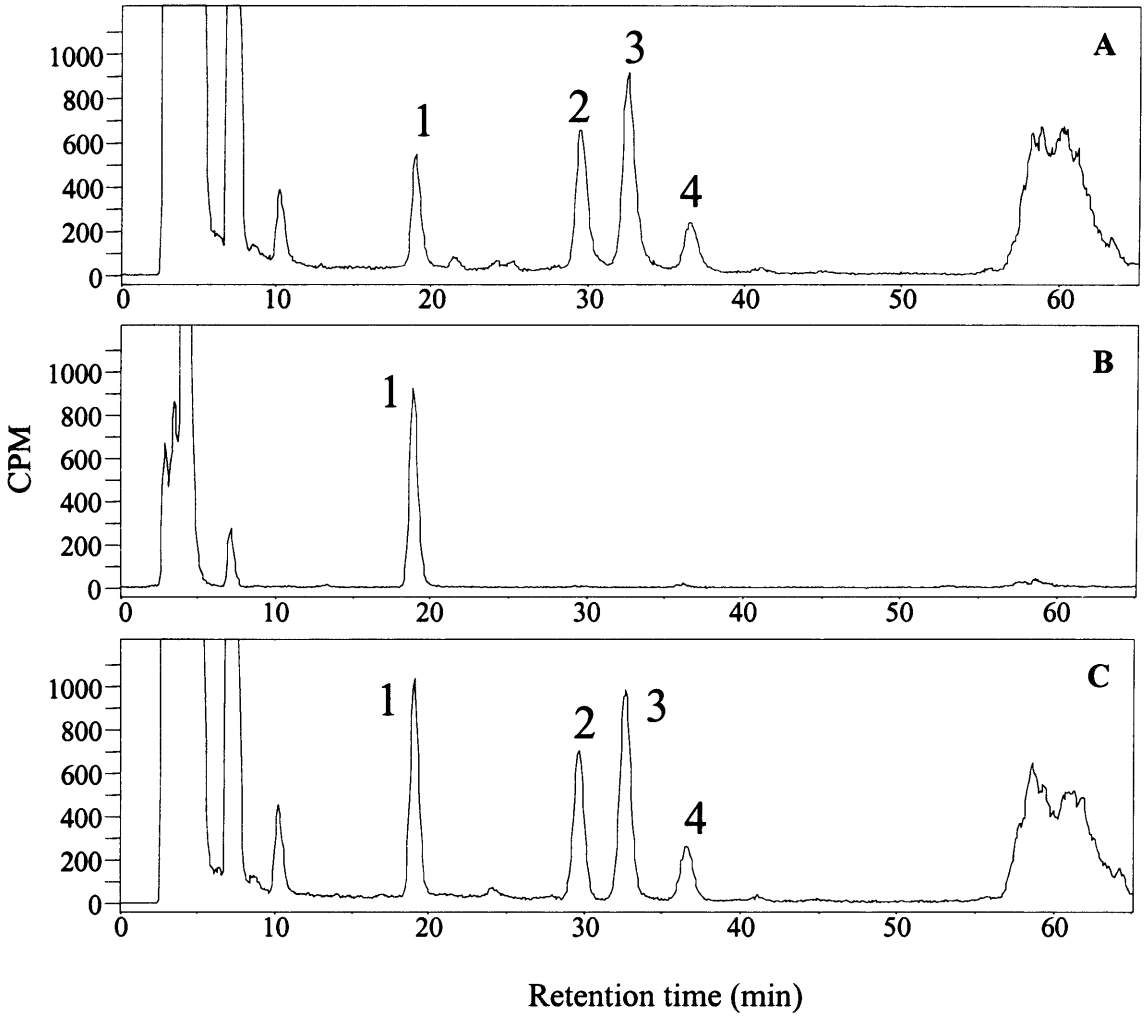


Figure 5.12: Typical HPLC chromatograms demonstrating adduct separation of ^{32}P-postlabelled *p*-BQ and HQ treated calf thymus DNA (**A**), the adduct formed *in vivo*, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (**B**) and co-elution of the two samples (**C**).
HPLC separation was performed using an isocratic system of 2 % acetonitrile in 2.0 M ammonium formate, pH 4.0 (0.27 mM EDTA).



5.6.8. ^{32}P-postlabelling of liver DNA from benzene treated mice

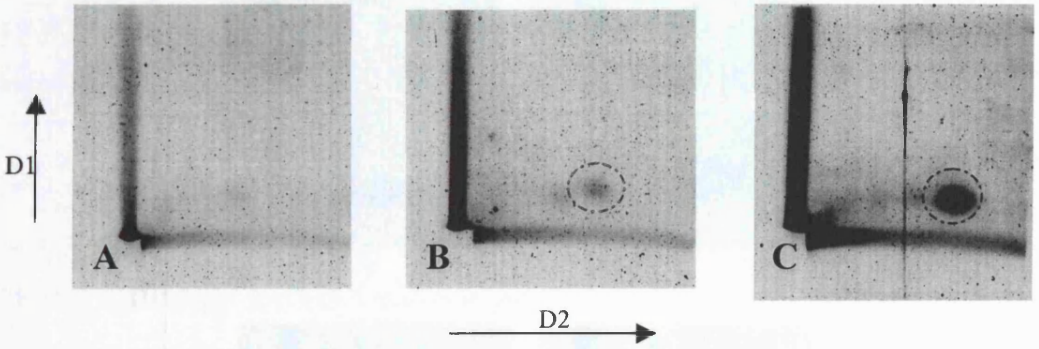
Liver DNA samples from benzene exposed p53W and 129svWT mice were analysed by ^{32}P-postlabelling coupled to the newly developed TLC method as detailed in Chapter 3. The animals were dosed by two different procedures; the p53W mice were dosed by inhalation (0 or 100 ppm) for 6 h/day, 5 days/week, for a total of 15 weeks. Each animal received a cumulative dose of 3000 ppm/week as described in Healey *et al.*, 2001 and the animals were sacrificed at 15 weeks. The 129svWT mice were dosed orally by gavage with 0, 0.2 or 1.0 mg benzene /g and the animals were sacrificed 24 h later. Typical TLC maps obtained from this experiment can be seen in Figures 5.13, A-B and 5.14, A-C, with comparison to the synthesised adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (Figures 5.13, C and 5.14, D).

In all the treated samples an adduct spot was apparent with similar mobility to the adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate. For the benzene exposed p53W mice the adduct level for this spot, ranged from 6.04 ± 3.68 to 12.19 ± 2.99 adducts / 10^8 nucleotides and for the 129svWT benzene exposed mice, adduct levels ranged from 6.86 ± 11.88 to 11.66 ± 4.64 and 4.59 ± 1.88 to 10.33 ± 3.19 adducts / 10^8 nucleotides for the animals dosed with 0.2 mg/g and 1.0 mg/g, respectively. Comparison to control samples for the p53W animals showed an increase in adduct levels in the treated DNA samples, however this increase was not significant, as shown in Figure 5.13, D.

Adduct levels in the 129svWT mice showed no overall increase when compared to control samples, probably a reflection in the dosing regime for these animals i.e. these animals received only a single dose of benzene.

Figure 5.13: Typical TLC maps of ^{32}P-postlabelled liver DNA from control p53W mice (**A**), p53W mice dosed by inhalation with 100 ppm benzene for 6 h/day, 5 days/week for 15 weeks (**B**) and co-elution of the liver DNA from p53W mice dosed by inhalation with 100 ppm benzene for 6 h/day, 5 days/week for 15 weeks with N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (**C**). Adduct of interest is highlighted by the dotted circle.
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).

Adduct numbers for the spot of interest / 10^8 nucleotides as determined by ^{32}P-postlabelling followed by TLC analysis for individual control (□) and benzene treated animals (100 ppm benzene for 6 h/day, 5 days /week for 15 weeks ■) (**D**). Data shown are mean ± SD (n=3).



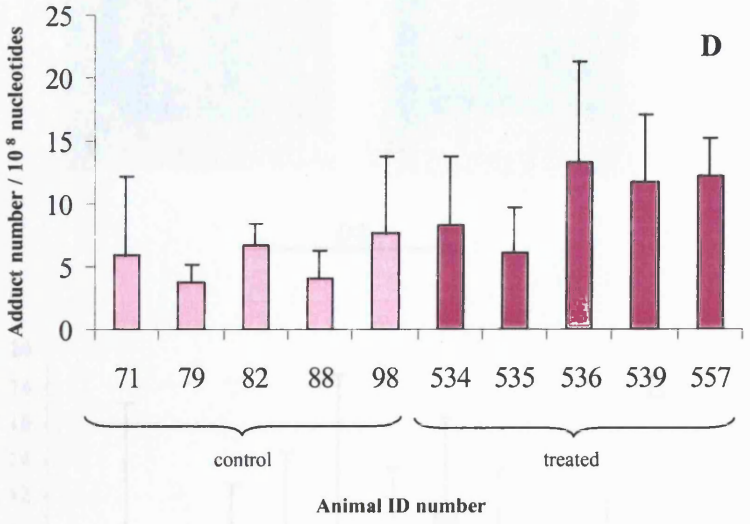
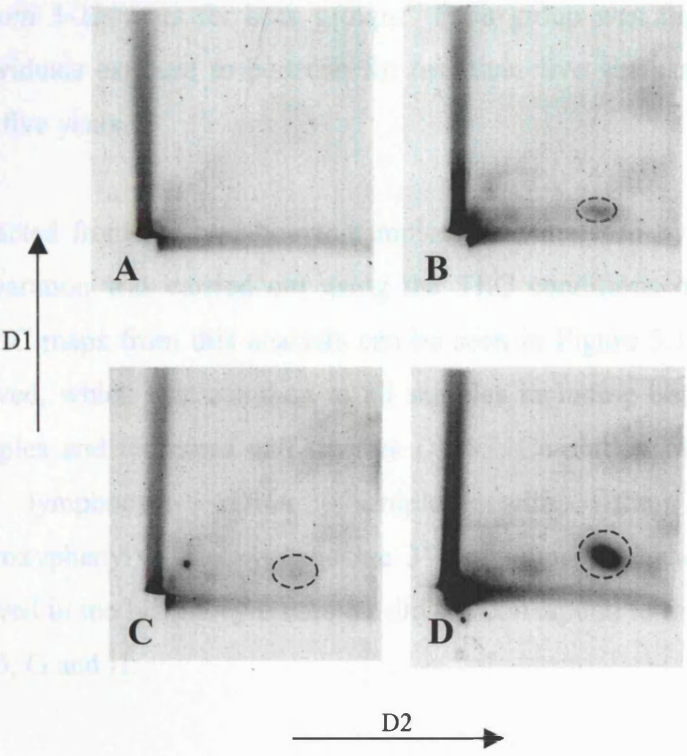
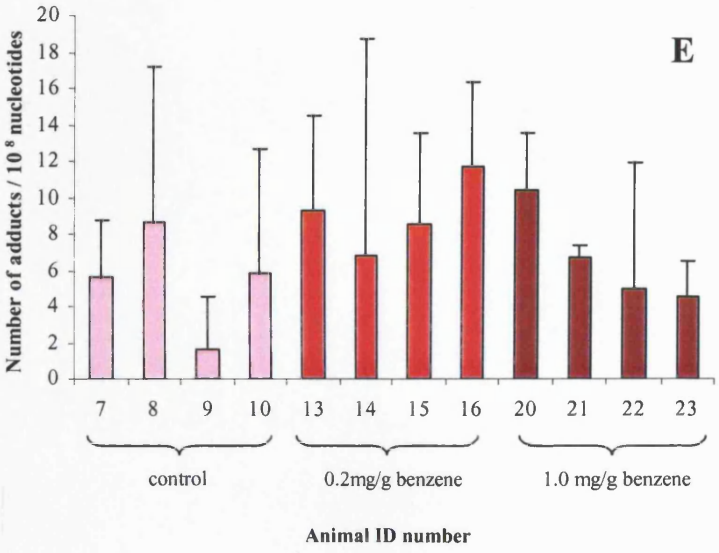


Figure 5.14: Typical TLC maps of ^{32}P-postlabelled liver DNA from control 129svWT mice (**A**), 129svWT mice dosed orally by gavage with 0.2 mg/g benzene (**B**) or 1.0 mg/g benzene (**C**) for one dose only and co-elution of liver DNA from 129svWT mice dosed with 1.0 mg/g benzene orally by gavage with the adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (**D**). Adduct of interest is highlighted by the dotted circle.
Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).

Adduct number for the spot of interest / 10^8 nucleotides as determined by ^{32}P-postlabelling followed by TLC analysis for individual control (□) and benzene treated animals (0.2 mg/g (■) or 1.0 mg/g (■)) (**D**). Data shown are mean ± SD (n=3).





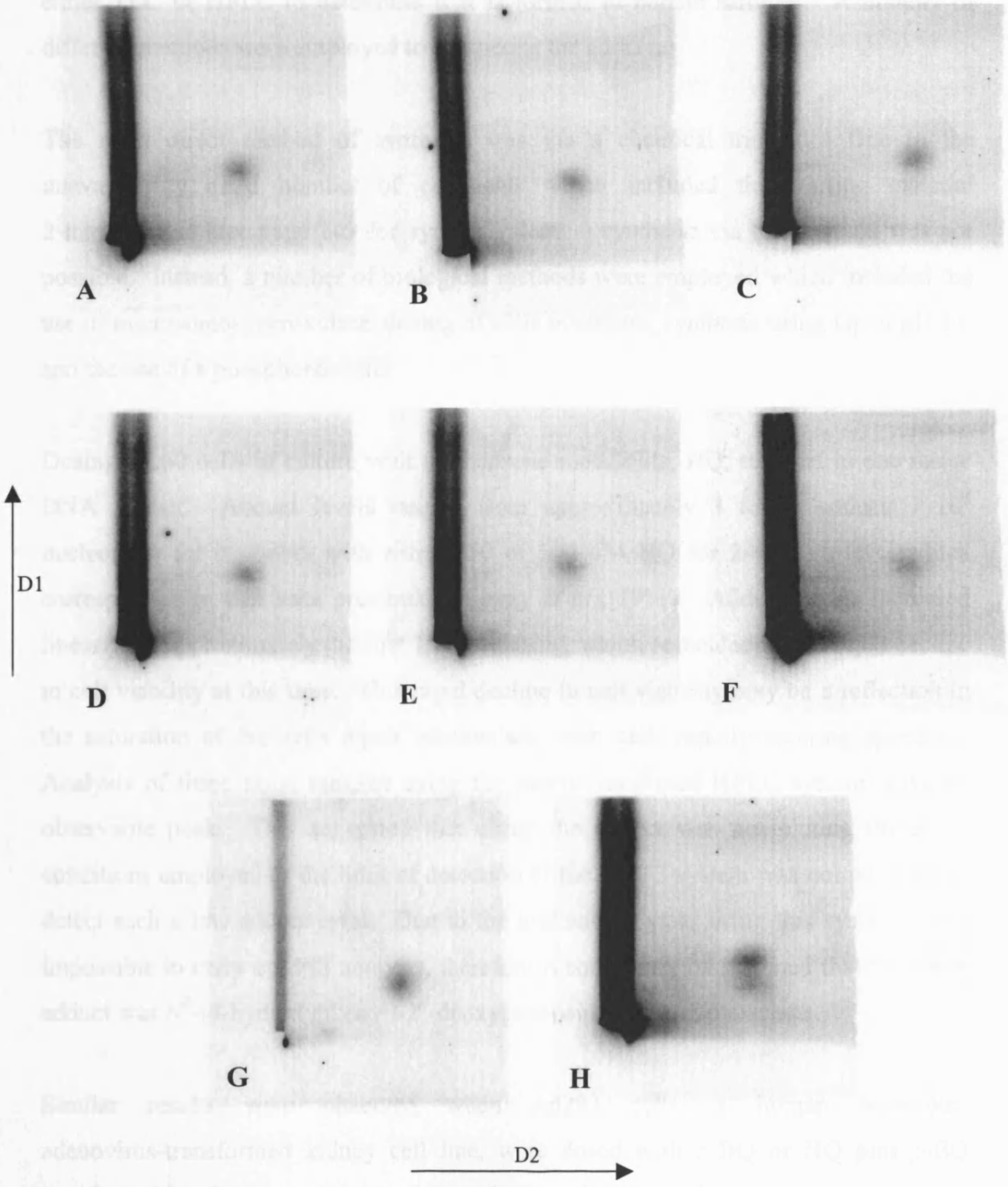
5.6.9. ^{32}P-postlabelling of lymphocyte DNA from occupationally exposed individuals

Lymphocyte samples were obtained from occupationally benzene-exposed smoking and non-smoking individuals from a petroleum refinery in Oporto, Portugal. Individuals were divided into two main groups; non-smokers and smokers. The age range and length of exposure were similar for both groups. The age range for non-smokers was 25-49, compared to 27 – 59 for smokers with the exposure duration ranging from 3-22 years for both groups. Each group was further sub-divided into those individuals exposed to benzene for less than five years and those exposed for more than five years.

DNA extracted from the lymphocyte samples were analysed by ^{32}P-postlabelling and adduct separation was carried out using the TLC conditions outlined in Chapter 3. Typical TLC maps from this analysis can be seen in Figure 5.15. One major adduct was observed, which was common to all samples including both control lymphocyte DNA samples and untreated calf thymus DNA. Co-elution of one typical benzene exposed lymphocyte DNA sample with the adduct standard, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate showed that the adduct spot observed in the lymphocyte samples did not correspond to this adduct, as shown in Figure 5.15, G and H.

Figure 5.15: Typical TLC maps of ^{32}P-postlabelled lymphocyte DNA from non-smoking individuals occupationally exposed to benzene for less than 5 yr (**A**), and more than 5 yr (**B**), smoking individuals occupationally exposed to benzene for less than 5 yr (**D**) and more than 5 yr (**E**), with comparison to calf thymus DNA control (**C**), control lymphocyte DNA from a smoking individual (**F**), and the synthesised adduct of interest, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (**G**). Co-elution was carried out with ^{32}P-postlabelled DNA from a benzene exposed individual and the adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (**H**).

Plates were developed for 3 h in 0.4 M sodium phosphate buffer, pH 6.8 (D1 direction) followed by a 4 h development in 1.7 M sodium phosphate, pH 6.0 (D2 direction).



5.7. Discussion

The main aim of this part of the work was to synthesise the major DNA adduct so far identified in the bone marrow DNA of rodents exposed to benzene. This adduct was identified and characterised as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (Pongracz and Bodell, 1996). Upon successful synthesis the adduct would be used as a standard in the newly developed ^{32}P-postlabelling assay coupled to either TLC or HPLC to determine if it is formed in human samples. A number of different methods were employed to synthesise the adduct.

The most direct method of synthesis was via a chemical method. Due to the unavailability of a number of chemicals which included the starting material 2-thioxanthine (see page 149 for synthesis details) synthesis via this method was not possible. Instead, a number of biological methods were employed which included the use of microsomes, peroxidase, dosing of cells in culture, synthesis using Gp at pH 1.0 and the use of a phosphoramidite.

Dosing HL60 cells in culture with the benzene metabolite, HQ, resulted in one major DNA adduct. Adduct levels ranged from approximately 3 to 73 adducts / 10^8 nucleotides for treatment with either 250 or 500 μM HQ for 2-48 h, a level which corresponded to that seen previously (Levay *et al.*, 1991). Adduct levels increased linearly for approximately the first 10 h of dosing, which coincided with a rapid decline in cell viability at this time. This rapid decline in cell viability may be a reflection in the saturation of the cells repair mechanism, with cells rapidly entering apoptosis. Analysis of these same samples using the newly developed HPLC system, gave no observable peak. This suggested that either the adduct was not eluting under the conditions employed or the limit of detection of the HPLC system was not adequate to detect such a low adduct level. Due to the low adduct yield using this system it was impossible to carry out MS analysis, therefore it could only be assumed that the major adduct was N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate.

Similar results were observed when Ad293 cells, a human embryonic adenovirus-transformed kidney cell line, were dosed with *p*-BQ or HQ plus *p*-BQ together. No adduct was observed in cells treated with the benzene metabolite, HQ,

due to the lack of peroxidase enzyme in this cell line. Peroxidase has been identified as the enzyme responsible for oxidising HQ to the more electrophilic metabolite, *p*-BQ, via a semi-quinone intermediate (Snyder *et al.*, 1993a). HL60 cells contain myeloperoxidase, hence the same adduct is formed when dosing with either HQ or *p*-BQ (Levay *et al.*, 1991). Similar results as to those seen here in the Ad293 cells, were observed by Levay *et al.*, 1993, in U-937 and Raji cells, two lymphoblastoid cell lines, which are deficient in peroxidase enzymes. The adduct level obtained in the Ad293 cell line was twice that reported for the HL60 cells. This higher level of adducts may be due to differences between the cells in regard to their repair mechanisms or may be a reflection of the metabolite used. Previous work with HL60 cells required a higher concentration and longer treatment time when dosing was carried out with HQ to achieve the same adduct number as reported in cells dosed with *p*-BQ (Levay *et al.*, 1991). However, as for the adduct observed in HL60 cells, without a fully characterised adduct standard it was difficult to determine whether the adduct observed was N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate.

Other biological systems employed included the use of peroxidase enzymes and microsomes prepared from the livers of C57Bl6 mice. Microsomes contain cytochrome P450 enzymes, including cytochrome P450 2E1, an enzyme involved in the metabolism of benzene. Samples generated using either system gave identical TLC maps to control samples. Both systems contained a high level of protein, hence reactive species formed may react directly with the protein rather than the DNA. Benzene metabolites form 9 to 43 fold greater protein adducts than DNA adducts (Robertson Creek *et al.*, 1997).

To determine whether the additional adduct seen in treated cells (compared to controls) was in fact N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate a different approach was required which would produce the adduct in sufficient yields to allow full identification and characterisation. Reaction of GMP with *p*-BQ at pH 1.0 has previously been reported to produce N^2-(4-hydroxyphenyl)-guanosine 3'-monophosphate (Pongracz and Bodell, 1996). This adduct has similar mobility on TLC to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (Pongracz and Bodell, 1996). Repeat of this work gave two products, which had similar but not identical retention times to the two adducts, (3"-hydroxy)-1,

N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate when separated by HPLC. This difference in retention was probably due to the extra hydroxyl group present on the Gp adducts. MS analysis identified one of the products as (3"-hydroxy)-1, N^2-benzetheno-guanosine 3'-monophosphate. The second product was not identified, but was assumed to be (3", 4"-dihydroxy)-1, N^2-benzetheno-guanosine 3'-monophosphate based on the retention times observed.

Strategies have been previously published which have used halogenated 2'-deoxyinosine phosphoramidites to synthesise adducts within oligonucleotides. This procedure avoids the need to carry out complex strategies for protection of adducted nucleotides prior to their incorporation into oligonucleotides. The hypothesis behind the procedure employed here involves a reversion of the natural polarity of the reaction. Under normal reaction conditions the heterocyclic base acts as the nucleophilic species whereas the adducting moiety is the electrophile, here this is reversed (Harris *et al.*, 1991). The reaction employed relies on the displacement of the halogen for an amino derivative of the carcinogen of interest, a technique that has previously been used by others (Decorte *et al.*, 1996 and Harris *et al.*, 1991).

2-Fluorodeoxyinosine was purchased as a phosphoramidite (fluorine is more easily displaced than chlorine or bromine (Harris *et al.*, 1991)) and was incorporated into a number of short oligonucleotides and also attached directly to the support matrix containing 3'-phosphate. Reaction of the incorporated phosphoramidite with 4-hydroxyaniline theoretically would displace the fluorine atom producing the N^2-(4-hydroxyphenyl) adduct. Reactions were carried out whilst the oligonucleotides or deoxynucleotide were still attached to the support matrix. Ammonium hydroxide which was used to deprotect and cleave the products in the synthesiser had the potential to displace the halogen atom producing 2'-deoxyguanosine 3'-monophosphate. Therefore the adducted oligonucleotides or deoxynucleotide required manual deprotection and cleavage from the support matrix, which was an added complication to the procedures.

Reaction with the amine, 4-hydroxyaniline, with the standard cleavage and deprotection steps as recommended by Glen Research (Sterling, Virginia, USA), resulted in no observable products when analysed by HPLC with UV detection. Analysis of the reaction supernatant however, gave a number of products, demonstrating that the amine not only produced the adduct but also cleaved and deprotected the oligonucleotide (or deoxynucleotide). The extent to which this was occurring and to what level deprotection was taking place was difficult to determine. Two products from the reaction supernatant gave significant peaks when analysed by ^{32}P-postlabelling and separated by HPLC. These products had similar retention times to the two adducts, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. The products were not available in sufficient quantity to allow MS analysis, so again it was difficult to conclude if N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate had successfully been synthesised.

