The Molecular Basis for the Non-Random Manifestation of DNA Phosphotriester Adducts

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by

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Rachel Le Pla

Abstract

Previously, in this laboratory it has been demonstrated that the manifestation of PTEs was non-random *in vivo*, but random *in vitro* with respect to normal base content. This was suggestive of either non-random formation *in vivo* or sequence specific repair (Guichard *et al.*, 2000).

In the present study the [^{32}P]-postlabelling protocol was further developed to allow for the more straight forward determination of nucleosides 5' to the PTEs. The molecular basis for the non-random manifestation of PTEs *in* vivo was studied using the improved assay.

To determine the influence of cellular processes upon the manifestation of PTEs in liver DNA taken from mice 5 hours to 56 days following a single dose of *N*-nitrosodiethylamine was analysed. Whilst there was an abrupt decline in the level of PTEs observed, possibly indicative of active repair, there was no appreciable change in the frequency of nucleosides located 5' to PTEs between 5 hours and 56 days following treatment.

It was demonstrated that the manifestation of PTEs in cells treated *in vitro* with diethylsulphate (DES) was non-random. To investigate the potential role of higher order packaging upon the manifestation of PTEs, nuclei and DNA were isolated from cells and treated *in vitro* with DES. The determination of the frequency of nucleosides found 5' to PTEs revealed the same non-random manifestation of PTEs observed in the whole cells treated with DES.

From these studies it would appear that neither DNA packaging nor sequence specific repair play a significant role. Studies using synthetic oligonucleotides indicate that the manifestation of PTEs may be influenced by the primary structure of DNA (i.e. its sequence).

In a separate study using specifically modified oligonucleotides it was demonstrated that the postlabelling assay was able to detect tandem PTE damage. However, PTEs separated by a single normal nucleotide appeared as single lesions.

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Abbreviations

5' NNA	5' nearest neighbour analysis
AAAF	*N*-acetoxy-2-acetylaminofluorene
Ada	O^6 methyl dG methyl transferase repair protein found in *E. coli*
AMP	adenosine monophosphate
AMPS	ammonium persulphate
AMS	accelerator mass spectrometry
ATP	adenosine triphosphate
AU	absorbance unit
BER	base excision repair
bp	base pair
BPDE	benzo(a)pyrene diol epoxide
CPD	cyclobutane pyrimidine dimers
CS	Cockayne syndrome
CSPD	calf spleen phosphodiesterase
CT-DNA	calf thymus DNA
dA	2'-deoxyadenosine
dC	2'-deoxycytidine
DES	diethylsulfate
dG	2'-deoxyguanosine
DMS	dimethylsulfate
DMSO	dimethylsulfoxide
dN	2'-deoxymononucleoside
DNA	deoxyribonucleic acid
dNp	2'-deoxy 3' mononucleotide
dNpdN	2'-deoxydinucleoside phosphate
dNpR	3'-phosphate alkylated 2'-deoxymononucleotide
dsDNA	double stranded DNA
dT	2' deoxythymidine
EDTA	ethylenediaminetetraaceticacid
ELISA	enzyme linked immunosorbent assay
EMS	ethylmethanesulphonate
ENU	*N*-ethyl-*N*-nitrosourea
FMR1	fragile X mental retardation 1
GG-NER	global genome nucleotide excision repair
HPLC	high performance liquid chromatography
LC-MS	HPLC linked mass spectrometry
MDS	multiply damaged sites
MEP	molecular electrostatic potential
MGMT	O^6 methyl dG methyl transferase repair protein
mM	millimolar
M	molar
MMS	methyl methane sulphonate
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MN	micrococcal nuclease
MNU	*N*-methyl-*N*-nitrosourea
MS	mass spectrometry
NDEA	*N*-nitrosodiethylamine
NDMAOAc	*N*-nitroso(acetoxymethyl)methylamine

NP1	nuclease P1
[^{32}P]-HPLC	HPLC with radioactivity detection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pdN	2' deoxy 5' mononucleotide
pdNpdN	2' deoxy dinucleotide
P*i*	inorganic phosphate species
PTE	phosphotriester
R	alkyl group
RIA	radioimmunoassay
RNA	ribonucleic acid
RPA	replication protein A
RpdN	5' phosphate alkylated 2' deoxy mononucleotide
ROH	alcohol
SAP	shrimp alkaline phosphatase
SVPD	snake venom phosphodiesterase
T4PNK	T4 polynucleotide kinase
TBE	tris borate EDTA buffer
TC-NER	transcription coupled nucleotide excision repair
TE	tris EDTA buffer
TEMED	*N, N, N', N'* – tetramethylethylene diamine
TLC	thin layer chromatography
U	units
USERIA	ultrasensitive radioimmunoassay
UV	ultraviolet
X	nucleophile (on DNA)
XP	Xeroderma pigmentosum
Y	leaving group of alkylating agent

Chapter 1:

General Introduction

1.1 Introduction

The formation of deoxyribonucleic acid (DNA) adducts is widely considered to be an important initial step in the process of genotoxic chemical carcinogenesis and the measurement of DNA adducts is one of several ways in which exposure can be monitored and, potentially, the risk assessed.

Since the carcinogenic and mutagenic effects of DNA adducts are widely considered to be the result of DNA base modification, the majority of studies to date have focussed upon DNA base adducts. However in addition to reacting with DNA base moieties, certain carcinogens react with the oxygen of the internucleotide phosphate moiety to form phosphotriesters (PTEs). The role of PTEs in carcinogenesis and mutagenesis is unknown (Berenek *et al.*, 1980; Beranek, 1990). However, the formation of PTEs parallels that of other oxygen alkylation products, such as O^6-alkylguanine and O^4-alkylthymine, which are known to be highly mutagenic. Consequently, PTEs may serve as surrogate markers of known mutagenic lesions (Shooter, 1978).

Several studies have shown that PTEs are chemically stable under physiological conditions, relatively abundant and resistant to DNA repair (Bannon and Verly, 1972; Beranek *et al.*, 1980; Beranek, 1990). Studies using rodents or human fibroblast cell lines exposed to alkylating agents have shown that PTE lesions have a half life that exceeds that of any other known DNA alkylation product *in vivo* (Shooter and Slade, 1977; den Englese *et al.*, 1986, 1987; Bodell *et al.*, 1979). For these reasons it has been suggested that PTEs may serve as ideal biomarkers for measuring cumulative genotoxic exposure (Shooter, 1978).

This Chapter will outline the role of DNA adducts in chemical carcinogenesis and mutagenesis, introduce the major chemical mechanisms behind the formation of

DNA adducts and review the methods available to study DNA base adducts and PTEs.

1.2 Chemical carcinogenesis

The term chemical carcinogenesis is generally defined as 'the induction or enhancement of neoplasia by chemicals' (Lu and Kacew, 2002). Chemical carcinogens may be classified according to their mode of action into genotoxic and non-genotoxic carcinogens.

1.2.1 Genotoxic carcinogens

Genotoxic agents can in fact react with a wide range of cellular macromolecules including protein, lipids and nucleic acids (DNA and ribonucleic acid (RNA)). However, DNA is generally considered to be the critical target with respect to mutagenesis and carcinogenesis. DNA adducts if not repaired prior to cell replication or if misrepaired can induce gene mutation. DNA adduct formation has been positively correlated to cell transformation and tumour induction (Beach and Gupta, 1992; Sukamar *et al.*, 1983; Balmain *et al.*, 1984; Marshall *et al.*, 1984; Knudsen, 1985). Genotoxic carcinogens may be divided into two groups; direct acting carcinogens (also known as primary carcinogens) and precarcinogens (also known as procarcinogens or secondary carcinogens).

Direct acting carcinogens are chemically and biologically reactive by virtue of their specific chemical structure. Direct acting carcinogenic agents are either electrophilic by nature or spontaneously hydrolyse *in vitro* to give the ultimate carcinogen electrophilic species that reacts with the DNA. Examples of direct acting carcinogens include a variety of alkylating agents (e.g. alkyl and aryl epoxides,

lactones, sulphate esters, nitrosamides, nitrosoureas and platinum amine chelates) and inorganic elements (e.g. Be, Cd, and Pb).

Precarcinogens require conversion *in vivo* to yield the ultimate carcinogen species. Most chemical carcinogens fall into this category including the polycyclic aromatic hydrocarbons, aromatic amines and nitrosamines. Characteristically these chemicals are chemically and biologically inert *in vitro*. While these chemicals may not react directly with nucleic acids *in vitro*, they require activation *in vivo* to yield the ultimate carcinogen species. Activation may occur either spontaneously under specific *in vivo* conditions (e.g. chemical hydrolysis in the stomach) or by an enzyme controlled reaction. Enzymes that have been implicated in the activation of precarcinogens include the cytochrome P-450 oxidases, epoxide hydrolyase and gluthionine-S-transferase (Blackburn and Gait, 1996;Watson *et al.*, 1994). Enzymatic activity may also be of bacterial origin, e.g. derived from the microflora in the intestinal tract. Chronic bacterial or parasitic infection may lead to the release of carcinogens that would not occur in a non-infected organ (Miller and Miller, 1979).

Figure 1.1 illustrates the initial key stages in genotoxic chemical carcinogenesis. It has been demonstrated that certain DNA adducts, if not repaired, may result in a mutation. This mutation may in turn result in the activation of an oncogene or the deactivation a tumour suppressor gene. It is believed that the transformation of a normal cell into a cancer cell may require the activation of several oncogenes and/or the deactivation of several regulatory or tumour suppressor genes. An oncogene is a gene associated with cancer derived from a precursor normal gene or a protooncogene. The proteins encoded by protooncogenes usually participate in

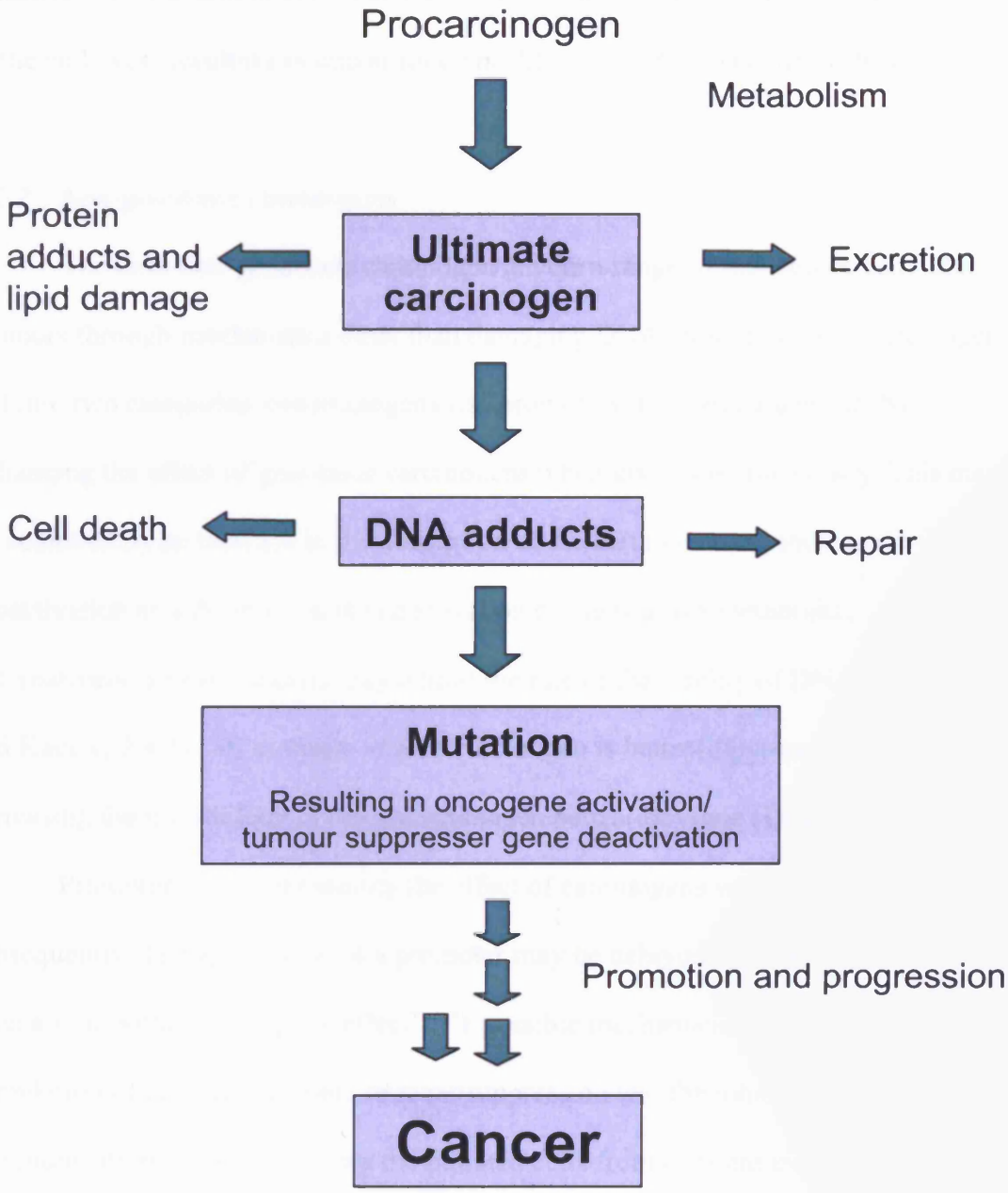


Figure 1.1: Key stages in chemical genotoxic carcinogenesis (adapted from Watson et al., 1994).

intracellular signalling pathways. Normal protooncogenes control cell growth, therefore an activated oncogene will give the cell a continuous signal to grow. Tumour suppressor genes are genes involved in the suppression of cell cycle. Deactivation of a tumour suppressor gene will result in the loss of negative feedback in the cell cycle resulting in uncontrolled proliferation (Watson *et al.*, 1994).

1.2.2 Non-genotoxic carcinogens

The term non-genotoxic carcinogen covers a range of substances that induce tumours through mechanisms other than damaging DNA. Non-genotoxic carcinogens fall into two categories: cocarcinogens and promoters. Co-carcinogens act by enhancing the effect of genotoxic carcinogens when given simultaneously. This may be achieved by an increase in the absorption of the carcinogen, an increase in the bioactivation or a decrease in the deactivation of the reactive metabolite. Alternatively, co-carcinogens may inhibit the rate or the fidelity of DNA repair (Lu and Kacew, 2002). An example of a co-carcinogen is benzo(e)pyrene which acts by increasing the metabolism of the precarcinogen benzo(a)pyrene (Lau and Baird, 1992)

Promoters act by increasing the effect of carcinogens when given subsequently. The application of a promoter may be delayed by several months or even a year without losing the effect. The possible mechanisms of action include stimulation of cell proliferation, immunosuppression and the inhibition of intercellular communication thereby releasing the initiated cells from restraint exercised by the surrounding normal cells. Examples of promoters include cytoxicants such as nitrilotriacetic acid, hormones, and a number of pesticides (e.g. dichloro diphenyl trichloroethane (DDT)) and pharmaceuticals (e.g. phenobarbital and diazepam) (Lu and Kacew, 2002).

1.3 Mechanism of action of genotoxic chemical carcinogens

Most genotoxic chemical carcinogens transfer to DNA either an alkyl, arylamino or aralkyl residue. Alkylating agents include derivatives of nitrosamines, aliphatic epoxides, aflatoxins, lactones, nitrosoureas, mustards, haloalkanes, alkyl triazenes and sultones. They react with DNA via nucleophilic substitution through a saturated carbon atom that is not conjugated to an aromatic system (Figure 1.2, I). Alkylating agents react with the ring nitrogens and exocyclic oxygen atoms on DNA bases and the phosphate oxygens of the sugar phosphate backbone of DNA (see Section 1.4).

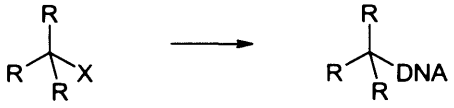
Arylaminating agents arise from amino azo dyes, aromatic amines, nitroaromatics and heterocyclic aromatic amines. These agents react with DNA either through the amino nitrogen itself or through an aromatic carbon conjugated with this nitrogen (Figure 1.2, II). Arylaminating agents primarily react with the C8 and amino group of guanine residues and occasionally with the C8 and amino group of adenine residues (Figure 1.3, II).

The aralkylating agents include derivatives of pyrrolizidine alkaloids, alkenyl benzenes, nitroaromatics, aralkyl halides and polycyclic aromatic hydrocarbons. Aralkylating agents react with DNA through a carbon atom that is conjugated with an aromatic system (Figure 1.2, III). Aralkylating agents react primarily with the amino groups on guanine, adenine and cytosine (Figure 1.3, III) (Dipple, 1995; Dipple and Lipinski, 1995).

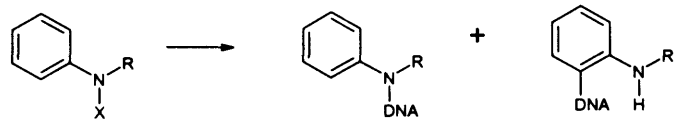
1.4 Formation of adducts by alkylating agents

Alkylating agents react at a variety of sites on the bases (primarily on the ring nitrogens and exocyclic oxygen atoms) and on the phosphate oxygens of the sugar

Alkylation (I)



Arylamination (II)



Aralkylation (III)

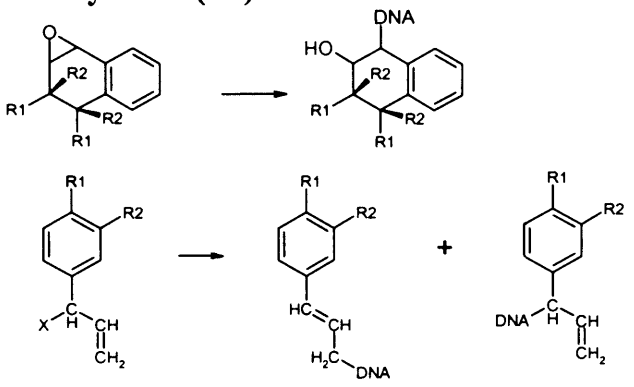
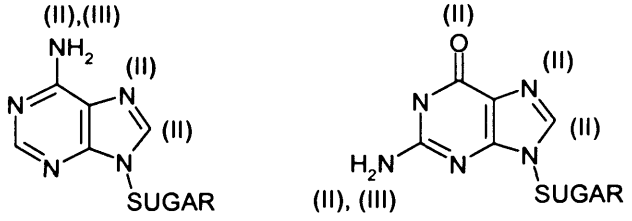
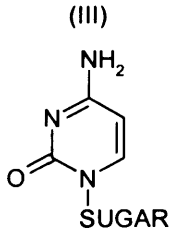


Figure 1.2: Schematic representation of the alkylating, arylaminating and aralkyating reaction mechanisms (Dipple and Lipinski, 1995). X = leaving group of electrophile.



No adducts formed



Thymidine

Deoxycytidine

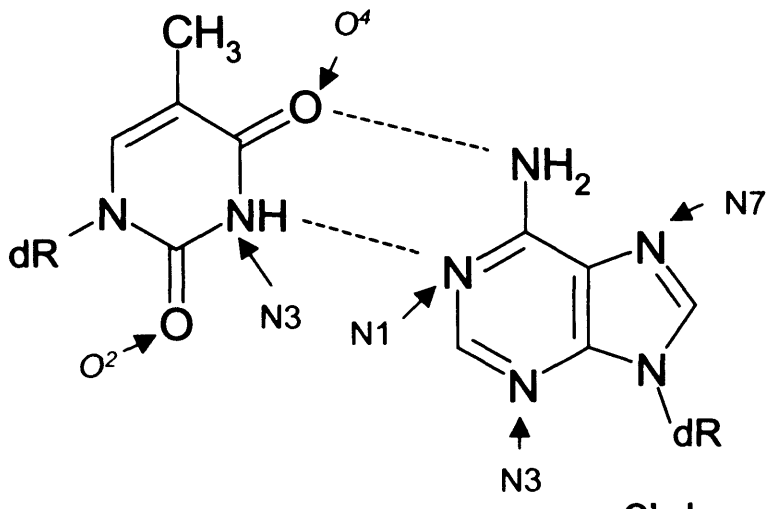
Figure 1.3: Summary of the potential sites of modification on the DNA bases by arylaminating (II) and aralkylating (III) agents (Dipple and Lipinski, 1995).

phosphate backbone of DNA (Figure 1.4). The N7 atom of deoxyguanosine (dG) is the most reactive site towards alkylation on the DNA bases. Other sites susceptible to alkylation on dG include O^6 and N3. The deoxyadenosine (dA) moieties in DNA can be alkylated at all three of the unsubstituted ring nitrogen atoms: N1, N3 and N7. Thymidine (dT) is alkylated at N3 and both exocyclic oxygens, O^4 and O^2. Alkylation may occur on the N3 and O^2 atoms of deoxycytidine (dC). In addition to this, alkylating agents may react with the internucleotide phosphate moiety to form phosphotriesters.

Alkylation of secondary phosphates, i.e., terminal phosphates, occurs rapidly under neutral conditions (Sun and Singer, 1975). Tertiary phosphates i.e., phosphodiesters, are less readily alkylated (Sun and Singer, 1975; Brown *et al.*, 1955). The relative proportion of the total alkylation products formed upon the phosphodiester depends upon the alkylating agent. The proportion of total alkylation products formed upon the phosophodiesters ranges from 55-57% for the S_N1 alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) to 0.8% for the S_N2 agent methylmethanesulphonate (MMS) (Beranek *et al.*, 1990).

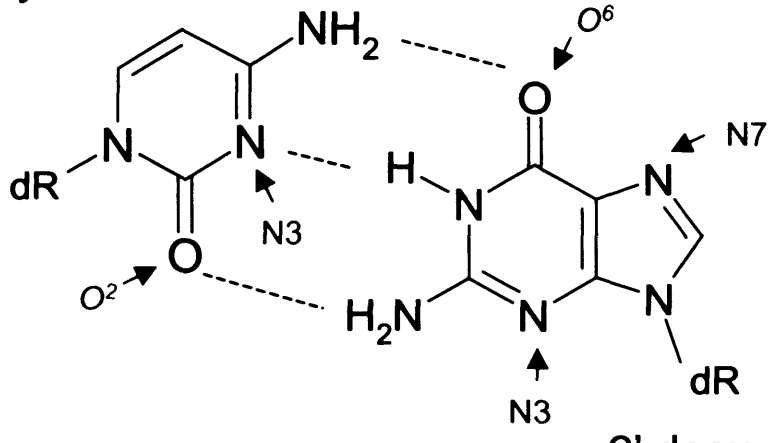
Not all the nucleophilic sites on DNA are susceptible to alkylation. In contrast with their ability to react with aromatic compounds, the exocyclic amino groups are not susceptible to modification by alkylating agents, this is probably due to their inherent low nucleophilicity (Swenson *et al.*, 1976). It is proposed that agents with aromaticity in their structures may form van der Waals bonds with the DNA that result in the electrophile being directed towards the exocyclic nitrogens. Alternatively the aromatic group upon the electrophile may act by delocalising the incipient charge upon the electrophile thereby lending it a more S_N1 character and permitting reaction with the exocyclic amino groups (Moschel *et al.*, 1979; Dipple *et al.*, 1982).

Thymidine

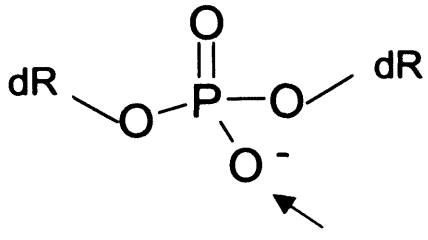


2' deoxyadenosine

2' deoxycytidine



2' deoxyguanosine



Phosphate

Figure 1.4: Sites of modification by alkylating agents upon DNA (adapted from Dipple and Lipinski, 1995). dR = deoxyribose.

Alkylation is suppressed upon all nitrogen atoms involved in base pairing in double stranded DNA, especially the N1 atom of dA, indeed it has been suggested that the extent of the reaction at this site could be used to measure the extent of single strandedness (Margison and O'Connor, 1979). The N1 of dG is unreactive even in free nucleotides, except in non-aqueous solution (Singer, 1985). The effect of secondary structure upon O-alkylation is much less marked due to the presence of a free electron pair not involved in hydrogen bonding (Margison and O'Connor, 1979).