Due to the problems associated with the synthesis of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, Dr. William Bodell of the University of California, San Francisco, USA (who originally identified and synthesised this adduct) kindly supplied some of the adduct. ^{32}P-postlabelling of this adduct and analysis by both TLC and HPLC gave an adduct spot (TLC) or peak (HPLC) which had similar mobility and retention time to the novel adduct, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Co-elution studies confirmed that both adducts migrated together on both TLC and HPLC. Modification of the HPLC conditions employed failed to separate the two adducts. Whether this was because one adduct was the product of the other, for example during the postlabelling procedure the N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was cyclising and undergoing further oxidation or whether the two adducts were different but with identical chromatographic properties is not known.

Using the synthesised adduct it was now possible to confirm that the major DNA adduct observed in HQ treated HL60 cells and *p*-BQ treated Ad293 cells did co-elute with N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate. One of the products synthesised in the GMP and phosphoramidite reactions was also identified as

having a similar retention time to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate. However, with the added complication of (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate co-eluting with N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate it cannot be assumed at this stage that the adduct observed in each reaction mechanism employed was N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, without further clarification by MS analysis.

Liver DNA from rodents exposed to benzene either by inhalation for 15 weeks with a cumulative dose of 3000 ppm/week as described by Healey *et al.*, 2001 or a single oral dose of 0, 0.2 or 1.0 mg benzene /g was analysed by ^{32}P-postlabelling. Adduct determination was carried out by TLC, due to its greater sensitivity over HPLC. One adduct spot was observed in benzene treated samples which had similar chromatographic properties to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate. Adduct levels were however extremely low and were not significantly different to control samples. Adduct levels ranged from approximately 4.5 – 12 adducts / 10^8 nucleotides for both groups of animals.

Previous work has reported no adduct formation when benzene was administered as a single dose to rodents (Bodell *et al.*, 1996; Levay *et al.*, 1996; Pathak *et al.*, 1995 and Reddy *et al.*, 1994). Adducts were only observed when benzene was administered twice daily (Bodell *et al.*, 1996; Levay *et al.*, 1996 and Pathak *et al.*, 1995). The difference between these studies and that carried out in our laboratory could be due to a number of factors. Previously published studies analysed DNA from the target tissue; bone marrow and white blood cells, the study carried out here was performed using liver DNA, a non-target tissue for benzene genotoxicity. Adduct differences could be due to the time taken for bio-accumulation of the reactive benzene metabolites in the tissues examined and due to the timing of sample collection. Studies carried out using the sensitive method of AMS determined that the adduct levels in the liver of rodents peaked within 30 min of exposure whereas in the bone marrow, adduct levels did not peak until 12-24 h after exposure (Robertson Creek *et al.*, 1997).

Analysis of DNA from the lymphocytes of workers in a petroleum refinery gave only one spot following TLC. This spot was thought to be formed from either endogenous processes or as a product of the postlabelling procedure. For example, products extracted from the eppendorf tubes used during the postlabelling procedure have been shown to be substrates for ^{32}P-labelling. The radioactive spot was common to both exposed and control workers and also the calf thymus DNA control sample. No adduct with similar chromatographic properties to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate was observed.

Lack of adduct formation detected in the human samples could be due to a number of factors. The fact that lymphocytes do not contain peroxidase may suggest that they are not the ideal surrogate tissue for the analysis of benzene DNA adducts. Neutrophils, which contain myeloperoxidase would be a more ideal surrogate tissue (Bodell *et al.*, 1993). Due to various moral and medical issues it is not advisable or allowed to sample the target tissue, bone marrow. Although a precise exposure level could not be determined, it was estimated that the volunteers were exposed to less than 2 ppm benzene, which is less than the present occupational exposure limit. This level could be below the threshold required for adduct formation. In the Chinese cohort study, protein adducts, which are formed in far greater quantity than DNA adducts (Robertson Creek *et al.*, 1997), were observed at all monitored doses, however a significant increase was observed at doses greater than 30 ppm (Yeowell –O' Connell *et al.*, 1998 and Yeowell –O' Connell *et al.*, 2001).

Other factors which may play a role in benzene genotoxicity in individuals include the age, sex and genetic makeup of the individuals monitored and exogenous factors which include lifestyle habits such as smoking and drinking. The reduced sensitivity of the ^{32}P-postlabelling assay developed here may also play a role in lack of adduct detection. Successful production of an antibody with specificity for the N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate adduct should improve the sensitivity of the assay. The antibody could be used in a purification step prior to ^{32}P-postlabelling.

Exposure of humans to low environmentally relevant doses and the distribution of the dose within the body could be monitored by the sensitive method of AMS. This procedure can determine the presence of DNA adducts at levels as low as 1-10 adducts/ 10^{12} nucleotides (Turteltaub *et al.*, 1993). However, this method requires the administration of radiolabelled benzene, which has a number of ethical issues to be considered in regard to the radioactivity administered, the fact that benzene is a classified human carcinogen, the route of exposure and the target tissue of benzene genotoxicity. AMS would hopefully answer the question as to whether DNA adducts are formed in tissues, in particular the bone marrow, of individuals exposed to benzene either occupationally or environmentally.

Chapter 6

The *supF* Assay

6.1. Introduction

Due to the efficient repair processes present in a cell, a mutation in DNA is a rare event. Mutations arise either spontaneously involving error prone polymerases during replication or by depurination, deamination or depyrimidation or by the direct action of endogenous or exogenous mutagens which give rise to DNA adducts. If a mutation is not successfully repaired, upon replication the mutation can become heritable and if present in a critical gene, may play a role in the early stages of carcinogenesis (Hemminki *et al.*, 2000 and Essigmann and Wood, 1993).

A DNA adduct, if not excised from DNA by efficient repair systems can affect several processes within the cell, which include both transcription and replication. During transcription of an active gene the presence of a DNA adduct can cause polymerase arrest, which hinders transcription and hence prevents the formation of fully functional proteins. During replication, a DNA adduct can cause slippage by DNA polymerase giving rise to deletions or lead to the insertion of an incorrect DNA base. If the mutation fails to be repaired it can become fixed during successive rounds of replication (Hemminki *et al.*, 2000). A mutation in a critical gene can affect the activity and function of crucial proteins, activate oncogenes and inactivate tumour suppressor genes (La and Swenberg, 1996). A number of factors exist which affect an adduct's ability to induce a mutation, these include orientation of the modifying group in respect to the base pairing strand, conformation and size of the lesion, sequence context and polymerase specificity (Singer, 1996).

6.1.1. Mutation types

A number of mutation types exist, these include point mutations which result from the substitution of one base pair for another, or the deletion/addition of a small number of base pairs which can affect for example, the function of a particular protein. On a larger scale, insertions, deletions, duplications and inversions can occur within any sequence of DNA and can encompass many genes. Such large-scale mutations brought about by nicks, gaps and double strand breaks can cause DNA rearrangement between chromosomes and even loss or duplication of part or whole chromosomes.

Point mutations most commonly result from exposure to a mutagenic chemical or physical agent (Friedberg *et al.*, 1995) and can be further sub-divided into base substitution mutations and frameshift mutations. Base substitution mutations include transition (purine to purine or pyrimidine to pyrimidine) or transversion mutations (purine to pyrimidine and vice versa) and can occur anywhere within the genome. Base substitution mutations which occur within the protein coding region of a gene are classed due to their phenotypic outcome as either being missense mutations (the change of a codon for one amino acid to another, which can have a wide range of effects on protein function), or nonsense mutations (change of an amino acid codon for a stop codon, which terminates protein synthesis prematurely). A change of one codon for another, which specifies the same amino acid is referred to as a silent mutation and is unlikely to affect the behaviour of the cell.

Frameshift mutations (loss or gain of one or more base pairs) can occur due to a number of factors, which include the DNA sequence context (normally occurring in repeat regions (Strauss, 2002), imbalance of the nucleotide pool or editing function of the DNA polymerase (Shibutani and Grollman, 1993). Frameshift mutations are caused by slippage of the primer or template strand during stalled replication. On slippage back to the original alignment, what was a normal base pair can now be a mispair resulting in a potential non-functional protein.

6.1.2. DNA repair

Repair of DNA lesions was first described in 1949 by Kelner and has been extensively studied since. In mammalian cells a number of repair mechanisms exist, these include base excision repair (BER), mismatch repair, direct reversal, recombinational repair and nucleotide excision repair (NER) (Bohr, 1995). The repair of DNA adducts is dependent on a number of factors which include the location of the lesion within the DNA sequence. Adducts in a coding region of DNA or on the transcribed strand of DNA are generally repaired faster than adducts in a non-coding region of DNA or on a non-transcribed strand (Hemminki, 1993 and van Zeeland *et al.*, 2001), hence most mutations generally occur in non- coding regions of DNA (Friedberg *et al.*, 1995).

Two main repair mechanisms exist for DNA lesions in mammalian cells, these are base excision repair and nucleotide excision repair and will be discussed in further detail below.

6.1.2.1. Base excision repair

Base excision repair (BER) of a DNA lesion is a process initiated by a class of enzymes called glycosylases which can excise quite specific DNA lesions, for example oxidised or methylated guanines. Glycosylases excise the damaged base by cleavage of the N-glycosyl bond, which results in another type of DNA damage known as apurinic or apyrimidinic (AP) sites. The AP sites are excised using AP endonucleases, which hydrolyse the phosphodiester bond immediately 5' or 3' to the AP site. Excision 5' to the site generates a 5'-deoxyribose-phosphate residue which can be removed by exonucleases. The gap is then filled with the correct base as determined by the opposite DNA strand and repair is completed by DNA ligase. Mismatch repair is a form of base excision repair but instead of repairing damaged bases, mispaired bases are excised and replaced (Friedberg *et al.*, 1995).

6.1.2.2. Nucleotide excision repair

Nucleotide excision repair (NER) is more complex and has much wider specificity than BER as it can remove mismatches, UV induced pyrimidine dimers and bulky adducts caused by exposure to carcinogenic/mutagenic agents. In mammalian cells, NER involves five main processes; recognition, incision, degradation, polymerisation and ligation and involves at least 17 different proteins (Bohr, 1995). Since NER takes time, cell cycle progression is delayed, a process mediated by p53, a tumour suppressor gene. As part of the incision process a number of proteins are involved which are referred to as the XP complement. The initial recognition step involves XPA, XPF and ERCC1 proteins followed by interaction with the general transcription factor, TFIIH. TFIIH, a protein complex, contains the two repair gene products, XPB and XPD and XPC and XPG. The XPG and ERCC1-XPF proteins are involved in the excision process, cutting 3' and 5' of the lesion, respectively, by hydrolysis of two phosphodiester bonds, thus creating an oligonucleotide fragment containing the lesion. The incision pattern is precise, with XPG cutting at 2-9 phosphodiester bonds 3' of the lesion, for both prokaryotes and eukaryotes. The second incision occurs 16- 25 phosphodiester bonds

5' of the lesion (Batty and Wood, 2000). The resulting single strand gap is then filled by DNA polymerase and joined by DNA ligase. One consequence of this enzyme system is that mismatched nucleotides can be excised. Unlike the mismatch repair system however, which can differentiate between the right and wrong strand, this system can excise the mismatched base from either strand hence leading to mutation fixation rather than avoidance.

6.1.2.3. Diseases associated with NER

In humans there are three diseases that are associated with nucleotide excision repair: xeroderma pigmentosum (*XP*), Cockayne's syndrome (CS) and trichothiodystrophy (TTD). *XP* patients have a predisposition to skin cancer when exposed to sunlight brought about by a defect in the initial incision step of NER (Bohr, 1995). There have been seven different complementation groups identified in *XP* patients each containing a different mutation in a different gene that are characterised by varying levels of sensitivity and deficiencies in repair. For example, *XP* cells, complementation group A, are unable to make an endonucleolytic incision in DNA (Seidman *et al.*, 1987). CS and TTD patients are not predisposed to skin cancer as in *XP* patients, but do suffer developmental problems, which include for CS patients, growth and mental retardation and photosensitivity. TTD patients also suffer mental retardation and brittle hair and neuroskeletal anomalies (Hanawalt, 1994 and Sancar, 1994). CS and TTD patients are defective in the preferential repair of transcriptionally active genes, which involves mutations in the XPB/ERCC6, XPD and XPG genes (Friedberg *et al.*, 1995 and Sancar, 1994).

6.1.3. Mutation assays

A number of assays exist which allow the detection and analysis of mutations, which arise as a consequence of exposure to a particular mutagenic agent and involve the use of prokaryotic or eukaryotic cells or whole animal systems. Mutations that revert a mutant phenotype to wild type or wild type phenotype to mutant are utilised in reversion and forward mutation assays, respectively. One example of a reversion mutation assay is the Ames test, which relies on the reversion of histidine auxotrophs of *S. typhimurium*. Genotoxic agents capable of reversing the mutation allow growth on histidine containing plates whereas mutants are incapable of growth.

Forward mutation assays use specific genes (reporter genes) as a target for mutagenesis. *In vitro* forward mutation assays include the hypoxanthine guanine phosphoribosyl transferase (*HPRT*) assay, the mouse lymphoma assay, the *lacI* system and the *supF* assay. The *HPRT* assay is routinely used in the initial assessment of genotoxicity of a test compound. V79 Chinese hamster cells have one functional copy of the gene, encoding the *HPRT* enzyme, an enzyme important for DNA synthesis. 6-Thioguanine, a toxic nucleoside, forms the basis of cell selection. Cells containing a mutant *HPRT* gene are resistant to 6-thioguanine and hence grow in selective medium. The mouse lymphoma assay uses the specific gene, thymidine kinase, which phosphorylates thymidine to thymidine 3'-monophosphate. A mutation in the thymidine kinase gene confers resistance to the cytotoxic effects of certain pyrimidine analogues, for example trifluorothymidine. The *lacI* system allows the analysis of nonsense mutations in the *lacI* gene of *E. coli*. The *lacI* gene codes for the repressor of the *lac* operon, which in turn codes for the proteins required for lactose metabolism. Mutants, having lost *lacI* function can therefore grow on the lactose analogue, phenyl-β-galactoside, allowing mutant selection (Friedberg *et al.*, 1995). The *supF* assay incorporates the use of a shuttle vector capable of replication in both eukaryotic and prokaryotic cells. The *supF* assay is the method of choice in this laboratory and is discussed in more detail in section 6.1.3.1.

The forward mutation assay has also been applied *in vivo,* in whole animal systems. These systems include transgenic animals that carry a bacterial gene, i.e. the *lacZ* or *lacI* gene, in all cells of their body. Following exposure to a mutagenic agent, the *lacZ* or *lacI* gene can be recovered and screened in *E. coli*, allowing direct analysis of mutagenesis. The *lacI* gene system has been used effectively to examine the mutations induced following exposure to tamoxifen (Davies *et al.*, 1997 and 1999).

6.1.3.1. Shuttle vector plasmids - The *supF* assay

Shuttle vector plasmids capable of replicating in both mammalian and bacterial cells are powerful tools for the study of mutations induced in eukaryotic cells after exposure to mutagenic agents (Seidman *et al.*, 1985). Such vectors consist of DNA sequences that allow both replication in mammalian cells and selection using bacterial cells, and a

target sequence of DNA, used for the monitoring of mutagenesis (Kraemer and Seidman, 1989).

The shuttle vector plasmid used here is pSP189, the structure of which can be seen in Figure 6.1, A. This vector contains the origin of replication and early gene region from SV40 virus and the origin of replication and drug resistance marker (ampicillin) from bacteria, which allow replication in both mammalian cells and bacteria, respectively (Seidman *et al.*, 1985 and Seidman, 1996). The reporter (target) gene in this vector is an *Escherichia coli* tyrosine amber suppressor tRNA gene, *supF,* which has been used as a mutagenic target in several shuttle vector plasmids and has been shown to be a sensitive target for mutagenic inactivation. The *supF* gene consists of a 35 base pair promoter, a 40 base pair pre-tRNA region, which has a function in RNA processing, an 85 base pair tRNA sequence and a short 17 base pair 3'-flanking sequence (Kraemer and Seidman, 1989). The sequence of the *supF* gene and its hypothetical secondary structure of a single-stranded DNA containing the *supF* tRNA gene sequence can be seen in Figures 6.1, B and C, respectively.

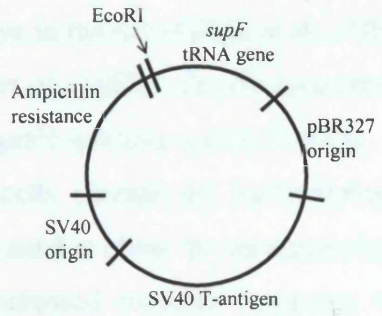
Upon transfection of a mutagen treated plasmid into mammalian cells, mutagenesis occurs within the first few rounds of replication and the resultant products are then amplified in subsequent rounds. Mutagen induced lesions within the plasmid are repaired by the cells own repair and replication system, hence alterations induced in the plasmid reflect those that would occur in the cells own genome. Recovered plasmid containing a potentially mutated *supF* gene can then be screened using an indicator strain of bacteria. The *E. coli,* bacterial strain, most commonly utilised, MBM7070, possesses an amber mutation in the *lacZ* gene, which codes for β-galactosidase. Transformation of *E. coli* with plasmid containing an active *supF* gene will suppress the *lacZ* amber mutation resulting in the synthesis of *β*-galactosidase. When grown on indicator agar containing isopropyl *β*-D-thiogalactoside (IPTG), an inducer of *β*-galactosidase and the indole derivative, 5-bromo-4-chloro-3-indolyl-*β*-D-galactoside (X-gal), which contains a *β*-galactoside linkage, only transformed bacteria will grow. Bacterial colonies containing a functional *supF* gene are capable of producing the enzyme, *β*-galactosidase, which in turn can cleave the *β*-galactoside linkage hence producing blue colonies. Colonies containing an inactive or partially active gene i.e. a

mutated gene are white or pale blue, respectively (Kraemer and Seidman, 1989). White colonies can then be selected for analysis of mutation spectra, made possible by the small size of the target gene. Only 1 base substitution is required to inactivate *supF* function.

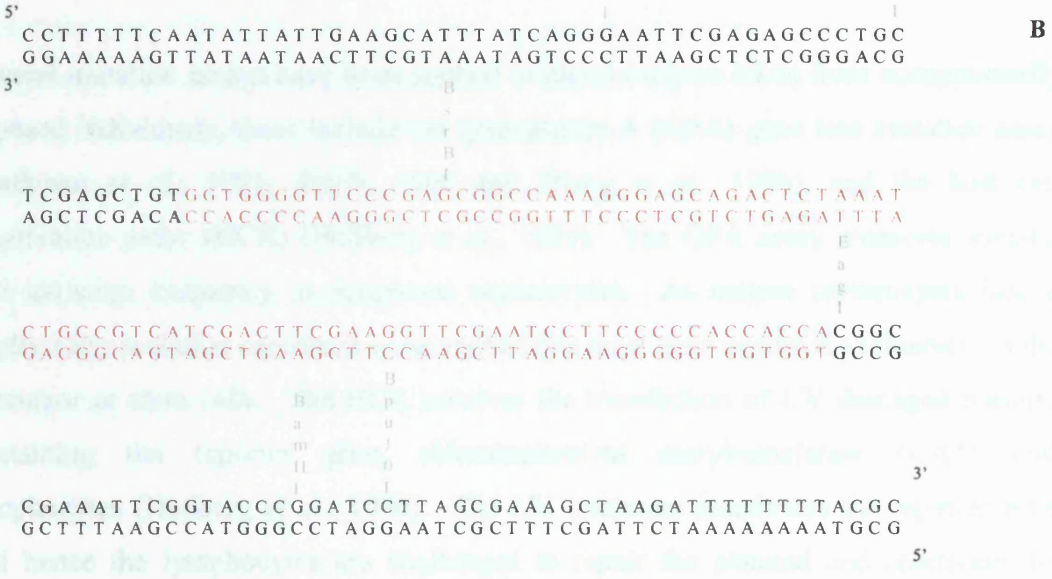
Identical mutations could represent individual mutational events or siblings of an initial event. To distinguish between the two possibilities, pSP189 contains a unique 8 base pair 'signature sequence' downstream of the *supF* target gene (Parris and Seidman, 1992), which has 4^8 (65536) sequence possibilities (Routledge *et al.*, 2001). The design of the plasmid and the small size of the target gene, decreases the probability of recovering spontaneous deletion mutations which occur at high frequency upon passage into mammalian cells (Seidman *et al.*, 1985 and Seidman, 1989).

The *supF* assay has been used in the determination of mutants induced by ultraviolet radiation (Routledge *et al.*, 2001; Seetharam and Seidman, 1991 and Seidman *et al.*, 1987) and a number of chemicals which include nitric oxide (Routledge *et al.*, 1993), nitrite (Routledge *et al.*, 1994), benzo[*a*]pyrene (Courtemanche and Anderson, 1999 and Routledge *et al.*, 2001), crotonaldehyde (Kawanishi *et al.*, 1998c), methyl-nitrosourea (MNU) (Sikpi *et al.*, 1990), acrolein (Kawanishi *et al.*, 1998b) 3-nitrobenzanthrone (Kawanishi *et al.*, 1998a), tamoxifen (McLuckie *et al.*, 2002) and aflatoxin (Courtemanche and Anderson, 1999), to name but a few examples.

Figure 6.1: Structure of the pSP189 shuttle vector plasmid as described by Parris and Seidman, 1992 (**A**). The plasmid contains origins of replication to allow replication in both mammalian and bacterial cells (SV40 and pBR322 origin), an ampicillin resistance gene, the SV40 T-antigen, which is necessary to activate the SV40 origin and the target gene for mutagenesis, *supF*. The sequence of the *supF* gene is shown in red (**B**) and its hypothetical secondary structure of single stranded DNA containing the *supF* tRNA gene sequence (**C**) are shown.

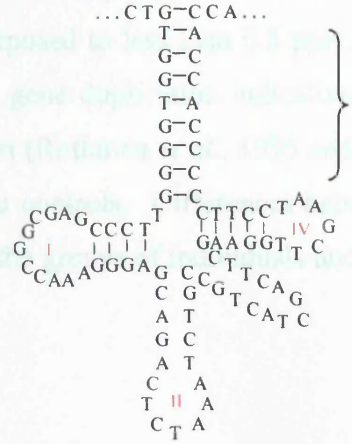


A



B

Hypothetical secondary structure of a single strand DNA containing the *SupF* tRNA gene sequence (modified from Kraemer and Seidman, 1989).



C

6.1.4. Benzene mutagenicity

Benzene and its metabolites have often been the subject of a number of mutation assays and have been shown to be weakly or non-mutagenic in most simple, short term, *in vitro* gene mutation assays in bacteria (Glatt *et al.*, 1989 and Martinez *et al.*, 2000) and mammalian cells (Glatt *et al.*, 1989). *In vivo* however, benzene has been shown to be mutagenic both in transgenic animals and in humans. Mutagenicity tests using whole animal systems whose cells contain the bacteriophage lambda, *lacI* transgene as a reporter gene, have been used to show the mutagenicity of inhaled benzene in a number of mouse tissues. An increased mutation frequency was observed in lung and spleen tissue, but not liver (Mullin *et al.*, 1995 and Mullin *et al.*, 1998).

Several mutation assays have been applied to blood samples taken from occupationally exposed individuals, these include the glycophorin A (GPA) gene loss mutation assay (Rothman *et al.*, 1995; Smith, 1996 and Zhang *et al.*, 1996), and the host cell reactivation assay (HCR) (Hallberg *et al.*, 1996). The GPA assay measures somatic cell mutation frequency in peripheral erythrocytes. As mature erythrocytes lack a nucleus any mutation expressed in an erythrocyte must have occurred exclusively in the precursor or stem cells. The HCR involves the transfection of UV damaged plasmid containing the reporter gene, chloramphenicol acetyltransferase (CAT) into lymphocytes (Hallberg *et al.*, 1996). The UV treatment inactivates the reporter gene and hence the lymphocytes are challenged to repair the plasmid and reactivate the reporter gene, resulting in the production of a detectable protein.

Both assays were applied to individuals occupationally exposed to benzene. The GPA assay was applied to heavily exposed individuals in Shanghai (mean level of 72.2 ppm), whereas the HCR assay was used in the analysis of samples from petrochemical workers in Texas, exposed to less than 0.3 ppm. In the GPA assay benzene exposure was shown to cause gene duplication indicative of recombination mechanisms rather than gene inactivation (Rothman *et al.*, 1995 and Smith, 1996) whereas the HCR assay gave no difference to controls. Differences between results could be due to exposure differences between the groups of individuals under investigation.

6.2. Aim

The benzene metabolite, *p*-BQ has often been associated with the toxic effects observed following benzene exposure and has been shown in an *in vitro* situation to produce four main DNA adducts as detailed in Chapter 2. Addition of HQ to this reaction has no overall significant effect on adduct production, but alone produces the single minor dGp adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Work carried out by others, for example with alkylating agents and vinyl chloride (reviewed in La and Swenberg, 1996) has shown that the minor adducts are responsible for the mutagenicity observed.