1.5 The mechanism by which alkylating agents react with DNA

Alkylation of biological macromolecules occurs on oxygen, nitrogen and sulphur atoms. The Ingold concept of nucleophilic substitution places alkylating agents into two groups depending upon their predominant mechanism of action: S_N1 and S_N2 (Streitweiser, 1956). The unimolecular, dissociative (S_N1) and bimolecular, associative (S_N2) mechanism of alkylation are illustrated in Figure 1.5.

The rate determining step for S_N1 type reactions is the formation of the carbocation (R^+) by the heterolytic cleavage of the alkylating agent (R-Y). The rate determining heterolytic cleavage step is reversible. S_N1 alkylation may be inhibited by an increase in the concentration of the leaving group of the alkylating agent (Y^-). Polar solvent molecules may facilitate the ionisation of the alkylating agent. Once formed the carbocation will react with any centre rich in electrons (Ross, 1962). Consequently, agents acting mainly via an S_N1 or S_N1-like mechanism show less selectivity towards nucleophilic strength; the carbocations are very reactive and have a short lifetime. S_N1 carbocations react nearly as frequently with oxygen atoms as with nitrogen atoms (Ross, 1962).

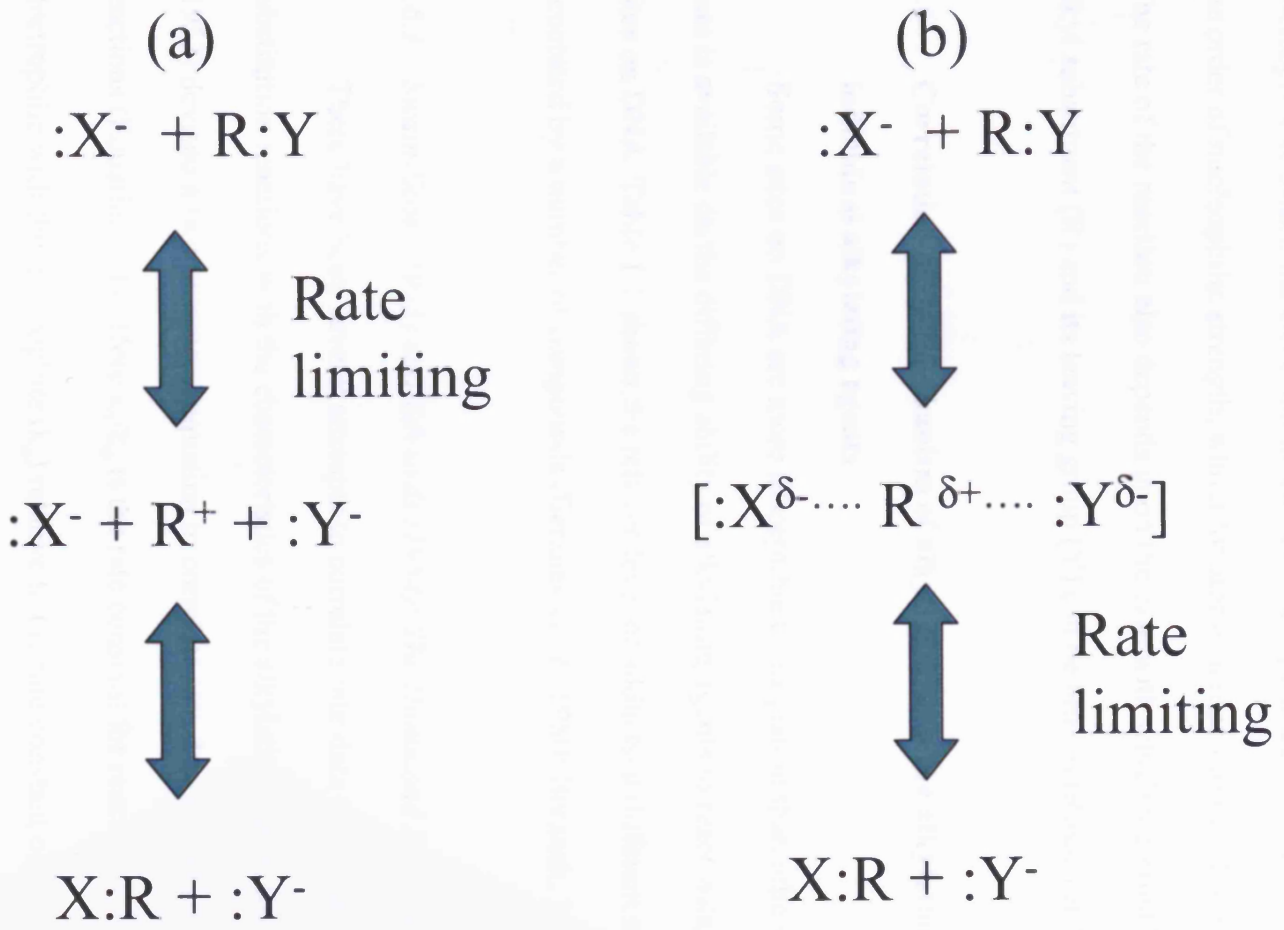


Figure 1.5: Schematic representation of the (a) S_N1 and (b) S_N2 reaction mechanisms of alkylation: X = nucleophile on DNA; R = alkyl substituent; Y = leaving group of alkylating agent.

In S_N2 alkylation a complex is formed between the alkylating agent and the nucleophile. The S_N2 mechanism depends upon the ability of the nucleophile (on the DNA) to attack the electrophilic carbon in the polarised molecule. The ability of a nucleophile to attack the electrophilic carbon in the polarised molecule increases with the order of nucleophilic strength, which for atoms in macromolecules is O< N< S. The rate of the reaction also depends upon the ease with which the bond between the alkyl substituent (R) and its leaving group (Y) can be broken (Ross, 1962).

1.6 Correlation of the mechanism of alkylation with the alkylation profile for individual alkylating agents

Some sites on DNA are more susceptible to alkylation than others. Extensive data is available on the differing ability of alkylating agents to react with nucleophilic sites on DNA. Table 1.1 shows the relative level of adducts at different sites in DNA generated by a number of compounds (Beranek *et al.*, 1980; Beranek, 1990).

1.6.1 Swain-Scott (1953) and Edwards (1954): The Hammond Postulate

There have been several attempts to correlate rate data for nucleophilic substitution reactions with the characteristics of the alkylating agent. Swain and Scott (1953) devised a two parameter equation to correlate rate data for substitution reactions (Equation 1.1). Here k_a/k_o is the rate constant for reaction of the electrophile with the nucleophile (k_a) relative to the rate constant of its reaction with water (k_o), *n* is the constant characteristic to the nucleophile (i.e. site on DNA) and *s* is the constant characteristic to the substrate (i.e. electrophile derived from alkylating agent).

Table 1.1: Relative levels of adducts induced at different sites in DNA by a variety of alkylating agents with different substrate constants (s). Adapted from Beranek (1990). A dash indicates that no data has been recorded, 'n.d.' means that adduct levels were below the detectable limit.

Site of alkylation		*Percentage of total DNA alkylation*					
		MMS (s = >0.83)	DMS (s = 0.81)	EMS (s = 0.67)	DES (s = 0.64)	MNU (s = 0.42)	ENU (s = 0.26)
Adenine	N1	1.9 – 3.8	1.0 – 3.0	1.7	2.0	0.7 – 1.3	0.2 – 0.3
	N3	10.4 – 11.3	15.0 – 18.0	4.2 – 4.9	10.0	8.0 – 9.0	2.8 – 5.6
	N7	1.8	2.0	1.1 – 1.9	1.5	0.8 – 2.0	0.3 – 0.6
Cytosine	O^2	n.d.	n.d.	0.3	n.d.	0.1	2.7 – 2.8
	N3	<1.0	<2.2	0.4 – 0.6	0.7	0.06 – 0.6	0.2 – 0.6
Gunaine	N3	0.6	1.1 – 1.3	0.3 – 0.9	0.9	0.6 – 1.9	0.6 – 1.6
	O^6	0.3	0.2 – 0.3	2.0	0.2	5.9 – 8.2	7.8 – 9.5
	N7	81.0 – 83.0	71.0 – 76.0	58.0 – 65.0	67.0	65.0 – 70.0	11.0 – 11.5
Thymine	O^2	n.d.	-	n.d.	n.d.	0.1 – 0.3	7.4 – 7.8
	N3	0.1	-	n.d.	n.d.	0.1 – 0.3	0.8
	O^4	n.d.	-	n.d.	n.d.	0.1 - 0.7	1.0 – 2.5
Phosphotriester		0.8	-	12.0 – 13.0	16.0	12.0 – 17.0	55.0 – 57.0

Equation 1.1 $\text{Log } k_a/k_o = sn$

The ability of a nucleophile to attack an electrophile increases with the order of nucleophilic strength, which for atoms in macromolecules is O< N< S ($n_O = 2$, $n_N = 4$, $n_S = 6$). Substrate constant decreases with increasing S_N1 behaviour. Electrophiles with large alkyl groups have lower *s* values (Ross, 1962). For example, ethylating agents frequently have a lower substrate constant (*s* value) than their methylating equivalent, as the larger alkyl group helps to stabilise the carbocation (Table 1.1) (Ross, 1962).

Edwards (1954) proposed an alternative four parameter equation to correlate rate data for reaction with the nucleophile relative to the rate constant with water (k_a/k_o) (Equation 1.2); where E_n is the constant for the nucleophile, H is the basicity constant derived from the pKa of the conjugate acid (the electrophile in alkylating reactions) and α and β are substrate constants.

Equation 1.2 $\text{Log } k_a/k_o = \alpha E_n + \beta H$

Both the Swain-Scott (1953) and Edwards (1954) models are based upon the Hammond postulate (also known as the reactivity selectivity principle). The Edwards (1954) model gives a closer agreement with experimentally determined rate values than the simpler Swain-Scott equation due to the fact that it incorporates two correction factors (α and β) calculated using a least squared analysis of the observed data.

A permutation of the Swain-Scott equation to compare two reactive sites is given in Equation 1.3; where s is the substrate constant of the alkylating agent, k_y and k_z are the rates for reaction at nucleophilic sites y and z, and n_y and n_z are the nucleophilic constants for the two potential sites of alkylation (Swenson and Lawley, 1978).

Equation 1.3 $\text{Log}(k_y/k_z) = s(n_y-n_z)$

From the equation, it can be seen that for two competing reactions, the effect of the relative nucleophilicities of the two groups (hence the selectivity) will be diminished as the substrate constant (s) approaches zero. This observation is backed up by experimental data. S_N1 type agents (low substrate constant) are less influenced by nucleophilic strength than S_N2 type alkylating agents (high substrate constant) (Beranek, 1990).

Alkylating agents with a strong S_N2 character primarily react at the nitrogen atoms in DNA (Table 1.1). The most nucleophilic site upon the DNA, N7-dG, is also the most frequent site of alkylation by S_N2 alkylating agents accounting for 58 - 83% of total alkylation products (Beranek, 1990). The proportion of alkylation products occurring upon the oxygen atoms increases with increasing S_N1 behaviour (Table 1.1). Oxygen base adducts (O^6-dG, O^2- dT, O^2- dC and O^4- dT) accounted for just 0.3% of total alkylation products formed by MMS (S_N2 alkylating agent). In contrast, oxygen base adducts (O^6-dG, O^2 -dT, O^2- dC and O^4 -dT) accounted for 18.9 - 22.6% of total alkylation products induced by ENU (S_N1 alkylating agent). It has been observed that the formation of PTEs parallels that of other oxygen alkylation products. PTEs accounted for just 0.8% of the total DNA alkylation products induced by the S_N2 type alkylating agent MMS, compared to 55 - 57 % of total alkylation products induced by ENU, an alkylating agent with a strong S_N1 character (Beranek, 1990).

1.6.2 The hard and soft acid and base theory

It has been proposed that the hard and soft acid and base theory of Pearson (1966) may better explain the outcome of DNA alkylation reactions (Saffhill *et al.*,

1985; Hathway and Kolar, 1980). According to this theory hard acids have a preference for hard bases, while soft acids prefer soft bases. The donor atoms of soft bases are of low electronegativity and high polarizability and are easy to oxidise. The donor atoms of soft bases hold their valence electrons loosely. In contrast, the donor atoms of hard bases are of high electronegativity and low polarizability. Hard bases hold their valence electrons tightly. Table 1.2a gives some examples of some hard and soft bases.

The acceptor atoms of soft acids are large, have a low positive charge and contain unshared pairs of electrons (*p* or *d* subshell) in their valence shells. Soft acids have high polarizability and low electronegatively. The acceptor atoms of hard acids are small, have a high positive charge and do not contain unshared pairs in their valence shells. They have low polarizability and high electronegativity. Table 1.2b gives some examples of some hard and soft acids.

In terms of hardness and softness, alkylating agents are considered to be intermediate acids. Similarly the oxygen and nitrogen atoms in purine and pyrimidine bases are considered to be intermediate bases. The electrophilic species formed from *N*-nitroso compounds (S_N1) are harder acids than those generated from alkane sulfonates (S_N2). According to Pearson's concept, oxygen atoms are harder bases than nitrogen atoms. Therefore, *N*-nitroso compounds will show a greater preference for reaction at oxygen atoms than the alkane sulfonates. The more branched the alkyl group, the harder acid the electrophile will be and the greater its preference for reaction at oxygen sites.

Table 1.2a: Hard and soft bases (Pearson, 1966)

Hard Bases	Intermediate Bases	Soft Bases
H_2O, OH^-, F^-, AcO^-, SO_4^{2-}, Cl^-, CO_3^{2-}, NO_3^-, ROH, RO^-, R_20, NH_3, RNH_2.	$ArNH_2$, C_2H_5N, N_3^-, Br^-, NO_2.	R_2S, RSH, RS^-, I^-, R_3P, $(RO)_3P$, CN^-, RCN, CO, C_2H_4, C_6H_6, H^-, R^-.

Table 1.2b: Hard and soft acids (Pearson, 1966)

Hard Acids	Intermediate Acids	Soft Acids
H^+, Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, Cr^{2+}, Fe^{3+}, BF_3, $B(OR)_3$, $AlMe_3$, $AlCH_3$, AlH_3, SO_3, RCO^+, CO_2, HX (hydrogen-bonding molecules).	Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+}, Sb^{3+}, Bi^{3+}, BMe_3, SO_2, R_3C^+, NO^+, GaH_3, $C_3H_5^+$	Cu^+, Ag^+, Pd^{2+}, Pt^{2+}, Hg^{2+}, BH_3, $GaCl_2$, I_2, Br_2, CH_2, carbenes.

1.6.3 Electronic factors that may influence the observed reaction patterns of alkylating agents with DNA

There is evidence that electronic factors may strongly influence the observed reaction patterns of methylating and ethylating agents. Early investigations into the influence of electronic factors upon the ability of small alkylating agents to react at different sites on DNA focused upon the molecular electrostatic potential (MEP). MEP is a good indicator of the reaction profile for the S_N1 type alkylating agents. The sites with most negative MEP are the phosphodiester oxygens followed by the N7 atom of dG (Pullman and Pullman, 1981), also happen to be the most favoured sites of reaction by alkylating agents with a strong S_N1 character (Beranek, 1990). However, whilst PTEs comprise a high proportion of the total alkylation products induced by agents with a strong S_N1 character, they form a relatively small proportion of alkylation products formed by S_N2 type alkylating agents (Table 1.1).

It has been proposed that the π ionisation energy of a site may be better predictor of the reaction profile of S_N2 alkylating agents than the MEP. Here the base π ionisation energy is a marker of the ease with which electronic rearrangement occurs that is necessary to accommodate significant charge transfer in the transition state. As π ionisation energies decrease, base π polarizability increases. S_N2 activation barrier height for reaction with DNA decreases as base polarizability increases (Kim *et al.*, 1999). A reasonable explanation of this relationship is that the transition state barrier heights are strongly influenced by the polarizability of the transition state.

1.7 The chemical and biological effects of DNA alkylation and their relation to mutagenesis and carcinogenesis

1.7.1 Chemical stability of adducts generated by alkylating agents

Ring nitrogen substituted products are all unstable to some extent, thus biological effects can be influenced by chemical transformations secondary to the initial DNA alkylation (Dipple, 1995). Quarternisation of the ring nitrogen by alkylation destabilises the glycosyl bond. There are two possible consequences of ring nitrogen modification (i) loss of the modified base by depurination/ depyrimidation or (ii) imidazole ring fission. The half lives for N3-methyldA, N7-methyldA and N7-methyldG *in vitro* at 37°C, pH 7 were determined to be 3 hours, ~26 hours and ~155 hours respectively (Singer and Grunberger, 1983). The rate of this hydrolysis was found to depend upon the nature of the attached alkyl group with ethylated DNA being more stable than methylated DNA (Lawley, 1966). Spontaneous depurination of the ring modified bases occurs at a rate 10^6-10^7 times greater than for unmodified bases (Singer, 1985).

Under alkaline conditions N1-methyldA rearranges to produce N^6-methyldA and the N7-alkylpurine nucleotides will undergo imidazole ring fission (O'Connor *et al.*, 1979). Ring fission has been reported to stabilise the glycosidic bond of ring nitrogen modified adducts in DNA (O'Connor *et al.*, 1979; Kohn and Spears, 1967; Chetsanga *et al.*, 1982, Degan *et al.*, 1988).

One important reason for the relatively late discovery of O-alkylation of pyrimidines is the extreme lability of the alkyl group, particularly in acid, but also in alkali (Singer *et al.*, 1978; Allore *et al.*, 1983). Kinetic studies of dealkylation at the O^4 position of dT suggest that under acidic conditions (pH<3) there is an initial protonation, presumably at N3, followed by a rate determining attack by water on the

alkyl group or at the alkoxylated ring carbon (Allore *et al.*, 1983). In alkali (pH>12) there is probably a rate determining attack of the hydroxyl ion upon the alkyl group or at the alkoxylated ring carbon (Allore *et al.*, 1983).

The glycosidic bond of O^2-alkyl deoxypyrimidines is destabilised in acid. The depyrimidation of O^2-alkyl deoxypyrimidines is believed to occur by direct hydrolysis of the glycosidic bond. This mechanism is supported by the finding that prolonged heating under anhydrous conditions did not cause detectable depyrimidination of O^2-alkyl dC, while the reaction is complete within 5 minutes in water (Singer *et al.*, 1978). Depyrimidation of O^2-alkyldC under physiological conditions occurs at a rate approximately 35 fold slower than that for N7- methyl dG (Singer, 1985).

DNA PTEs are very stable under neutral and acidic conditions with the alkyl group only being released by strong acid (e.g. 70% $HClO_4$, 100°C, 1 hour) (Bannon and Verly, 1972; Sun and Singer, 1975). PTEs are labile under alkaline conditions. The site of attack depends upon the character of the nucleophile. Hard nucleophiles (e.g. OH-) will preferentially attack the phosphorus (P) atom of the PTE. Attack at P results in a penta-coordinated P with no clear preference for either of the three ester groups to act as the leaving group, resulting in the cleavage of any of the three PTE bonds with approximately the same priority. In contrast, soft nucleophiles (e.g. NH_3) preferentially attack the primary carbon in the alkyl group resulting in the cleavage of the alkyl oxygen bond (Swenson and Lawley, 1978). The rate of hydrolysis of PTEs is much greater than for phosphodiesters (Shooter, 1976).

1.7.2 Repair of alkylative damage

A variety of DNA damage repair pathways have been identified in eukaryotic and prokaryotic systems. The major repair pathways include base excision repair

(BER), nucleotide excision repair (NER), double strand break repair (DSB), mismatch repair (MMR) and direct repair (Yu *et al.*, 1999). The major repair pathways known to be involved in the repair of alkylative damage include BER, direct repair and NER.

The majority of base adducts have been shown to be subject to rapid repair *in vivo*. Exceptions to the rule include PTEs (in higher organisms) and O^2-alkyldT. BER is the major repair pathway for adducts generated by simple alkylating agents and oxidative damage. There are two modes of BER: short patch (involved in the repair of small lesions and abasic sites) and long patch (involved in the repair of single strand breaks). Figure 1.6 illustrates the mechanism of short patch BER. Modified, damaged or fragmented bases are removed from the DNA by one of a variety of DNA glycosylases that recognise damaged bases. The DNA glycosylase hydrolyses the sugar-base glycosidic bond leaving an abasic site. The resulting abasic site may also occur by spontaneous hydrolysis. The core BER reaction is initiated by an incision adjacent to the abasic site by APE 1 (apurinic/ apyrimidinic endonuclease 1). The initial incision of DNA during BER *in vivo* is presumed to take place mainly, if not exclusively, 5' to abasic sites by AP endonucleases, which leaves a 3'-OH and a 5' deoxyribose-phosphate termini (Friedberg *et al.*, 1995). Then the phosphodiesterase activity of DNA polymerase β-XRCC1 (X-ray cross complementing group 1) cuts 3' to generate a one nucleotide gap. Finally, DNA polymerase β-XRCC1 fills in the gap at the exposed 3' hydroxy group and DNA ligase 3-XRCC1 seals the remaining nick (Yu *et al.*, 1999; Hoeijmakers, 2001).

Another important repair pathway for alkylative damage is direct repair. The O^6-methyl dG methyl transferase (MGMT) repair protein removes the alkyl groups from the premutagenic lesions O^6-alkyldG and O^4-methyldT, by transferring the alkyl group irreversibly to a cysteine residue. MGMT is a 'suicide' protein, that is it

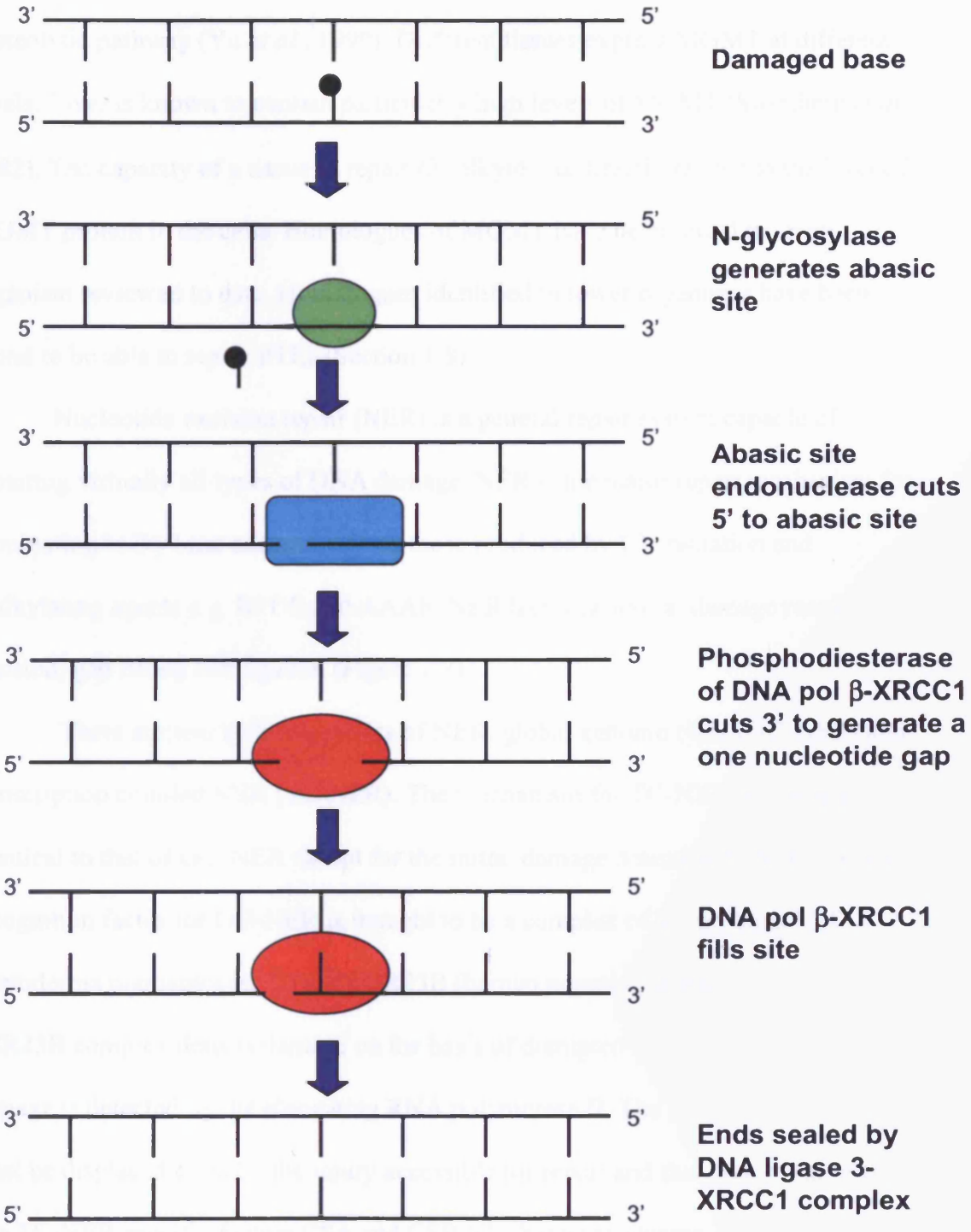


Figure 1.6: General scheme for short patch base excision repair (adapted from King, 2000 and Hoeijmakers, 2001).

cannot regenerate itself once it has removed an alkyl group, therefore one protein repairs just one lesion. The inactivated protein is degraded via the ubiquitin proteolytic pathway (Yu *et al.*, 1999). Different tissues express MGMT at different levels. Liver is known to contain particularly high levels of MGMT (Swenberg *et al.*, 1982). The capacity of a tissue to repair O^6-alkyldG is directly related to the level of MGMT protein in the cells. Homologues of MGMT have been found in every organism reviewed to date. Homologues identified in lower organisms have been found to be able to repair PTEs (Section 1.8).

Nucleotide excision repair (NER) is a general repair system capable of repairing virtually all types of DNA damage. NER is the major repair mechanism for eliminating bulky base adducts such as those produced by UV radiation and aralkylating agents e.g. BPDE and AAAF. NER has four stages: damage recognition, incision, gap filling and ligation (Figure 1.7).

There are two different modes of NER: global genome NER (GG-NER) and transcription coupled NER (TC-NER). The mechanism for TC-NER is essentially identical to that of GG-NER except for the initial damage detection step. The damage recognition factor for GG-NER is thought to be a complex of the proteins XPC (Xeroderma pigmentosum C) and hHR23B (human homologue Rad23). The XPC-hHR23B complex detects damage on the basis of disrupted base pairing. In TC-NER damage is detected by the elongating RNA polymerase II. The stalled polymerase must be displaced to make the injury accessible for repair and this requires at least two TC-NER specific factors CSA and CSB (Cockayne syndrome A and B).

Following damage recognition a multi-subunit transcription factor, TFIIH, is recruited to the damaged site. The XPB (Xeroderma pigmentosum B) and XPD (Xeroderma pigmentosum D) helicases of TFIIH catalyse the partial opening of the

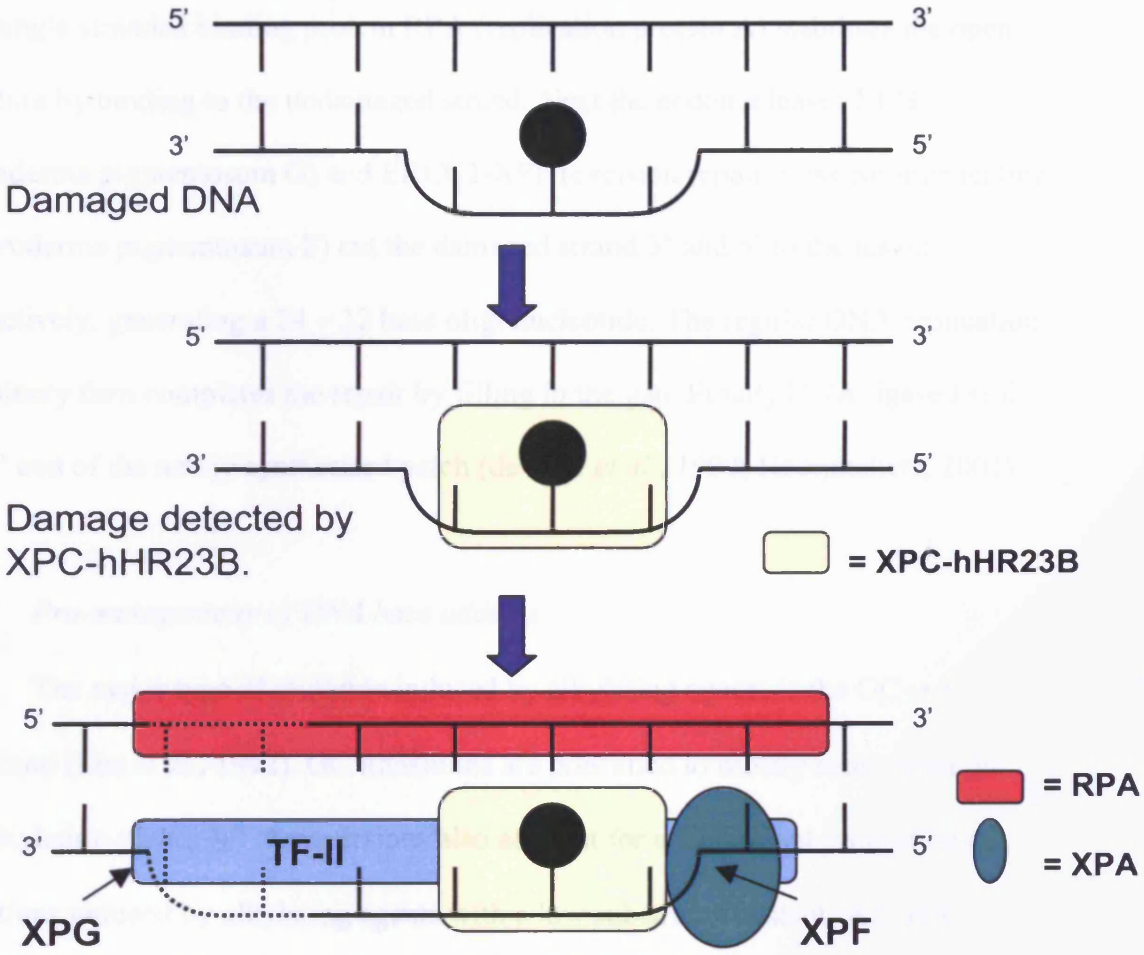


Figure 1.7: General scheme for global genome nucleotide excision repair (adapted from King, 2000; Hoeijmakers, 2001).

helix ~ 30 base pairs around the lesion. XPA probably confirms the presence of damage by probing for abnormal backbone structure and when absent aborts NER. The single stranded binding protein RPA (replication protein A) stabilises the open structure by binding to the undamaged strand. Next the endonucleases XPG (Xeroderma pigmentosum G) and ERCC1-XPF (excision repair cross complementing 1- xeroderma pigmentosum F) cut the damaged strand 3' and 5' to the lesion respectively, generating a 24 – 32 base oligonucleotide. The regular DNA replication machinery then completes the repair by filling in the gap. Finally DNA ligase I seals the 5' end of the newly synthesised patch (de Laat *et al.*, 1999; Hoeijmakers, 2001).

1.7.3 Pro-mutagenicity of DNA base adducts

The major type of mutation induced by alkylating agents is the GC→AT transition (Lee *et al.*, 1992). GC transitions are presumed to mostly result from the O^6-alkylation of dG. AT transversions also account for a significant proportion of mutations induced by alkylating agents with a low substrate constant. AT→GC transitions are mostly attributed to O^4-alkyldT, whilst AT→TA transversions are mostly attributed to O^2 alkylation of dT (Eckert *et al.*, 1988).

When alkyl modifications in DNA are considered in terms of their pro-mutagenic properties, three classes can be recognised. The first consists of modifications that alter the tautomeric form of the base leading to incorrect base pairing. This group includes O^4-alkyldT and O^6-alkyldG.

Loveless (1969) first observed that the mutagenic and carcinogenic action of methylating agents was correlated to their ability to form O^6-methyldG in DNA. O^6-ethyldG, O^6-propyldG and O^6-butyldG have also been shown to be promutagenic *in vitro* (Saffhill *et al.*, 1985). Similarily the mutagenic and carcinogenic potential of O^4-

alkyldT has been confirmed using both RNA polymerase and bacterial and mammalian DNA polymerases (Saffhill *et al.*, 1985). Even though O^4-alkyldT and O^6-alkyldG comprise only a tiny proportion of total adducts formed they represent the most significant group of alkylation products with respect to the mutagenic and carcinogenic properties of alkylating agents.

The second class of base lesions consists of modifications that cause steric interference with the base pairing regions, but are not significantly mutagenic. These include the 3-alkylpyrimidines, 1-alkylpurines and O^2-alkylpyrimidines. They strongly inhibit DNA polymerases and may play a role in determining the cytotoxicity of the agents. Whilst base lesions in this class do not induce a significant number of errors during the DNA synthesis they do give rise to a significant number of errors during RNA synthesis (Saffhill *et al.*, 1985).

The third class consists of those modifications at positions not involved in hydrogen bonding. This class includes the N7- and N3-alkylpurines. Base modifications at these sites do not pose a problem for DNA polymerase. For these lesions it has been proposed that it is the generation of abasic sites either by base excision repair or spontaneous hydrolysis of the glycosidic bond that induce mutation (Vogel and Nivard, 1994). Abasic sites are non-instructive sites. When DNA polymerase is faced with a non-instructive lesion it will insert a random nucleotide, usually a deoxyadenosine (pdA) (Tessman, 1976). Insertion of pdA opposite an abasic site may lead to either a GC→TA or a AT→TA transversion (Loeb and Preston, 1986).

It was found that oligonucleotides containing a DNA PTE did not direct the misincorporation of bases during DNA synthesis. This is not unexpected since

modification of the sugar phosphate backbone does not influence base pairing or result in the formation of abasic sites (Jensen and Reed, 1978; Miller *et al.*, 1981).

1.8 Manifestation and biological significance of DNA phosphotriesters

The biological significance of PTEs remains unknown. PTEs are known to be stable *in vivo* and resistant to repair in higher eukaryotes. Studies using rodents or human fibroblast cell lines exposed to alkylating agents have shown that methyl and ethyl PTE lesions have a half life that exceeds that of any other known alkylation product *in vivo.* The half life of methyl PTEs was found to be approximately 16 days in mice. Ethyl PTEs were much more stable *in vivo* than the methyl derivative with a reported half life of 10 – 15 weeks in mice (Shooter and Slade, 1977). The observed half life of PTEs has been found to vary between different studies. Den Englese *et al.*, (1986) reported a half life of 7 and 32 days, for methyl-PTEs and ethyl-PTEs respectively in adult rats treated with either ENU or *N*-nitrosodimethylamine.

Most DNA PTEs are stable under physiological conditions. However, PTEs containing an O, N or S atom in a β position, intramolecular rearrangement may occur that will result in a strand break in the DNA, as for 2-hydroxyethyl PTEs formed by ethylene oxide (Bannon and Verly, 1972; Conrad *et al.*, 1986).

Miller *et al.* (1974) suggested that a single PTE group in the backbone of a nucleic acid could potentially lead to conformational changes and/or changes in the stability of the nucleic acid helix in the region of modification due to the loss of the negative charge on the sugar phosphate backbone. However, it was found that overall many of the physical characteristics of DNA were not significantly affected by the presence of a single ethyl PTE (Broido and Mezei, 1990; Kan *et al.*, 1988). In contrast, Lawrence *et al.* (1987) did report significant local perturbations in duplex

stability and conformation in oligonucleotides containing an isopropyl PTE. However, the DNA remained in B-DNA conformation in the presence of either an ethyl or isopropyl PTE (Broido and Mezei, 1990; Kan *et al.*, 1988; Lawrence *et al.*, 1987). PTE containing oligonucleotides can form base paired complexes with complementary oligonucleotides. However, these complexes have a greater themal stability than for similar complexes formed between normal oligonucleotides, presumably due to the removal of phosphate backbone charge repulsion (Miller *et al.*, 1974; Broido and Mezei, 1990).

The PTE group is asymmetric and can adopt one of two configurations, R*p* and S*p* (Figure 1.8). In the S*p* diastereomeric configuration the alkyl group points perpendicularly out from the helix of dsDNA. In the R*p* configuration the alkyl group points into the major groove. Melting temperature for oligonucleotides containing an R*p* ethyl-PTE were found to be lower than for oligonucleotides containing an S*p* ethyl-PTE (Broido and Mezei, 1990).

PTEs have been reported to interfere with the activity of several enzymes. Miller *et al.* (1982) used oligonucleotides containing a single ethyl PTE modification as PCR template to investigate the effect on DNA polymerase I activity. The rate and extent of polymerisation directed by the modified templates was found to be 25% lower (for isomer I) and 50% lower (for isomer II) than that of the unmodified template. The observation that polymerisation occurs at a slower rate with isomer II suggests that the orientation of the ethyl relative to the rest of the template backbone is an important factor in determining the effectiveness of inhibition. Miller *et al.* (1982) did not determine which isomers correspond to the R*p* and the S*p* PTE stereoisomers.

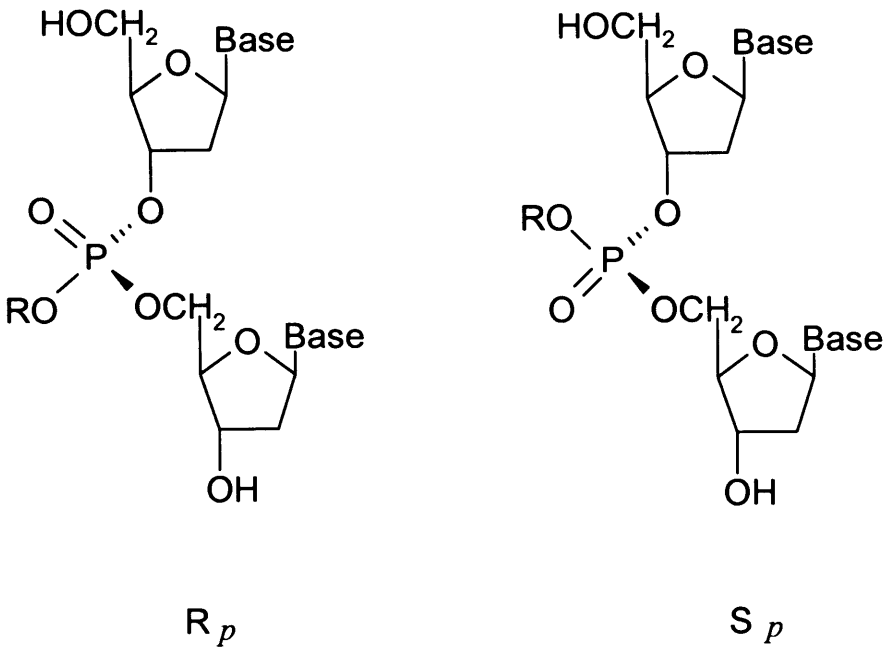


Figure 1.8: Structure of the two alkyl-PTE stereoisomers

Yashiki *et al.* (1992) reported that the presence of an isopropyl PTE in the template inhibited DNA chain elongation by *Escherichia coli* DNA polymerase I *in vitro*. The level of the inhibition depended upon the base 5' to the PTE. A greater level of inhibition was observed for oligonucleotides with a dT 5' to the PTE than for oligonucleotides with a dA 5' to the PTE. The action of human DNA polymerase α, T4 DNA polymerase and Sequenase (modified T7 DNA polymerase lacking 3' to 5' exonuclease activity) on the templates containing an isopropyl PTE linkage were also examined. For all these polymerases, inhibition of DNA chain elongation was observed, although the extent of inhibition varies between the polymerases.

Rasouli-Nia *et al.* (2000) reported the results of a study on the effects of DNA phosphate alkylation upon the activity of several nucleases including: (i) the 3' to 5' exonuclease activity of phage T4 DNA polymerase, (ii) *E. coli* exonuclease III (5' to 3' dsDNA specific exonuclease and AP endonuclease activity) and (iii) lambda exonuclease (5' to 3' exonuclease with a preference for dsDNA). The presence of a PTE significantly inhibited the digestion of the PTE containing strand for all of the tested nucleases. The digestion of the complementary strand was also inhibited for *E. coli* exonuclease III. No significant difference was observed between the substrates containing PTEs of either the R*p* or S*p* configuration. The fact that the AP endonuclease activity of E. coli exonuclease III was inhibited by the presence of a PTE raises questions as to whether other excision repair enzymes could be affected by the presence of a PTE adjacent to a damaged base.

1.9 Phosphotriester repair in prokaryotes and lower eukaryotes

PTEs are considered to be resistant to repair in eukaryotes. However, repair mechanisms have been found in lower organisms. The alkyl group of the PTEs are

removed by the Ada protein in *E. coli* (McCarthy *et al.*, 1983). The Ada protein is a homologue of the MGMT repair protein found in humans. The alkyl group from the PTE lesion is transferred to Cys-69 found on the amino terminal domain of Ada (McCarthy and Lindahl, 1985; Sedgwick *et al.*, 1988). The Ada protein can only remove PTEs of the S*p* configuration (approximately 50% of total PTEs) (Weinfeld *et al.*, 1985).

The Ada protein plays a central role in the up-regulation of repair in *E. coli* in response to alkylating agents (McCarthy *et al.*, 1983; Nakamura *et al.*, 1988). Methylation at the Cys-69 residue converts the Ada protein into an efficient transcriptional activator of its own gene and several other repair genes including *alkA, alkB* and *aidB* (Teo *et al.*, 1984 and 1986; Kataoka and Sekiguchi, 1985; Volkert and Ngyuen, 1984). The methylated form of Ada promotes transcription by binding to a sequence referred to as the 'Ada box'.

The Ada protein also repairs O^6-alkyl guanine and O^4-methyl thymine by transferring the alkyl group irreversibly to Cys-321 located on its carboxyl terminal domain (Teo *et al.*, 1984 and 1986; Demple *et al.*, 1985). Ada is a 'suicide' protein, meaning that it cannot regenerate itself once it has removed an alkyl group, therefore one protein can repair only a single lesion.

Homologues of Ada have been found in many species. A homologue of Ada found in *Aspergillus nidulans*, a lower eukaryote, has also been shown to be able to repair methyl phosphotriesters (Baker *et al.*, 1992). However a mechanism for the repair of alkyl phosphotriesters has yet to be demonstrated in higher eukaryotic species. It has been demonstrated that human MGMT does not repair PTEs (Yarosh *et al.*, 1985; Dolan *et al.*, 1984).

Studies using rodents or human fibroblast cell lines exposed to alkylating agents have shown that methyl and ethyl phosphotriester lesions have a half life that exceeds that of any other known alkylation product *in vivo* (Shooter and Slade, 1977; Shooter, 1978). Previously it has been suggested that the long term stability of PTEs *in vivo* suggests that they are not repaired in higher organisms and as such may serve as good markers of cumulative exposure to genotoxic carcinogens.

1.10 Modern methods of DNA adduct detection

Some extremely sensitive and highly sophisticated methods for assessing and quantifying DNA adducts have been developed over the past thirty years. The major approaches that have been employed include (i) immunoassays; (ii) fluorescence assays; (iii) mass spectrometry based methods and (iv) postlabelling.

1.10.1 Immunoassays, fluorescence assays and mass spectrometry based methods

Immunological techniques employed for adduct detection include competitive radioimmunoassay (RIA), solid phase competitive or non-competitive enzyme linked immunosorbent assay (ELISA) and ultrasensitive radioimmunoassay (USERIA). These assays are typically sensitive enough to detect 1 adduct per 10^8 nucleotides using 25-50 μg of DNA. This approach has been used for the detection of many base adducts. The major limitations of immunoassays include cross-reactivity with other antigens, the requirement for a new antibody for each adduct and the inability to detect adducts from unknown chemicals (Phillips, 1990).

Fluorescence assays utilise the inherent fluorescent properties of some adducts, notably adducts formed by the many polycyclic aromatic hydrogens. Sensitivity is reported to be between 3 and 10 adducts per 10^8 nucleotides.

Disadvantages include a lack of specificity due to broad peaks and cross-detection with other fluorophores (Phillips, 1990).

The most powerful methods for the unequivocal chemical characterisation and quantitation of DNA adducts employ mass spectrometry (MS) (Marnett and Burcham, 1993). Recent developments in MS technology have heralded the development of various MS based sequencing techniques. DNA enzymatic laddering and MS sequencing have been adapted to be used for direct adduct localisation within a sequence. One limitation of DNA laddering sequencing is that enzyme digestion may be slowed by DNA modification, for example simple methylation increases the required digestion time 5-fold over that of the unmodified oligonucleotide (Glover and Sweetman, 1996; Sweetman *et al.*, 1998; Bentzley *et al.*, 1996). For this reason MS fragmentation sequencing has proved to be the more popular sequencing technique for the purpose of adduct localisation. Although in principle it should be possible to locate adducts within unknown sequences using MS sequencing, in practice it is much simpler to use oligonucleotides of known sequence. This practice makes fragmentation sequencing much simpler especially for smaller adducts, which can produce modified bases that have the same mass as an unmodified base e.g., 8-hydroxyadenine has the same mass as unmodified guanine (Sweetman *et al.*, 1997; Wunchel *et al.*, 1997).

1.10.2 Postlabelling detection of adducts

The [^{32}P]-postlabelling assay is the most widely used approach for DNA adduct determination. The [^{32}P]-postlabelling assay was developed by Randerath and co-workers (Randerath *et al.*, 1981; Gupta *et al.*, 1982). [^{32}P]-postlabelling is noted for its sensitivity (potentially 1 adduct per 10^{10} nucleotides) and its applicability to a

wide range of structurally diverse adducts (Beach and Gupta, 1992). The [^{32}P]-postlabelling assay comprises of three major steps: digestion, labelling and separation of the labelled products using chromatography. There are many different versions of the [^{32}P]-postlabelling assay, however all versions exploit the inability of certain nucleases to digest the internucleotide phosphodiester bond at or close to the site of an adduct. T4 polynucleotide kinase (T4PNK) has several substrate requirements including the presence of a free 5' hydroxyl group, a 3' phosphate group (either mono or diester, but not triester linkage) and ideally an unmodified 5' base. Unmodified DNA is ultimately reduced by digestion to mononucleosides, which are not labelled by T4PNK. Modified DNA is resistant to enzymatic digestion and therefore provides substrates for [^{32}P]-end labelling by T4PNK. Several methods are used to separate the various labelled compounds including TLC, HPLC and PAGE.

A major limitation of [^{32}P]-postlabelling is that it does not offer any structural information about a product and identification relies upon the availability of a synthesised standard. Some adducts are known to be unstable as mononucleotides or are poor substrates for T4 polynucleotide kinase. Problems may also be encountered if digestion is incomplete leading to errors in adduct quantitation (Phillips, 1997).

Other forms of postlabelling include [^{14}C] postlabelling, [^{3}H] postlabelling and fluorescence postlabelling. [^{14}C] postlabelling and [^{3}H] postlabelling involve the chemical modification of adducts with radiolabel containing chemicals following digestion. The radiolabelled adducts may then be analysed by liquid scintillation and/or accelerator mass spectrometry (AMS) (Hemminki *et al.*, 1988; Phillips, 1990). The sensitivity of AMS greatly exceeds that of any other method capable of detecting carcinogen interactions with biological macromolecules (1 adduct per 10^{12} nucleotides). AMS does not give any direct information regarding the nature of the

compound as the molecule is destroyed in the sample preparation process (Sweetman *et al.*, 1998).