The main aim of this part of the work was to determine the mutagenic relevance of benzene-DNA adducts formed from the benzene metabolites, HQ and *p*-BQ as observed in mammalian cells. By the use of repair deficient cells it was also hoped to determine the mechanism of repair for these adduct types.

Overall, by observing mutagenesis caused by benzene-DNA adducts it was hoped that a role for benzene-DNA adducts in the development of leukaemia in occupationally exposed individuals could be proposed.

6.3. Materials

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Melford Laboratories Ltd (Ipswich, Suffolk). Fugene transfection reagent was obtained from Roche (Lewes, East Sussex). Cell culture medium, glutamax and foetal calf serum (FCS) were obtained from Life Technologies (Paisley, UK). Plasmid and genomic DNA extraction kits were purchased from Qiagen (Crawley, West Sussex). The Restriction enzyme, DpnI and NEBuffers were obtained from New England Biolabs (Hitchin, Hertfordshire).

All other chemicals and materials were purchased from either Sigma (Poole, Dorset) or Fisher (Loughborough, Leicestershire) and were of the highest grade available.

6.3.1. Cell lines

HL60 cells (a human promyelocytic cell line) were purchased from the European Collection of Cell Cultures (ECACC), Centre for Applied Microbiology and Research, Salisbury, Wiltshire. Human embryonic adenovirus-transformed kidney cells (Ad293 cells) were a kind gift from the late Dr. A. Dipple, National Cancer Institute, Frederick, MD, USA. Normal human SV40 transformed fibroblast cells (GM00637) and SV40 transformed, *Xeroderma pigmentosum* fibroblast cells, complementation group A (GM04429) were purchased from NIGMS Human Genetic Cell Repository, Camden, NJ, USA.

The plasmid, pSP189 (generated from pS189 by the inclusion of a randomly generated 8 bp signature sequence 3' of the *supF* gene (Parris and Seidman, 1992), providing a possible 4^8 (65536) sequence possibilities (Routledge *et al.*, 2001)) containing the *supF* gene and the *E. coli* strain, MBM7070 (which carries a *lacZ* amber mutation) were gifts from Dr. Michael Seidman, National Institute of Aging, NIH, Baltimore, MD, USA.

6.4. Methods

6.4.1. Cell culture

HL60 cells, a human promyelocytic cell line, were grown in RPMI medium supplemented with 10 % FCS and 1 % glutamax at 37 °C, 5 % CO_2.

Ad293 cells were grown in Dulbecco's MEM supplemented with 10 % FCS. GM00637 and GM04429 cells were grown in MEM (Eagles) containing 15 % or 10 % FCS, respectively. All cells were grown at 37 °C, 5 % CO_2.

6.4.2. Preparation of pSP189 plasmid

An aliquot of pSP189 transformed *E. Coli,* MBM7070 (1 mL; 15 % glycerol in LB broth (v/v)), was grown in LB medium (10 mL) supplemented with ampicillin (100 μg/mL in water) for 8 h at 37 °C, in a shaking incubator at 250 rpm (Multitron, INFORS UK Ltd, Reigate, Surrey). The cell suspension was transferred into a flask containing LB broth (600 mL) supplemented with ampicillin (100 μg/mL in water) and the cells were grown further at 200 rpm, for 15 h, 37 °C.

Glycerol stocks were prepared at this time (1 mL aliquots; 15 % glycerol in LB broth). Plasmid was prepared from the remaining cell suspension using a Qiagen Endofree plasmid mega kit. The cells were pelleted by centrifugation at 6000 rpm for 20 min at 4 °C and the resulting cell pellet was resuspended in Qiagen buffer P1 (10 mL; 50 mM Tris HCl, pH 8.0; 10 mM EDTA; 100 μg /mL RNase A) and Qiagen buffer P2 (10 mL; 200 mM NaOH, 1 % SDS (w/v)). The samples were inverted several times and then incubated at room temperature for 5 min. Qiagen neutralization buffer P3 (10 mL; 3.0 M potassium acetate, pH 5.5) was added to the suspension and the sample was again inverted a number of times.

The cell suspension was poured directly onto a Qiagen Mega cartridge attached to a sterile 1 L bottle and a vacuum pump. The suspension was incubated at room temperature for 10 min and then the liquid was drawn through the cartridge into the duran bottle. Qiagen wash buffer FWB (30 mL; 1.0 M potassium acetate, pH 5.0) was stirred into the precipitate and the vacuum was reapplied. Qiagen buffer ER (7.5 mL;

proprietary formulation) was added to the resultant lysate, and the solution was inverted several times to ensure thorough mixing. The lysate was incubated on ice for 30 min.

A Mega Qiagen column was equilibrated with Qiagen buffer QBT (35 mL; 750 mM NaCl; 50 mM MOPs, pH 7.0; 15 % isopropanol (v/v); 0.15 % Triton X-100 (v/v)). The lysate from the Qiagen Mega cartridge was applied to the Qiagen column. The Qiagen column was washed with Qiagen buffer QC (200 mL; 1.0 M NaCl, 50 mM MOPS, pH 7.0; 15 % isopropanol (v/v)) and the DNA was eluted from the Qiagen column by the addition of Qiagen buffer QN (35 mL; 1.6 M NaCl; 50 mM MOPS, pH 7.0; 15 % isopropanol (v/v)). The plasmid DNA was precipitated by the addition of isopropanol (24.5 mL) and pelleted by centrifugation at 16, 000 rpm for 30 min at 4 °C. The plasmid DNA pellet was washed twice with 70 % ethanol in water (v/v) followed by one wash with ethanol.

The plasmid DNA pellet was air dried briefly and resuspended in an appropriate volume of TE buffer, pH 8.0 (10 mM Tris HCl, pH 8.0; 1 mM EDTA).

DNA yield, purity and concentration was assessed using the method outlined in Chapter 2, section 2.5.1.

6.4.3. Preparation of electrocompetent cells (MBM7070)

An aliquot of *E. coli* MBM7070 cells (1 mL; 15 % glycerol in LB broth(v/v)) was grown for 15 h in LB medium (10 mL) in an orbital shaking incubator at 37 °C, 250 rpm. Cells were transferred into a larger volume of LB medium (600 mL) and cells were grown at 37 °C, 200 rpm until an optical density between 0.5 and 0.7 at 600 nm was obtained. Cells were cooled on ice for 30 min to halt growth. Glycerol stocks were prepared at this time (1 mL aliquots; 15 % glycerol in LB broth(v/v)) and the remaining cells were pelleted by centrifugation at 6000 rpm for 20 min. The cell pellet was washed twice by resuspension in ice-cold sterile water followed by two washes in 10 % glycerol in sterile water (v/v). Repelleting by centrifugation, as described above, was carried out between each wash step. After the last wash step the supernatant was poured off and the cell pellet was resuspended in an equivalent pellet volume of 10 % glycerol in sterile water (v/v). Cells were stored as 100 µL aliquots, at –80 °C.

6.4.4. Treatment of pSP189 plasmid with benzene metabolites

pSP189 (100 μg) was treated on three separate occasions with either 0, 5, 10 or 20 mM HQ, *p*-BQ or HQ plus *p*-BQ in ammonium formate buffer (500 μL; 30 mM, pH 7.0) for 15 h at 37 °C. The DNA was precipitated by the addition of 0.1 volume of sodium acetate (2 M) and an equal volume of ice-cold ethanol. The DNA was recovered by centrifugation at 12, 000 rpm for 60 min. The DNA pellet was washed twice with 70 % (v/v) ethanol in water followed by one wash with ethanol. The DNA was air dried briefly and resuspended in an appropriate volume of SSC buffer (1:100 dilution with water; 150 mM NaCl, 15 mM trisodium citrate).

The DNA recovery was assessed using a UV spectrophotometer (GeneQuant, Pharmacia Biotech, St Albans, Herts) as detailed in Section 2.5.1, Chapter 2.

An aliquot of DNA (5 μg) from each treatment, was analysed by ^{32}P-postlabelling as detailed in section 3.5.2, Chapter 3.

6.4.5. Transfection of eukaryotic cells

Eukaryotic cells (Ad293, GM00637 and GM04429 cells) were plated into 9 cm^2 transfection plates and grown to approximately 60 % confluency in the appropriate medium. Several hours prior to transfection, the medium was replaced with fresh medium. In the following order; unsupplemented medium appropriate for the cell line (500 μL), fugene transfection reagent (20 μL) and plasmid from the appropriate treatment (10 μg), were aliquoted into 1.5 mL tubes. The samples were inverted several times to mix and then incubated for 20 min at room temperature. The mix was added dropwise to the appropriate transfection plate. The plates were swirled to mix and incubated at 37 °C, 5 % CO_2. Plasmid was recovered 48 h after transfection.

Alternatively, HL60 cells were transfected by electroporation, based on the methods of Sokoloski *et al.*, 1986 and Spencer, 1991. Confluent cells were assessed for cell density using the trypan blue method outlined in Chapter 5, Section 5.5.1.1. Aliquots of cells (4-8 x 10^6 cells) were pelleted and resuspended in cold PBS (1 mL). Plasmid (20 μg-50 μg) was added to each cell suspension and cells were electroporated using cold gene pulser cuvettes with a 0.4 cm gap (Biorad, Gene pulser) at 2 kV and 1 or 25

μF capacitance. Cells were transferred directly into T25, medium sized flasks each containing supplemented medium (19 mL). Cells were maintained for 48 h prior to dosing.

6.4.6. Dosing of transfected HL60 cells

Transfected cells were pelleted and washed with PBS to remove free plasmid, prior to re-pelleting. The cell pellet was resuspended in fresh medium and maintained for a further 15 h prior to dosing. Cells were dosed with 0-2 mM HQ and assessment of cell viability and plasmid recovery was carried out at timed intervals.

6.4.7. Small scale plasmid recovery from transfected eukaryotic cells

Transfected eukaryotic cells were extracted using a Qiagen plasmid mini kit in a similar manner to that described in section 6.4.2, with several alterations. The volumes used of the initial solutions, P1, P2 and P3 were scaled down 25 fold and the washing of the resultant precipitate was omitted. Instead, after the addition of Qiagen buffer P3, samples were incubated for 10 min at –20 °C. Samples were centrifuged at 14,000 rpm for 10 min and the supernatant was applied to a Qiagen mini column equilibrated with buffer QBT (1 mL). Qiagen columns were washed with Qiagen buffer QC (4 x 1 mL) and the DNA was eluted by the addition of buffer QF (0.8 mL).

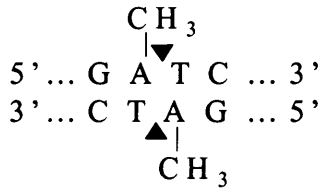
The plasmid was precipitated by the addition of 0.7 volumes of isopropanol (room temperature). The DNA was pelleted by centrifugation at 14,000 rpm for 60 min and washed and dried as described in section 6.4.4. The DNA pellet was resuspended in TE buffer, (20 μL; pH 8.0).

6.4.8. Digestion of unreplicated pSP189 plasmid

Recovered, transfected pSP189 plasmid underwent treatment with the restriction endonuclease, Dpn1. This enzyme removes any unreplicated plasmid by cleavage at a methylated recognition site as shown in Figure 6.2. DNA present in *E. coli* contains methylated bases, which are lost upon replication in eukaryotic cells, and hence the DNA is no longer a substrate for the enzyme. Only repaired or mutated DNA is left intact.

Recovered plasmid was incubated with DpnI (2 U) in diluent NEBuffer B (300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 μg/mL BSA and 50 % glycerol, pH 7.4) and NEBuffer 4 (20 mM tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, pH 7.9) for 2 h at 37 °C. The enzyme was heat inactivated by incubation at 80 °C for 20 min and the DNA was re-precipitated and washed as described in section 6.4.4. Dried DNA was resuspended in TE buffer (20 μL; pH 8.0).

Figure 6.2: Representation of the recognition site for the restriction endonuclease, Dpn 1.



6.4.9. Transformation of electrocompetent cells (*E. coli*, MBM7070)

Recovered, DpnI treated plasmid (2 μL) was transferred into an aliquot of *E. coli*, MBM7070 (100 μL aliquots, approx. 2 x 10^6 cells) and stored on ice prior to transformation. Samples were transferred into a pre-chilled electroporation cuvette (0.2 cm electrode gap) and the cells were electroporated at 2.5 kV, 25 μF and 200 ohms using a Biorad Gene Pulser (time constant 3.9 ms). The cells were transferred into SOC medium (1 mL; 2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ and 20 mM glucose) and incubated in vented tubes for 30-45 min at 37 °C, 250 rpm.

6.4.10. Screening for mutant colonies

Transformed MBM7070 cells (300 μL aliquots) were plated onto LB agar plates (525 cm^2) containing ampicillin (100 μg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal; 75 μg/mL) and isopropyl β-D-thiogalactoside (IPTG; 25 μg/mL). Plates were incubated for 24 h at 37 °C. The total numbers of colonies were counted and

categorised as blue, pale blue or white and the mutation frequency was calculated as follows:

$$\text{Mutation frequency} = \frac{\text{number of white or pale blue colonies (mutant)}}{\text{total number of colonies}}$$

Pale blue and white colonies were restreaked onto small 9 cm^2 LB agar plates (as above) and plates were again incubated for 24 h at 37 °C. Colonies were restreaked and regrown until a single mutant colony could be isolated.

6.4.11. Isolation and sequencing of mutant pSP189 plasmid

Single, mutant colonies (1 per 9 cm^2 agar plate) were transferred into TE buffer (20 μL; pH 8.0). Samples were vortexed vigorously and an aliquot of each sample (3 μL) was transferred into a fresh tube (0.2 mL). Samples underwent PCR amplification using the Templiphi amplification kit (Amersham Biosciences, Little Chalfont, Buckinghamshire). Sample buffer (5 μL; proprietary formulation) was added to each sample and samples were incubated at 95 °C for 3 min followed by cooling to 4 °C. Reaction buffer (5 μL; proprietary formulation) and enzyme mix (0.2 μL; proprietary formulation) were added to each sample and samples were incubated for 6 h at 30 °C, followed by incubation at 65 °C for 10 min. Samples were then cooled to 4°C.

Amplified DNA supplied with the primer 5'-GGCGACACGGAAATGTTGAA-3' (10 μL/sample; 1 pmol/ μL) was sent to the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester for sequencing. Sequencing was carried out using an Applied Biosystems Model 377 DNA sequencer using BigDye version 1.0 sequencing chemistries. Mutants with identical signature sequences (siblings) were excluded from the analysis. The spectrum of mutations was assessed, and any positions within the sequence where the number of mutations were four fold or higher than that expected were considered hotspots (as determined by Poisson distribution). Mutation spectra were compared using the Cariello Hyperg program ($p \leq 0.05$ indicates significant difference; Cariello *et al.*, 1994).

6.5. Results

Plasmid (pSP189) was dosed with the benzene metabolites HQ or *p*-BQ or a combination of both to assess the potential mutagenicity of these metabolites. Treated plasmid was transfected into three different human cell lines; Ad293, an adenovirus transformed kidney cell line, GM00637, a normal SV40 transformed fibroblast cell line and GM04429, a repair deficient SV40 transformed, *Xeroderma pigmentosum* (*XP*) fibroblast cell line (complementation group A).

Repaired or mutated DNA recovered from the three cell lines was transformed into the indicator bacteria, MBM7070 and colonies containing mutant pSP189, or more specifically a mutated *supF* gene as indicated by their white or pale blue colour, were selected for sequencing.

6.5.1. ^{32}P-postlabelling of benzene metabolite treated pSP189

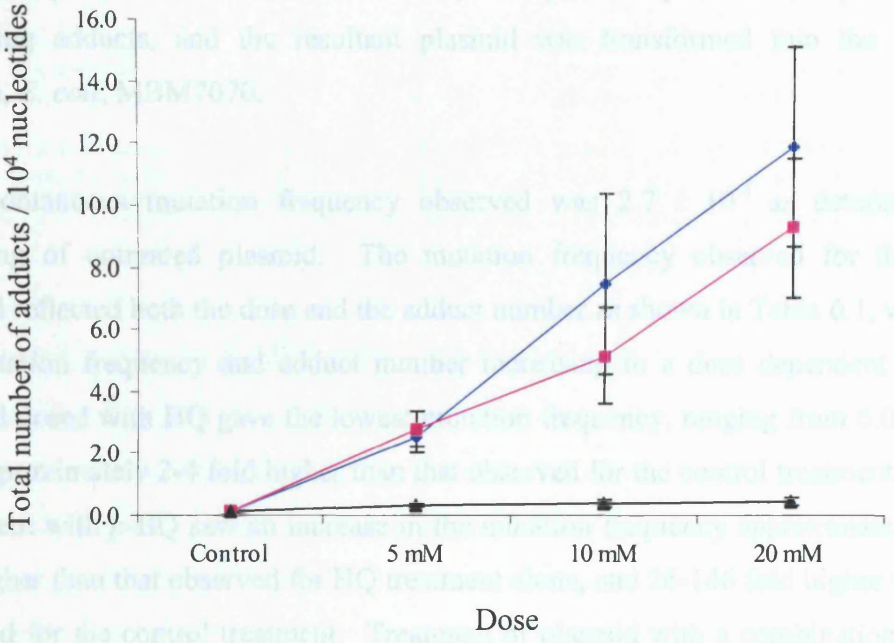
Prior to transfection of human cells, pSP189 containing the reporter gene, *supF*, was treated *in vitro,* with HQ, *p*-BQ or both metabolites together at four different dose levels (0, 5, 10 and 20 mM). An aliquot of plasmid (5 μg), from each treatment group underwent ^{32}P-postlabelling to determine the level of modification. The overall adduct numbers are shown in Figure 6.3. As expected, based on the observations seen for calf thymus DNA treated with HQ, *p*-BQ or both metabolites together in varying ratios (see section 3.5.5, Chapter 3), HQ treatment alone gave the lowest adduct number, whilst treatment with *p*-BQ (and HQ) gave an adduct number which reflected the increasing dose. Treatment with HQ alone gave a single adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, the level of which did not significantly increase with dose, this level ranged from 0.31 ± 0.08 to 0.45 ± 0.12 adducts / 10^4 nucleotides (n =3), for the doses ranging from 5 to 20 mM i.e. less than one adduct per plasmid.

Treatment of plasmid with the benzene metabolite, *p*-BQ, gave an adduct level which ranged from 2.52 ± 0.47 to 11.87 ± 3.23 adducts / 10^4 nucleotides (n =3) for doses ranging from 5 to 20 mM. A combination of the two metabolites gave an adduct range similar to *p*-BQ alone, which ranged from 2.79 ± 0.59 to 9.28 ± 2.25 adducts / 10^4

nucleotides (n =3). In both cases, overall adduct formation was linear as shown in Figure 6.3 (R^2 = 0.9822 and 0.9835 for *p*-BQ and both metabolites in combination, respectively). The adduct values obtained for the combination of metabolites were not significantly different from those obtained for treatment with *p*-BQ only (as determined by oneway ANOVA followed by Tukey's post hoc test).

Again, as observed for the treatment of calf thymus DNA with *p*-BQ and both *p*-BQ and HQ together (Chapter 3), the major adduct formed following reaction with pSP189 was (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate followed by (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate. The two, deoxyguanosine 3'-monophosphate adducts, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate again formed the minor products.

Figure 6.3: Graphical representation of the adduct level observed following dosing of pSP189 plasmid with 0, 5, 10 or 20 mM HQ (▲), *p*-BQ (◆) or both metabolites together (■).
Adduct numbers were assessed using ^{32}P-postlabelling coupled to HPLC. The data shown are mean ± SD (n =3).



6.5.2. The *supF* assay in Ad293 cells – mutation frequency

Ad293 cells, a human kidney cell line, selected for their low spontaneous mutation frequency, were transfected with plasmid that had undergone no treatment (control), or treatment with HQ, *p*-BQ or both metabolites in combination. The plasmid was recovered 48 h after transfection, allowing for repair and replication of any of the benzene modifications to occur. The recovered plasmid was treated with the restriction enzyme, DpnI, to allow removal of any unreplicated plasmid i.e. plasmid still containing adducts, and the resultant plasmid was transformed into the indicator bacteria, *E. coli*, MBM7070.

The spontaneous mutation frequency observed was 2.7 / 10^{-4} as determined by screening of untreated plasmid. The mutation frequency observed for the dosed plasmid reflected both the dose and the adduct number as shown in Table 6.1, with both the mutation frequency and adduct number increasing in a dose dependent manner. Plasmid dosed with HQ gave the lowest mutation frequency, ranging from 6.02-9.78 x 10^{-4}, approximately 2-4 fold higher than that observed for the control treatment (water). Treatment with *p*-BQ saw an increase in the mutation frequency approximately 11-40 fold higher than that observed for HQ treatment alone, and 26-146 fold higher than that achieved for the control treatment. Treatment of plasmid with a combination of both metabolites (HQ and p-BQ) saw a reduction in mutation frequency when compared to *p*-BQ, with only a 14-79 fold higher increase observed compared to the control sample. This result reflected the adduct numbers observed, which also showed a reduced, but not significantly different, level of adducts compared to the *p*-BQ treatment alone.

Table 6.1: Mutation frequency observed after treatment of pSP189 plasmid with varying doses of HQ, *p*-BQ and the two metabolites together with transfection into Ad293 cells and subsequent transformation into *E. coli*, MBM7070.

Treatment	Number of colonies screened	Number of mutants	Mutation frequency /10^4 colonies	Adduct number / 10^4 nucleotides (± SD)
Control	56191	15	2.67	0 ± 0
5 mM HQ	29899	18	6.02	0.31 ± 0.08
10 mM HQ	13513	16	11.84	0.38 ± 0.13
20 mM HQ	42934	42	9.78	0.45 ± 0.12
5 mM *p*-BQ	8221	57	69.33	2.52 ± 0.47
10 mM *p*-BQ	4544	84	184.86	7.48 ± 2.90
20 mM *p*-BQ	2074	81	390.55	11.87 ± 3.23
5 mM HQ + *p*-BQ	33199	121	36.45	2.79 ± 0.59
10 mM HQ + *p*-BQ	14349	103	71.78	5.14 ± 1.54
20 mM HQ + *p*-BQ	16703	354	211.94	9.28 ± 2.25

6.5.3. *supF* assay in Ad293 cells - mutation type

Only the highest dose for each treatment was analysed further to determine the mutation types induced by each metabolite or combination of metabolites. For treated plasmid (all types) the mutations observed were predominantly single base substitutions (65-84 %), as shown in Table 6.2. Only 36 % of the mutations induced in the control sample were single base substitutions. A small number of tandem base substitutions (substitutions at adjacent bases) were detected for the dosed plasmid (less than 3 %), with none observed for the control sample. Of the mutations detected for the HQ treatment, 27 % were frameshift mutations, with the majority of these being a deletion of more than 2 base pairs. A small percentage of frameshift mutations (6 %) were detected for the *p*-BQ treatment and none were observed for the two metabolites in combination, although some frameshift mutations are included in the 'other' category (multiple mutations involving a combination of base substitutions and frameshift mutations). The majority of the mutations detected for the control sample were frameshift mutations (56 %), and as with the HQ treatment, the majority of these were deletions involving more than 2 base pairs.

Multiple base substitutions (2 or more base substitutions at non-adjacent sites along the *supF* gene) accounted for a low percentage of the total mutations detected for treatment with either HQ or *p*-BQ (1 and 5 %, respectively). For the combined treatment, the

number of multiple mutations detected had increased approximately three fold compared to *p*-BQ treatment only.

The majority of the single and tandem base substitutions observed, were transversions as seen for the control sample and the two individual treatments of HQ and *p*-BQ, as shown in Table 6.3. For the combined treatment, the numbers of base substitutions were split equally between transversions and transition mutations. For the control sample the major mutation was the GC→CG transversion. A similar result was observed for the HQ treatment with the majority of transversions also being the GC→CG base substitution. For this treatment however, the major base substitution was a GC→AT transition. For the *p*-BQ treatment the majority of base substitutions affected GC base pairs, which included GC→TA transversions and GC→AT transitions. These two mutations accounted for a total of 80 % of the single and tandem base substitutions observed for this treatment. Compared to *p*-BQ treatment alone, the major base substitution for the combination of metabolites also affected GC base pairs but unlike with *p*-BQ treatment alone, GC→AT transitions predominated. The level of GC→TA transversions, although being the next major base substitution for the combined treatment was actually showing a reduced level to that observed for the lone treatment of *p*-BQ.

6.5.4. *supF* assay in Ad293 cells-mutation spectra

The spectrum of mutations for the single and tandem base substitutions induced by the various treatments can be seen in Figure 6.4. A number of hotspots for mutation were detected and these all occurred at GC base pairs within the *supF* sequence. Hotspots occurred at position 129 in the control sample, positions 105 and 122 for the HQ treatment and positions 123 and 159 for the *p*-BQ treatment. No hotspots were observed for the combined treatment. The base substitutions at the hotspots accounted for 23 %, 18 % and 21 % of the total single and tandem base substitutions detected for the control, HQ and *p*-BQ treatments, respectively and the majority of these involved substitution for a thymine or adenine base.

Of all the mutation spectra analysed, the majority of multiple base substitutions observed occurred after the combined HQ plus *p*-BQ treatment as shown in Table 6.2. The types of base substitutions induced in these spectra are shown in Table 6.4. In all cases, except for the HQ treatment, the majority of the multiple base substitutions were transversions. As only a small number of multiple base substitutions were observed for the control and the two individual treatments (HQ or *p*-BQ) it is difficult to comment on the mutation type and spectra observed. Interestingly for the combined metabolite treatment, the major individual base substitution was a GC→AT transition, a result similar to that seen for the single base substitutions shown in Table 6.3. The spectrum of mutations for the multiple base substitutions can be seen in Figure 6.5. In contrast to the single base substitution spectra, a hotspot was now observed for the combined treatment at position 145, which was at a TA base pair and involved substitution for a guanine base for all mutations detected at this hotspot.

The spectra of frameshift mutations observed for all treatments can be seen in Figure 6.6, the majority being detected for the control and HQ treatment. In 75 % and 87 % of cases, the majority of the frameshift mutations involved deletions of more than 2 base pairs and in 10 % and 22 % of cases this involved deletion of the entire *supF* gene, for control and HQ treatment, respectively. A number of insertions were also detected for the control and HQ treatment group, the inserts ranged from 2 non-adjacent bases, to 20 to 278 base pairs which involved both random and repeat sequences. Treatment with *p*-BQ gave only 6 % frameshift mutations, all of which involved deletions, 50 % being single base deletions and 50 % being deletions of more than 2 base pairs. The combined treatment resulted in two individual single base pair deletions but these occurred as a multiple mutation, occurring alongside a single base and a tandem base pair substitution, respectively.

Table 6.2: Mutation types observed in the *supF* gene following treatment of pSP189 plasmid with 20 mM HQ, *p*-BQ or both metabolites together after transfection into Ad293 cells and subsequent transformation into *E. coli*, MBM7070

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Base substitutions	**13 (36)**	**56 (67)**	**58 (92)**	**67 (97)**
Single	13 (36)	54 (65)	53 (84)	54 (78)
Tandem	0 (0)	1 (1)	2 (3)	2 (3)
Multiple	0 (0)	1 (1)	3 (5)	11 (16)
Frameshifts	**20 (56)**	**23 (27)**	**4 (6)**	**0 (0)**
Single base deletion	2 (6)	1 (1)	2 (3)	0 (0)
>2 bases deleted	15 (42)	20 (24)	2 (3)	0 (0)
Single base insertion	1 (2)	0 (0)	0 (0)	0 (0)
>2 bases inserted	2 (6)	2 (2)	0 (0)	0 (0)
[a]other	**3 (8)**	**5 (6)**	**1 (2)**	**2 (3)**
Total number sequenced	**36 (100)**	**84 (100)**	**63 (100)**	**69 (100)**

[a]Other mutation types involve a combination of both base substitutions and frameshift mutations seen in the same spectra

Table 6.3: Types of single and tandem base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in Ad293 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**11 (85)**	**32 (57)**	**33 (58)**	**29 (50)**
GC → TA	4 (31)	9 (16)	23 (40)	13 (23)
GC →CG	7 (54)	15 (27)	5 (9)	7 (12)
AT → TA	0 (0)	6 (11)	5 (9)	6 (10)
AT → CG	0 (0)	2 (3)	0 (0)	3 (5)
Transitions	**2 (15)**	**24 (43)**	**24 (42)**	**29 (50)**
GC →AT	2 (15)	19 (34)	23 (40)	23 (40)
AT → GC	0 (0)	5 (9)	1 (2)	6 (10)
Total number of single and tandem base substitutions	**13 (100)**	**56 (100)**	**57 (100)**	**58 (100)**

Table 6.4: Types of multiple base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in Ad293 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**4 (80)**	**1 (25)**	**5 (62.5)**	**17 (63)**
GC → TA	2 (40)	0 (0)	3 (37.5)	6 (22)
GC →CG	0 (0)	1 (25)	0 (0)	3 (11)
AT → TA	1 (20)	0 (0)	2 (25)	2 (8)
AT → CG	1 (20)	0 (0)	0 (0)	6 (22)
Transitions	**1 (20)**	**3 (75)**	**3 (37.5)**	**10 (37)**
GC →AT	1 (20)	2 (50)	3 (37.5)	10 (37)
AT → GC	0 (0)	1 (25)	0 (0)	0 (0)
Total number of multiple base substitutions	**5 (100)**	**4 (100)**	**8 (100)**	**27 (100)**

Figure 6.4: Mutation spectra showing single and tandem base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into Ad293 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown in blue.

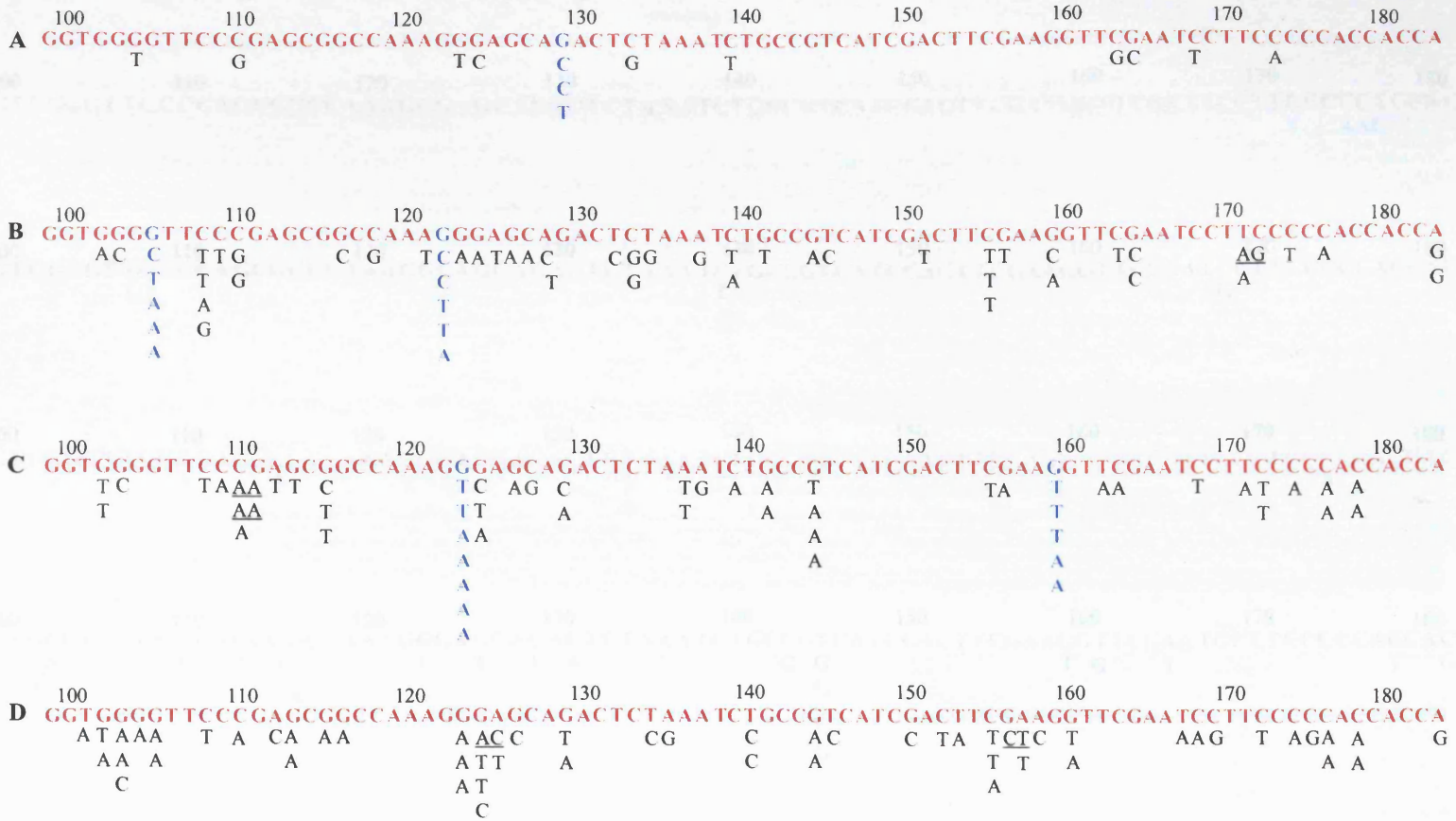


Figure 6.5: Mutation spectra showing multiple base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into Ad293 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown on the sequence in blue.
Related base substitutions i.e. those from the same DNA sequence, are highlighted in the same colour.

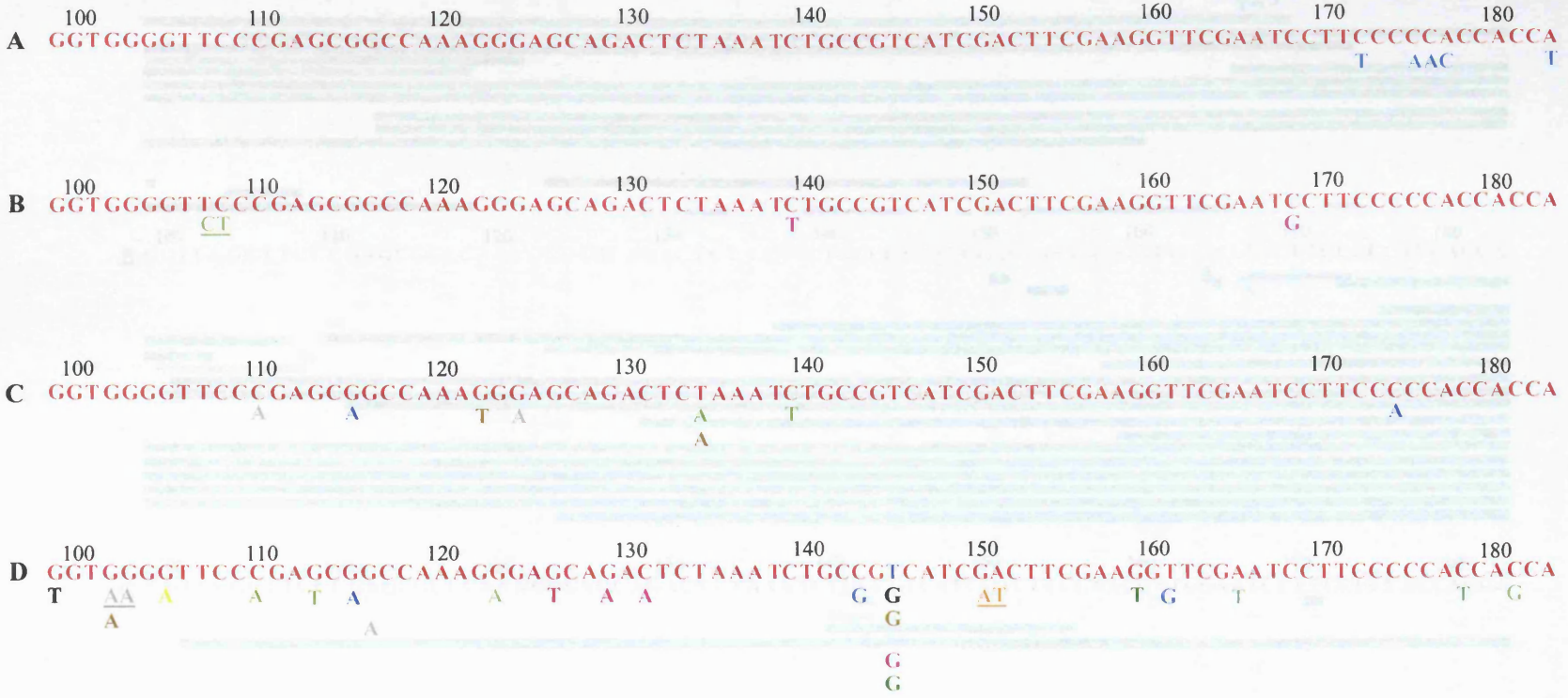
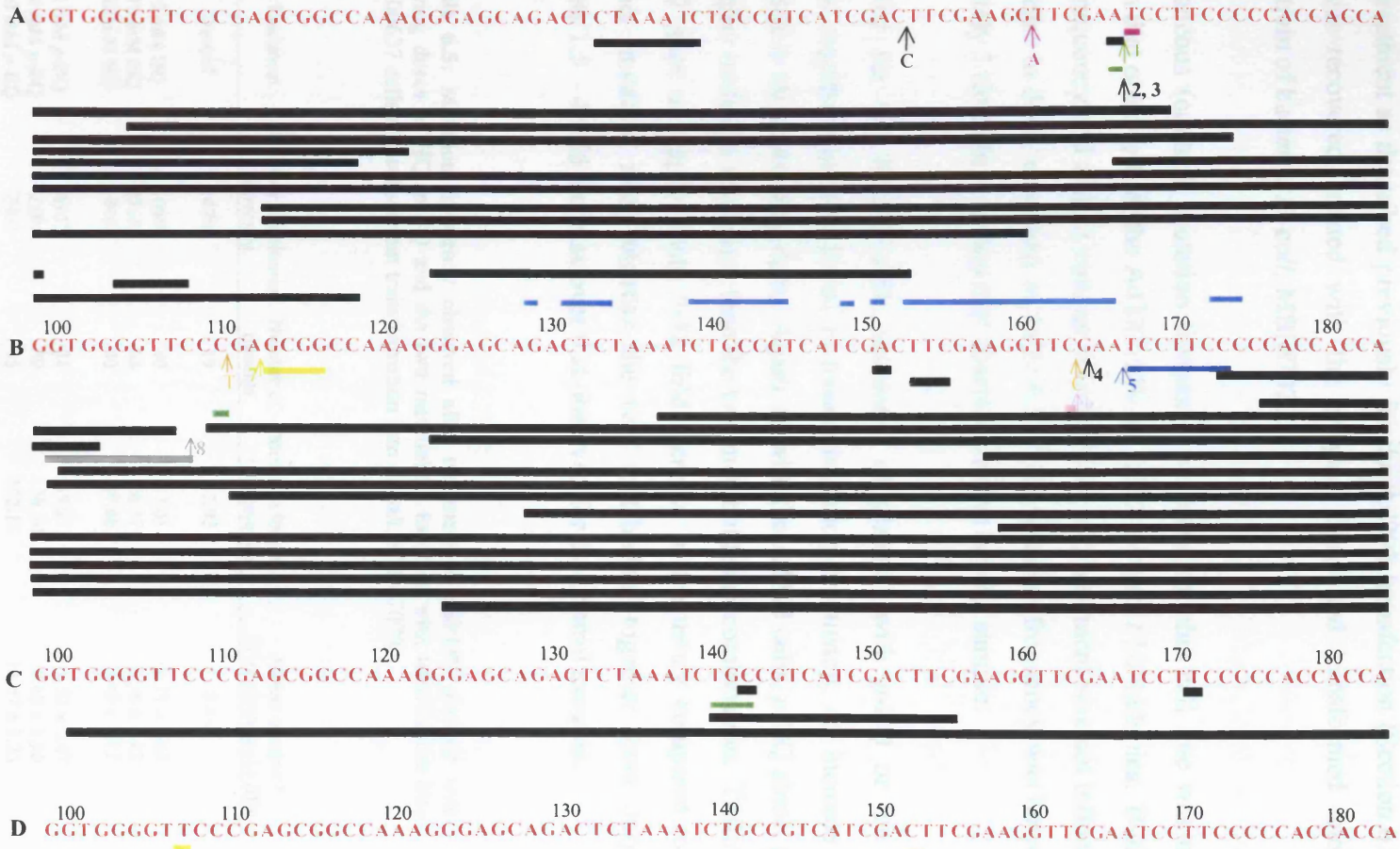


Figure 6.6: Mutation spectra showing frameshift mutations induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into Ad293 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Bars indicate where the deletion has occurred.
Related mutations i.e. from the same DNA sequence are highlighted in the same colour (some may relate to base substitutions shown in Figure 6.5).



1 signifies a random 42 bp insert, 2 a 58 bp repeat sequence, 3 a 20 bp repeat sequence, 4, a 74 bp insert including a 30 bp repeat sequence. 5, a 139 bp random insert, , a 98 bp random insert, 7, a random 278 bp insert and 8, a random 173 bp insert

6.5.5. *supF* assay in GM00637 cells – mutation frequency

GM00637 human fibroblast cells were transfected with plasmid, which had undergone the same treatment as described previously for the Ad293 transfection (Section 6.5.2). Plasmid was recovered, treated with the enzyme DpnI and transformed into the indicator strain of bacteria, *E. coli,* MBM7070.

The spontaneous (control) mutation frequency observed for this cell line was much higher than that observed for the Ad293 cells, at 12.92 mutants / 10^4 colonies. Both the mutation frequency and adduct number detected after HQ treatment was not influenced by an increase in dose, as shown in Table 6.5. The mutation frequency was however, approximately 3 times higher than that observed for the control sample.

As observed for the Ad293 cells, treatment of plasmid with *p*-BQ or the two metabolites together resulted in an increased mutation frequency, an increase that corresponded to the increasing dose. Again, as with the Ad293 cells, *p*-BQ alone, gave a much higher mutation frequency than the two metabolites in combination. Treatment with *p*-BQ gave an approximate 4-12 fold increase in frequency compared to the spontaneous mutation rate, whereas the two metabolites together gave only an approximate 1.5 – 4 fold increase over that observed for the control samples.

Table 6.5: Mutation frequency observed after treatment of pSP189 plasmid with varying doses of HQ, *p*-BQ and the two metabolites together with transfection into GM00637 cells and subsequent transformation into *E. coli*, MBM7070.

Treatment	Number of colonies screened	Number of mutants	Mutation frequency /10^4 colonies	Adduct number / 10^4 nucleotides (± SD)
Control	14703	19	12.92	0 ± 0
5 mM HQ	10803	40	37.03	0.31 ± 0.08
10 mM HQ	25686	68	26.47	0.38 ± 0.13
20 mM HQ	13484	40	29.66	0.45 ± 0.12
5 mM *p*-BQ	6152	34	55.27	2.52 ± 0.47
10 mM *p*-BQ	2194	30	136.74	7.48 ± 2.90
20 mM *p*-BQ	2301	35	152.11	11.87 ± 3.23
5 mM HQ + *p*-BQ	6149	12	19.52	2.79 ± 0.59
10 mM HQ + *p*-BQ	1418	4	28.21	5.14 ± 1.54
20 mM HQ + *p*-BQ	2189	12	54.82	9.28 ± 2.25

6.5.6. *supF* assay in GM00637 cells – mutation type

For each treatment regimen (20 mM dose only), base substitutions were the predominant mutation type, and of these, single base substitutions were the major type, as reported in Table 6.6. No tandem mutations were detected for the control and combined metabolite treatment although this mutation type did feature in two of the multiple mutations observed for both samples. For the individual metabolite treatments, HQ and *p*-BQ, tandem mutations accounted for 2 % and 7 % of the total mutations detected, respectively. Similar levels of multiple base substitutions were recovered for each treatment type, with levels ranging from 14-18 % of all mutation types. Similar to the findings after transfection into Ad293 cells, 21 % of the mutants screened for the HQ treatment contained a frameshift mutation, the majority of which involved a deletion of more than 2 base pairs. Few frameshifts (< 5%) were detected for the *p*-BQ and combined treatment.

The control sample gave an approximately similar level of base substitutions compared to frameshift mutations. As with the mutants that arose from transfection of untreated plasmid into Ad293 cells, the majority of the mutants were either single base substitutions or deletions of more than 2 base pairs. The control sample gave a similar level of multiple mutations as observed for each treatment group.

Of the single and tandem base substitutions recorded, as shown in Table 6.7, the number of transversion mutations were slightly higher than transition substitutions with, for each treatment group plus control, GC→TA mutations predominating. For the transition mutations, the same result was observed for each treatment group (including control) with GC→AT mutations being the major type, accounting for greater than 86 % of the transition mutations observed.

Similar to the findings with the single and tandem base substitutions, the majority of the multiple base substitutions observed, as shown in Table 6.8, were due to a transversion. However, unlike the single and tandem base substitutions, a difference was observed in regard to the major base substituted. For the control sample, the majority of multiple mutations were due to either an AT→CG transversion or a GC→AT transition. For the two individual treatments the major mutations were due

either to GC→TA or AT→CG transversions or a GC→AT transition. The combined treatment gave a similar finding except, the AT→CG transversion did not feature as a major mutation.

6.5.7. *supF* assay in GM00637 cells -spectrum of mutations

The mutation spectra, which include single, tandem and multiple base substitutions and frameshift mutations are shown in Figures 6.7 to 6.9. Many more mutation hotspots were detected for this cell line, compared to the Ad293 cells, with again all single and tandem base substitution hotspots occurring at GC base pairs. In the control sample hotspots were observed at positions 127, 133 and 150. The HQ treatment gave mutation hotspots at positions 108, 109, 123, 129 and 175 with the strongest mutation hotspot being at position 133. Treatment with *p*-BQ gave mutation hotspots at different positions to those detected for the HQ treatment, with these being at 115, 122 and 155. The combined treatment gave three mutation hotspots at positions highlighted as hotspots in the individual treatments, these were at positions 115, 122 and 123. Base substitutions at the hotspots accounted for 53 %, 21 % and 30 % of the total single and tandem base substitutions detected and of these, 77 %, 92 % and 94 % involved substitution for either an adenine or thymine base, for treatment with HQ, *p*-BQ and the combined treatment, respectively.

A number of mutation hotspots also arose in the multiple mutation spectra, although only being detected for the two individual treatments, HQ and *p*-BQ. Treatment with HQ gave two hotspots at positions 133 and 145, which involved both a GC and a TA base pair and accounted for 11 % and 19 % of all the multiple base substitutions, respectively. Five hotspots in the *p*-BQ treated sample were detected, one occurring at the same site as the main hotspot in the HQ treatment (position 145). Three of these hotspots were clustered at the 5' end of the *supF* gene at positions 102, 103 and 105, and all involved mutations at a GC base pair, whilst the other two mutations occurred at positions 145 and 154 and involved mutations at TA base pairs. Mutations at these hotspots accounted for a total of 49 % of all the multiple base substitutions observed. At the hotspots observed here, only 9 % and 53 % involved substitution for either adenine or thymine bases for HQ and *p*-BQ treatments, respectively.

The distribution of frameshift mutations are shown in Figure 6.9, and such mutations accounted for 45 %, 21 %, 4 % and 3 % of all the mutations observed for control, HQ, *p*-BQ and the combined treatment, respectively. All frameshift mutations involved a deletion, and in the majority of cases for each treatment this involved deletion of more than 2 base pairs. For the control, HQ and *p*-BQ treatment, deletion of the entire *supF* gene occurred in 23 %, 27 % and 100 % of cases for deletions of more than 2 base pairs.

Table 6.6: Mutation types observed in the *supF* gene following treatment of pSP189 plasmid with 20 mM HQ, *p*-BQ or both metabolites together after transfection into GM00637 cells and subsequent transformation into *E. coli*, MBM7070

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Base substitutions	**15 (52)**	**69 (79)**	**72 (93)**	**65 (93)**
Single	11 (38)	53 (61)	53 (69)	54 (77)
Tandem	0 (0)	2 (2)	5 (7)	0 (0)
Multiple	4 (14)	14 (16)	14 (18)	11 (16)
Frameshifts	**13 (45)**	**18 (21)**	**3 (4)**	**2 (3)**
Single base deletion	0 (0)	3 (4)	1 (1)	1 (1.5)
>2 bases deleted	13 (45)	15 (17)	2 (3)	1 (1.5)
Single base insertion	0 (0)	0 (0)	0 (0)	0 (0)
>2 bases inserted	0 (0)	0 (0)	0 (0)	0 (0)
[a]other	**1 (3)**	**0 (0)**	**2 (3)**	**3 (4)**
Total number sequenced	**29 (100)**	**87 (100)**	**77 (100)**	**70 (100)**

[a]Other mutation types involve a combination of both base substitutions and frameshift mutations seen in the same spectra

Table 6.7: Types of single and tandem base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in GM00637 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**6 (55)**	**30 (53)**	**34 (54)**	**27 (50)**
GC → TA	4 (37)	17 (30)	24 (38)	22 (41)
GC →CG	2 (18)	12 (21)	3 (5)	4 (7)
AT → TA	0 (0)	0 (0)	3 (5)	0 (0)
AT → CG	0 (0)	1 (2)	4 (6)	1 (2)
Transitions	**5 (45)**	**27 (47)**	**29 (46)**	**27 (50)**
GC →AT	5 (45)	24 (42)	28 (44)	23 (43)
AT → GC	0 (0)	3 (5)	1 (2)	4 (7)
Total number of single and tandem base substitutions	**11 (100)**	**57 (100)**	**63 (100)**	**54 (100)**

Table 6.8: Types of multiple base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in GM00637 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**8 (57)**	**27 (75)**	**22 (63)**	**21 (72)**
GC → TA	2 (14)	9 (25)	9 (26)	11 (38)
GC →CG	1 (7)	6 (17)	1 (3)	4 (14)
AT → TA	0 (0)	0 (0)	3 (8)	3 (10)
AT → CG	5 (36)	12 (33)	9 (26)	3 (10)
Transitions	**6 (43)**	**9 (25)**	**13 (37)**	**8 (28)**
GC →AT	5 (36)	9 (25)	12 (34)	8 (28)
AT → GC	1 (7)	0 (0)	1 (3)	0 (0)
Total number of multiple base substitutions	**14 (100)**	**36 (100)**	**35 (100)**	**29 (100)**

Figure 6.7: Mutation spectra showing single and tandem base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM00637 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown in blue.