The practical problems of handling relatively large quantities of radioactive material has led some researchers to explore other means of postlabelling detection. Fluorescent postlabelling is achieved either by the attachment of a fluorescent tag or by conversion of the adduct into a fluorescent derivative. Methods that involve the attachment of a fluorescent molecule to the adduct require that the unreacted excess of reagent be completely removed in order to reduce background fluorescence. Methods that involve the conversion of the adduct into a fluorescent derivative generally do not have this problem as the precursors are usually only weakly or non-fluorescent. Analysis may then be carried out using HPLC with fluorescence detection. It is difficult to express precisely the absolute sensitivity of a fluorescence assay using a given fluorophore, since there is no fluorescence equivalent of an extinction coefficient. Absolute sensitivity is affected by a combination of factors, e.g., fluorometer noise, correct selection of excitation and emission wavelengths, the emission filters and the quantum yield of fluorescent derivative. Nevertheless, it is clear that fluorescence assays can be extremely sensitive and are capable of measuring adducts at the sub-femtomolar level (Shuker *et al.*, 1993; Kelman *et al.*, 1988; Lobazov *et al.*, 1986).

1.11 Other methods of phosphotriester detection

Several methods exist for the study of PTEs. One of the first methods for their detection relied on the use of radiolabelled alkylating agents coupled with the radiochemical detection of eluates from ion-exchange chromatography of enzymatic DNA digests (den Englese *et al.*, 1986; Swenson *et al.*, 1976). However, due to the

levels of activity required these methods were not appropriate for the detection of PTEs *in vivo* in human DNA and were unsuitable for the detection of PTEs that may be produced endogenously.

PTEs have also been quantified by detection of alkali-induced strand breaks at the site of PTE lesions. This method exploits the fact that the sedimentation coefficient of DNA increases as the number strand breaks increases. Treatment of alkylated DNA with NaOH may result in the hydrolysis of any one of the three PTE bonds, in approximately equal proportions. Only 60-70% the PTEs hydrolyse to give a break in the DNA chain with the remainder reverting to the phosphodiester by the loss of the alkyl group (Shooter and Merrifield, 1976). This is a relatively non-specific approach of quantifying PTEs that is also confounded by the presence of other alkali-labile lesions in the DNA, i.e. abasic sites, which may lead to the overestimation of the level of PTEs (Shooter, 1978; Shooter and Merrifield, 1976; Bannon and Verly, 1972; Snyder and Regan, 1981).

Haglund *et al.* (1997, 2000, 2001) have developed another method for studying PTEs. The method uses a strong nucleophile such as thiosulphate or coba(I)amin to remove the PTE alkyl group from the DNA. The method is fast and specific. The alkyl group from the PTEs is transferred exclusively to cob(I)amin within minutes. 7-Methyl dG was stable for 2.5 hours and 3-methyl dA and O^6-methyl dG were stable for 1.3 – 1.5 hours in the presence of cob(I)alamin. In the reactions between cob(I)alamin and synthetic dinucleotides containing a PTE (methyl- and ethylthymidyl phosphates), dTpdT was the only detectable leaving group. Recent studies have shown that SVPD/NP1 digestion of the DNA prior to reaction with coba(I)amin significantly improves the yield of PTEs detected (Haglund *et al.*, 2000). The detection limit for the assay was estimated to be between 0.01 and

0.05 nmol of adduct (Haglund *et al.*, 2000). More recently the method has been developed to be used in conjuction with LC-MS (Alsberg *et al.*, 2001). This method enables both the sensitive detection of PTEs and the identification of the PTE alkyl group. However this method does not offer any information on the sequence context in which the PTEs lie.

1.12 The phosphotriester postlabelling assay

The SVPD/NP1 postlabelling assay is a modified version of the SVPD postlabelling assay used to detect a wide variety of oxidative lesions, cyclobutane pyrimidine dimers and abasic sites (Weinfeld *et al.*, 1989a and b; Weinfeld and Soderlind, 1991; Weinfeld and Buchko, 1993). Figure 1.9 illustrates the SVPD/ NP1 postlabelling protocol for the detection of PTEs.

DNA is digested by a 'cocktail' of nucleases: DNase I, snake venom phosphodiesterase I (SVPD) and nuclease P1 (NP1) in the presence of shrimp alkaline phosphatase (SAP). DNase I, an endonuclease, cleaves the DNA into smaller fragments enabling SVPD to work more efficiently. SVPD, a 3' to 5' exonuclease, hydrolyses nucleic acids down to 5' mononucleotides. However, SVPD cannot hydrolyse either the internucleotide phosphate moiety containing a PTE or the phosphodiester 5' to certain modified nucleosides. However, NP1 is able to hydrolyse the phosphodiester 5' to a modified base. Thus, digestion with DNase I, SVPD and NP1 results in a digest containing a mixture of PTE containing dinucleotides and 5' mononucleotides. SAP then removes the terminal phosphates resulting in a mixture modified and unmodified nucleosides (dX/dN) with a few PTE containing dinucleoside phosphates (dNp(R)dN).

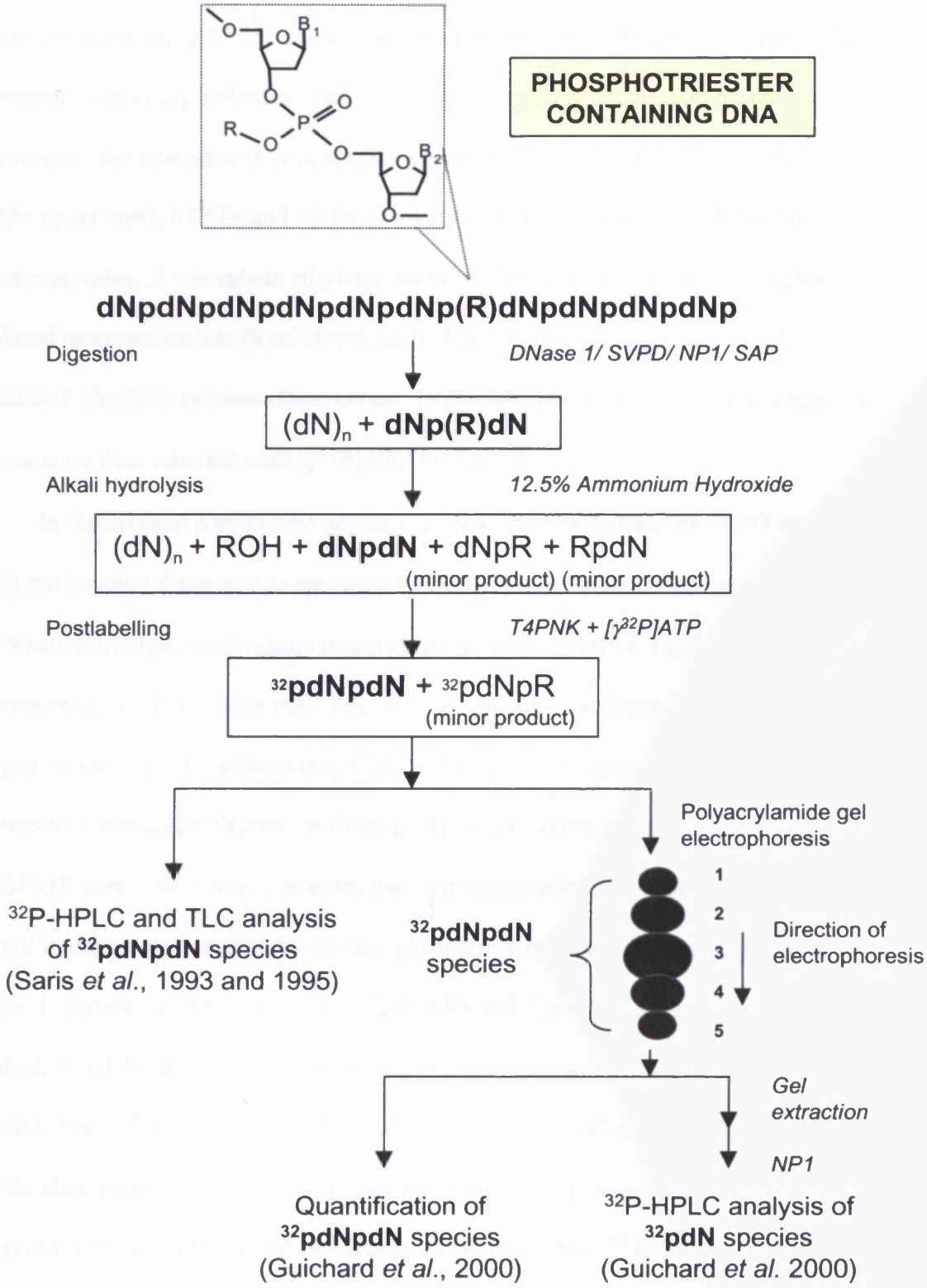


Figure 1.9: SVPD/NP1 postlabelling detection and quantification of PTE adducts

In order to permit subsequent ^{32}P postlabelling of the dNp(R)dN species, it is necessary to remove the alkyl group (R) from the dinucleoside phosphate species by alkali hydrolysis using 12.5% aqueous ammonium hydroxide. Potentially, any of the three ester bonds may be hydrolysed, however the major product of ammonia hydrolysis is the unmodified dinucleoside phosphate (dNpdN) which accounts for ~100% of all methyl-PTEs and ~80% of all ethyl-PTEs. In ethyl-PTE containing oligonucleotides, 3' phosphate ethylated mononucleotide (dNpR) and 5' phosphate ethylated mononucleotide (RpdN) species both accounted for approximately 10% of the alkali hydrolysis products (Saris *et al.*, 1995). The resulting dNpdN and dNpR species were then labelled with [$\gamma^{32}P$]ATP by T4PNK.

In the original SVPD/NP1 protocol as described by Saris *et al.* (1993 and 1995) the labelled dinucleotide species (^{32}pdNpdN) were analysed using a combination of high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). However, this two step process was inappropriate for routine analysis of samples. To address this, Guichard *et al.* (2000) developed a method for the detection and quantification of PTEs using polyacrylamide gel electrophoresis (PAGE) (Figure 1.9). PAGE separates the 16 dinucleotides into 5 bands (1-5). Their relative intensity as measured by storage phosphorimage analysis, was approximately 1:4:6:4:1. Bands 1 and 5 contain only ^{32}pdGpdG and ^{32}pdCpdC species respectively. Bands 2, 3 and 4 consist of labelled dinucleotides containing the base sequences [GA, GT, AG, TG], [CG, GC, AT, TA, TT, AA] and [TC, CT, AC, CA] respectively. The radiolabelled products were visualised using autoradiography and/ or phosphorimaging. Furthermore the identity of the nucleotide 5' to the phosphotriester lesion was obtained by the treatment of the gel isolated ^{32}pdNpdN species with NP1

to release ^{32}pdN species which may then be analysed by HPLC with radioactivity detection (^{32}P-HPLC) (Guichard *et al.*, 2000) (Figure 1.9).

1.13 The manifestation of phosphotriesters is non-random *in vivo*

Using the SVPD/NP1protocol Guichard *et al.* (2000) reported that the frequency of nucleosides 5' to the site of PTE lesions was non-random *in vivo.* The frequency of dT 5' to the site of PTE lesions in the liver DNA of mice treated *in vivo* with NDEA was significantly greater and the frequency of dG 5' to the site of PTE lesions was found to be significantly lower than expected with respect to normal nucleoside content (Fig. 1.10). In contrast, in studies using calf thymus (CT-DNA) treated *in vitro* with either DES or DMS, the frequency of bases 5' to PTEs was random with respect to normal nucleoside content (Fig. 1.11). These findings were suggestive of the non-random manifestation of PTEs *in vivo* being the result of an intrinsic *in vivo* factor.

1.14 Aims and objectives of the present study

The overall aim of the present study was to investigate the molecular basis behind the non-random manifestation of PTE lesions observed *in vivo.* Specifically we investigated:

- The influence of multiply damaged sites (MDS) upon the SVPD/NP1 postlabelling assay (Chapter 3).
- Change in the manifestation of PTEs over time *in vivo* (Chapter 4).
- The influence of higher DNA packing upon the manifestation of PTEs (Chapter 5).

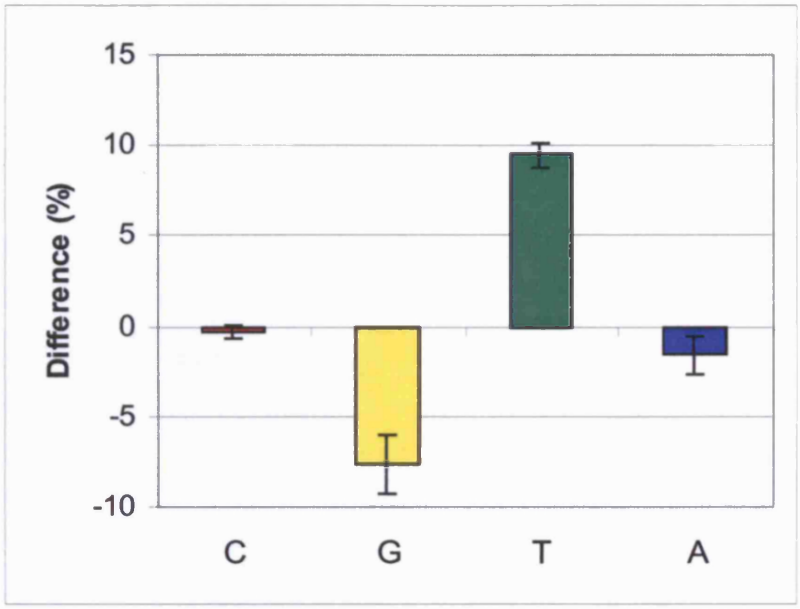


Figure 1.10: Difference between the frequency of nucleosides found 5' to PTEs and the normal nucleotide content of liver DNA taken from mice treated in vivo with NDEA (Guichard et al., 2000). Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

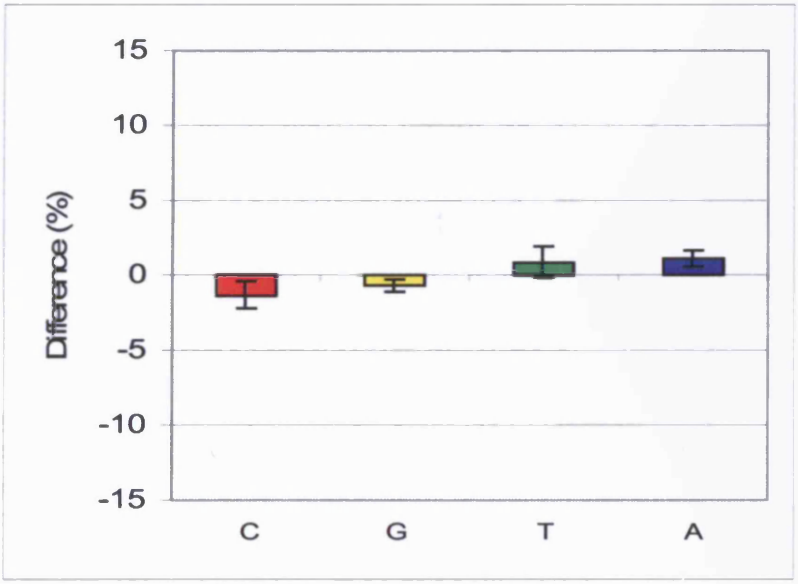


Figure 1.11: Difference between the frequency of nucleosides found 5' to PTEs and the normal nucleotide content of CT-DNA treated in vitro with DES (Guichard et al., 2000). Figures are the mean of two independent experiments. Error bars = +/- standard deviation.

- The influence of DNA base sequence and the alkylating agent used upon the manifestation of PTEs *in vitro* (Chapter 6).

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Bromophenol blue (cat. no. B-5525); calcium chloride (min. 96%, cat. no. C-4902); calf thymus DNA (CT-DNA, type I DNA, cat. no. D-1501); diethyl sulfate (DES, cat. no. D3263); dimethyl sulfate (DMS, 98% +, cat. no. D5279); ethylene diamine tetraacetic acid (EDTA, practical grade, 99%, cat. no. ED4S), formamide (min. 99.5%, cat. no. F-9037), *N*-ethyl-*N*-nitrosourea (ENU, cat. no. N-3385), magnesium chloride (anhydrous, min. 98%, cat. no. M-8266); *N*-methyl-*N*-nitrosourea (MNU, cat. no. N-1517), *N*-nitrosodiethylamine (NDEA, cat. no. N-0258), nucleotide and nucleoside standard kit (min. 98%, cat. no. DNN-8-1), 3M sodium acetate, pH5.2 (cat. no. S-7899); sodium acetate (99%, cat. no. S-7545); sodium phosphate (dibasic, anhydrous, 99.4%, cat. no. S-9763); Trizma™ base (99.9%, cat. no. T-8524); Tris HCl (reagent grade, cat. no. T-3253); sucrose (>99.5%, cat. no. S-7903); xylene cyanol (cat. no. X-4126) and zinc chloride (>98%, cat. no. Z-4875) were purchased from Sigma-Aldrich Corporation (Poole, UK).

[γ-^{32}P]ATP (Redivue™, ~110 TBq/mM, > 3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

Ammonium persulfate (ultra pure™, min. 98%, cat. no. 15523-012), *N, N, N', N'*-tetramethylethylene-diamine (TEMED, ultra pure™, 99%, cat. no. 15713-019) and urea (ultra pure™, >99.4%, cat. no. 15505-027) were purchased from Invitrogen (Paisley, UK).

40% Polyacrylamide (cat. no. EC-850) and 10x TBE buffer (cat. no. EC-860) were supplied by National Diagnostic (Hull, UK).

Aqueous ammonium hydroxide (35%, cat. no. A/3290/P308), isopropanol (99.99%, HPLC grade, cat. no. P/7507/17); methanol (>99.5%, HPLC grade, cat. no.

M/3950/17) and sodium succinate (cat. no. S/6490/48) were obtained from Fisher Scientific (Loughborough, UK).

Sodium phosphate dihydrate (monobasic, min. 99%, cat. no. 27175-0010) was purchased from Acros Organics (Geel, Belgium).

Genomic 'Midi' blood and cell culture genomic tip kits (cat. no. 13343) were purchased from Qiagen (Crawley, UK).

Orthophosphoric acid (AnalR™, 85%, cat. no. 10173) and triethylamine (AnalR™, min. 99.5%, cat. no. 104095) were purchased from BDH (Poole, Dorset, UK).

2.1.2 Enzymes

Apyrase (from potato, grade VII) was purchased from Sigma Aldrich Corporation (cat. no. A-6535). Apyrase was resuspended in deionised water to a concentration of 20 mU/µl and stored at -20°C. One unit will liberate 1 µmol of inorganic phosphate per minute at 30°C, pH 6.5. *

Deoxyribonuclease I (DNAse I, type II from bovine pancreas) was purchased from Sigma Aldrich Corporation (cat. no. D4527). Deoxyribonuclease I was resuspended in deionised water to a concentration of 40 mU/µl and stored at -20°C. One unit will produce a ΔA_{260} of 0.001 per minute using DNA type I or III as a substrate under standard assay conditions (37°C, pH 5, 4.2 mM $MgCl_2$).*

* Unit as defined by manufacturer

Micrococcal Nuclease (MN) was purchased from Sigma Aldrich Corporation (cat. no. N5386). MN was resuspended in deionised water at a concentration of 0.4 U/µl and stored at -20°C. One unit will produce 1 µmol of acid soluble polynucleotides from native DNA per minute at 37°C, pH 8.8. *

Nuclease P1 (NP1) was obtained from Boehringer Mannheim (Lewes, UK) (cat. no. 236225). NP1 was resuspended in SAZ buffer (see section 2.1.6) to a concentration of 0.3 U/µl and stored at -20°C. *3'→5' phosphodiesterase activity*: 1 unit will catalyse the hydrolysis of 1 µmol equivalent of phosphodiester linkages in yeast RNA in 1 minute at 37°C. *3' phosphomonoesterase activity*: 1 unit will catalyse the formation of 1 µmol of inorganic phosphate from 3'AMP per minute at 37°C. *

Phosphodiesterase I from snake venom ((SVPD, type IV from Crotalus atrox) was purchased from Sigma Aldrich Corporation (cat. no. P4506). SVPD was resuspended in deionised water to a concentration of 12 mU/µl and stored at -20°C. One unit will hydrolyse 1 µmol of bis(p-nitrophenyl) phosphate per minute at 37°C, pH 8.8. *

Phosphodiesterase II from calf spleen (CSPD) was purchased from Calbiochem (Nottingham, UK) (cat. no. 524710). CSPD was subjected to dialysis in deionised water overnight at 4°C. The final concentration was calculated to be 17 mU/µl. One unit is defined as the amount of enzyme that will hydrolyse 1 µmol of thymidine-3'-p-nitrophenyl phosphate per minute at 25°C, pH 7. *

* Unit as defined by manufacturer

Protease K was supplied as part of the Qiagen blood and cell culture genomic tip kit and prepared according to manufacturers instructions (Qiagen) (cat. no. 13343).

RNase A (0.108 U/µl) was purchased from Sigma Aldrich Corporation (cat. no. R-4642). RNase A was stored at -20°C. One unit will produce acid soluble oligonucleotides equivalent to a ΔA_{260} of 1 in 30 minutes under standard assay conditions (37°C, pH 7.5).*

RNase T_1 (549.9 U/µl) was purchased from Sigma Aldrich Corporation (cat. no. R-1003). RNase T_1 was stored at 4°C. One unit will produce acid soluble nucleotides equivalent to a ΔA_{260} of 1 in 15 minutes under standard assay conditions (37°C, pH 7.5).*

Shrimp alkaline phosphatase (SAP) (1 U/µl) was purchased from Amersham Pharmacia Biotech (cat. no. E70092X). SAP was stored at -20°C. One unit catalyses the hydrolysis of 1 µmole of 3' AMP per minute at 37°C, pH 7.2.*

T4 polynucleotide kinase (T4PNK) (30 U/µl) was purchased from Amersham Pharmacia Biotech (cat. no. E70031X). T4PNK was stored at -20°C. One unit incorporates 1 nmol of ^{32}P from [γ-^{32}P]ATP into MN treated DNA in 30 minutes at 37°C under standard assay conditions.*

* Unit as defined by manufacturer

T4 polynucleotide kinase (T4PNK) (10 U/µl, certified 3' phosphatase free) was obtained from Boehringer Mannheim (cat. no. 838292). T4PNK was stored at -20°C. One unit will incorporate 1 nmol of [^{32}P] from [γ-^{32}P]ATP into acid precipitable products within 30 minutes at 37°C. *

2.1.3 Oligonucleotides

All oligonucleotides were prepared via automated phosphoramidite DNA oligonucleotide synthesis using a ABI 394 DNA/ RNA synthesiser (Applied Biosystems, Warrington, Cheshire, UK) by the Protein and Nucleic Acids Chemistry Laboratory (PNACL, Hodgkin Building, University of Leicester, UK).

Unmodified oligonucleotides were synthesised using dA bz (cat. no. 401159), dC bz (cat. no. 401160), dG ibu (cat. no. 401161) and dT (cat. no. 401162) phosphoramidites purchased from Applied Biosystems. Cleavage and deprotection was carried out using 35% ammonium hydroxide.

Oligonucleotides containing a methyl-PTE at a specific position were synthesised using dT-CE phosphoramidites (cat. no.10-1030-20) and dT-Me (cat. no. 10-1130-05) purchased from Glen Research (Sterling, VA, USA) according to manufacturers instructions. Cleavage and deprotection was carried out using 0.05 M potassium carbamate in methanol for 17 hours at room temperature. Oligonucleotides were desalted using NAP-5 columns (cat. no. 17-0853-01) purchased from Amersham Pharmacia Biotech and dried down using a centrifugal evaporator (SC110A, Savant, Farmingdale, NY, USA.).

* Unit as defined by manufacturer

2.1.4 Cell lines and media

h2E1/OR (cat. no. C123) were obtained from Gentest Corporation (Woburn, MA, USA.). h2E1/OR cells were grown in RPMI medium 1640 (with 2 mM L-histidinol, without L-glutamine and L-histidine) with 10% horse serum (Gentest, cat. no. M202) and 2mM glutamax-1 (Invitrogen, cat. no. 35050-038).

HeLa cells were provided by Dr. Y. Guichard (Dept. of Oncology, University of Leicester). HeLa cells were grown in Dulbecco's MEM with glutamax-l (Invitrogen, cat. no. 61965 – 026) with 10% foetal calf serum (PAA laboratories Ltd., Linz, Austria).