Figure 6.8: Mutation spectra showing multiple base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM00637 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown on the sequence in blue.
Related base substitutions i.e. those from the same DNA sequence, are highlighted in the same colour.

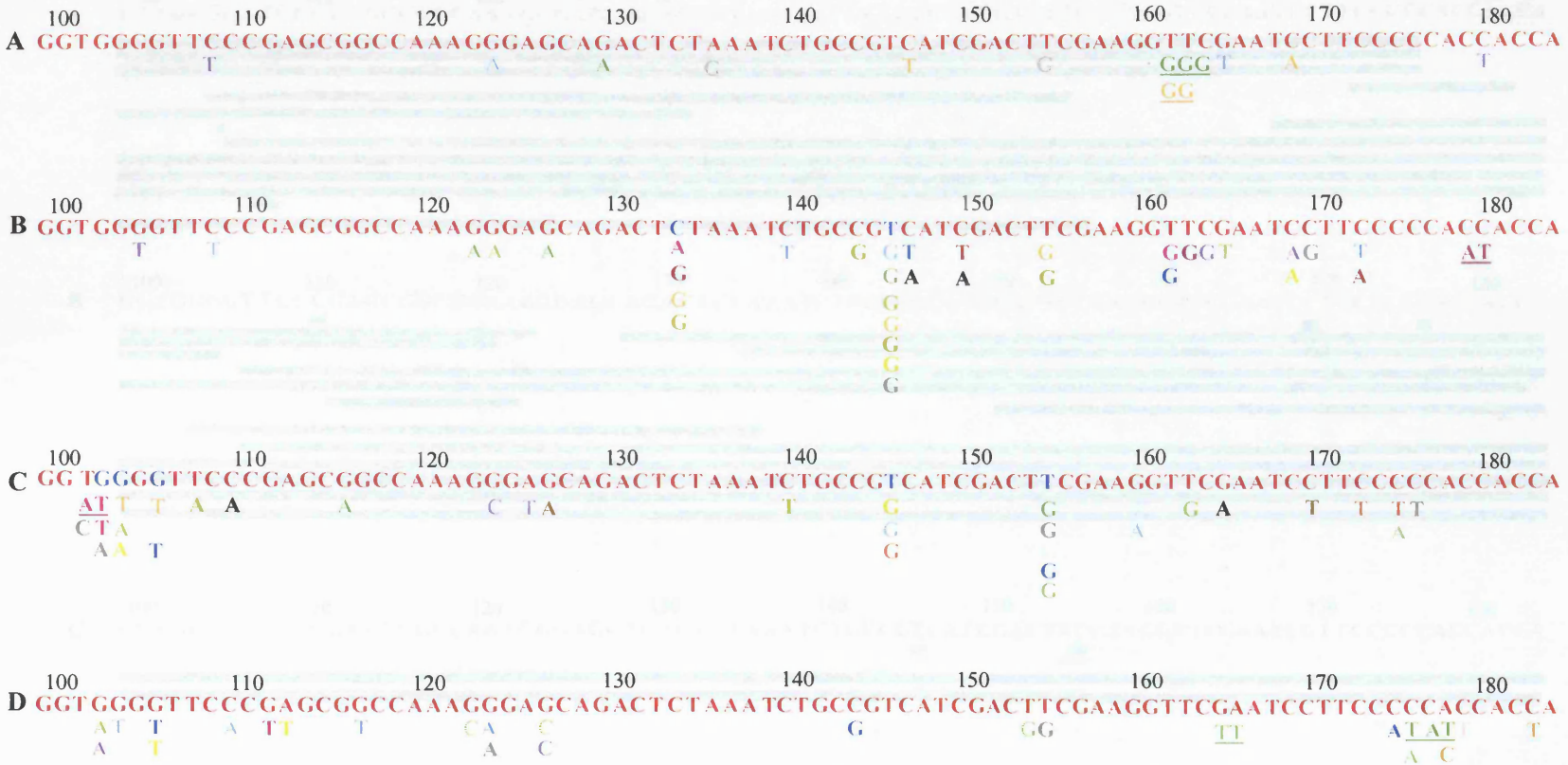
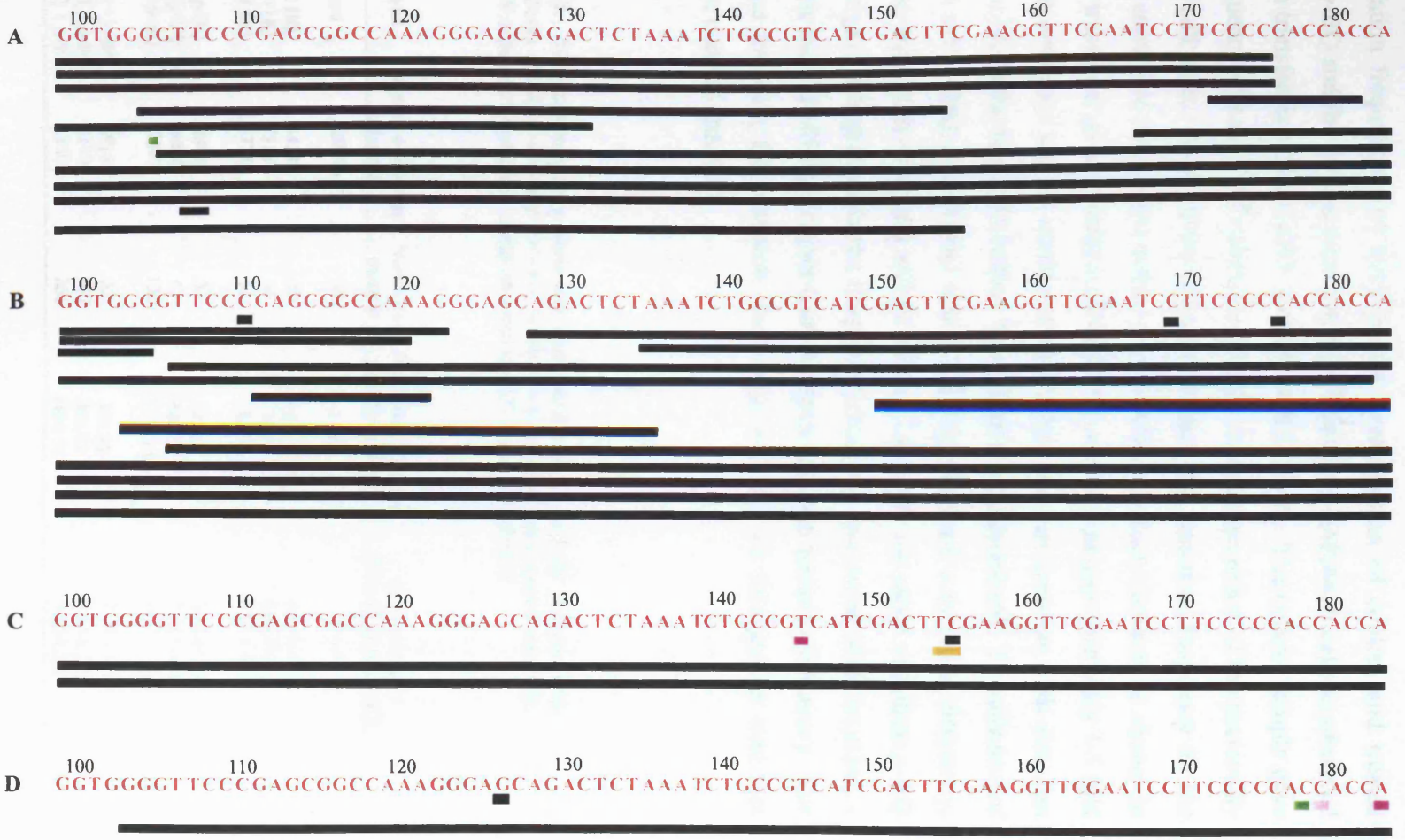


Figure 6.9: Mutation spectra showing frameshift mutations induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM00637 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Bars indicate where the deletion has occurred.
Related mutations i.e. from the same DNA sequence are highlighted in the same colour. (some may relate to base substitutions shown in Figure 6.8).



6.5.8. *supF* assay in GM04429 cells – mutation frequency

A similar mutation frequency was detected for transfection of control and treated plasmid (HQ, *p*-BQ and the two metabolites together) into GM04429 cells as observed previously with transfection into Ad293 and GM00637 cells. The control sample gave a mutation frequency of 1.65 / 10^4 colonies, a value lower than either of the previously two mentioned cell lines. As with the other cell lines, the mutation frequency for the individual and combined treatments reflected the DNA adduct number, as shown in Table 6.9. HQ treatment gave a mutation frequency which was approximately 3-5 fold higher than that observed for the control sample, but did not increase with dose, an observation also made for the DNA adduct level that was determined. Transfection of plasmid treated with *p*-BQ or both HQ and *p*-BQ together saw a mutation frequency, which increased with both dose and adduct level. As with the other cell lines *p*-BQ treatment alone gave a higher mutation frequency than the two metabolites together, a frequency which was 30-108 fold higher than the spontaneous mutation frequency. For both metabolites together, the mutation frequency was 6 – 63 fold greater than that observed for the control sample.

Table 6.9: Mutation frequency observed after treatment of pSP189 plasmid with varying doses of HQ, *p*-BQ and the two metabolites together with transfection into GM04429 cells and subsequent transformation into *E. coli*, MBM7070.

Treatment	Number of colonies screened	Number of mutants	Mutation frequency /10^4 colonies	Adduct number / 10^4 nucleotides (± SD)
Control	229796	38	1.65	0 ± 0
5 mM HQ	54288	30	5.53	0.31 ± 0.08
10 mM HQ	73167	56	7.65	0.38 ± 0.13
20 mM HQ	87788	51	5.81	0.45 ± 0.12
5 mM *p*-BQ	6990	35	50.07	2.52 ± 0.47
10 mM *p*-BQ	8880	61	68.69	7.48 ± 2.90
20 mM *p*-BQ	7297	130	178.16	11.87 ± 3.23
5 mM HQ + *p*-BQ	49169	52	10.58	2.79 ± 0.59
10 mM HQ + *p*-BQ	41794	147	35.17	5.14 ± 1.54
20 mM HQ + *p*-BQ	25504	266	104.30	9.28 ± 2.25

6.5.9. *supF* assay in GM04429 cells – mutation type

The level of mutation types detected for GM04429 cells, as shown in Table 6.10, were comparable to the values observed for GM00637 cells, with the majority of mutations observed being base substitutions. Of this type of mutation, single base substitutions predominated. The numbers of tandem mutations observed, except in one case (*p*-BQ), equalled the number of multiple mutations detected. For the *p*-BQ treatment, only 3 tandem mutations were observed, which accounted for 5 % of the total mutations induced. Compared to GM00637 cells, fewer multiple base substitutions were observed, with this type of mutation accounting for 10 % or less of the total mutations detected. Treatment with *p*-BQ only, gave the most mutiple base substitutions (10 %), followed by the combined treatment (7 %), treatment with HQ gave less than 2 % multiples and none were observed for the control sample.

Frameshift mutations accounted for approximately 50 % and 24 % of the mutations observed in the control and HQ treated sample, respectively. Few frameshift mutations were detected for the *p*-BQ and combined treatment, a pattern similar to that observed for the other two cell lines. In all cases, the major frameshift mutation involved deletions of more than 2 base pairs. Insertions were only detected in the control and *p*-BQ treatment.

The detail regarding the types of single, tandem and multiple base substitutions can be seen in Tables 6.11 and 6.12. Similar findings were observed in this cell line as compared to GM00637 when analysing the individual substitution types. In both treatments involving *p*-BQ the majority of base substitutions were transversions whereas in the control and HQ treatment transitions predominated. In all cases, the majority of both transversions and transitions involved mutation at a GC base pair. The majority of transversions for all three treatments involved a GC→TA substitution whereas for the control GC→TA and GC→CG transverions were equally predominant. The major transition mutation in all 4 cases was the GC→AT substitution.

The predominant base substitutions observed for the multiple mutations differed to those for the single and tandem base substitutions as transversions now predominated for the control and combined dose, whereas for the two individual treatments,

transitions now dominated. Only 1 and 3 multiple base substitutions were detected for the control and HQ treatment respectively, so, although the majority of transversion mutations were AT→TA, more data would be required to determine whether this was a significant observation. The multiple base substitutions occurring in the *p*-BQ and combined treatment were predominantly at GC base pairs. For the *p*-BQ treatment the major transversion mutation was the GC→TA substitution whereas for the combined treatment the major mutation was the GC→CG substitution. For the transition mutations GC→AT substitutions were dominant.

6.5.10. *supF* assay in GM04429 cells - mutation spectra

The distribution of mutations achieved by transfection of treated plasmid (HQ, *p*-BQ or both metabolites together), into the repair deficient cell line GM04429 are shown in Figures 6.10 – 6.12. In the single and tandem base substitution spectra (Figure 6.10), no mutational hotspots were observed in the control sample. Four hotspots were observed for the HQ treated sample, at positions 108, 129, 139 and 155 and the substitutions at these sites accounted for 31 % of the total single and tandem base substitutions. All four hotspots occurred at GC base pairs, 94 % of these base substitutions were for either an adenine or thymine base.

Only one hotspot was observed for the *p*-BQ treatment, which occurred at the GC base pair position 155, a hotspot already detected for the HQ treatment (see above). Three of the four base substitutions at this site were either a thymine or adenine base.

Two hotspots were observed for the combined metabolite treatment, which were located centrally in the *supF* gene at positions 132 and 139, which corresponded to a TA and a GC base pair, respectively. Substitutions were 50 % thymine or adenine and 50 % guanine or cytosine.

The mutation spectra for the multiple mutations can be seen in Figure 6.11. Few mutations were detected and hence no hotspots were observed. Substitutions in the spectra for all three treatment groups were predominantly at GC base pairs with, in the majority of cases, substitution being for a thymine or adenine base.

As seen previously with the Ad293 and GM00637 cell lines, the majority of the frameshift mutations were observed for the control and HQ treatment. Frameshift mutations accounted for 46 %, 23.5 %, 8 % and 5 % of the total mutation type for control, HQ, *p*-BQ and the combined treatment, respectively. In all cases the majority of the mutations were deletions, with deletions of more than 2 base pairs being dominant. For the control and HQ treatment, total deletion of the *supF* gene accounted for 25 % and 18 % of the deletions of more than 2 base pairs, respectively.

Table 6.10: Mutation types observed in the *supF* gene following treatment of pSP189 plasmid with 20 mM HQ, *p*-BQ or both metabolites together after transfection into GM04429 cells and subsequent transformation into *E. coli*, MBM7070

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Base substitutions	**14 (50)**	**51 (75)**	**54 (90)**	**53 (93)**
Single	14 (50)	49 (72)	45 (75)	45 (79)
Tandem	0 (0)	1 (1.5)	3 (5)	4 (7)
Multiple	0 (0)	1 (1.5)	6 (10)	4 (7)
Frameshifts	**13 (46)**	**16 (23.5)**	**5 (8)**	**3 (5)**
Single base deletion	0 (0)	1 (1.5)	1 (1.5)	1 (2)
>2 bases deleted	12 (43)	15 (22)	3 (5)	2 (3)
Single base insertion	0 (0)	0 (0)	1 (1.5)	0 (0)
>2 bases inserted	1 (4)	0 (0)	0 (0)	0 (0)
[a]other	**1 (4)**	**1 (1.5)**	**1 (2)**	**1 (2)**
Total number sequenced	**28 (100)**	**68 (100)**	**60 (100)**	**57 (100)**

[a]other mutation types involve a combination of both base substitutions and frameshift mutations seen in the same spectra

Table 6.11: Types of single and tandem base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in GM04429 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**4 (29)**	**23 (45)**	**29 (57)**	**32 (62)**
GC → TA	2 (14.5)	15 (29)	15 (29)	21 (40)
GC →CG	2 (14.5)	5 (10)	9 (18)	8 (15)
AT → TA	0 (0)	3 (6)	5 (10)	3 (5)
AT → CG	0 (0)	0 (0)	0 (0)	0 (0)
Transitions	**10 (71)**	**28 (55)**	**22 (43)**	**20 (38)**
GC →AT	10 (71)	25 (49)	17 (33)	15 (29)
AT → GC	0 (0)	3 (6)	5 (10)	5 (9)
Total number of single and tandem base substitutions	**14 (100)**	**51 (100)**	**51 (100)**	**52 (100)**

Table 6.12: Types of multiple base substitution mutations observed in the *supF* gene after treatment of pSP189 with 20 mM HQ, *p*-BQ or both metabolites together followed by transfection and replication in GM04429 cells and subsequent transformation into *E. coli*, MBM7070.

	Number of plasmids with mutations (%)			
Mutations	Control	20 mM HQ	20 mM *p*-BQ	20 mM HQ + *p*-BQ
Transversions	**1 (100)**	**1 (33)**	**7 (47)**	**5 (63)**
GC → TA	0 (0)	0 (0)	5 (33)	2 (25)
GC →CG	0 (0)	0 (0)	1 (7)	3 (38)
AT → TA	1 (100)	1 (33)	1 (7)	0 (0)
AT → CG	0 (0)	0 (0)	0 (0)	0 (0)
Transitions	**0 (0)**	**2 (67)**	**8 (53)**	**3 (37)**
GC →AT	0 (0)	2 (67)	8 (53)	3 (37)
AT → GC	0 (0)	0 (0)	0 (0)	0 (0)
Total number of multiple base substitutions	**1 (100)**	**3 (100)**	**15 (100)**	**8 (100)**

Figure 6.10: Mutation spectra showing single and tandem base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM04429 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown in blue.

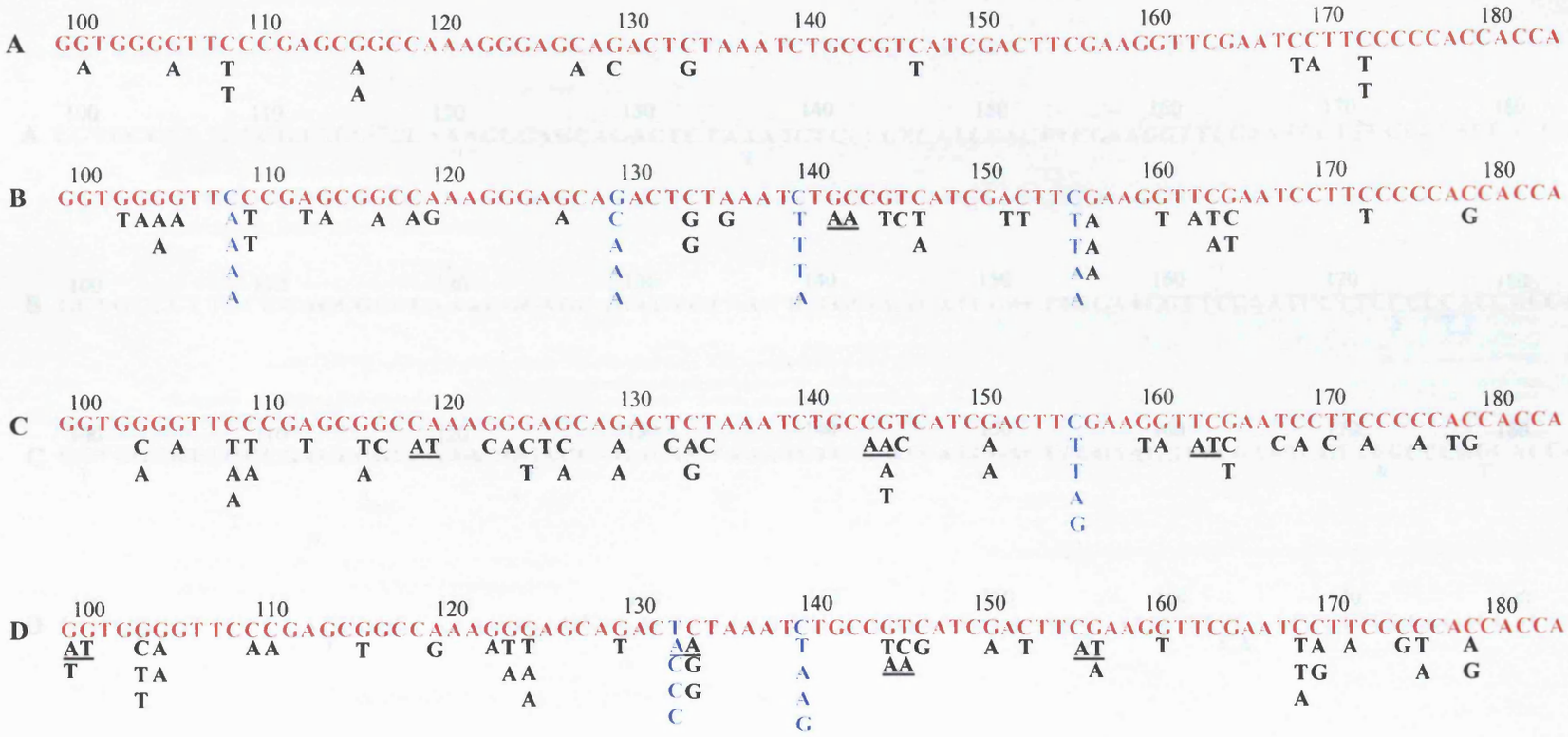


Figure 6.11: Mutation spectra showing multiple base substitutions induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM04429 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Hotspots are shown on the sequence in blue.
Related base substitutions i.e. those from the same DNA sequence, are highlighted in the same colour.

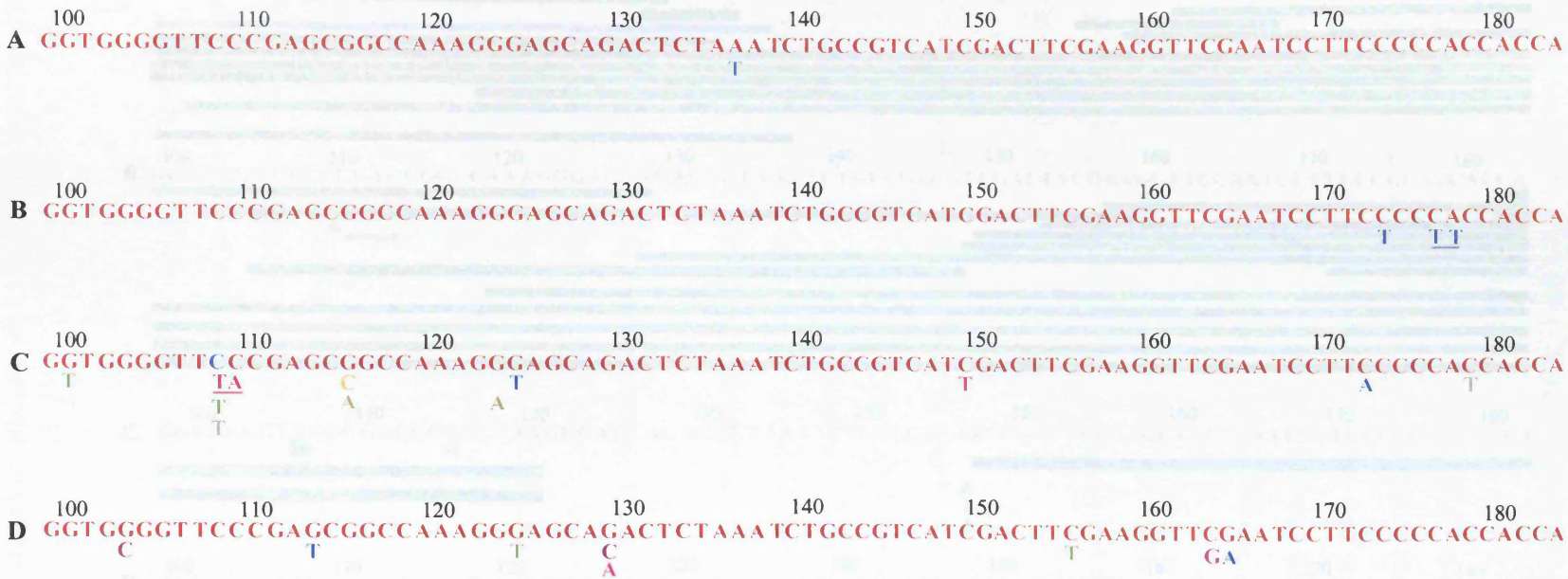


Figure 6.12: Mutation spectra showing frameshift mutations induced in the *supF* gene. pSP189 plasmid containing the *supF* gene was transfected into GM04429 cells after no treatment (control) (**A**), or treatment with 20 mM HQ (**B**), 20mM *p*-BQ (**C**) or both HQ and *p*-BQ together (**D**) with subsequent transformation into the indicator host, *E. coli*, MBM7070. Bars indicate where the deletion has occurred.
Related mutations i.e. from the same DNA sequence are highlighted in the same colour (some may relate to base substitutions shown in Figure 6.11).

I signifies a random 84 bp insert

6.5.11. Comparison of mutation spectra

Mutation spectra were compared using the Cariello Hyperg program (Cariello *et al.*, 1994). The mutation spectra containing single and tandem base substitutions from Ad293 transfected plasmid for both the control and HQ treated samples were significantly different to the *p*-BQ treatment ($p < 0.05$). For plasmid transfected into GM00637 cells again the spectra obtained for the HQ treatment was shown to be significantly different to the *p*-BQ treatment ($p < 0.05$). No difference was observed for any of the spectra obtained with plasmid transfected into GM04429 cells.

Comparison of spectra achieved with GM00637 and GM04429 cells showed a significant difference between the spectra for the HQ treatment ($p < 0.05$). No significant difference was observed for any of the other treatments.

6.5.12. Transfection of HL60 cells prior to plasmid treatment

To form the benzene DNA adduct, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, which is the main adduct observed in treated cells and is the only adduct considered to form following *in vivo* exposure to benzene (see Chapter 5 for further details), transfected cells were dosed with benzene metabolites.

It has been demonstrated that this adduct forms during treatment of HL60 cells with either HQ or *p*-BQ (Levay *et al.*, 1991 and Pongracz and Bodell, 1996) and it was postulated that HL60 cells could be transfected with plasmid and then treated with benzene metabolites in order to induce this adduct on plasmid DNA, and in particular within the *supF* gene.

HL60 cells, a human, promyelocytic cell line, which have no SV40 replicative ability were transfected by electroporation with pSP189 plasmid. Adaptations of the transfection method involved electroporation of cells at differing capacitance (μF) in order to determine the best procedure to allow the greatest influx of plasmid into the cells with minimal cell death.

Electroporation of HL60 cells with pSP189 proved to be successful at either 1 μF or 25 μF, although 24 h after transfection, cell death was in the range 12-14 % and 50-60 %,

respectively. Over time cell viability recovered and 96 h after electroporation, cell viability was in the range 92-99 %. Extraction of plasmid at 24 h time intervals resulted in a time dependent decline of colony numbers upon transformation of recovered plasmid into *E. coli,* MBM7070 (results not shown), suggesting destruction of the plasmid by the HL60 cellular environment.

Due to the initial cell death observed, routine transfection of HL60 cells was carried out at 1 μF capacitance. Cells electroporated with pSP189 plasmid were allowed to recover for up to 72 h, prior to further insult by dosing with benzene metabolites. Due to the increased cytotoxicity observed with *p*-BQ (Levay *et al.*, 1991), treatment of transfected cells was carried out with HQ (0-2 mM) for 2 to 48 h.

After an 8 h treatment, cell viability ranged from 97 %, 78 %, 21 % and 2 % for control, 500 μM, 1 mM and 2 mM HQ treatment, respectively. The yield of recovered plasmid increased both with dose and duration of treatment. Analysis of these samples by agarose gel electrophoresis resulted in no apparent bands corresponding to plasmid in any of the treated samples. Recovered plasmid was only observed in control samples suggesting that the DNA recovered was fragmented genomic DNA caused by the HQ treatment.

6.6. Discussion

The work described here details the use of the shuttle vector forward mutation assay involving the *supF* reporter gene for the analysis of mutations induced by the benzene metabolites HQ and *p*-BQ and both metabolites in combination. Mutagenesis was assessed using three different human cell lines; adenovirus transformed kidney cells (Ad293), due to their low spontaneous mutation background (Lebkowski *et al.*, 1984). and two fibroblast cell lines, GM00637 and GM04429. GM00637 is a repair proficient cell line and GM04429, a homozygous *Xeroderma pigmentosum* fibroblast complementation group A cell line, which is highly deficient in DNA repair.

Treatment of DNA with the two benzene metabolites HQ and *p*-BQ or *p*-BQ on its own, resulted in the formation of four major adducts as discussed in detail in Chapters 2 and 3. These four adducts were identified as (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Treatment of pSP189 plasmid with *p*-BQ or both HQ and *p*-BQ in combination resulted in the formation of these same four adducts. The 2'-deoxycytidine 3'-monophosphate adduct was the major adduct formed, with the two, 2'-deoxyguanosine 3'-monophosphate adducts being formed to a lesser extent. Adduct levels increased dose dependently for both treatment types, a result reflected in the mutation frequency as discussed earlier. Treatment of pSP189 plasmid with HQ alone, gave a low adduct number, ranging from 0.31 ± 0.08 to 0.45 ± 0.12 adducts / 10^4 nucleotides, a value which corresponded to less than one adduct per plasmid. The major adduct resulting from this reaction was (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate.

6.6.1. The *supF* assay in Ad293 cells

The spontaneous mutation frequency reported here for transfection into Ad293 cells was 2.67 / 10^4 colonies, approximately 10 fold higher than that reported by Seidman, 1989 and McLuckie *et al.*, 2002, who observed a spontaneous mutation frequency of 3 /10^5 colonies and 2/10^5 colonies, respectively. The mutation frequency observed for the 10 mM *p*-BQ dose following transfection into Ad293 cells (185 /10^4 colonies) was

similar to that observed by Nakayama *et al.*, 1999 and 2000, who reported a mutation frequency of 140 /10^4 colonies and approximately 240 /10^4 colonies for human and mouse cells, respectively. In general, the mutation frequency achieved for each dose corresponded to the adduct levels observed, which increased dose dependently for the *p*-BQ and combined treatment, a result not observed for the HQ treatment.

Spontaneous mutations reported in a number of publications have been pooled to obtain an overall spectrum. This spectrum showed that spontaneous mutations are non-randomly distributed with the majority of substitutions occurring at cytosines (positions 108-110) or guanines (positions 102-105, 122-124) and CpG sites especially within TCGA sites within the *supF* gene (Lewis *et al.*, 2001). Although a low level of spontaneous base substitutions were recorded here for transfection into Ad293 cells, mutations were observed at similar sites to those reported by Lewis *et al.*, 2001, which included positions 104, 110, 123 and 124.

Transfection of Ad293 cells with treated plasmid gave a predominance of single base substitutions at GC base pairs, predominantly GC→TA transversions or GC→AT transitions for the *p*-BQ and combined treatment. For the HQ treatment, GC→CG transversions and GC→AT transitions, dominated. Work carried out by Nakayama *et al.*, 1999 and 2000 showed similar findings following transfection of *p*-BQ (9.3 mM) treated plasmid into both mouse and repair proficient human fibroblast cells. As observed here, they saw a predominance of base substitutions at GC base pairs with the major mutations being GC→AT transitions followed by GC→TA transversions. Following propagation into human fibroblast cells, hotspots for mutation (or cold spots for repair) were observed at positions 113 and 123 for pMY189 plasmid and 150 and 158 for pNY200 plasmid (Nakayama *et al.*, 2000). For *p*-BQ treated plasmid transfected into the Ad293 cells, similar hotspot positions were observed at positions 123 and 159. Quite interestingly, the hotspots observed for single and tandem base substitutions for treated plasmid propagated in Ad293 cells, occurred at positions where two or more guanine bases were adjacent to each other, possibly due to the increased negative electrostatic potential brought about by this sequence context (La and Swenberg, 1996).

The majority of the single and tandem base substitutions reported here were for an adenine or thymine base and this occurred at a frequency of 61 %, 89 % and 73 % respectively for HQ, *p*-BQ and both metabolites in combination. This high frequency lends support for the 'A' rule, a mechanism by which damaged lesions are mispaired with adenine during DNA repair with certain mammalian DNA polymerases (reviewed in Strauss, 2002). This suggests that several of the mutations reported actually occurred on the non-transcribed DNA strand.

For the HQ treatment, which predominantly gave the minor benzene-DNA adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, it would be expected that all the base substitutions detected would be located at GC base pairs. This was not the case for approximately one third of the mutated bases. This result suggests that either, HQ treatment produces the other major DNA adducts which are produced in such low yield they are not detectable by ^{32}P-postlabelling, the adduct is having effect some distance downstream of the actual lesion or another mechanism i.e. oxidative damage, is causing the additional mutations. The major product of oxidative damage, 8-hydroxyguanine, causes G→T and A→C transversion mutations (Cheng *et al.*, 1992 and reviewed by Grollman and Moriya, 1993). Comparison of the mutation spectra using the Cariello Hyperg program (Cariello *et al.*, 1994) demonstrated a significant difference between the spectra obtained for the HQ treatment compared to the *p*-BQ treatment ($p < 0.05$), supporting the idea that another mechanism may be responsible for the mutagenicity observed. Jeong *et al.*, 1999 analysed mutations induced by hydroquinone-glutathione conjugates in Ad293 replicated plasmid and as observed here for the unconjugated metabolites, a predominance of GC base substitutions were observed. They compared this finding to that observed for hydroxyl radical induced mutations.

A number of multiple mutations were observed for each treatment group, these were included on a separate spectrum as it is thought that multiple mutations occur due to a separate mechanism to single or tandem mutations, possibly due to error prone polymerases during repair or translesional DNA synthesis (Courtemanche and Anderson, 1999 and Seidman *et al.*, 1987). The majority of multiple mutations were observed for the combined metabolite treatment, with a mutational hotspot occurring at

position 145. The multiple mutations could have arisen due to the possibility of more than one adduct occurring within the *supF* gene sequence, as an average 9 or 12 adducts were present on the plasmid for *p*-BQ and the combined treatment, respectively. Due to the large size of the plasmid (4952 base pairs) the likelihood of more than one hit on the reporter gene is quite low and the possibility of the multiple mutations occurring because of a different mechanism is supported by the presence of a hotspot at position 145, a thymine base. As described in Chapters 2 and 3, adducts forming with thymine are highly unlikely. It is possible that the adduct occurred on the opposite strand at the adenine base but as all the base substitutions were guanine i.e. the 'A' rule does not apply, this suggests either a different polymerase or mechanism is at work.

Multiple mutations have been correlated with an increased level of single strand breaks either as a consequence of the treatment regimen or as part of the repair mechanism (Seidman *et al.*, 1987). For the HQ treatment alone, a high percentage of the mutations detected (27 %) were frameshift mutations, with deletions of more than 2 base pairs predominating. Again, this result may imply a role for strand breaks (Kanbashi *et al.*, 1997). These results are similar to those reported by Hirakawa *et al.*, 2002 who observed that HQ caused not only DNA strand breaks but also DNA damage resulting from base modification at guanine and thymine residues, thought to be due to oxidative mechanisms.

Several other groups have carried out transfection of Ad293 cells with control and treated plasmid, containing the *supF* reporter gene. Joseph *et al.*, 1998, carried out transfection with either HQ or *p*-BQ treated plasmid. They concluded from their work that HQ caused a specific deletion of cytidine in the 5'-172-CCCCC-176-3' region of the *supF* gene resulting in a frameshift mutation, a result they did not observe with *p*-BQ. This work does not agree with the findings presented here, deletion of cytidine did occur in this study, but only as part of a larger deletion normally incorporating a large number of base pairs.

6.6.2. The *supF* assay in GM00637 and GM04429 cells

Work done by Singer and co-workers has demonstrated the role of AP endonucleases, without the need for glycosylases in the repair of the benzene-DNA adducts; (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate (Hang *et al.*, 1996 and Singer and Hang, 1999), (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate (Hang *et al.*, 1998 and Singer and Hang, 1999) and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate (Singer and Hang, 1999). These enzymes are utilised in an unusual mode of action which involves the enzyme directly incising the oligonucleotide 5' to the adduct without generation of an AP site. The adduct is actually left as a 'dangling base' on the 5' terminus (Hang *et al.*, 1996) and it is this localised effect on the DNA sequence, i.e. sequence distortion, rather than the adduct itself which allows recognition of the repair enzyme (Hang *et al.*, 1997). Work carried out with the dAp adduct demonstrated the importance of sequence context in regard to enzymatic action (Hang *et al.*, 1998). This work demonstrates the role of a BER-like process in the repair of benzene-DNA adducts. However, this work was carried out *in vitro* using a short sequence of double stranded DNA and therefore may not reflect the repair processes *in vivo*, when the complexity of the DNA structure, position of the adduct (major groove, minor groove, sequence context, etc) and the cellular environment may play a role in the recognition process of repair enzymes.

Mutational analysis was carried out in a repair proficient human fibroblast cell line, GM00637, and comparison was made to the mutation spectra obtained using the repair deficient cells, GM04429. Background mutation frequency in GM04429 cells was 1.65 /10^4 colonies compared to 5 / 10^5 colonies as reported by Seidman *et al.*, 1987. This group also reported a much higher spontaneous mutation frequency for the repair proficient cell line, GM00637, 7 / 10^4 colonies (Seidman *et al.*, 1987) which compared to the level observed here, 12.92 / 10^4 colonies. Comparison of mutation frequencies saw a 3-7 times lower level of mutation frequency for the HQ treatments in the repair deficient compared to repair proficient cell lines, which fits with the findings of Bredberg *et al.*, 1986, who studied the repair of UV damage in repair deficient and proficient cells. However, this result does not fit with the role of NER in repair of this damage, as an increased mutation frequency would be expected for the deficient cells compared to proficient cells. For the *p*-BQ and combined treatment no obvious pattern

in changes in mutation frequency were observed between the two different cell lines. These results could indicate a differing mechanism for the repair of HQ damage compared to the bulky damage induced by *p*-BQ.

Transfection of treated plasmid into both cell lines gave a predominance of single base substitutions in each case. In all situations the majority of these mutations occurred at GC base pairs, with GC→TA transversions and GC→AT transitions predominating. Again, as observed for transfection of treated plasmid into Ad293 cells, the majority of base substitutions for each treatment group were for either an adenine or thymine base. This result again suggests the application of the 'A' rule.

Hotspots of mutation for the single and tandem base substitutions occurred at positions 108, 109, 123, 129, 133 and 175 for HQ treated plasmid transfected into repair proficient cells compared to 108, 129, 139 and 155 for the repair deficient cells. Comparison of these two spectra using the Cariello Hyperg program (Cariello *et al.*, 1994) demonstrated a significant difference ($p < 0.05$). Transfection of *p*-BQ treated plasmid into GM00637 cells resulted in hotspots at positions 115, 122 and 155 whereas for GM04429 cells only one hotspot was recorded, again at position 155. These results were again similar to the findings of Nakayama *et al.*, 2000 who reported mutation hotspots in similar regions of the *supF* gene. For the combined treatment, different hotspots were observed for each cell type, positions 115, 122 and 123 and positions 132 and 139 for repair proficient and deficient cells, respectively. Two of the hotspots observed for *p*-BQ and the combined treatment in repair proficient cells were common to both treatments, these were located at positions 115 and 122. One hotspot observed in the GM04429 cells (combined treatment) was at position 132, a thymine base. A hotspot at a thymine base was not recorded in either GM00637 or Ad293 cells for single or tandem base substitutions.

No common pattern in regard to sequence context was observed for the hotspots observed in the spectra recorded for the single and tandem base substitutions. In the GM00637 cells, HQ treatment resulted in 3 hotspots being observed at multiple cytidine sites, suggesting that the mutations may have originated on the non-transcribed strand, one hotspot occurred within 3 adjacent guanine bases and the remaining two

were flanked either by adenine or thymine bases, respectively. A similar finding was observed for the same treatment following transfection into GM04429 cells. For the hotspots detected for the repair proficient cells, 2 out of 3 and 3 out of 3 hotspots were observed in multiple guanine sites for the *p*-BQ and combined treatment, respectively. For the repair deficient cells, (combined treatment) each hotspot was flanked by either cytidine or thymine bases, respectively.

Tandem base substitutions have been demonstrated to be products of pyrimidine dimers or intrastrand crosslinks and in particular a tandem mutation occurring at a CC base pair has been identified as a potential marker for oxidative damage during carcinogenesis (Reid *et al.*, 1994). All the tandem mutations occurring in GM00637 cells transfected with HQ treated plasmid occurred at CC base pairs. As observed for the mutations induced in the Ad293 cells, a high percentage of the mutations induced by treatment with HQ alone were frameshifts, accounting for 21 % and 23.5 % of the total for GM00637 and GM04429 cells, respectively.

A number of multiple mutations were detected for all treatments for both cell lines, with a higher percentage occurring in the repair proficient cells compared to repair deficient cells. A similar observation was made by other workers after passage of a UV-irradiated or aflatoxin B_1 treated shuttle vector into NER deficient cells. They suggested from this result that multiple mutations occur during NER by a gap filling error prone polymerase (Seidman *et al.*, 1987) and the secondary mutation is thought to occur 3' to the original lesion during DNA replication (Courtemanche and Anderson, 1999). A number of hotspots were recorded for the individual treatments with HQ and *p*-BQ in the repair proficient cells. As observed in the Ad293 cells, the majority of base substitutions at these hotspots were for a guanine base, suggesting that as the 'A' rule does not apply to this situation a different polymerase or repair mechanism is involved.

6.6.3. Transfection of HL60 cells prior to plasmid treatment

The use of shuttle vectors has proved an important system in the study of mutagenesis for a number of different mutagens as it is often found that the mutation types observed in simple systems reflect those occurring in tumours of mammals exposed to

carcinogenic agents (Essigmann and Wood, 1993). The mutation spectrum differs depending on cancer type and the carcinogen involved (Perera, 1996). For example, most polycyclic aromatic hydrocarbons give GC→TA and AT→TA transversions (Bigger *et al.*, 2000) whereas oxidative damage in the form of 8-hydroxyguanine, gives predominantly G→T and A→C base substitutions (Cheng *et al.*, 1992). G→T transversions in the p53 gene of lung, breast, stomach and liver cancers are consistent with DNA adducts following exposure to bulky carcinogens or oxidative damage (Perera, 1996).

To date, only one major benzene-DNA adduct has been identified *in vivo* and hence analysis of its mutagenicity may aid the determination of whether this adduct plays an important role in benzene toxicity. The adduct has been identified as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate and has been observed following treatment of HL60 cells with the benzene metabolites, HQ or *p*-BQ. To induce this adduct on plasmid DNA, it was postulated that HL60 cells could be transfected with plasmid and then treated with metabolites. Both the transfection procedure and the metabolite treatment proved to be detrimental to the cells causing a decline in recovered plasmid probably due to the recognition of the plasmid as 'foreign' to the cell hence leading to its digestion and an increase in the amount of fragmented genomic DNA, probably due to the clastogenic effects of HQ. The discovery that dosing Ad293 cells with a combined treatment of HQ and *p*-BQ gave an adduct with similar chromatographic properties to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate or the synthesis of this adduct as a phosphoramidite to be utilised in site specific assays (see Chapter 5 for further details) may be a potential solution to this problem .

6.6.4. Summary

The work described here provides direct information regarding the nature of benzene metabolite induced mutations after processing through replication systems in human cells *in vitro* which were both proficient (Ad293 and GM00637) and deficient (GM04429) in nucleotide excision repair. The study also demonstrates the potential difference in both the repair and mutagenicity of the DNA damage induced by HQ and *p*-BQ. HQ, but not *p*-BQ, proved positive in the oxidative mutagenicity test (Martinez

et al., 2000) and has been shown to indirectly damage DNA by reacting with oxygen forming semi-quinone radicals and reactive oxygen species (Gut *et al.*, 1996a) whereas *p*-BQ can react directly with DNA, forming DNA adducts.

The role of oxidative damage induced either by HQ directly or from the semi-quinone intermediate produced during the redox cycling of HQ and *p*-BQ has been demonstrated in a number of ways here. The high frequency of frameshift mutations and the presence of base substitutions not at GC base pairs in HQ treated plasmid, the presence of CC tandem mutations in GM00637 cells, propagated with HQ treated plasmid and the high incidence of multiple mutations occurring at thymine, a base not known to produce a benzene-DNA adduct have all been characterised as mutations occurring due to oxidative damage (Hirakawa *et al.*, 2002; Kanbashi, *et al.*, 1997, and Reid *et al.*, 1994).

Such oxidative damage induced by quinones, semi-quinones and oxygen radicals can lead to strand breaks, inhibition of topoisomerase and microtubule assembly with the potential consequence of chromosomal damage (Smith 1996). A whole range of chromosomal effects following exposure to benzene and its metabolites has been reported in the literature and in some cases has been linked to the oxidative damaging potential of HQ. Administration of HQ has been shown to cause an increased number of strand breaks in human lymphocytes *in vitro* (Andreoli *et al.*, 1997 and 1999), an increase in micronuclei formation *in vitro* (Glatt *et al.*, 1989 and Yager *et al.*, 1990) and in mouse cells *in vivo* (Jagetia *et al.*, 2001 and Tunek *et al.*, 1982) and sister chromatid exchange *in vitro* (Erexson *et al.*, 1985). These effects could be linked to the chromosomal damage observed following benzene exposure in both test animals and occupationally exposed individuals. These observations include chromosomal loss and breakage in mouse bone marrow and spleen cells *in vivo* (Chen *et al.*, 1994), and both structural and numerical chromosomal abnormalities in the peripheral blood lymphocytes of occupationally exposed individuals, which include part or whole deletion of chromosomes 5 and 7 and translocations between chromosomes 8 and 21 (Forni, 1996; Rothman *et al.*, 1995; Zhang *et* al., 1998 and Zhang *et al.*, 2002). If benzene produces a mutation 'signature' in shuttle vectors which reflects the mutation types observed in the cells of benzene exposed mammals, then it may be of interest to

analyse certain genes involved in leukaemia development for example the AML gene and in particular, genes on chromosomes 5 and 7.

The role of bulky DNA adducts in the mutagenicity of benzene however, cannot be ruled out. Jowa *et al.*, 1986 reported that adducts at the N^2 position, as the deoxyguanosine 3'-monophosphate adducts are, may be resistant to repair (Jowa *et al.*, 1986) and the work described here does demonstrate that these adducts are mutagenic. How these results relate to the damage observed in occupationally exposed individuals is difficult to assess here. Some genotoxic agents are capable of inducing multiple adducts, and it is often assumed that the major adduct types are the most mutagenic, which is often not the case. For example, in the case of alkylating agents, the minor lesions occurring at exocyclic oxygen sites are more mutagenic than the major lesions occurring at ring nitrogen positions (Basu and Essigmann, 1990; Singer and Essigmann, 1991 and Singer, 1996). In the study here, the major benzene DNA adduct is located on a cytidine base whereas the minor products are on guanine bases. As the majority of base substitutions occurred at GC base pairs, taking the 'A' rule into account the mutagenicity of the two adduct types was similar. The use of the polymerase stop assay or site specific adduct analysis may give further insight into the role of these individual adducts.

The polymerase stop assay is based on the observation that polymerization stops at the adducted base and can therefore be used to determine the position of a lesion within a DNA sequence. This assay was utilised by Nakayama *et al.*, 1999, for the analysis of *p*-BQ treated plasmid, which was transfected into mouse cells. They observed that the sites of the adducts did not correspond exactly to the position of the mutation. A mutation may in fact occur to the left or right of the actual site of the adduct, or the lesion could have an effect from a distance as demonstrated by UV damage (Levy *et al.*, 1996). The site specific mutation assay incorporates a DNA adduct at a specific site within a known DNA sequence (Singer and Essigmann, 1991). Both assays allow sequence context to be investigated, a factor thought to play an important role in mutagenesis (Sikpi *et al.*, 1990). Sites of relatively low frequency of modification but high level of mutagenicity, as well as sites which are frequently modified but

infrequently mutated and those which are frequently modified and mutated can be determined (Seetharam and Seidman, 1991).

Chapter 7
Discussion

7.0. Discussion

Benzene has been under investigation for a number of years due to its ability to induce solid tumours in rodent models and its link to the induction of leukaemia in humans. The mechanism by which benzene exerts its genotoxic and carcinogenic effect has yet to be elucidated but it is considered that more than one metabolite is involved that acts through multiple mechanisms on more than one cellular target.