2.1.5 Miscellaneous

Octadecylsiloxy (ODS) reversed phase C18 column (5μm; 4.6 x 250 mm; cat. no. 30105-060) was purchased from ThermoHypersil (Runcorn, UK).

ODS reversed phase C18 column (Apex, 5 μm; 4.6 x 250 mm; cat. no. 112120 was purchased from Jones Chromatography Ltd. (Hengoed, UK).

C18 guard column cartridges (Techsphere, 5 μm; 3 x 10 mm; cat. no. GTS-E03501) was purchased from HPLC Technology Co. Ltd. (Macclesfield, UK).

PEI cellulose TLC sheets (cat. no. 105579) were purchased from BDH.

Kodak x-omat K film (35 x 43 cm; cat. no. F-5263) was purchased from Sigma-Aldrich Corporation.

2.1.6 Commonly used solutions and buffers

The following solutions were used throughout this study. Solutions were prepared using deionised water from a Maxima Ultra Pure water system (ELGA, High Wycombe, Buckinghamshire, UK).

Denaturing polyacrylamide gel loading buffer: 95% formamide, 0.02% bromophenol blue and 0.02% xylene cyanol in TBE (see below).

DNA digestion buffer: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$ and 2 mM $ZnCl_2$.

Glutamax-1: 200 mM glutamax-1 in 6.5% NaCl.

Non-denaturing polyacrylamide gel loading buffer: 0.025% bromophenol blue and 0.025% xylene cyanol in 40% sucrose.

Phosphate buffered saline(PBS), pH 7.2: 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 in deionised water. Purchased from Oxoid (Basingstoke, UK). Autoclaved before use.

Qiagen buffer C1: 1.28 M sucrose, 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$ and 4% Triton X-100 (supplied with Qiagen genomic tip kits).

Qiagen buffer G2: 800 mM guanidine HCl, 30 mM Tris-HCl (pH 8.0), 30 mM EDTA (pH 8.0), 5% Tween-20 and 0.5% Triton X-100 (supplied with Qiagen genomic tip kits).

Qiagen buffer QBT: 750 mM NaCl; 50 mM MOPS (pH 7.0), 15% isopropanol and 0.15% Triton X-100 (supplied with Qiagen genomic tip kits).

Qiagen buffer QC: 1 M NaCl, 50 mM MOPS (pH 7.0) and 15% isopropanol (supplied with Qiagen genomic tip kits).

Qiagen buffer QF: 1.25 M NaCl, 50 mM Tris-HCl (pH 8.5) and 15% isopropanol (supplied with Qiagen genomic tip kits).

SAP reaction buffer: 200mM Tris-HCl (pH8), 100 mM $MgCl_2$ (supplied with enzyme from Amersham).

Sodium acetate zinc chloride buffer (SAZ): 10 mM sodium acetate (pH 5.3) and 1 mM $ZnCl_2$.

Sodium succinate calcium chloride buffer (SSCC): 100 mM sodium succinate, 50 mM $CaCl_2$, pH 6.

T4PNK reaction buffer (x 10): 0.5 M Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol (supplied with enzyme from Amersham).

T4PNK dilution buffer: 50 mM Tris-HCl (pH 8)(supplied with enzyme from Amersham).

T4PNK 3' phosphatase free reaction buffer (x 10): 0.5 M Tris-HCl, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM DTT and 1 mM spermidine (pH 8.2 at 25°C) (supplied with enzyme from Boeringer Mannheim).

Tris-borate EDTA buffer (TBE) (x 10): 890 mM Tris Borate (pH8.3), 20 mM EDTA (10 x TBE was purchased from National Diagnostics and diluted 10-fold with deionised water before use).

Tris EDTA buffer (TE): 10 mM Tris (pH 7.4) and 1mM EDTA (pH 8).

Trypsin EDTA (x 10): 5 g trypsin, 5.4 mM EDTA and 145 mM NaCl. Diluted in sterile PBS before use.

2.2 Quantification of DNA

DNA was quantitated using UV spectrometry (DU 7500, Beckman, C.A., USA) with a quartz cuvette (length of path 1cm, Helma Supersil™, Fisher Scientific). The concentration of DNA was calculated using the formula (ΔA_{260}x 50)/ dilution factor (for genomic DNA) or (ΔA_{260}x 33)/ dilution factor (for oligonucleotides).

2.3 Isopropanol precipitation of DNA

DNA samples were precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 7) and 1 volume of isopropanol, followed by incubation overnight at

-20°C. The DNA was pelleted by centrifugation (21000g, 15 minutes, 4°C). The DNA pellet was washed twice with 70% ethanol. The pellet was then dried using a centrifugal evaporator and dissolved in deionised water to a concentration of 1mg/ml.

2.4 Extraction of DNA from cells using Qiagen genomic tips

2.4.1 Preparation of nuclei

Cells were resuspended to a concentration of 1x 10^7 cells/ml and lysed using 1 volume of ice cold buffer C1, followed by 3 volumes of ice cold distilled water and incubated on ice for 10 minutes. The lysed cells were then centrifuged (15 minutes, 1300g, 4°C) and the supernatant was discarded. The pellet was resuspended in 1 ml buffer C1 and 3 ml of ice cold distilled water. The sample was centrifuged (15 minutes, 1300g, 4°C) and the supernatant was discarded. At this point the pellet (containing the nuclei) was frozen and stored at -20°C.

2.4.2 Extraction of DNA from nuclei

The nuclei were resuspended in 5ml of buffer G2 and then incubated with 95µl proteinase K (600 AU/ml), 162 µl RNAse A (2.5 U/µl) and 0.5 µl RNAse T_1(800U/µl) for 1 hour at 50°C.

Qiagen genomic midi tips (100/G) were equilibrated using 4 ml of buffer QBT. The DNA samples were vortexed for 10 seconds before being applied to the equilibrated tips and allowed to enter the resin by gravity flow. Next the genomic tips were each washed with 25 ml of buffer QC and the DNA eluted with 5 ml buffer QF (warmed to 50°C).

The DNA was precipitated by adding 0.7 volume of isopropanol. The samples were inverted 20 times and kept at -20°C overnight. The DNA was pelleted (>5000g,

15 minutes, 4°C) and the supernatant discarded. The pellet was then washed with 2 ml of cold 70% ethanol and dried using a centrifugal evaporator (low heat, 10 – 15 minutes). The pellet was dissolved in 1 ml of distilled water overnight on a shaker (4°C).

2.5 Alkylation of calf thymus DNA

CT- DNA treated with 0.1 - 10 mM diethyl sulfate (DES) in sodium phosphate buffer (0.5 M, pH 6.0) at room temperature for 8 hours, was provided by Dr. Yves Guichard (Dept. of Oncology, University of Leicester, UK).

CT-DNA (1 mg/ ml) was also treated with 2 - 10 mM DES in PBS for either 3 hours at 37°C or 8 hours at room temperature. The DNA was precipitated by isopropanol precipitation (Section 2.3) and resuspended in deionised water to a concentration of 1 mg/ml.

2.6 Treatment of mice with NDEA

Mouse liver DNA was provided by Drs. R. Singh and V. Oreffo. (MRC Toxicology Unit, University of Leicester, UK). Briefly, the liver DNA was obtained from SWR, Balb/c and C57BL/6J mice treated *in vitro* with a single intraperitoneal dose of NDEA (90 mg/ kg). Control mice were given a single intraperitoneal dose of adjuvant. Mice were sacrificed 5 hours, 10 hours, 24 hours, 4 days, 7 days, 28 days and 56 days after the single dose of NDEA. Livers were taken and the DNA extracted as described by Oreffo *et al.* (2000).

2.7 Treatment of cells with DES

h2E1/OR cells were treated with DES (0.5 – 10 mM final concentration in 25ml of the appropriate cell media) for 2 hours at 37°C, 5% CO_2. HeLa cells (~ 4 x 10^7 cells) were treated with DES (0.5 – 10 mM final concentration in 75 ml of the appropriate cell media) for 3 hours at 37°C, 5% CO_2. The cell suspension was centrifuged for 10 minutes (1500g, 4°C) and the supernatant discarded. The cell pellet was washed twice with PBS (10 minutes, 1500g, 4°C). DNA was extracted using Qiagen genomic midi tips as described in section 2.4.

2.8 Treatment of isolated nuclei and DNA with DES

Nuclei were obtained from cells using the Qiagen tip protocol (section 2.4.1) and integrity assessed via fluorescence microscopy (*data not shown*). The nuclei were washed twice with 1ml PBS and resuspended in PBS to a concentration equivalent to 1mg/ml DNA (calculated according to the amount of DNA extracted from an equivalent number of cells harvested on the same day). DES (diluted in DMSO) was added to give a final concentration of 1, 5 or 10 mM DES. Control samples were treated with DMSO alone. The nuclei were incubated for 3 hours at 37°C. Following incubation the nuclei were pelleted by centrifugation and the supernatant discarded. The nuclei were washed twice with 5 ml PBS. The DNA was then extracted from the nuclei using Qiagen genomic tips (section 2.4.2).

DNA extracted from cells (section 2.4) was dissolved in PBS to a concentration of 1mg/ml. DES (1 - 10% v/v solution diluted in DMSO) was added to give a final concentration of 1, 5 or 10 mM DES. Control samples were treated with DMSO alone. The DNA was incubated for 3 hours at 37°C. The DNA was

precipitated using isopropanol precipitation and dissolved in deionised water to a concentration of 1mg/ml.

2.9 Treatment of oligonucleotides with alkylating agents

2.9.1 Preparation of duplexs

The prepared oligonucleotides were dissolved in PBS buffer at a concentration of 10 μg/μl. The oligonucleotides were annealed together in a ratio of 1:0, 1:1, 1:2 and 1:5 using a thermal cycler (PTC-200, MJ Research, MA, USA): 90°C for 5 mins; 85°C for 15 mins; 80°C for 15 mins; 75°C for 15 mins; 70°C for 20 mins; 65°C for 20 mins; 60°C for 20 mins; 55°C for 15 mins; 50°C for 15 mins; 45°C for 15 mins; 40°C for 15 mins; 30°C for 15 mins; 20°C for 15 mins. The oligonucleotides were cooled very slowly in order to prevent any unusual secondary structures forming.

2.9.2 Evaluation of the extent of oligonucleotide duplex formation

An aliquot of the oligonucleotides was labelled with [γ-^{32}P]ATP by T4PNK prior to annealing (section 2.9.1). Each kinase reaction mixture (10 μl) contained kinase buffer (1μl of the x 10 buffer supplied), 0.6 pmol of oligonucleotide, 5 μCi of [^{32}P]ATP (approximately 1.65 pmol) and T4 PNK (7.5 units). The samples were incubated for 1 hour at 37°C.

The kinase mix was used directly in the preparation of duplexes (Section 2.9.1). Following annealing, the oligonucleotide mix was diluted with an equal volume of non-denaturing loading buffer. The single and double stranded oligonucleotides were separated upon a 30% non-denaturing polyacrylamide gel. The gel electrophoresis equipment employed consisted of a power supply (EPS 3500XL, Pharmacia Biotech) together with a Model S2 Sequencing Gel Electrophoresis System

unit using 33.5 cm x 41.5 cm glass plates, 0.4 mm spacers and a 16 tooth comb (Life Technology™, Invitrogen). Electrophoresis was carried out at 600V until the bromophenol blue and xylene cyanol markers were approximately 4 cm apart. The radiolabelled products were visualised by autoradiography on Kodak x-omat K film (35 x 43 cm) and storage phosphorimage analysis (Molecular Dynamics, model 42SE, using ImageQuant™ software version 3.2, Sunnyvale, CA, USA).

2.9.3 Standard treatment of oligonucleotidse with alkylating agents

The single and double stranded oligonucleotides (1 μg/ μl) were treated in PBS with 0, 5, 10 and 50 mM of ENU, MNU, DES or DMS (0.24 – 32.9 % solution diluted in DMSO) for 3 hours at 37°C. Control samples were treated with DMSO alone. The oligonucleotides were precipitated using isopropanol precipitation (Section 2.3). The pellet was washed twice with 95% ethanol and dried using a centrifugal evaporator. The oligonucleotides were dissolved in deionised water to a concentration of 1mg/ ml.

2.10 Phosphotriester postlabelling assay

2.10.1 Digestion

DNA was digested as described in Guichard *et al.* (2000). Samples of DNA, methyl PTE containing oligonucleotide or methyl PTE containing dinucleoside phosphate (10 μg) were incubated overnight at 37°C with DNAse I (0.4 U), SVPD (0.04 U), NP1 (0.6 U) and SAP (0.4 U) in 30μl of digestion buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 2 mM $ZnCl_2$). The enzymes were then precipitated by addition of 3 volumes of ice-cold ethanol followed by storage at

-20°C for at least 1 hour. The enzymes were pelleted by centrifugation (21000g, 15 minutes, 4°C) and a 100 µl aliquot of the supernatant removed and dried using a centrifugal evaporator. The resulting residue was resuspended in deionised water (100µl), boiled for 10 minutes to remove any residual enzymatic activity and then stored at -20°C.

2.10.2 Alkali Hydrolysis of PTE containing dinucleoside phosphates

Hydrolysis with ammonium hydroxide was carried out according to the method described by Saris *et al.* (1995). An equal volume of 25% NH_4OH/H_2O was added to an aliquot of the digest (50 µl) giving a final concentration of 12.5% NH_4OH. The reaction was incubated in a tightly capped vial at 70°C for 20 - 24 hours. The reaction mixture was evaporated to dryness using a DNA centrifugal evaporator. The residue was dissolved in deionised water (50 µl) and stored at -20°C. Digests of the modified oligonucleotides synthesised containing one or more methyl-PTEs were further diluted prior to labelling to give a concentration of 1 pmol/ 5µl of labellable material to ensure that an excess of [γ^{32}P]ATP would be present in the labelling reaction.

2.10.3 [^{32}P]-labelling

Each kinase reaction mixture (10 µl) contained kinase buffer (1µl of the x 10 buffer supplied), 5 µl of the digested sample (treated or untreated with alkali), 5 µCi of [^{32}P]ATP (approximately 1.65 pmol), T4PNK (7.5 U) and where appropriate 100 fmol of poly dT_8 (<u>not</u> in samples to be analysed by 5' nearest neighbour analysis, Section 2.11). The samples were incubated for 1 hour at 37°C. The reaction mixture

was then incubated with an additional 3.75 U T4PNK and 1 µl of deionised water for 30 minutes. An optional step involved the addition of poly dT_{16} (5 U) in the second stage of the kinase reaction to consume the excess [γ^{32}P]ATP (not in samples to be analysed by 5' nearest neighbour analysis, Section 2.11).

2.10.4 Polyacrylamide gel electrophoresis of the [^{32}P]-labelled compounds

Prior to loading on to the gel an equal volume of formamide loading buffer was added. Samples (5 µl) were loaded onto a 20% polyacrylamide/7M urea gel. The gel electrophoresis equipment the power supply and electrophoresis apparatus was as described in Section 2.9.2, but using 0.8 mm spacers and a 32 tooth comb (Life Technology™, Invitrogen). Electrophoresis was carried out at 1200 V until the bromophenol blue marker had migrated 11-12 cm (~3 hours). Radiolabelled products were visualised by autoradiography and storage phosphorimage analysis as described in Section 2.9.2.

2.10.5 External quantification standard and internal labelling reference

The dinucleoside phosphates in the samples were quantified by comparing the integrated volume of a mixture of 16 dinucleoside phosphate standards (50 fmol on the gel, 3.125 fmol of each) against the integrated volume of the dinucleoside phosphate species of the samples: (integrated volume of ^{32}pdNpdN obtained from the sample)/ (integrated volume of ^{32}pdNpdN obtained from the standard) x 50. When poly dT_8 was used as an internal labelling reference, the obtained value was adjusted by the following correction factor: (integrated volume for poly dT_8 in standard)/ (integrated volume for poly dT_8 in sample).

2.11 Analysis of the 5' labelled nucleotide of the ^{32}pdNpdN species

2.11.1 Preparation of samples for HPLC analysis: the 'direct' method

Labelled digests (containing 0.125 - 0.625 μg digested DNA) were incubated with 2μl of apyrase (40 mU) for 30 minutes at 37°C. Samples were heated to 100°C for 15 minutes to remove residual apyrase activity and then incubated with NP1 (0.6 U in 50μl of SAZ buffer) to release the 5' labelled mononucleotides (^{32}pdN).

2.11.2 HPLC analysis of 5' labelled nucleotides: the 'direct' method

Aliquots of the apyrase-boiled-nuclease P1 treated sample were mixed with 40 μl of 5' mononucleotides UV marker mix (200 mM each). Total injection volume was 100μl. HPLC was performed using a Varian Star 9012 pump system (Varian U.K. Ltd., Surrey, U.K.) connected to a UV-Vis detector (Varian Star 9050) and a radioactivity detector (β-ram, LabLogic, Sheffield, UK). Analysis was performed at 30°C (Column temperature control system, Jones Chromatography Ltd.) at a flow rate of 1ml/min using a reversed phase ODS C18 column (5μm; 4.6 x 250 mm) (ThermoHypersil) fitted with a C18 guard column (Techsphere, 5 μm; 3 x 10 mm; HPLC Technology Co. Ltd.). The elution conditions were as follows: solvent A (83 mM triethylammonium phosphate buffer (pH 4), 1% methanol); solvent B (83 mM triethylammonium phosphate buffer (pH 4) 20% methanol); gradient: 0 minutes - 100% A; 20 minutes - 55% A, 45% B; 28 minutes - 100% B; 33 minutes - 100% B; 35 minutes - 100% A. The frequency of nucleosides 5' to PTEs was independently determined a minimum of three times.

2.11.3 HPLC analysis of 5' labelled nucleotides as used by Guichard et al. (2000)

Samples were mixed with 40 µl of 5' mononucleotides UV marker mix (200 mM each). Total injection volume was 100µl. HPLC was performed using a reversed phase C18 column (Apex, 5 µm; 4.6 x 250 mm; Jones Chromatography Ltd.) fitted with a C18 guard column (Techsphere, 5 µm; 3 x 10 mm; HPLC Technology Co. Ltd.). The HPLC conditions were as follows: solvent A (50 mM ammonium acetate pH 4.5); solvent B (methanol/ water (80:20)); gradient: 0 minutes - 100% A; 5 minutes - 100% A; 20 minutes - 90% A, 10% B; 25 minutes - 100% A.

2.12 Labelling 3' mononucleotides

T4 PNK supplied by Amersham Pharmacia Biotech is known to contain 3' phosphatase activity and therefore is not appropriate for the labelling of 3' mononucleotides. Therefore T4 PNK (certified 3' phosphatase free) was obtained from Boehringer Mannheim. Each kinase reaction mixture (20µl) contained 2µl kinase buffer supplied, 1 pmol of dNp, 5µCi of [γ^{32}P]ATP (approximately 1.65 pmol) and T4 PNK (5 U). The samples were incubated for 1 hour at 37°C.

2.13 Determination of the normal nucleotide content

DNA (10 µg) was digested with MN (0.4 U), CSPD (60 mU) in sodium succinate (16 mM), calcium chloride (8 mM) buffer (pH 6). Samples were digested overnight at 37°C. The enzymes were then precipitated by the addition of 3 volumes of ice-cold ethanol and removed by centrifugation (21000g, 15 minutes, 4°C). The supernatant was removed and evaporated using a centrifugal evaporator. The resulting residue was dissolved in water (100 µl), heated at 100°C for 10 minutes to remove residue enzymatic activity and then stored at -20°C.

The digest was diluted 375-fold and a 5 µl aliquot was labelled as described in section 2.12. Half the [^{32}P]-labelled mixture (10µl) was then incubated with 2 µl apyrase (40 mU) at 37°C for 1 hour to remove excess [γ^{32}P]ATP. Aliquots (2 µl) were spotted onto PEI cellulose sheets (20 cm x 20 cm). The sheets were developed in 0.12M sodium phosphate (pH 6.8) for 2 hours. Radiolabelled products were visualised by autoradiography and storage phosphorimage analysis as in Section 2.9.2.

2.14 Statistical Analysis

Data was analysed using one-way analysis of variance (ANOVA) using Minitab software (Version 13, Cleocom Ltd., Birmingham, UK).

2.15 Safety Aspects

All guideline and regulations relating to the handling of radioactivity were adhered to in the use of [γ^{32}P]ATP. All work was carried out behind perspex screens. The user wore eye protection, double gloves (latex) and overalls. The area was monitored for contamination before and after use with a geiger counter.

Special precautions were also taken in the handling of the carcinogens *N*-nitrodiethylamine, diethylsulfate, dimethylsulfate, *N*-ethyl-*N*-nitrosourea and *N*-methyl-*N*-nitrosourea. All procedures involving these chemicals were carried out inside a fume hood. The user wore eye protection, double gloves (latex), overalls, plastic apron and an anti-vapour face mask.

Chapter 3:

Further development of the postlabelling method for detecting alkyl-phosphotriesters

3.1 Introduction

One of the reasons so little work has previously been undertaken to investigate DNA PTEs has been the lack of suitable analytical methods. Saris *et al.* (1993 and 1995) developed a SVPD/NP1-based [^{32}P]-postlabelling approach for the detection of PTEs. The technique is based on the inability of all known nucleolytic enzymes to digest the internucleotide PTE bond. Consequently, complete digestion of alkylated DNA with these nucleases in the presence of an alkaline phosphatase yields PTE-dinucleoside phosphates (dNp(R)dN). These species are then converted to corresponding dinucleoside phosphates (dNpdN), by treatment with alkali, to permit subsequent [^{32}P]-labelling (see Section 1.12).The method was further developed by Guichard *et al.* (2000) to permit the determination of the frequency of the nucleosides located 5' to the PTE lesions. The identity and frequency of the nucleosides located 5' to the PTE lesions were obtained by NP1 digestion of the gel-isolated ^{32}pdNpdN species and analysis of the released ^{32}pdN species by HPLC with radioactivity detection ([^{32}P]-HPLC) (Figure 3.1).

This method was been applied to the analysis of CT-DNA treated *in vitro* with dimethylsulfate (DMS) or diethylsulfate (DES) and liver DNA from mice treated *in vivo* with *N*-nitrosodiethylamine (NDEA). Results obtained from CT-DNA treated with DMS or DES showed that the frequency of the four detected nucleotides reflected the normal nucleoside content of CT-DNA, implying a random formation of methyl and ethyl PTE adducts in DNA modified *in vitro*. However, for liver DNA extracted from mice treated *in vivo* with NDEA, the frequency of 2' deoxyguanosine (^{32}pdG) was found to be significantly lower and the frequency of thymidine (^{32}pdT) significantly greater than expected with respect to the normal nucleoside content of mouse liver DNA. The frequency of 2'deoxycytosine (^{32}pdC) and 2'deoxyadenosine

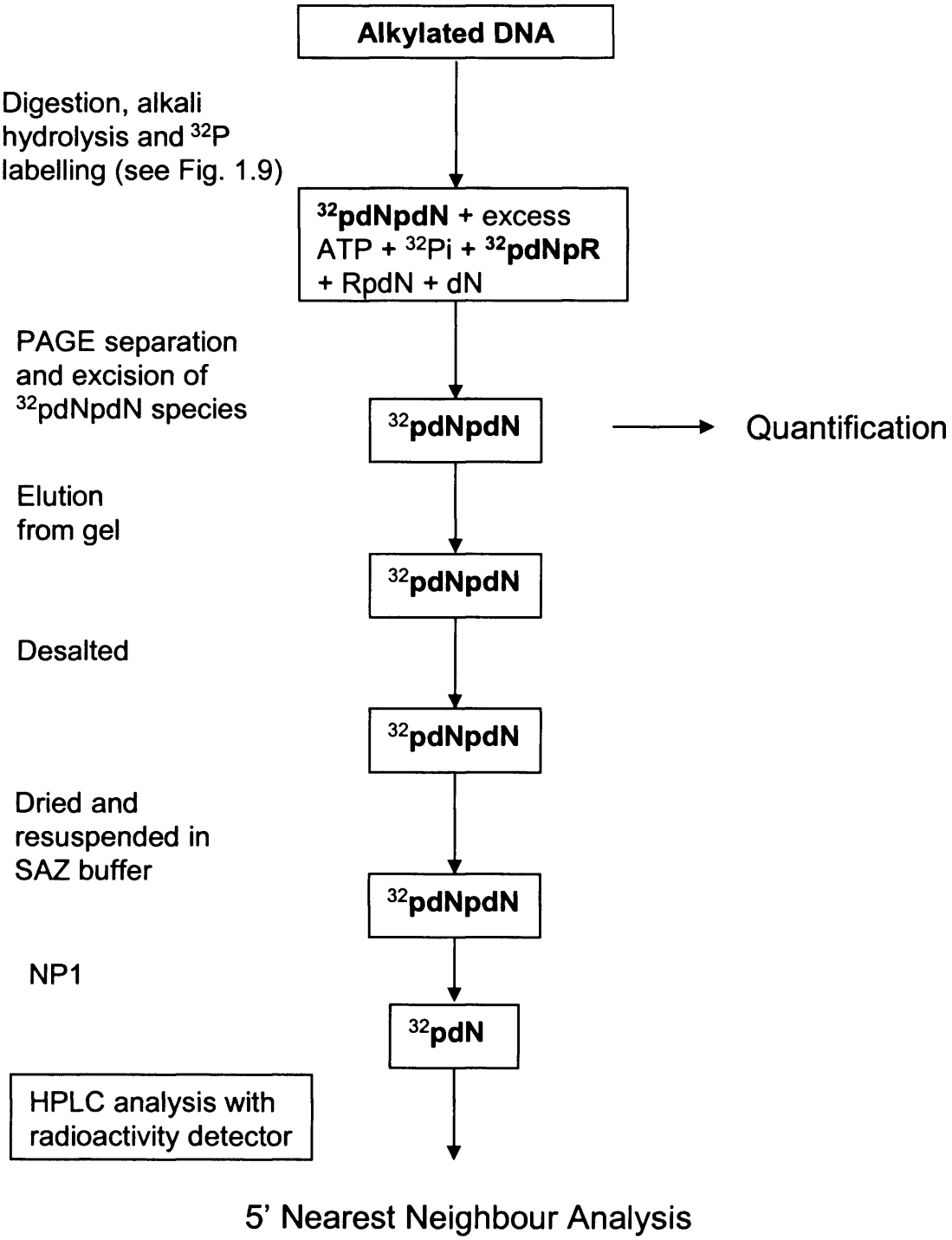


Figure 3.1: Scheme illustrating the original method of PTE quantification and the preparation of samples for 5' NNA, as described by Guichard et al. (2000)

(^{32}pdA) in the liver DNA treated *in vivo* with NDEA were found to reflect the normal nucleoside content of mouse liver DNA. These results were indicative of either (i) the non-random formation of ethyl PTE *in vivo* and/or (ii) base sequence-specific ethyl PTE repair (Guichard *et al.*, 2000).

The original 5'NNA protocol as described by Guichard *et al.* (2000) was both time consuming, highly manipulative (involving the direct handling of radiolabelled material of high specific activity), and frequently resulted in the loss of labelled material, particularly during the desalting of the gel-isolated material prior to NP1 digestion. Consequently it was desirable to develop and establish an alternative protocol for PTE 5' NNA avoiding the gel-isolation of the ^{32}pdNpdN species.

In a second research theme, the ability of the SVPD/NP1 postlabelling assay to detect DNA multiply damaged sites (MDS) was evaluated. Numerous cytotoxic and mutagenic agents are proposed to exert their deleterious effects via the formation of lesions and adducts in genomic DNA. As well as producing 'simple' individual lesions/adducts, a number of agents such as ionising radiation and chemotherapeutic drugs are proposed to generate 'complex' multiply damaged sites, where more than one moiety in a local region (i.e., 1 helical turn or 10 bp) is damaged. It has been proposed that MDS are lesions of heightened biological significance due to the greater challenge they present to repair systems (Ward, 1988; Donald *et al.*, 1999; Venkhataraman *et al.*, 2001). However, other than indirect measures, there are few analytical techniques that enable the detection of MDS in DNA. In this particular study the potential of the SVPD/NP1 postlabelling assay to detect tandem PTE damage, a form of MDS, where two PTE lesions are located immediately adjacent to one another on the same DNA strand was investigated. The specificity of the assay in

dealing with pairs of PTE lesions on the same strand of DNA separated by a single normal nucleotide was also investigated.

3.2 Specific aims of the study

- To develop a new protocol that eliminates the need for gel purification of the ^{32}pdNpdN species prior to 5'NNA analysis.
- To establish the new method by comparing the results obtained from analysis of CT-DNA treated *in vitro* with DES and mouse liver DNA extracted from mice treated *in vivo* with NDEA using the new 'direct' 5'NNA protocol, with those previously reported by Guichard *et al.* (2000).
- Evaluate the ability and specificity of the SVPD/NP1 postlabelling method to the detect tandem PTE lesions in synthetic oligonucleotides.

3.3 Results

3.3.1 Development of a new 'direct' method for the 5' nearest neighbour analysis of PTEs to eliminate the requirement for the gel purification of ^{32}pdNpdN species.

Studies were undertaken using labelled ^{32}pdNpdN species, prepared by the ^{32}P-end labelling of an equimolar mix of the 16 possible dinucleoside monophosphates (dNpdN). Initial studies involving the direct ^{32}P-HPLC analysis of the kinase mix following NP1 treatment to release the labelled 5' mononucleotides (^{32}pdN), revealed that the residual excess [γ-^{32}P] ATP present after labelling interfered with the detection of ^{32}pdT (Figure 3.2). To address this it was proposed that the NP1 digested samples be treated with apyrase. Apyrase catalyses the hydrolytic breakdown of ATP and ADP to AMP and inorganic phosphate (P*i*). However, analysis of the

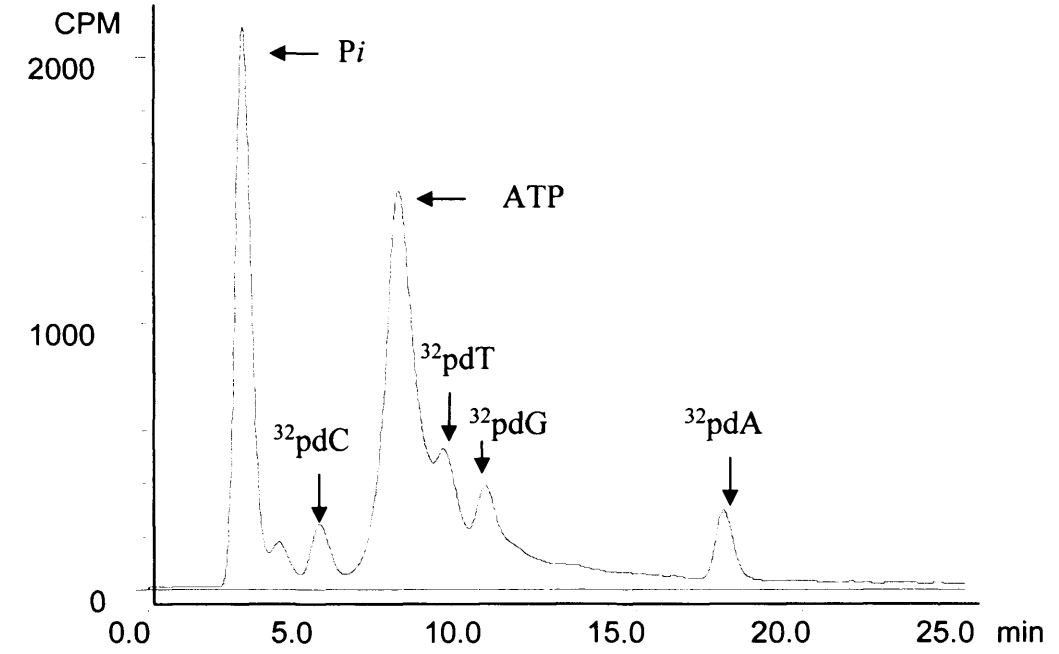


Figure 3.2: Typical [^{32}P]-HPLC chromatogram for ^{32}pdNpdN species treated with NP1 and then applied straight to the HPLC column without PAGE purification. HPLC conditions as described in section 2.11.3.

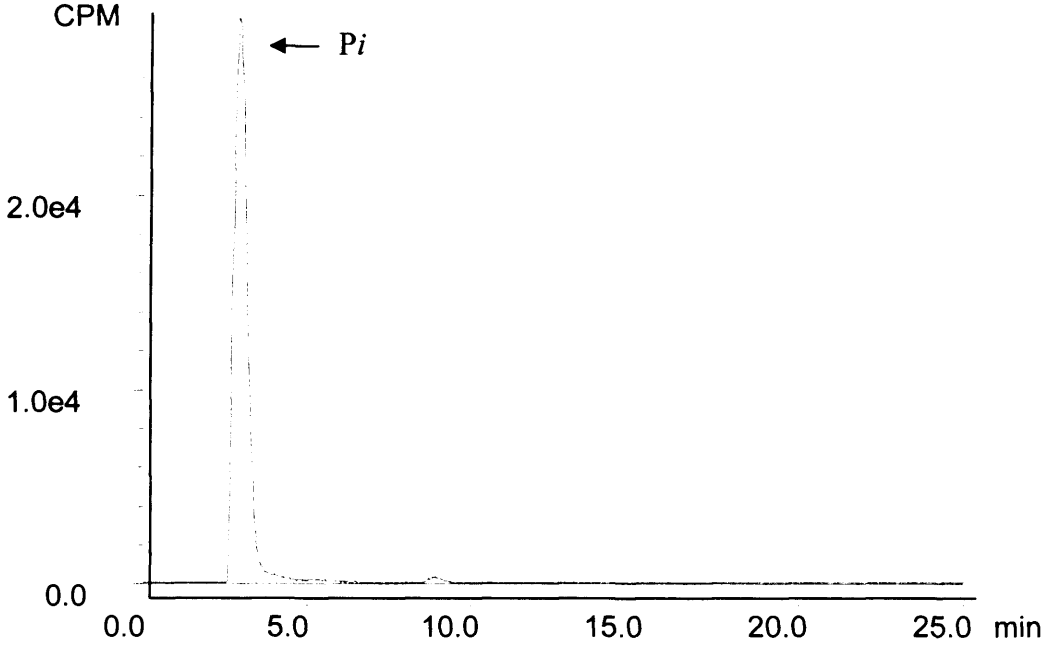


Figure 3.3: [^{32}P]-HPLC chromatogram for ^{32}pdNpdN mix treated firstly with NP1 and then apyrase. HPLC conditions as described in section 2.11.3.

resulting labelled products revealed the complete loss of the anticipated ^{32}pdN species, with only the activity from P*i* apparent (Figure 3.3).

This phenomenon is further demonstrated in Figure 3.4. Figure 3.4a depicts an autoradiograph of the PAGE separated labelled products obtained after various treatments of the sixteen ^{32}pdNpdN species with NP1 and/or apyrase. Lane 1 shows the sixteen labelled dinucleotide species as separated by PAGE, resolving as the five clearly distinct bands as described previously in Section 1.11 (Guichard *et al.*, 2000). Bands 1 and 5 contain only the ^{32}pdGpdG and ^{32}pdCpdC species respectively; bands 2, 3 and 4 consist of labelled dinucleotides containing the base-sequences [GA, GT, AG, TG], [CG, GC, AT, TA, TT, AA,] and [TC, CT, AC, CA], respectively. Digestion of the ^{32}pdNpdN species with NP1 (lane 6) cleaves the labelled dinucleotides to yield the four ^{32}pdN species that migrate as three bands on PAGE analysis. Band A contains ^{32}pdG, band B contains ^{32}pdA plus ^{32}pdT, and band C contains ^{32}pdC (plus the residual excess [γ-^{32}P] ATP). However, subsequent treatment of the NP1 generated ^{32}pdN species with apyrase (lane 7), supposedly to remove the excess [γ-^{32}P]ATP, led to the dramatic loss of all of the radiolabelled nucleotide species. Reversing the order of treatment (apyrase treatment followed by NP1 digestion)(lane 5) did lead to the observation of some of the ^{32}pdN species but there was still a substantial loss activity for all of the labelled species (>80%; see Figure 3.4b), but particularly for ^{32}pdG (band A). This activity appeared to be specific for the 5' mononucleotides, as treatment of the labelled dinucleotides with apyrase (lane 2) led to only a small decrease in the level of the ^{32}pdNpdN species (~5%; see Figure 3.4b), whilst entirely converting the excess [γ-^{32}P]ATP to ^{32}P*i* and AMP. From these observations it is deduced that the apyrase preparation contained some adventitious activity that catalyses the hydrolytic dephosphorylation of 5' mononucleotides to

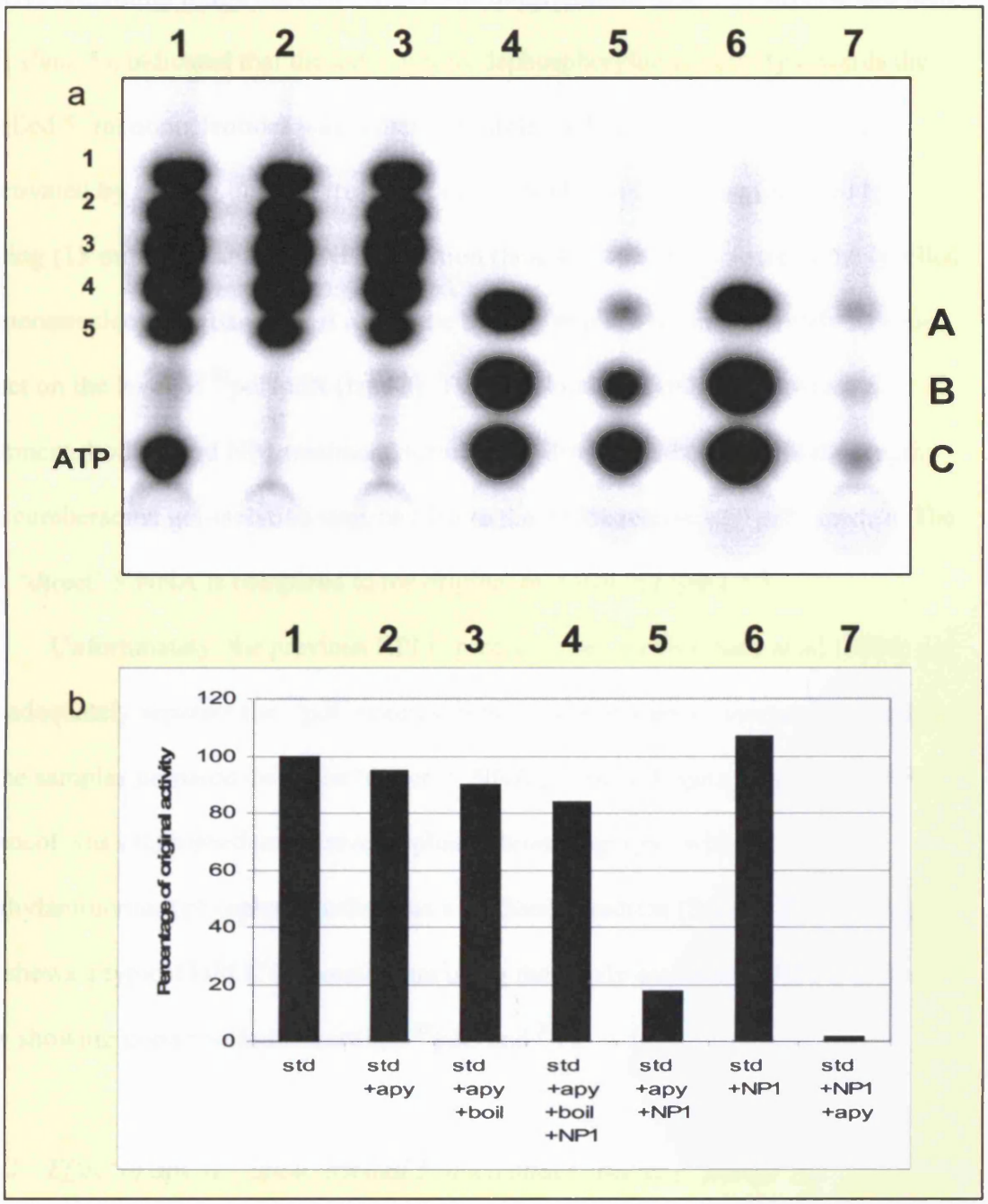


Figure 3.4: The effect of various treatments upon the ^{32}pdNpdN mix: (a) autoradiograph showing the effect of various treatments upon the ^{32}pdNpdN species; (b) histogram showing the effect of various treatments: Lane 1, ^{32}pdNpdN (40 fmol); lane 2, ^{32}pdNpdN treated with apyrase; lane 3 ^{32}pdNpdN treated with apyrase, then boiled; lane 4, ^{32}pdNpdN treated with apyrase, boiled and then treated with NP1; lane 5, ^{32}pdNpdN treated with apyrase and then NP1; lane 6, ^{32}pdNpdN treated with NP1; lane 7, ^{32}pdNpdN treated with NP1 and then apyrase.

nucleosides and ^{32}Pi. However, the observation of there being some residual ^{32}pdN species remaining following treatment of the dinucleotides first with apyrase and then NP1 (lane 5), indicated that the adventitious dephosphorylating activity towards the labelled 5' mononucleotides was somewhat labile, and therefore may be readily inactivated by heating. Indeed, treatment of ^{32}pdNpdN with apyrase followed by boiling (15 minutes), and then NP1 digestion (lane 4), led to the release of the labelled 5' mononucleotides (bands A, B & C): the boiling step not having an overly adverse effect on the level of ^{32}pdNpdN (lane 3). The developed procedure of apyrase treatment, boiling and NP1 treatment, termed the 'direct' method, avoids the lengthy and cumbersome gel-isolation step, and led to the >80% release of ^{32}pdN species. The new 'direct' 5'NNA is compared to the original protocol in Figure 3.5.

Unfortunately, the previous HPLC protocol used by Guichard *et al.* (2000) did not adequately separate the ^{32}pdC species from the vast excess of inorganic phosphate in the samples prepared using the 'direct' 5'NNA protocol (Figure 3.6). A new HPLC protocol was established using reverse phase chromatography with a triethylammonium phosphate buffer plus a methanol gradient (Section 2.11.2). Figure 3.7 shows a typical HPLC chromatogram using the newly established HPLC method now showing good resolution between ^{32}pdC and ^{32}Pi.

3.3.2 Effect of apyrase upon labelled 3' nucleotides species (^{32}pdNp)

Apyrase is used to remove excess [γ-^{32}P]ATP following ^{32}P end labelling in the traditional MN/CSPD postlabelling assay. The MN/CSPD postlabelling assay digests damaged DNA to give a mixture of modified 3' mononucleotides (dXp) and unmodified nucleosides (dN). The modified 3' mononucleotides (dXp) are substrates for ^{32}P end labelling (Reddy *et al.*, 1981; Randerath *et al.*, 1981; Gupta *et al.*, 1982).

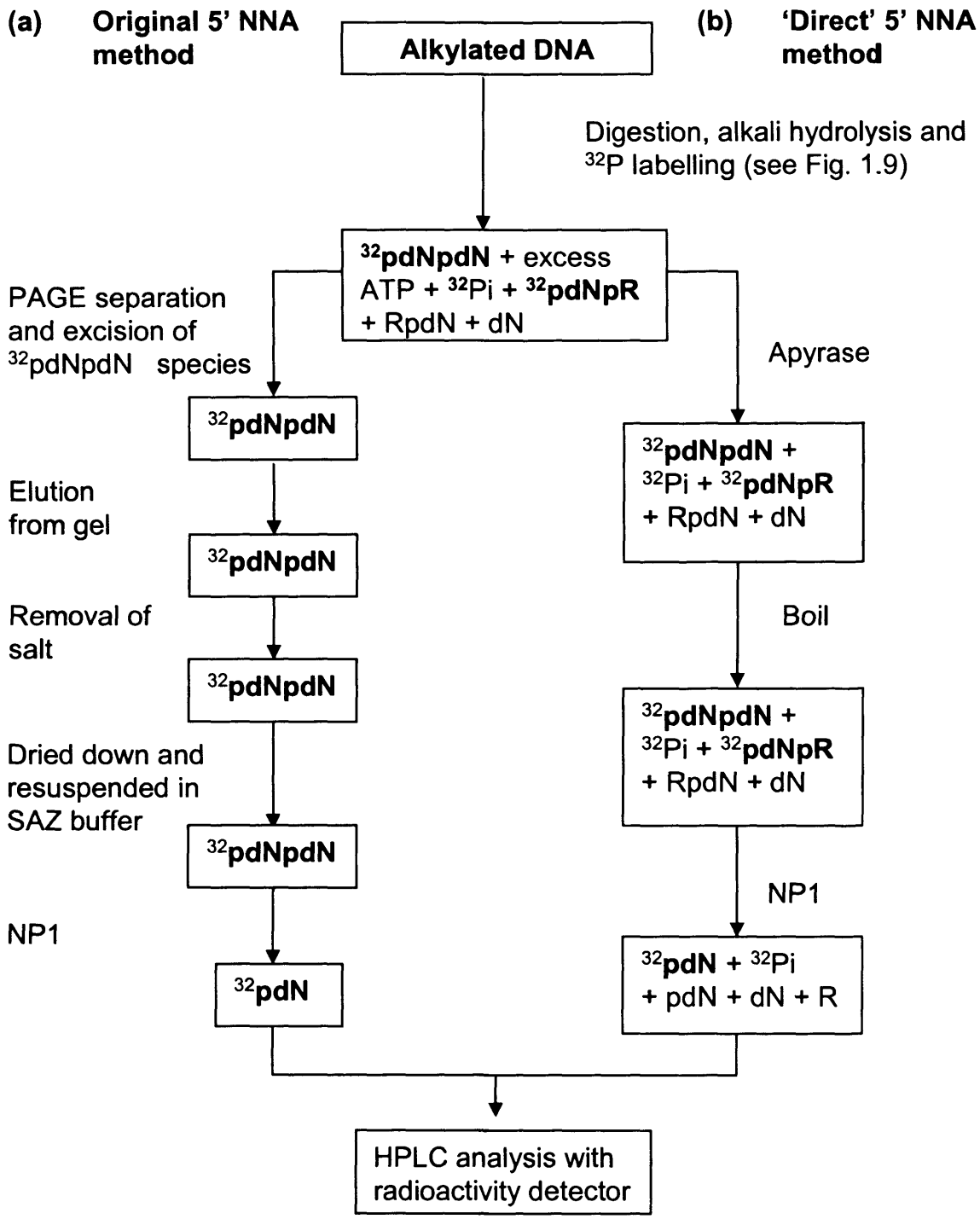


Figure 3.5: Scheme illustrating the two methods used to prepare samples for 5' nearest neighbour analysis (5'NNA) by ^{32}P-HPLC (a) the original 5' NNA protocol as described by Guichard et al. (2000) and (b) the new 'direct' 5'NNA protocol.