The main aim of the work described in this thesis was to determine whether DNA adducts could be detected in DNA samples from occupationally exposed individuals and whether these adducts could provide a mechanistic insight into the link between benzene and leukaemia. The most sensitive and widely available technique for DNA adduct detection is ^{32}P-postlabelling (accelerator mass spectrometry is more sensitive, but not as widely available, or applicable to unlabelled compounds). The technique of ^{32}P-postlabelling has been applied to the biological monitoring of individuals occupationally exposed to a number of chemicals. These include complex mixtures of polycyclic aromatic hydrocarbons in coke oven and aluminium plant workers (Kriek *et al.*, 1993; Lewtas *et al.*, 1993 and Schoket *et al.*, 1993) and styrene oxide in a group of lamination (reinforced plastic) workers (Vodicka *et al.*, 1999).

Before the analysis of any DNA samples from benzene-exposed individuals several modifications to the ^{32}P-postlabelling assay were required. These modifications would hopefully improve both the sensitivity and selectivity of the assay. Initially, four benzene-DNA adduct standards were synthesised, one of which was a novel adduct, characterised and identified as (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. These adducts were used to develop TLC and HPLC systems for ultimately analysing tissue DNA samples. The chromatography systems developed allowed for the clear separation of all four DNA adducts. A number of methods were employed to synthesise, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, the only adduct so far identified following *in vivo* exposure to benzene in rodents. Synthesis of this adduct was complicated by several factors. The ideal method of synthesis would have been a chemical one, but as several important chemicals for its synthesis were no longer available, which included the starting material 2-thioxanthine, a number of biological

methods were employed. Several of these methods consistently produced two adducts, one of which had similar retention to the adduct, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and the other, to the adduct (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. However, insufficient material was available to confirm the structure of these products by mass spectrometry. The adduct N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate, was eventually obtained from Dr. William Bodell, University of California. ^{32}P-postlabelling of this adduct standard showed that it co-eluted with the newly identified adduct, (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate, using both the TLC and HPLC systems. It was difficult to assess at this time whether one adduct was the product of the other due to some further reaction occurring during the ^{32}P-postlabelling procedure or whether the two adducts were indeed structurally different but with identical chromatographic properties, which is a more feasible explanation. If this were true however, it asks the question as to whether the DNA adduct that has previously been observed *in vivo* (Bodell *et al.*, 1996) and identified as N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate is indeed this adduct and not (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Further work would need to be carried out to determine which adduct is indeed formed *in vivo*.

Attempts were made to raise an antibody to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate either as part of a polyclonal sera or as a monoclonal antibody. In both cases, the antibody produced recognised either the linking group between the DNA adduct and the carrier protein or required both the adduct and carrier protein to elicit a response, respectively. The antibody, if successful would have been used in an enrichment step prior to ^{32}P-postlabelling, improving the assay's selectivity to this particular adduct. It was also proposed that the antibody could be used in an immunoslot blot assay, when DNA samples are limited, which is quite often the case in human biomonitoring studies.

However, since the production of an antibody was not successful, ^{32}P-postlabelling of benzene-DNA adducts was reliant on the traditional method of adduct enhancement using the enzyme, nuclease P1. Nuclease P1 digests unmodified nucleotides to

nucleosides. The adducts under investigation, probably due to their small size, were shown to also be substrates for this digestion and hence modifications, which included a reduction in the time allowed for digestion, were required to ensure full adduct recovery. Other steps in the ^{32}P-postlabelling procedure, for example, digestion to the nucleotide components and labelling efficiencies were also assessed to ensure the procedure was optimal.

The ^{32}P-postlabelling method was applied to the analysis of DNA samples from benzene-exposed mice and occupationally exposed individuals. A DNA adduct was observed in liver DNA samples from benzene exposed mice. This adduct had similar mobility on TLC to N^2-(4-hydroxyphenyl)-2'-deoxyguanosine 3'-monophosphate (and (3", 4"-dihydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate). The adduct levels however were extremely low, ranging from 4.5 – 12 adducts / 10^8 nucleotides. Analysis of DNA from the human lymphocyte samples, resulted in no observable DNA adducts, except one radioactive spot, which was common to all samples including the control DNA samples.

Lack of DNA adduct formation in the human samples may be a reflection of a number of factors. The volunteers were employed in a petroleum refinery, and their benzene exposure was estimated to be less than 2 ppm. The current occupational exposure limit to benzene stands at 10 ppm per 8 h working day. Therefore at the 2 ppm level the risk from benzene exposure may be minimal. The major target organ of benzene toxicity is the bone marrow and therefore ideally this tissue would be required to allow successful DNA adduct analysis. In the absence of the target tissue (due to ethical issues) a surrogate tissue was investigated, in this case, lymphocytes were utilised. However, neutrophils, which contain myeloperoxidase, the enzyme that metabolises hydroquinone (HQ) to its theoretical more toxic metabolite, *para*-benzoquinone (*p*-BQ), would have been more relevant but the amount of DNA available just from neutrophils would have been limiting. Successful production of an antibody, which was selective for one or more of the benzene-DNA adducts would improve adduct detection immensely. Therefore further work would be required to improve assay sensitivity before any definite statement in regard to benzene-DNA adduct formation in humans could be made.

If a sensitive method could be developed that allowed DNA adducts to be observed in human tissues, the relevance of these adducts in regard to benzene carcinogenicity would be difficult to assess based on this method alone. As an extension to the work described here, the forward mutation assay, *supF*, was employed to determine the consequence of benzene-DNA adducts in regard to their mutagenicity. Two repair proficient and one repair deficient cell line was employed. Treatment of plasmid, pSP189 with the benzene metabolite, *p*-BQ, produced four adducts which were also used for ^{32}P-postlabelling method development; (3"-hydroxy)-3, N^4-benzetheno-2'-deoxycytidine 3'-monophosphate, (3"-hydroxy)-1, N^6-benzetheno-2'-deoxyadenosine 3'-monophosphate, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate and (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. Treatment of plasmid, pSP189 with the benzene metabolite, HQ, resulted in only one adduct, (3"-hydroxy)-1, N^2-benzetheno-2'-deoxyguanosine 3'-monophosphate. The majority of the mutations induced for the treatments and for the three cell lines, were base substitution mutations for both compounds. The majority of mutations following treatment with *p*-BQ were GC→TA transversions and GC→AT transitions, whereas for HQ treatment GC→AT transitions predominated, for all the cell lines investigated.

The results obtained suggested that the two benzene metabolites induced mutations by two different mechanisms. The major mutations observed following treatment with *p*-BQ could be due to either the major dCp adduct or either of the minor dGp adducts. Further analysis using for example, the polymerase stop assay, would help determine which adduct was the more mutagenic. HQ treatment however, gave a number of results, which were indicative of oxidative DNA damage. These results included a high frequency of frameshift mutations, a high incidence of multiple mutations occurring at thymine and base substitutions not occurring at GC base pairs. A difference was also observed in the repair mechanisms between the two treatments. Bulky benzene-DNA adducts are reportedly repaired by base excision repair. The results obtained here suggest the damage incurred following HQ treatment is repaired by nucleotide excision repair.

Work carried out by a number of other groups have demonstrated a link between HQ treatment, oxidative damage and strand breaks which could be linked to the chromosomal damage observed in benzene exposed individuals. The chromosomal damage includes part or whole deletion of chromosomes 5 and 7 and translocations between chromosomes 8 and 21. The results obtained here suggest a role for oxidative damage induced by HQ in benzene genotoxicity, however the role of the more bulky DNA lesions induced by *p*-BQ cannot be ruled out at this stage. Further mutational work using a more relevant target gene, for example the AML gene located on chromosome 21, or one of the genes associated with haematopoiesis on chromosome 5 may be more beneficial to the long term study of benzene genotoxicity.

Chapter 8
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Identification and Characterization of (3″,4″-Dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-Monophosphate, a Novel DNA Adduct Formed by Benzene Metabolites

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Reaction of 2′-deoxyguanosine 3′-monophosphate with mixtures of the benzene metabolites *p*-benzoquinone (*p*-BQ) and hydroquinone (HQ) in an aqueous solution at pH 6.0 gave two main products which were isolated from the reaction mixture using reversed-phase HPLC and characterized using UV spectroscopy, negative ion electrospray mass spectrometry, and ^{1}H NMR. Variation of the ratio of *p*-BQ to HQ in the reaction mixture caused an increase in yield of one of the products. The two products were identified as (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate and a new product, not previously characterized, (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate. Similar products were isolated from identical reactions with 2′-deoxyguanosine. Reaction of calf thymus DNA with HQ and *p*-BQ (1:1, w/w) resulted in four main products as identified by ^{32}P-postlabeling coupled with HPLC. The relative abundances of these adducts were 9%, 60%, 27%, and 4%, respectively. Co-chromatography of ^{32}P-postlabeled (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate and (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate with the ^{32}P-postlabeled adducted calf thymus DNA identified these as the two minor products of the calf thymus DNA reaction.

Introduction

Benzene is a ubiquitous environmental pollutant present in automobile exhaust fumes and cigarette smoke and is used extensively in the chemical industry. Benzene exposure has been associated with bone marrow toxicity and leukemia in humans (*1*) and is a known rodent carcinogen causing malignancy in a number of organs in rats and mice (*2*).

Benzene is initially metabolized in the liver to form benzene oxide, which can undergo spontaneous rearrangement to form phenol. Phenol can be metabolized by cytochrome P450 2E1 to form the hydroxylated compounds hydroquinone (HQ),[1] catechol, and 1,2,4-benzenetriol. The hydroxylated compounds, HQ and catechol, are thought to be transported (if not excreted in the form of glucuronide or sulfate conjugates in the urine) (*3*) to the bone marrow, the target tissue for benzene toxicity. Once in the bone marrow, HQ can undergo further oxidation by the action of peroxidases, in particular myeloperoxidase, to form the reactive metabolite *p*-benzoquinone (*p*-BQ). The metabolites, HQ and *p*-BQ, have been shown to induce sister chromatid exchanges in vitro in human lymphocytes (*4*) and cause micronuclei formation in human lymphocytes in vitro and mouse erythrocytes in vivo (*5, 6*) and mutations in V79 cells (*7*). The formation of DNA adducts by HQ and/or *p*-BQ may be one mechanism responsible for the genotoxic effects of benzene. Several studies have been carried out to demonstrate covalent binding of benzene metabolites to DNA in vivo in rat liver (*8*) and mouse bone marrow (*9*) although these studies failed to determine which metabolite was responsible.

In vitro, the formation of three benzene DNA adducts has been reported, (3″-hydroxy)-3,N^4-benzetheno-2′-deoxycytidine 3′-monophosphate[2] (*10*), (3″-hydroxy)-1,N^6-benzetheno-2′-deoxyadenosine 3′-monophosphate (*11*), and (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate (*12*–*14*). These adducts are formed from the reaction of DNA or individual nucleotides or nucleosides with the benzene metabolites *p*-BQ and HQ. In DNA, the approximate yields of these three adducts as reported by Pongracz and Bodell (*11*) are 74.4 ± 4.5%, 22.4 ± 4.1%, and 1.1 ± 0.9%, respectively.

In our laboratory, when repeating the reaction of calf thymus DNA with HQ and *p*-BQ, we were aware of four major adducts as observed by ^{32}P-postlabeling coupled with HPLC. Three of the adducts were identified as those

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[1] Abbreviations: HQ, hydroquinone; *p*-BQ, *p*-benzoquinone.

[2] The names of these compounds have been changed compared to those given in the literature in order to clarify the position of hydroxylation. Thus, 3′, as in the literature (which might be interpreted as a position on the sugar), has been replaced by 3″.

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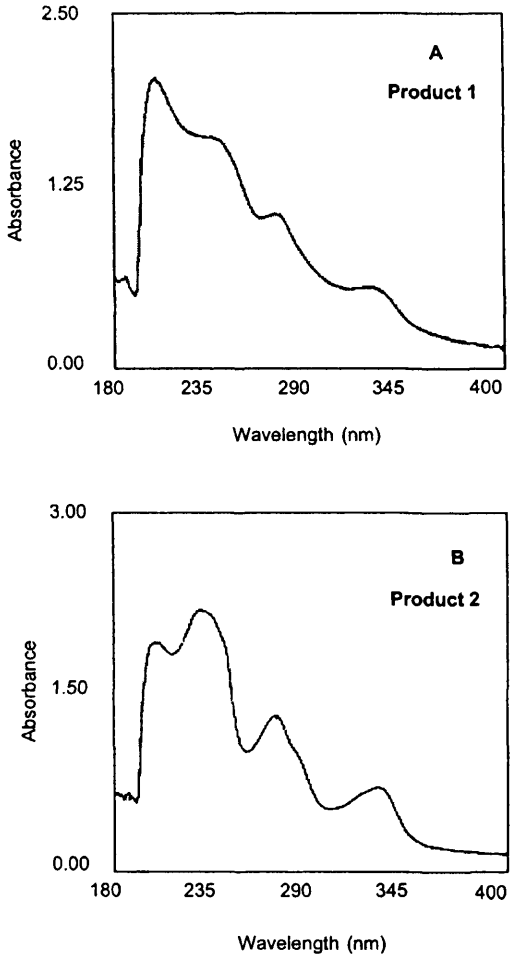


Figure 1. Typical UV absorption spectra of (A) product 1 and (B) product 2 from the reaction of 2′-deoxyguanosine 3′-monophosphate with hydroquinone and *p*-benzoquinone. Reaction was carried out in 10 mM ammonium formate, pH 6.0 at 37 °C, overnight. Products were isolated following HPLC analysis, and UV absorption spectra were obtained using a Uvikon 860 spectrophotometer (Kontron Instruments, Zurich, Germany).

described above (*10*–*14*), and one is a previously unidentified adduct. Snyder et al. (*15*) and Rushmore et al. (*16*) have reported that incubating rat liver mitoplasts and rabbit bone marrow cell mitoplasts with both radiolabeled dGTP and benzene resulted in at least six deoxyguanosine adducts in the rat cells and seven in the rabbit cells as revealed by Sephadex column chromatography. These results imply that there is a high probability that the unidentified adduct observed here may be found on deoxyguanosine. In this paper, we confirm this and describe the characterization of the adduct formed, which was eventually determined to be (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate.

Materials and Methods

Chemicals and Reagents. Calf thymus DNA, 2′-deoxyguanosine 3′-monophosphate, 8-hydroxy-2′-deoxguanosine, nuclease P1, micrococcal nuclease, and HQ were purchased from Sigma (Poole, Dorset, U.K.). 2′-Deoxyguanosine and *p*-BQ were obtained from Fluka (Gillingham, U.K.). [γ-^{32}P]ATP (>185 TBq/mmol, >5000 Ci/mmol) was purchased from Amersham (Amersham, Bucks, U.K.). T4 polynucleotide kinase (3′-phosphatase-free) and calf spleen phosphodiesterase were obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). All other chemicals used were of the highest grade available and were obtained from Fisher Scientific Ltd. (Loughborough, Leicestershire, U.K.).

Caution: *[γ-^{32}P]ATP is a hazardous radioactive compound and should be handled with sufficient protection and shielding. HQ and p-BQ are harmful and must be handled appropriately.*

Instrumentation. Analytical UV HPLC was conducted using a Gilson 715 system (Gilson, Middleton, WI) using a Hypersil, C18, 5 μm, 4.6 × 250 mm reversed-phase column; semipreparative HPLC was carried out using a Hypersil, C18, 5 μm, 10 × 250 mm reversed-phase column. UV absorbance was monitored at 254 nm.

UV spectra of the isolated products were recorded with a Uvikon 860 spectrophotometer (Kontron Instruments, Zurich, Germany).

Mass spectra of the isolated products were recorded on a Micromass Quattro BioQ (Micromass, Manchester, U.K.) tandem quadrupole mass spectrometer with an electrospray interface. Solutions were introduced by constant infusion using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Edenbridge, U.K.) and a Hamilton 1 mL gastight syringe pumped at a flow rate of 10 μL/min. Samples were dissolved in 1:1 methanol/water (v/v). Samples were analyzed using the negative ionization mode.

^{1}H NMR spectra of the isolated products were recorded at room temperature on a Bruker ARX 250 (250 MHz) instrument. Samples were dissolved in d_4-methanol.

HPLC of ^{32}P-postlabeled samples was carried out using a Varian system with Star 9012 pump (Varian Analytical Instruments, Surrey, U.K.) and a Hypersil, C18, 5 μm, 4.6 × 250 mm reversed-phase column. Eluate passed through a radiochemical detector (β-ram, LabLogic, Sheffield, U.K.) fitted with a solid-phase cell (500 μL). Data analysis was performed using Laura, a Microsoft Windows package (LabLogic, Sheffield, U.K.).

1. Reaction of 2′-Deoxyguanosine 3′-Monophosphate with HQ and *p*-BQ. 2′-Deoxyguanosine 3′-monophosphate (5 mg), HQ (5 mg), and varying amounts of *p*-BQ (0–10 mg), or alternatively 2′-deoxyguanosine 3′-monophosphate (5 mg), varying amounts of HQ (0–10 mg), and *p*-BQ (5 mg), were dissolved in 1 mL of 10 mM ammonium formate buffer, pH 6.0, and incubated overnight at 37 °C (*14*). The reaction mixture was subjected to ethyl acetate extraction to remove unreacted benzene metabolites prior to HPLC separation. Two main products were separated by analytical HPLC (t_R 15.5 and 17.5 min) using a linear gradient of methanol (solvent B) in 10 mM ammonium formate (solvent A), pH 5.1 (0–70% B, 30 min; 70% B, 30–35 min; 70–0% B; 35–40 min; 0% B, 40–45 min), flow rate 1 mL/min.

The 3′-phosphate group was removed from the isolated adducted products (dried HPLC fractions) by incubating the samples with nuclease P1 (2 μg/μL in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5.0) for 24 h at 37 °C.

2. Reaction of 2′-Deoxyguanosine with HQ and *p*-BQ. 2′-Deoxyguanosine (5 mg), HQ (5 mg), and varying amounts of *p*-BQ (0–10 mg), or alternatively 2′-deoxyguanosine (5 mg), varying amounts of HQ (0–10 mg), and *p*-BQ (5 mg), were dissolved in 1 mL of 10 mM ammonium formate buffer, pH 6.0, and incubated overnight at 37 °C. The reaction was scaled up (20-fold) for NMR analysis. The reaction mixture was subjected to ethyl acetate extraction to remove unreacted benzene metabolites prior to HPLC separation. Two main products were separated by HPLC (t_R 19.5 and 22 min) using a linear gradient of methanol (solvent B) in 50 mM ammonium formate (solvent A), pH 5.4 (0–35% B, 15 min; 35% B, 15–25 min; 35–70% B, 25–35 min; 70–0% B, 35–40 min; 0% B, 40–45 min), flow rate 1 mL/min for analytical analysis and 5 mL/min for semi-preparative analysis.

3. Reaction of 8-Hydroxy-2′-deoxyguanosine with HQ and *p*-BQ. 8-Hydroxy-2′-deoxyguanosine (5 mg), HQ (5 mg), and 0.5 mg of *p*-BQ were dissolved in 1 mL of 10 mM ammonium

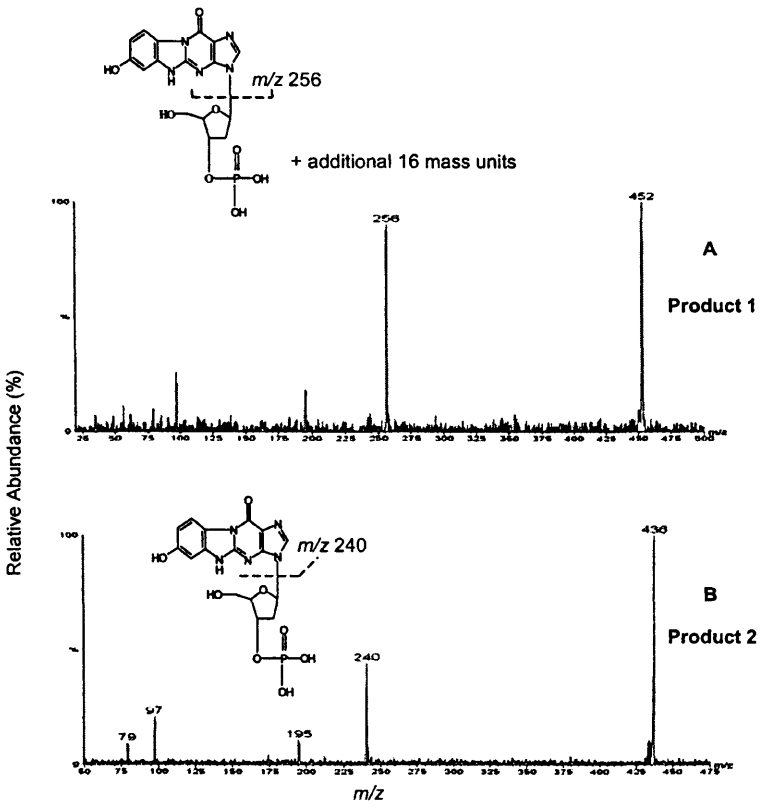


Figure 2. Typical MS/MS analyses of products 1 and 2 from the reaction of 2′-deoxyguanosine 3′-monophosphate with hydroquinone and *p*-benzoquinone in 10 mM ammonium formate, pH 6.0 at 37 °C, overnight. Product 1 (A) has a molecular ion $[M - H]^-$ *m/z* 452, with a fragment ion *m/z* 256. Product 2 (B) has a molecular ion $[M - H]^-$ *m/z* 436, with a fragment ion *m/z* 240. Fragment ions *m/z* 79, 97, and 195 relate to PO_3^-, phosphate group, and deoxyribose plus phosphate moieties, respectively.

formate buffer, pH 6.0, and incubated overnight at 37 °C. The reaction mixture was extracted with ethyl acetate to remove unreacted benzene metabolites prior to HPLC separation. Two main products were separated by HPLC (t_R 20.5 and 23 min) using the conditions described for reaction 2 with a flow rate of 1 mL/min.

4. Reaction with DNA. Calf thymus DNA (1 mg) was treated with *p*-BQ (1 mg) and HQ (1 mg) in 1 mL of 30 mM ammonium formate, pH 7.0 at 37 °C, overnight. The reacted DNA was precipitated by the addition of 0.8 volume of 2-propanol. DNA was washed twice with 70:30 ice-cold ethanol/water (v/v) followed by 1 wash with ice-cold ethanol. DNA was dissolved in 1.5 mM sodium chloride, 0.15 mM trisodium citrate.

^{32}P-Postlabeling Analysis. Adducted calf thymus DNA (5 μg) from reaction 4 was enzymatically digested to deoxyribonucleoside 3′-monophosphates using micrococcal nuclease (175 milliunits) and calf spleen phosphodiesterase (3 milliunits) at 37 °C, overnight. DNA underwent adduct enrichment using the nuclease P1 enhancement method described by Reddy and Randerath (*17*). Adducted nucleotides, which included the two products from reaction 1, were radiolabeled by 5′-phosphorylation using [γ-^{32}P]ATP (62.5 μCi) and T4 polynucleotide kinase (6.25 units). Incubation was carried out for 1 h at 37 °C.

HPLC analysis of the radiolabeled samples was carried out using an isocratic separation of acetonitrile (solvent B) in 2 M ammonium formate, pH 4.0, containing 270 μM EDTA (solvent A) (2% B, 50 min; 2–50% B, 50–65 min), flow rate 1 mL/min.

Statistical Analysis. Reactions of 2′-deoxyguanosine 3′-monophosphate (5 mg) with either HQ (5 mg) and varying amounts of *p*-BQ (0–10 mg), or alternatively 2′-deoxyguanosine 3′-monophosphate (5 mg), varying amounts of HQ (0–10 mg), and *p*-BQ (5 mg) were performed a minimum of 3 times. Standard statistical methods were used to calculate means and their standard errors. Significance of differences was examined using one-way analysis of variance followed by Tukey's posthoc test. The level of confidence required for each selected comparison was set in advance to $p < 0.05$.

Results

Reaction of 2′-Deoxyguanosine 3′-Monophosphate with HQ and *p*-BQ. The reaction of 2′-deoxyguanosine 3′-monophosphate with *p*-BQ and HQ, at pH 6.0, resulted in the synthesis of two major products with t_R of 15.5 (product 1) and 17.5 min (product 2) when isolated by reversed-phase HPLC. The UV absorption spectrum of product 1 gave a peak at 203 nm and shoulders at approximately 235, 270, and 330 nm, and product 2 gave similar UV spectra except peaks denoting UV_{max} are more defined with peaks at 203, 229, 271, and 330 nm and no shoulders (Figure 1).

Tandem MS analysis of these two products using continuous-infusion, negative ion electrospray resulted in molecular ions $[M - H]^-$ *m/z* 452 and 436 and fragment ions *m/z* 256 and 240, respectively (Figure 2). These data are consistent with products of molecular weights 453 and 437, with a mass difference between the two products of 16. The data from the fragment ions are consistent with the loss of the deoxyribose and phosphate moieties from the molecular ion for both compounds. Again, a mass difference of 16 is observed which suggests the extra 16 mass units on product 1 is present on the adducted base rather than the deoxyribose. This verifies that product 1 is not due to contaminating guanosine 3′-monophosphate in the starting material.