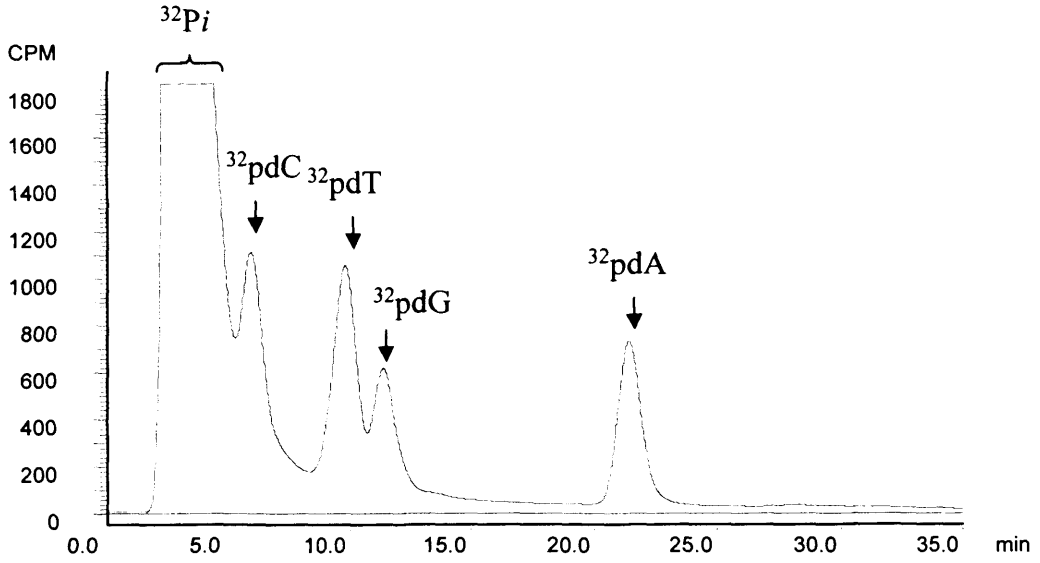


Figure 3.6: Separation of the 5' mononucleotides prepared using the new 'direct' method of 5' NNA sample preparation using the HPLC protocol as described in Guichard et al. (2000).

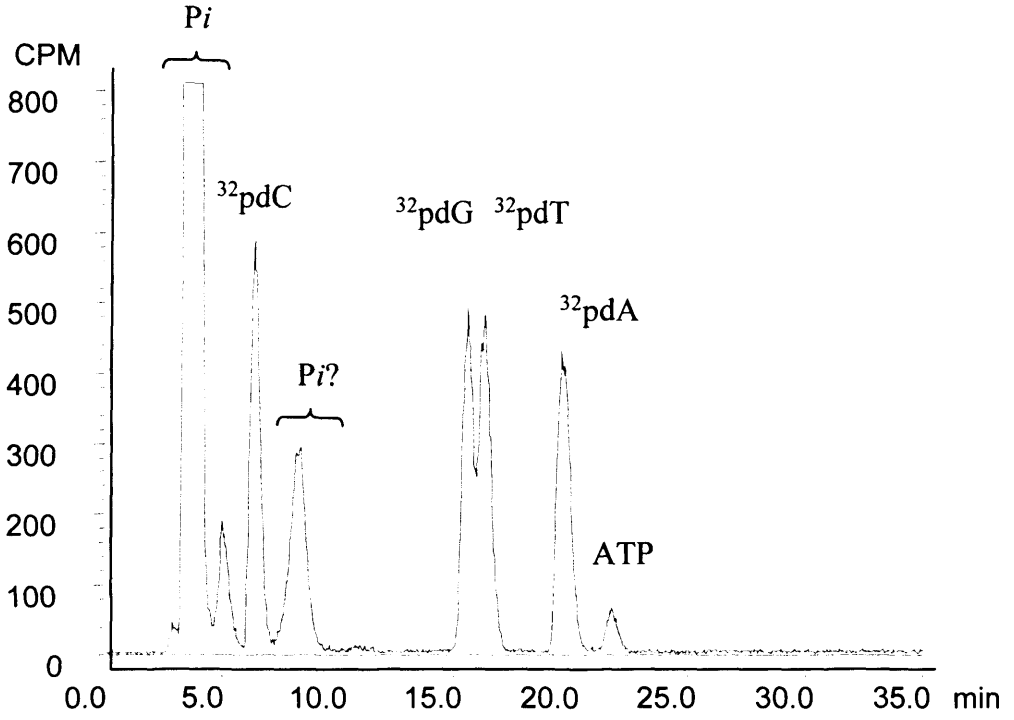


Figure 3.7: [^{32}P]-HPLC chromatogram using the newly established triethylamine phosphate based HPLC method for the 'direct' 5' nearest neighbour analysis of an equimolar mix of ^{32}pdNpdN standards (section 2.11.2)

Previously it has been demonstrated that the apyrase preparation contained some adventitious activity that catalyses the hydrolytic dephosphorylation of 5' mononucleotides, resulting in the complete loss of the ^{32}pdN species (see Section 3.3.1). In contrast, the ^{32}pdNpdN species were not found to be substrates for hydrolytic dephosphorylation. Consequently, it was of interest to determine whether the ^{32}pdNp species, products of the traditional MN/CSPD postlabelling protocol, would be affected by apyrase treatment or not.

Studies were undertaken using labelled ^{32}pdNp species, prepared by the ^{32}P end labelling of the four normal 3' nucleotide standards (dNp) in the presence of a limiting amount of [γ^{32}P]ATP. Figure 3.8 depicts an autoradiograph showing the separation of the postlabelled products by PAGE. Table 3.1 compares the relative intensity of the bands upon the polyacrylamide gel before and after apyrase treatment. There was a slight decrease (0.6 – 2%) in the level of ^{32}pdNp species upon treatment with apyrase. The increase in the intensity of the bands corresponding to the inorganic phosphate species was primarily a result of the hydrolysis of residual [γ^{32}P]ATP present in the kinase reaction mixture.

3.3.3 Establishing the 'direct' 5' nearest neighbour analysis protocol.

To establish the 'direct' method for the 5'NNA of PTE adducts, archived samples (stored at -80°C), prepared and analysed as part of the earlier study (Guichard *et al.*, 2000), were re-analysed using the newly developed 'direct' method for PTE 5'NNA.

Figure 3.9 shows the results for the 5'NNA of PTEs for the *in vitro* DES-treated CT-DNA, with Figure 3.9a comparing the frequency of the nucleoside bases found 5' to PTEs with the determined normal nucleoside content of CT-DNA, and

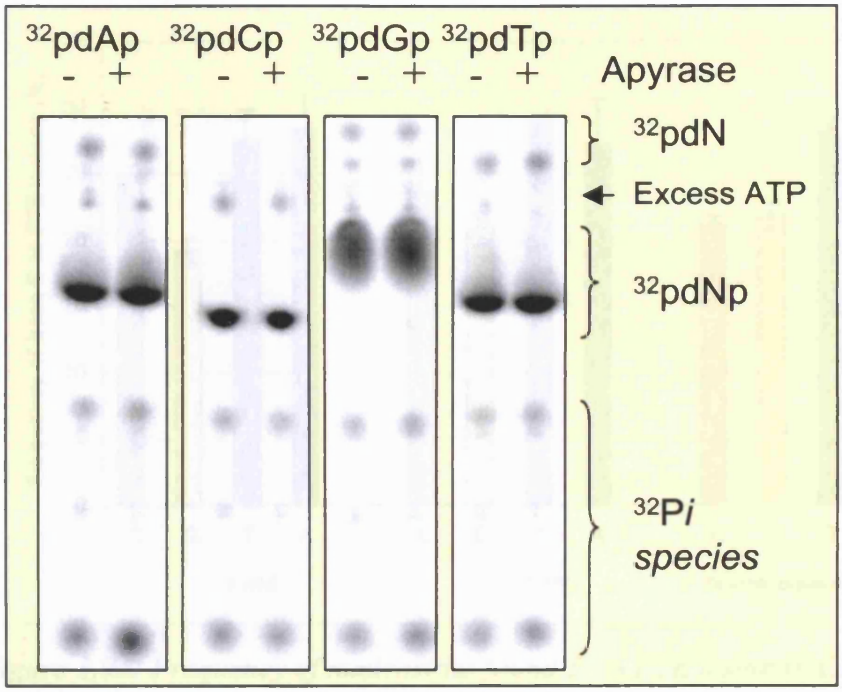


Figure 3.8: Effect of apyrase treatment upon the level of the ^{32}pdNp species.

Table 3.1: Distribution of radiolabelled products before and after apyrase treatment of the ^{32}pdNp species.

		Percentage of radioactivity in lane	
		- apyrase	**+ apyrase**
^{32}pdAp	^{32}pdN + ATP	2.71	2.32
	^{32}pdNp	84.82	83.15
	^{32}Pi	12.48	14.54
^{32}pdCp	^{32}pdN + ATP	4.35	1.42
	^{32}pdNp	87.28	86.58
	^{32}Pi	8.37	12.00
^{32}pdGp	^{32}pdN + ATP	0.83	0.37
	^{32}pdNp	88.43	87.93
	^{32}Pi	10.74	11.70
^{32}pdTp	^{32}pdN + ATP	4.19	2.16
	^{32}pdNp	87.69	86.55
	^{32}Pi	8.12	11.29

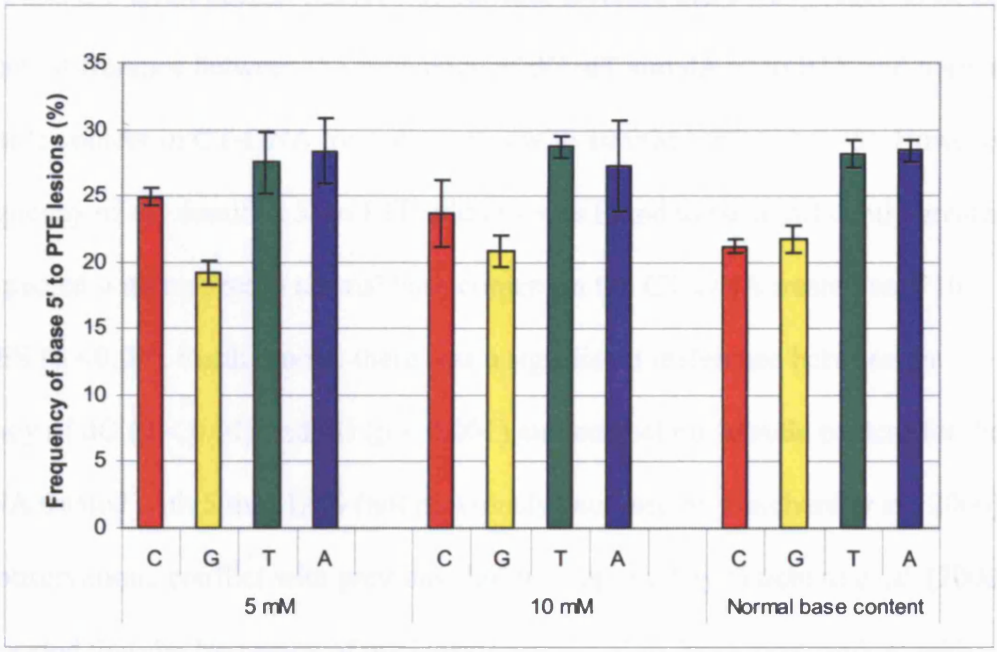


Figure 3.9a: Frequency of nucleosides found 5' to PTE lesion in CT-DNA treated in vitro with DES. Figures are the mean of 8 independent experiments. Error bars = +/- standard deviation.

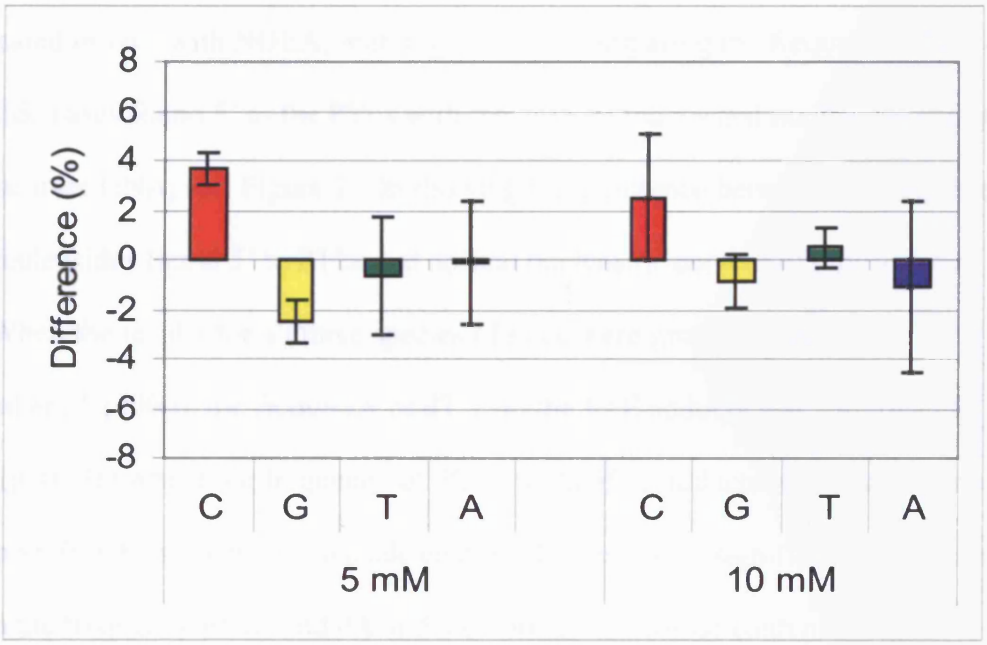


Figure 3.9b: Difference between the frequency of nucleosides found 5' to PTEs in CT-DNA treated in vitro with DES and the normal nucleosides content of CT-DNA. Figures are the mean of 8 independent experiments. Error bars = +/- standard deviation.

Figure 3.9b showing the difference between the frequency of the nucleosides found 5' to PTEs and CT-DNA normal nucleoside content. Overall, there was judged to be no significant difference between the frequency of dG, dT and dA 5' to PTE and normal nucleoside content in CT-DNA treated *in vitro* with 10 mM DES ($p > 0.05$). However, the frequency of dC manifest 5' to PTE adducts was found to be significantly greater than expected with respect to normal base content in the CT-DNA treated with 10 mM DES ($p < 0.05$). Furthermore, there was a significant difference between the frequency of dC ($p < 0.01$) and dG ($p = 0.001$) and normal nucleoside content for the CT-DNA treated with 5 mM DES (not previously analysed by Guichard *et al.*, 2000). These observations conflict with previous findings reported by Guichard *et al.* (2000) who reported that the frequency of nucleosides found 5' to PTEs was random with respect to normal nucleoside content.

Figure 3.10 shows the results for the 5'NNA of PTEs for liver DNA from mice treated *in vivo* with NDEA, with Figure 3.10a comparing the frequency of the nucleoside bases found 5' to the PTEs with the determined normal nucleoside content of mouse liver DNA, and Figure 3.10b showing the difference between the frequency of the nucleosides found 5' to PTEs and normal nucleoside content of mouse liver DNA. When the results for all three species of mice were grouped together, as in Guichard *et al.* (2000), the frequency of dT 5' to the PTE adducts was significantly greater ($p < 0.01$) whilst the frequency of dG 5' to the PTE adducts was significantly lower ($p < 0.01$) than normal nucleoside content. There was no significant difference between the frequency of dC and dA and the normal nucleoside content of mouse liver DNA. These results are similar to those reported previously by Guichard *et al.* (2000), with the conclusion that the manifestation of PTEs is non random in mouse liver DNA treated *in vivo* with NDEA.

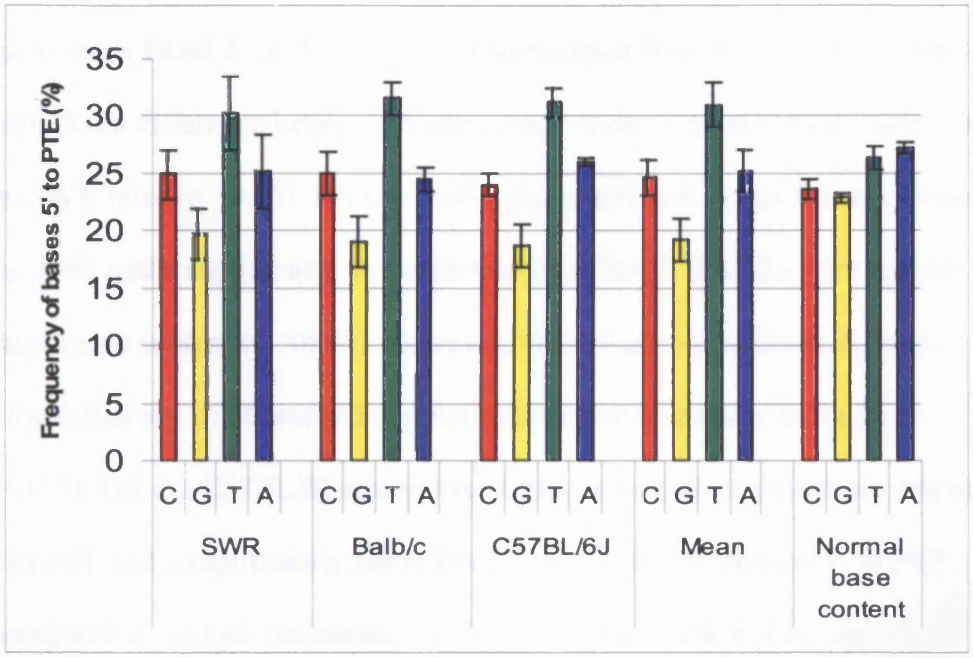


Figure 3.10a: Frequency of nucleosides found 5' to PTEs in mouse liver DNA treated in vivo with NDEA (5 hours, 90 mg/kg) extracted from 3 species of mice. Figures are the mean of 3 independent experiments. Error bars = +/- standard deviation.

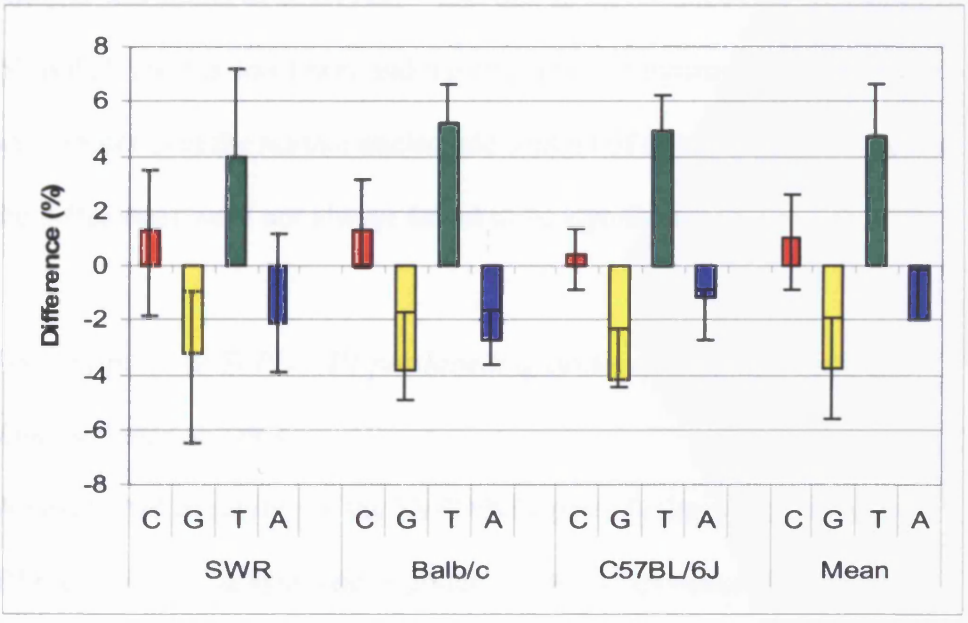


Figure 3.10b: Difference between the frequency of nucleosides found 5' to PTEs and the normal nucleoside content for mouse liver DNA treated in vivo with NDEA (5 hours, 90 mg/kg) extracted from 3 species of mice. Figures are the mean of 3 independent experiments. Error bars = +/- standard deviation.

There were subtle differences in the 5' NNA results between the three species of mice. For the SWR mice there was no significant difference between the frequency of any nucleosides found 5' to PTEs and normal nucleoside content ($p > 0.05$). There was no significant difference between the frequency of dC and the normal nucleoside content in SWR mice ($p > 0.05$). For the Balb/c mice there was a significantly higher frequency of dT and a significantly lower frequency of dA 5' to PTEs with respect to normal nucleoside content ($p < 0.05$). However, there was no significant difference between the frequency of dC and dG and normal nucleoside content in the Balb/c mice ($p > 0.05$). For the C57BL/6J mouse liver DNA there was a significantly higher frequency of dT and a significantly lower frequency of both dG and dA 5' to PTE lesions compared to normal nucleoside content ($p < 0.05$). There was no significant difference between the frequency of dC and the normal nucleoside content in C57BL/6J mice ($p > 0.05$). For all three mouse strains the frequency of nucleosides 5' to PTE adducts was found to be dT>dC ~ dA>dG. In all instances the frequency of purines 5' to PTE lesions was lower and the frequency of pyrimidines 5' to PTE lesions was greater than the normal nucleoside content of mouse liver DNA, even though the differences were not always found to be significant.

3.3.4 The ability of the SVPD/NP1 postlabelling protocol to detect multiply damaged sites on DNA.

We evaluated the ability of the SVPD/NP1 postlabelling assay to detect tandem PTE damage using synthetic oligonucleotides. Oligonucleotides were synthesised containing either one or two methyl-PTEs either immediately adjacent or separated by a single normal nucleotide (as described in section 2.1.3). Table 3.2 shows the oligonucleotides used in the study.

Table 3.2: Oligonucleotides used to evaluate the ability of the SVPD/NP1 postlabelling assay to detect tandem PTE damage

A	**dTp(Me)dT**	**2TM**
B	**dTpdTpdTp(Me)dTpdTpdTpdTpdTpdTpdT**	**11TM**
C	**dTp(Me)dTp(Me)dT**	**3TM2**
D	**dTpdTpdTp(Me)dTp(Me)dTpdTpdTpdTpdTpdT**	**11TM2**
E	**dTp(Me)dTpdTp(Me)dT**	**4TM2(s)**
F	**dTpdTpdTp(Me)dTpdTp(Me)dTpdTpdTpdTpdT**	**11TM2(s)**

The methyl-PTE containing oligonucleotides were digested with SVPD, DNase I, NP1 and SAP. The digests, either treated or untreated with alkali, were end labelled with [γ^{32}P]ATP by T4 polynucleotide kinase (T4PNK) and the products separated by PAGE (as described in Section 2.10). The labelled digests were run alongside labelled ^{32}pdTpdT (2T), ^{32}pdTpdTpdT (3T) and ^{32}pdTpdTpdTpdT (4T) oligonucleotide standards.

Figures 3.11a and 3.11b show the outcome of the analysis of oligonucleotides containing a single PTE (oligonucleotides A and B, respectively). Lane -/- shows the untreated labelled oligonucleotides (no digestion, no alkali treatment). Lane -/+ shows the labelled products of digestion (no alkali treatment). Lane +/+ shows the labelled products of digestion following alkali treatment. Digestion of the oligonucleotides containing a single methyl-PTE yielded dinucleoside phosphates that became labellable after alkali treatment (a PTE alkyl group on the phosphodiester bond at the 5' end of DNA inhibits end-labelling by T4PNK).

Figures 3.11c and 3.11d show the outcome of the analysis of oligonucleotides containing two PTEs immediately adjacent to one another (oligonucleotides C and D, respectively). It is evident that there was a mixture of dimethylated, monomethylated and presumably unmethylated material in the samples due to the presence of multiple bands (looks like one broad band) in the lane containing the untreated oligonucleotide D (Figure 3.11d, lane -/-) and F (Figure 3.11f, lane -/-). Consequently, two products were produced by the digestion of the oligonucleotides containing two adjacent methyl-PTEs: ^{32}pdNpdN (from the monomethylated oligonucleotide) and ^{32}pdNpdNpdN (from the dimethylated oligonucleotide).