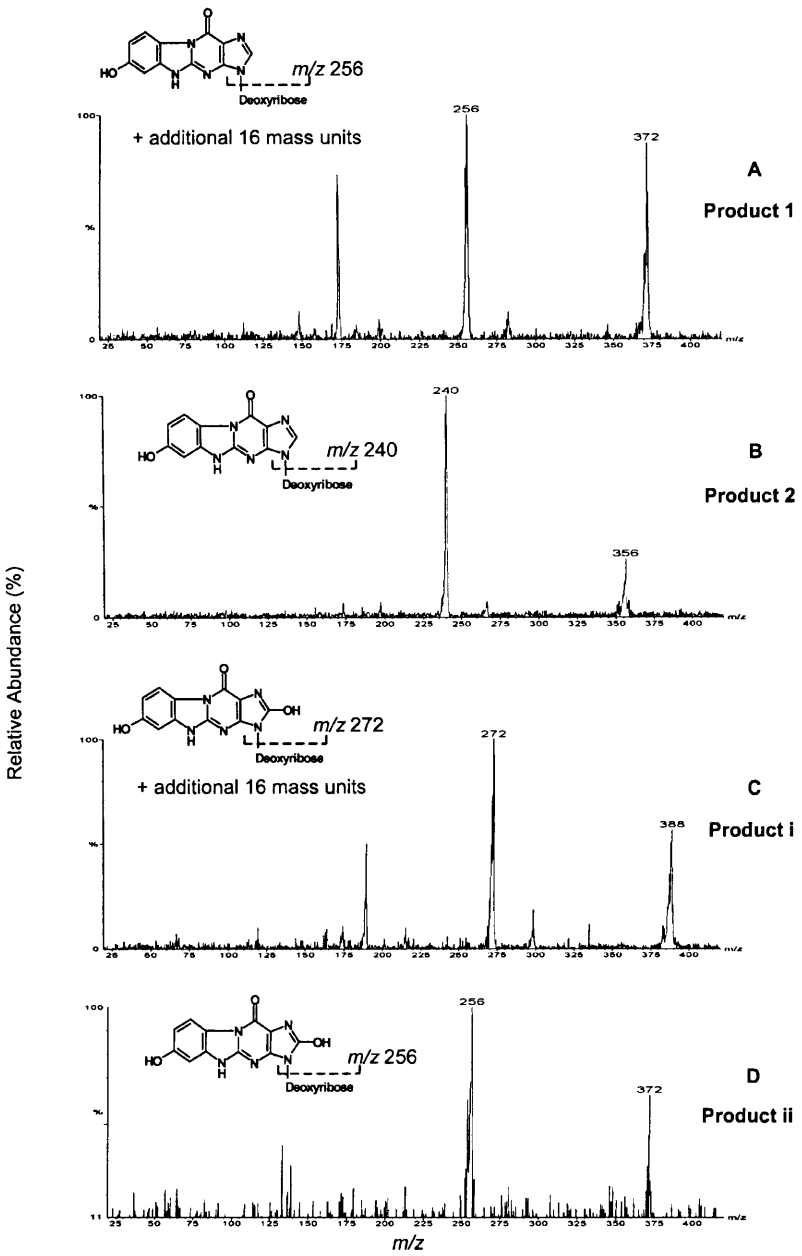


Figure 3. Typical MS/MS analyses of the main products from the reaction of 2′-deoxyguanosine or 8-hydroxy-2′-deoxyguanosine with hydroquinone and *p*-benzoquinone in 10 mM ammonium formate, pH 6.0 at 37 °C, overnight. Product 1 from the 2′-deoxyguanosine reaction (A) has a molecular ion $[M - H]^-$ *m/z* 372, with a fragment ion of *m/z* 256. Product 2 from the 2′-deoxyguanosine reaction (B) has a molecular ion $[M - H]^-$ *m/z* 356, with a fragment ion of *m/z* 240. Product i from the 8-hydroxy-2′-deoxyguanosine reaction (C) has a molecular ion $[M - H]^-$ *m/z* 388 with a fragment ion of *m/z* 272. Product ii from the 8-hydroxy-2′-deoxyguanosine reaction (D) has a molecular ion $[M - H]^-$ *m/z* 372 with a fragment ion of *m/z* 256.

Reaction of 2′-Deoxyguanosine with HQ and *p*-BQ. Reaction of 2′-deoxyguanosine with *p*-BQ and HQ resulted in the formation of two main products with t_R of 19.5 (product 1) and 22 min (product 2), respectively, when isolated using reversed-phase HPLC. Tandem MS analysis of these two products using continuous-infusion, negative ion electrospray resulted in molecular ions $[M - H]^-$ *m/z* 372 and 356 and fragment ions *m/z* 256 and 240, respectively (Figure 3 A,B). These data are consistent with products of molecular weights 373 and 357, respectively, again with a mass difference between the two molecules of 16. The data from the fragment ions again suggest loss of the deoxyribose moiety; this is similar to the results obtained with the nucleotide.

Jowa et al. (*12, 13*) have identified an adduct with a similar UV spectra and molecular weight to product 2. They identified this adduct as (3″-hydroxy)-1,N^2-benzetheno-deoxyguanosine.

Investigation of the Formation of Product 1. When 2′-deoxyguanosine 3′-monophosphate is reacted with equal amounts (w/w) of HQ and *p*-BQ, then the (3″-hydroxy)-1,N^2-benzetheno adduct (product 2) is the major product formed. When the amount of *p*-BQ in the reaction is decreased relative to HQ, then a significant ($p < 0.05$) increase in yield of product 1 occurs (Figure 4A). If the amount of HQ in the reaction is decreased in relation to

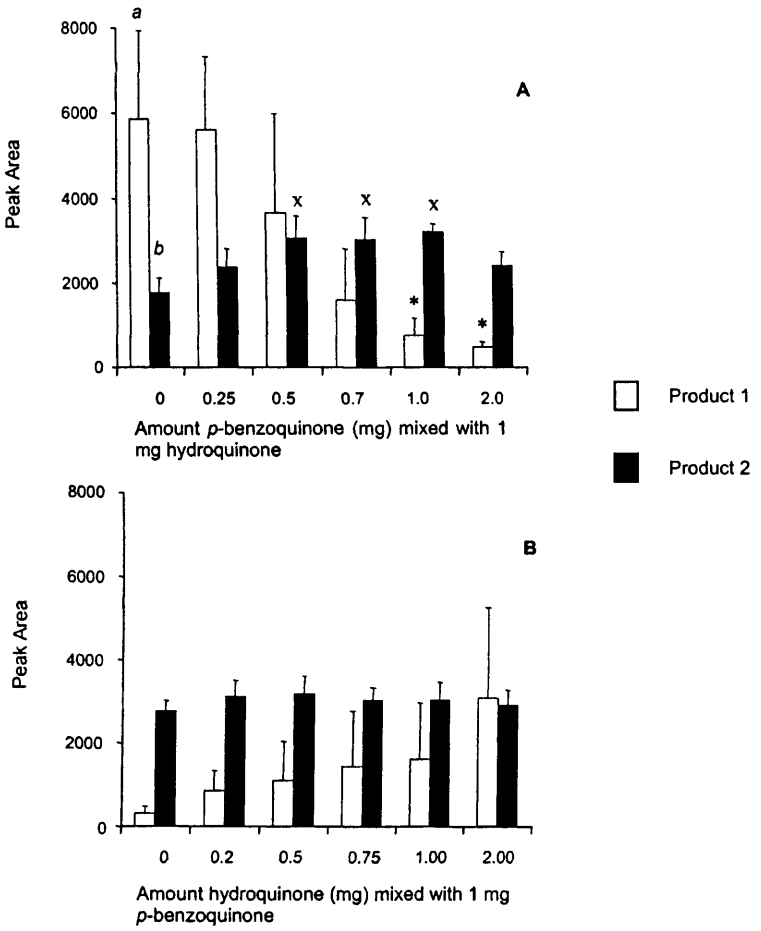
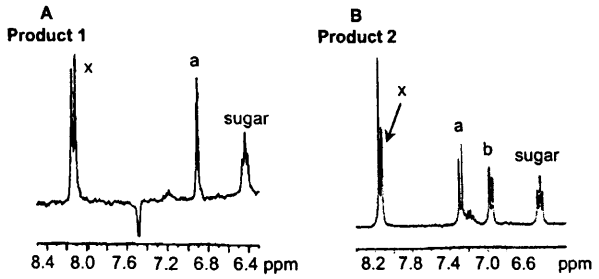


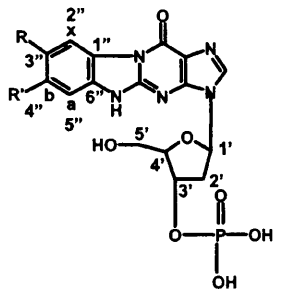
Figure 4. Comparison of product formation from the reaction of 2′-deoxyguanosine 3′-monophosphate with varying amounts of the two metabolites hydroquinone and *p*-benzoquinone. Analysis of samples was carried out using UV–HPLC (A_{254}) by comparison of peak areas. (A) Graphical representation of the pattern of adduct formation when the amount of *p*-benzoquinone in the reaction is varied and the amount of hydroquinone is constant. (B) Pattern of adduct formation when the amount of hydroquinone is varied and *p*-benzoquinone is kept constant. Data shown are mean ± SD ($n = 3$). An asterisk indicates significant difference from *a* ($p < 0.05$), and a superscript x indicates significant difference from *b* ($p < 0.05$) as determined by Oneway ANOVA followed by Tukey's posthoc test.

p-BQ, no significant effect on product 1 formation is observed (Figure 4B, $p < 0.05$). Similar findings were also observed when the 2′-deoxyguanosine 3′-monophosphate was substituted for 2′-deoxyguanosine (data not shown).

The formation of product 2, (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate, maintains a similar level of production for the range of HQ:*p*-BQ ratios tested (Figure 4B). Some significance ($p < 0.05$) in product 2 formation is observed when the amount of *p*-BQ in the reaction is decreased relative to HQ, but this is not concentration-dependent (Figure 4A).

Reaction of 8-Hydroxy-2′-deoxyguanosine with HQ and *p*-BQ. The molecular weights of the isolated nucleoside products from the reaction of 2′-deoxyguanosine with HQ and *p*-BQ, as determined by MS, were 373 and 357, respectively ($[M - H]^-$ *m/z* 372 and 356) (Figure 3A,B). The mass difference between the two products is 16 units. The evidence presented by the UV spectra (Figure 1) suggests that the two products are quite similar. It was thought possible that product 1 may be formed by monooxygenation of product 2, (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate, for example at the C8 position, which is a common lesion associated with oxidative damage. To investigate this possibility, simultaneous reactions were carried out with 2′-deoxyguanosine or 8-hydroxy-2′-deoxyguanosine with HQ and *p*-BQ (10:10:1, w/w). Reversed-phase HPLC was carried out. Two main products were observed in each reaction by reversed-phase HPLC, and subsequent tandem MS analysis identified one product from each reaction with identical molecular and fragment ions *m/z* 372 and 256, respectively (Figure 3A,D). HPLC cochromatography confirmed the two products of identical molecular weight did not coelute, thus suggesting that they were structurally not identical. The product with molecular weight 373 (product ii), from the 8-hydroxy-2′-deoxyguanosine reaction, was known to have the extra monooxygenation at the C8 position which indicates that the product we are attempting to identify with the same molecular weight (product 1) is not hydroxylated at the C8 position. Further evidence to support this theory comes from the first product (product i), from the reaction of 8-hydroxy-2′-deoxyguanosine with the two metabolites, HQ and *p*-BQ. MS/MS analysis of this product gave a molecular ion with $[M - H]^-$ *m/z* 388 and a fragment ion with *m/z* 272 (Figure 3C). These data are consistent with a compound of molecular weight 389, which is a further addition of 16 mass units. These data suggest we





	R(3")	R'(4")
Product 1	OH	OH
Product 2	OH	H

Figure 5. Typical ^{1}H NMR spectrum recorded in d_4-methanol of (A) product 1 and (B) product 2 from the reaction of 2′-deoxyguanosine with hydroquinone and *p*-benzoquinone in 10 mM ammonium formate, pH 6.0 at 37 °C, overnight. Proposed structures of product 1 and structure of product 2 are shown.

have a compound containing the (3″-hydroxy)-1,N^2-benzetheno adduct, plus hydroxylation at the C8 position plus a further oxygenation somewhere on the molecule. The fragment ion *m/z* 272 again supports the theory that the additional monooxygenation is present on the adducted base.

^{1}H NMR Analysis. The two main products from the reaction of 2′-deoxyguanosine with HQ and *p*-BQ (10:10:1, w/w) were isolated and analyzed by ^{1}H NMR, and the results are shown in Figure 5.

The data for product 1 were as follows: 8.16 (s, 1H, H-8), 8.11 (s, 1H, ring-2″), 6.92 (s, 1H, ring-5″), 6.44 (t, 1H, H-1′), 4.61 (m, 1H, H-3′), 4.07 (m, 1H, H-4′), 3.82 (m, 2H, H-5′), 2.81 (m, 1H, H-2′$_b$), 2.44 (m, 1H, H-2′$_a$).

The data for product 2 were as follows: 8.15 (s, 1H, H-8), 8.11 (d, 1H, ring-2″, J = 2.32 Hz), 7.27 (d, 1H, ring-5″, J = 8.66 Hz), 6.95 (dd, 1H, ring-4″, J = 2.37, 8.62 Hz), 6.43 (t, 1H, H-1′), 4.60 (m, 1H, H-3′), 4.06 (m, 1H, H-4′), 3.83 (m, 2H, H-5′), 2.81 (m, 1H, H-2′$_b$), 2.45 (m, 1H, H-2′$_a$).

Nuclease P1 Treatment of the Products from the Nucleotide Reaction. To aid characterization of products 1 and 2, ^{1}H NMR analysis was carried out on the products from the 2′-deoxyguanosine reaction. To confirm that adduction of the products from the nucleotide reaction was identical to that occurring on the nucleoside

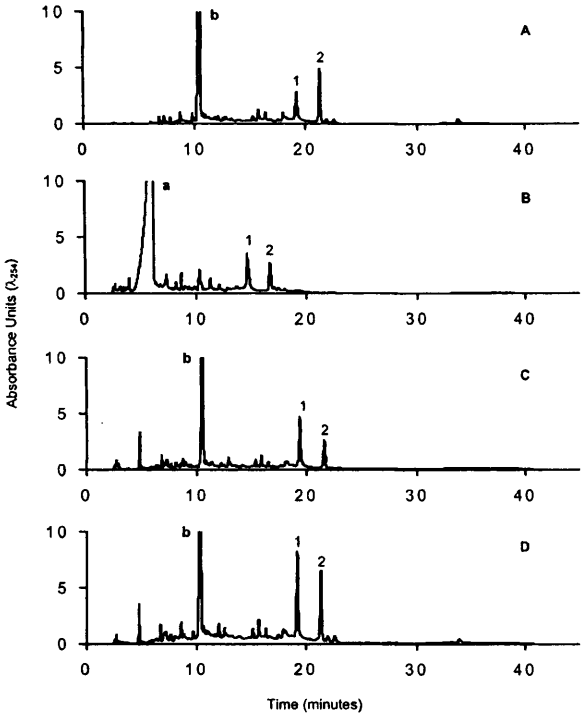


Figure 6. (A) UV–HPLC chromatograms of the products from the reaction of 2′-deoxyguanosine or (B) 2′-deoxyguanosine 3′-monophosphate with hydroquinone and *p*-benzoquinone (10:10:1, w/w). (C) Chromatogram of the products from the nucleotide reaction after nuclease P1 treatment and (D) coelution of the nucleoside adducts with the products from the nuclease P1-treated nucleotide adducts. Identical HPLC conditions were used for both nucleotide and nucleoside reactions: linear gradient of methanol (solvent B) in 50 mM ammonium formate (solvent A), pH 5.4 (0–35% B, 15 min; 35% B, 15–25 min; 35–70% B, 25–35 min; 70–0% B, 35–40 min; 0% B, 40–45 min), flow rate 1 mL/min. Peaks a and b represent unreacted nucleotide and nucleoside, respectively.

adducts, dephosphorylation was performed on the nucleotide adducts. The two products from the nucleotide reaction were treated with nuclease P1 (an enzyme which removes the 3′-phosphate moiety), and the resulting products underwent reversed-phase HPLC, as shown in Figure 6C. Comparison to nucleotide and nucleoside products confirmed that the nuclease P1-treated products now eluted approximately 5 min later than the nucleotide adducts (Figure 6B,C) but appeared to elute at the same time as the nucleoside adducts (Figure 6A). Coelution was performed to confirm that the nuclease P1-treated products had the same retention time as the nucleoside adducts as shown in Figure 6D.

^{32}P-Postlabeling Analysis. ^{32}P-postlabeling coupled with HPLC detected four major and several minor adducts from the reaction of calf thymus DNA with HQ and *p*-BQ (1:1, w/w). The total adduct level was 2.5 ± 0.2 adducts/10^4 nucleotides (mean ± SD, n = 3). The relative abundances of the main adducts formed were 9%, 60%, 27%, and 4%, respectively. Elution of peaks can be seen in Figure 7. The major adducts formed from this reaction were identified as (3″-hydroxy)-3,N^4-benzetheno-2′-deoxycytidine 3′-monophosphate (peak 2, Figure 7A) (60%) and (3″-hydroxy)-1,N^6-benzetheno-2′-deoxyadenosine 3′-monophosphate (peak 3, Figure 7A) (27%). Adducts were identified by individual adduct synthesis using the methods reported by Pongracz et al. (*10, 11*), followed

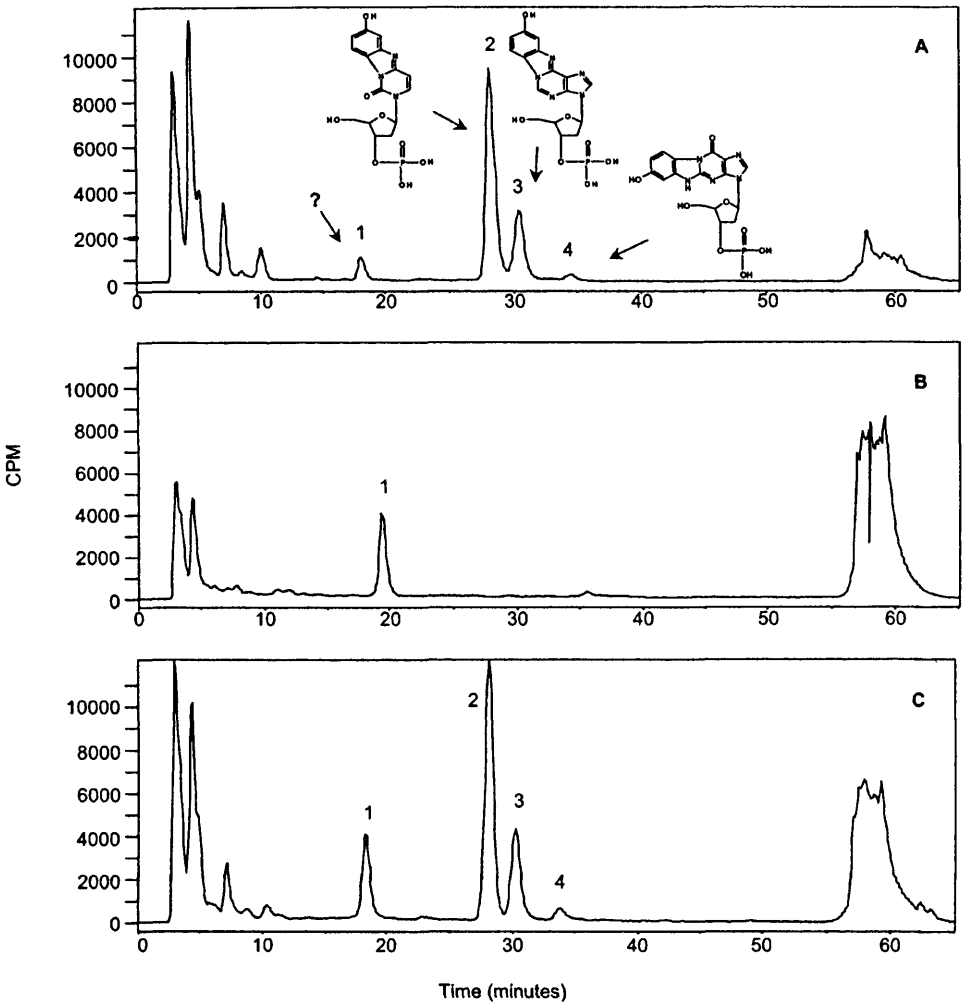


Figure 7. Typical HPLC chromatograms of ^{32}P-postlabeled adducts formed from (A) the reaction of calf thymus DNA with hydroquinone and *p*-benzoquinone, (B) elution of ^{32}P-postlabeled product 1 from the reaction of 2′-deoxyguanosine 3′-monophosphate with hydroquinone and *p*-benzoquinone, and (C) coelution of ^{32}P-postlabeled product 1 with ^{32}P-postlabeled adducted DNA. Peaks 2, 3, and 4 have been identified as (3″-hydroxy)-3,N^4-benzetheno-2′-deoxycytidine 3′-monophosphate, (3″-hydroxy)-1,N^6-benzetheno-2′-deoxyadenosine 3′-monophosphate, and (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate, respectively, by coelution studies. Chromatography was carried out using acetonitrile (solvent B) in 2 M ammonium formate, pH 4.0, containing 270 μM EDTA, (2% B, 50 min; 2–50% B, 50–65 min), flow rate 1 mL/min.

by postlabeling and HPLC coelution. Following ^{32}P-postlabeling, product 1 had a similar retention on HPLC as the adduct identified as 1 in Figure 7A; this was confirmed by co-chromatography with the ^{32}P-postlabeled products from the reaction of calf thymus DNA with HQ and *p*-BQ (Figure 7C). ^{32}P-postlabeled (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate (product 2) co-chromatographed with adduct 4 of the ^{32}P-postlabeled calf thymus DNA adducts.

Discussion

In this study, we have demonstrated the production of two adducts from the reactions of 2′-deoxyguanosine or 2′-deoxyguanosine 3′-monophosphate with HQ and *p*-BQ. After dephosphorylation with nuclease P1, the two compounds from the nucleotide reaction were confirmed to be identical to the two products from the nucleoside reaction.

Product 2 has been identified as (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate by comparison of the UV spectra and MS data with previously published work (*12, 13*). Product 1 was characterized in this study. The mass difference between the two adducts is 16, which suggests the new compound, product 1, has undergone a monooxygenation. MS/MS eliminated the possibility of this extra oxygenation to be due to contamination of the starting material with guanosine 3′-monophosphate, and reaction with 8-hydroxy-2′-deoxyguanosine ruled out the possibility of a hydroxyl substitution at the C8 position.

Using mass spectral and ^{1}H NMR analysis, we have confirmed that the oxygenation has taken place on the adducted part of the molecule and identified product 1 as a new benzetheno adduct. We have assigned the name (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate to this compound, and the proposed structure can be seen in Figure 5. The yield of this adduct can be increased by decreasing the amount of *p*-BQ in the reaction in comparison to HQ (w/w).

Reports in the literature (*10–15*) suggest that the benzetheno adducts including (3″-hydroxy)-1,N^2-benze-

theno-2′-deoxyguanosine 3′-monophosphate are formed by reaction of *p*-BQ with nucleotide, resulting in loss of water. Work carried out by Smart and Zannoni (*18*) investigating the influence of DT-diaphorase and peroxidase on the covalent binding of benzene metabolites confirmed that it was *p*-BQ and not its semiquinone intermediate which was responsible for adduct formation. At physiological pH, HQ can undergo spontaneous autoxidation to form *p*-BQ (*19*), an observation also made in our laboratory at pH 6.0. Autoxidation of HQ is thought to result in the formation of a semiquinone intermediate and superoxide radicals as well as *p*-BQ (*19*). In the work described here, autoxidation of HQ to *p*-BQ and the presence of *p*-BQ already in the reaction are thought to be responsible for the formation of product 2 [(3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate]. With a decrease of *p*-BQ in the reaction mixture, the equilibrium of the two metabolites (HQ and *p*-BQ) may have been shifted in favor of the formation of reactive oxygen radicals which may be present in the solution. These radicals may react with HQ, forming a compound which may subsequently react with nucleotide to produce the new adduct. Alternatively, the new adduct, (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate, may be formed initially via (3″-hydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate followed by a single oxidation on the additional ring.

In conclusion we have shown that the adduct (3″,4″-dihydroxy)-1,N^2-benzetheno-2′-deoxyguanosine 3′-monophosphate is formed in vitro following the reaction of 2′-deoxyguanosine 3′-monophosphate or calf thymus DNA with HQ and *p*-BQ. We are currently investigating whether this adduct is formed in vivo, and hence whether it would be a reliable biomarker of benzene exposure.

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