Figures 3.11e and 3.11f show the outcome of the analysis of oligonucleotides containing two PTEs separated by one normal internucleotide phosphate moiety

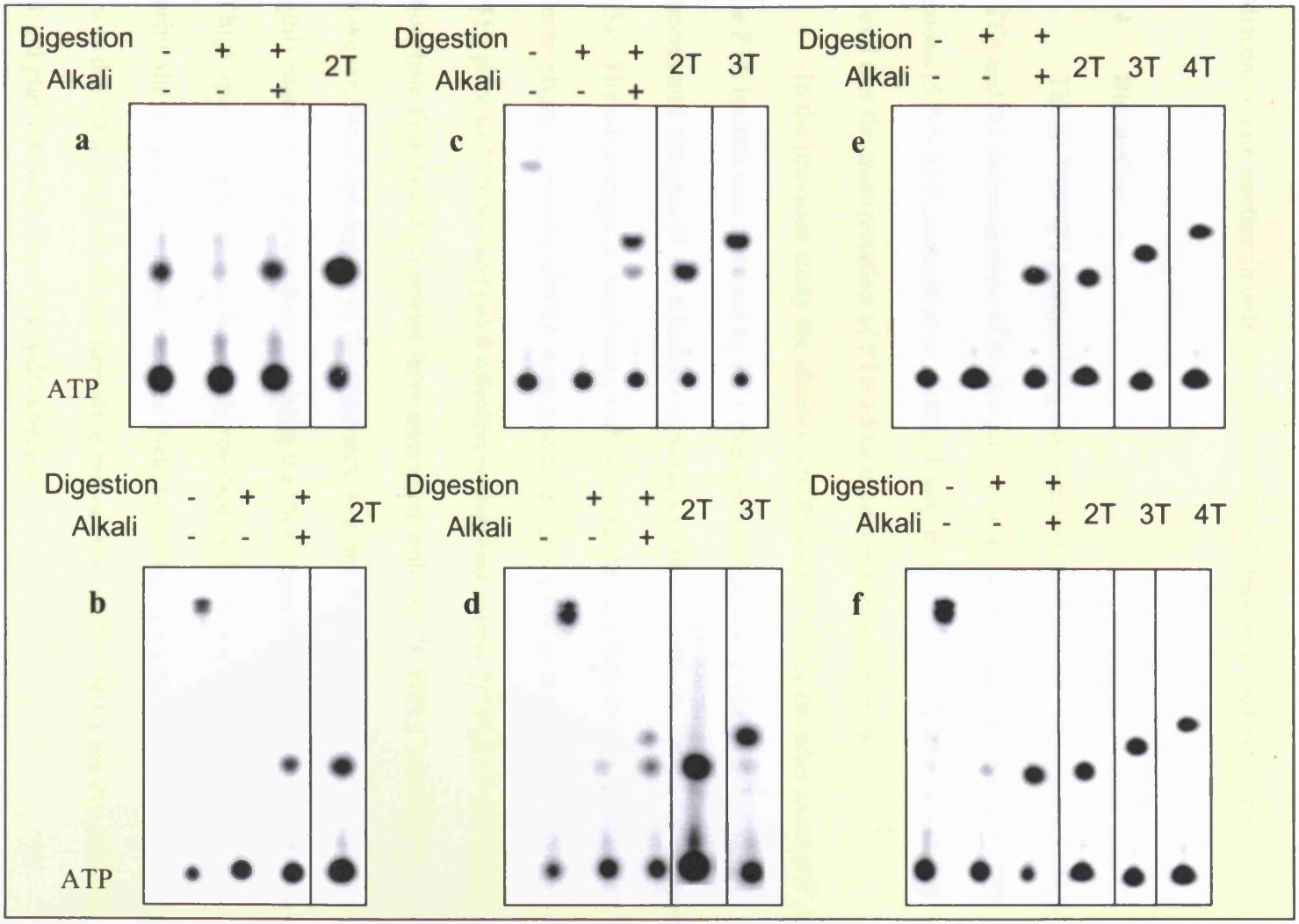


Figure 3.11: Autoradiographs showing the products generated by the SVPD/NP1 PTE postlabelling assay for oligonucleotides A-F.

(oligonucleotides E and F respectively). Digestion of the oligonucleotides synthesised containing two PTEs separated by a normal nucleotide yielded only the 2T species (^{32}pdNpdN). Therefore, it is mandatory to have the two PTE lesions immediately adjacent to one another in order to generate the 3T species (^{32}pdNpdNpdN).

3.4 Discussion

The SVPD/NP1 postlabelling assay permits the specific detection of alkyl-PTEs and the determination of the frequency of the nucleosides located 5' to PTE lesions (5'NNA) (Guichard *et al.*, 2000). Using this assay Guichard *et al.* (2000) found that the manifestation of PTE adducts was non-random *in vivo*.

In the previous study the identity and frequency of nucleosides located 5' to the PTE lesions was obtained by NP1 digestion of the PAGE isolated ^{32}pdNpdN species and analysis of the released mononucleotides (^{32}pdN) by ^{32}P-HPLC (Figure 3.5a). The advantages of separating and isolating the radiolabelled species by gel electrophoresis prior to 5'NNA were two fold: (i) it allowed the quantification of PTEs prior to 5' NNA and (ii) it effectively removed excess [γ^{32}P]ATP and inorganic phosphate that would otherwise have interfered with the ^{32}P-HPLC analysis. However, the disadvantage of this approach was that it was a very time consuming, highly manipulative procedure involving the direct handling of radiolabelled material of high specific activity and frequently resulted in the loss of material particularly during the desalting of the gel isolated material prior to NP1 digestion. Consequently it was desirable to develop an alternative protocol for PTE 5'NNA ideally avoiding the gel purification of the ^{32}pdNpdN species.

In the present study an alternative approach to PTE 5'NNA, namely the 'direct' method (Figure 3.5b) has been developed. Using prepared ^{32}pdNpdN species

we demonstrated that the sequential use of apyrase, boiling and NP1 digestion led to the >80% release of the ^{32}pdN species. The boiling step was introduced to destroy the 5' dephosphorylating activity present in the apyrase preparation used. This activity was found to only affect 5' mononucleotides and not the ^{32}pdNpdN or ^{32}pdNp species. The advantage of the 'direct' method is that it is a simpler, and less time consuming method of preparing samples for 5'NNA. It avoids the direct handling of radioactive material and allows for all of the reactions to be carried out within a single tube so lessening the likelihood of material loss. The direct protocol also allows for the inclusion of any dNpR species into the 5'NNA assessment (see Fig. 3.5). These species generated by the alkali treatment of dNp(R)dN, are radiolabelled by incubation with [γ^{32}P]ATP/ T4PNK, but are separated from the ^{32}pdNpdN species by PAGE and so were not included in the original 5'NNA protocol (Guichard *et al.*, 2000). The inclusion of the dNpR species via the direct method allows for a more complete determination of PTE 5'NNA.

With the 'direct' 5'NNA method developed, we sought to establish the protocol through the analysis of samples previously studied. Analysis of liver DNA from mice treated *in vivo* with NDEA revealed a higher frequency of dT and a lower frequency of dG manifest 5' to PTEs with respect to normal nucleotide content of mouse liver DNA. These observations were identical to those reported by Guichard *et al.* (2000). However the analysis of CT-DNA treated *in vitro* with DES revealed significant differences between the frequency of nucleosides 5' to PTEs and the normal nucleoside content. There was a significantly higher frequency of dC in CT-DNA treated with 10 and 5 mM DES and a significantly lower frequency of dG manifest 5' to PTEs in the CT-DNA treated with 5 mM DES. This is contrary to the previous findings that the *in vitro* treatment of CT-DNA with DES led to the random

manifestation of PTE adducts with respect to the 5' nearest neighbour (Guichard *et al.*, 2000). There are a number of factors that could account for differences in the results obtained for the two respective 5'NNA methods. Firstly, there is the inclusion of the dNpR species in the new 'direct' 5'NNA assessment, however dNpR species only account for 0 – 10% of the products generated by alkali hydrolysis of the dNp(R)dN species (Saris *et al.*, 1993 and 1995). Secondly there were differences in the normal nucleotide content between the two respective studies, with the values obtained by the present study being much closer to literature values. However whilst this may account for the present observation of there being a significantly higher frequency of dC 5' to PTEs , it does not account for the significantly lower frequency of dG manifest 5' to PTEs. Perhaps most significant is the fact that in the present study the data generated is the result of eight independent determinations allowing for rigorous statistical analysis. In the previous study the data is presented as two single determinations, this not being suitable for any form of statistical analysis (Guichard *et al.*, 2000).

A limitation of using ^{32}P-HPLC for 5'NNA is that certain species may be preferentially labelled by T4 PNK, which in turn could directly influence the outcome of the 5'NNA. Van Houten *et al.* (1998) reported that the labelling efficiency of oligonucleotides by T4 PNK depends upon the 5' nucleotide. Labelling efficiency of a set of oligonucleotides that differed only in their 5' nucleotide ranged between 5 and 65%. Oligonucleotides with a 5' purine nucleotide labelled the most efficiently. The oligonucleotide with a 5' dG labelled the most efficiently (60 ± 5%), whilst the oligonucleotide with dC as the 5' base labelled the least efficiently (10 ± 5%). Oligonucleotides with either dA or dT as the 5' base labelled more or less with the same efficiency (45 ± 5%). An excess of ATP did not improve labelling efficiencies

significantly (personal communication). In contrast, Guichard *et al.* (2000) reported a similar labelling efficiency of each dinucleoside phosphate species of 50 ± 15%. As the results of the present study and of Guichard *et al.* (2000) show, the frequency of dG is consistently lower and dT consistently greater 5' to PTEs than expected with respect to normal nucleoside content. Furthermore, the figures for the normal nucleoside content used in the present study were determined by a postlabelling protocol (Section 2.13). Therefore it is concluded that the non-random manifestation of PTEs as determined by 5'NNA, is not the consequence of the preferential labelling of certain dNpdN species in the kinase reaction.

The term MDS describes a wide range of complex lesions where several moieties in a local region of the DNA (~10 bp) are damaged. One type of MDS that has been the focus of extensive study is DNA double strand breaks (DSB). However, many other types of MDS are possible including those consisting of pairs of lesions near or immediately adjacent (tandem) to each other on the same strand of DNA. Studies using oligonucleotides synthesised with two methyl-PTEs either immediately adjacent to one another or separated by a single normal phosphodiester linkage revealed that it was necessary to have the two PTEs immediately adjacent to one another in order for the postlabelling digestion to generate a SVPD/NP1 resistant trimer species (^{32}pdNpdNpdN). Digestion of the oligonucleotides synthesised containing two PTEs separated by a normal nucleotide yielded only the dimer species (^{32}pdNpdN). Therefore it is concluded that the SVPD/NP1 postlabelling assay is limited to being able to detect tandem damage. It has also been found that it is mandatory to have two abasic or two oxidative (thymine glycol) lesions immediately adjacent to one another in order to generate the trimer species (Bowman *et al.*, 2001). In order to generate the higher molecular weight species by postlabelling digestion it

is necessary to have a run of two (or more) modified nucleotides immediately adjacent to one another. The SVPD/NP1 postlabelling assay treats lesions separated by as few as one normal nucleotide moiety as independent lesions.

Chapter 4:

The persistence of the non-random manifestation of PTEs *in vivo*: Is repair contributing towards the non-random manifestation of PTEs *in vivo*?

4.1 Introduction

Previously it has been established that the manifestation of PTEs was non-random in mouse liver DNA treated *in vivo* with NDEA (Guichard *et al.*, 2000). The observed non-random manifestation of PTEs *in vivo* was taken as being indicative of either: (i) non-random formation *in vivo*, perhaps due to the influence of DNA-protein interactions (investigated in Chapter 5), or (ii) non-random repair of PTE lesions.

In the present study, the frequency of nucleotides found 5' to PTEs in liver DNA taken from mice 5 hours – 56 days following a single dose of NDEA was determined using the new 'direct' 5'NNA protocol. NDEA is a proximate carcinogen. The genotoxic properties of NDEA are mediated via intermediates formed during the detoxification and excretion of *N*-nitrosodialkylamines (Figure 4.1). The initial step in the metabolism of *N*-nitrosodialkylamines requires hydroxylation by cytochrome P450 enzymes CYP2E1 and/ or CYP2A6 to generate a α-hydroxynitrosamine. α-hydroxynitrosamine breaks down spontaneously to give an alkyl diazohydroxide. The alkyl diazohydroxide may protonate to lose water generating a diazonium ion. Both the alkyl diazohydroxide and the diazonium ion may react with cellular DNA to form adducts (Magee *et al.*, 1975).

4.2 The higher ordered structure of DNA *in vivo*

DNA does not exist as a free entity *in vivo*, but within a dynamic complex with numerous proteins. DNA is tightly packed within intact cells. A compaction of about 50,000 fold is required for the DNA to fit into the nucleus (Bohr *et al.*, 1987). The first level of compaction is the formation of the nucleosomes (Figure 4.2). The

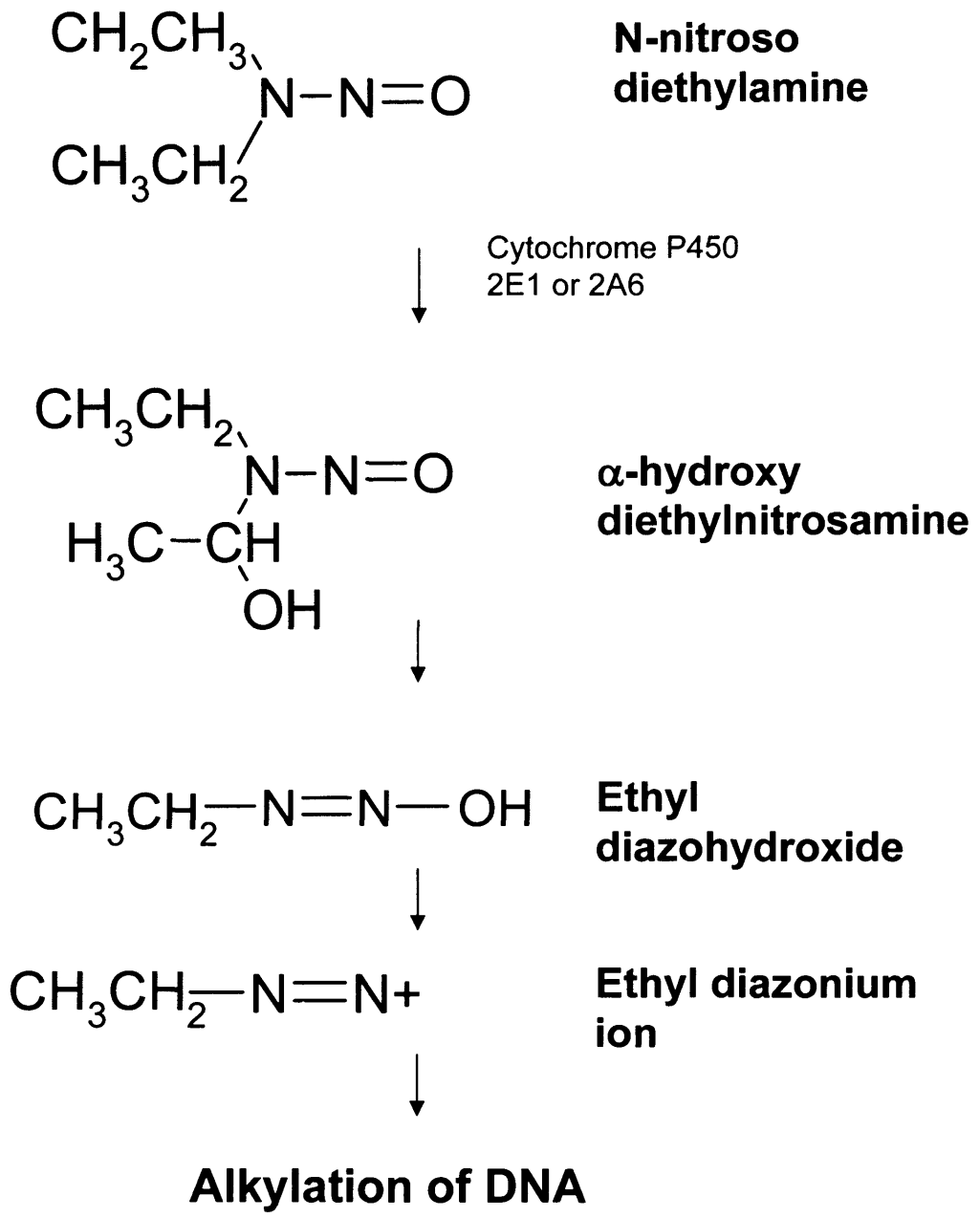


Figure 4.1: Metabolic activation of the proximate carcinogen N-nitroso diethylamine (NDEA)

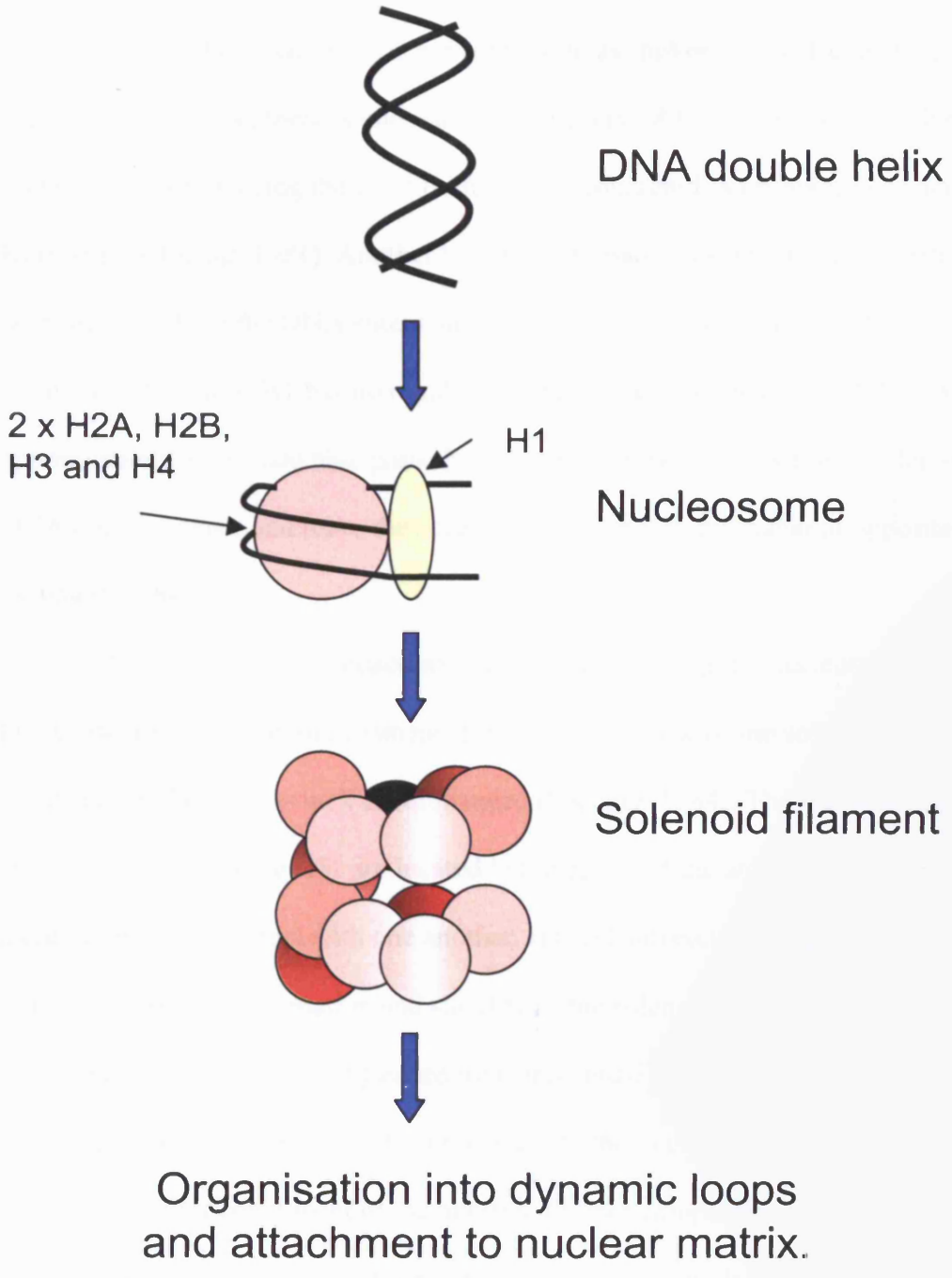


Figure 4.2: Structures involved in the packaging of the DNA into the nucleus

nucleosome core is a heart shaped particle consisting of eight histones (two molecules each of H2a, H2b, H3 and H4) around which ~146 bp of DNA is wrapped. The regions of DNA between histones are referred to as 'linker' DNA. Depending on species and cell type, there is one nucleosome every 180 – 250 bp, with a value of 200 bp per nucleosome being the most frequently encountered (Kornberg, 1977 and 1974; Kornberg and Klug, 1981). Another histone, H1, associates with the core particle at or near the site where the DNA enters and leaves the nucleosome (Figure 4.2). Chromatin devoid of H1 has no regular structure. Nucleosomes are still distributed approximately every 200 base pairs, however their appearance is less regular and DNA does not enter and leave the core at the same point, but rather at opposite sides (Saenger, 1984).

The next level of compaction is achieved by coiling the nucleosome packed DNA into a 30 nm solenoid filament (Fig. 4.2). The sense of the solenoid helix is not fixed and can be right as well as left handed (Saenger, 1984). The nucleosomes are arranged so that histones H1 are located in the centre of the solenoidal helix and therefore in close contact with one another. H1- H1 interactions appear to play an important role in the formation and stability of the solenoid filament (Saenger, 1984). These filaments are further organised into large and dynamic loops each containing ~100 kbp. The loops are closed by attachment to the nuclear matrix, the proposed internal structural component of the nucleus. Further compaction occurs during cell division and in the formation of dense heterochromatin involving additional proteins, many of which are still unknown (Tyler, 2002).

4.3 Influence of nuclear structure on DNA repair

Preferential repair of adducts at the nucleotide level is thought to be comprised of two factors: (i) DNA-protein interactions/ nuclear structure and (ii) DNA sequence. Nuclei structure alters the accessibility of DNA for repair. Harris *et al.* (1974) examined repair synthesis in the euchromatin and the heterochromatin of human fibroblasts treated with *N*-acetoxy-2-acetylaminofluorene (AAAF), 7 bromomethylbenz(a)anthracene, MNU and UV radiation. They found twice as much repair synthesis in euchromatic regions as in the heterochromatic regions of the nucleus.

Nucleotide excision repair (NER) is a general repair system for removing virtually all types lesions from DNA and is the major repair mechanism for eliminating bulky base adducts such as those produced by UV radiation and aralkylating agents, e.g. benzo(a)pyrene diol epoxide (BPDE) and AAAF. The nucleosome severely inhibits damage recognition and excision of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts by excision repair (Schieferstein and Thoma, 1998; Hara *et al.*, 2000).

Subtle alterations in DNA molecules, such as oxidised or alkylated bases, are mainly repaired by the base excision repair (BER) pathway. Ye *et al.* (1998) reported that DNA-protein interactions interfere with N7-methyldG repair in the *PGK1* gene of human fibroblasts. In regions known to have bound transcription factors the half life of N7-methyl dG adducts was found to be greater in whole cells than in naked DNA *in vitro* (in the presence of N-methyl purine DNA glycosylase). Elsewhere on the gene there was a good correlation between N7-methyldG half lives in whole cells and naked DNA.

4.4 DNA sequence influences the rate of repair

There is evidence that the rate of direct repair of O^6-methyldG is influenced by local sequence. The rate of repair of O^6-methyldG varied depending upon the sequence flanking the methyl guanine *in vitro* (Dolan *et al.*, 1988; Georgiadis *et al.*, 1991). Dolan *et al.* (1988) found that the rate of repair by either HT29 colon carcinoma cell extract or purified *E. coli* O^6-methyl guanine transferase for the methylated dodecamer 5'- TATACG<u>C</u>G^{me}TATA – 3' was 1.5 to 2.1-fold greater than for 5'- TATACC<u>G</u>G^{me}TATA - 3'. Georgiadis *et al.* (1991) found that the rate of repair by *E.coli* cell extract of O^6-methyldG with a dT 5' to the modified base, was 25-fold greater than the rate of repair of O^6-methyldG preceded 5' by a dG. Substitution of the second guanine of the TG^{me}<u>G</u>AG sequence to give TG^{me}<u>A</u>AG reduced the rate of repair by 2.6 fold. While bases sequence beyond the adjacent bases or on the complementary strand may also exert an influence upon repair, the major influence is still expected to come from the adjacent bases. The rate of repair may also reflect the accessibility of the O^6-methyldG, with the most rapidly repaired adducts also being those most readily bound by the antibody used for the detection of O^6-methyldG (Georgiadis *et al.*, 1991).

In contrast, the rate of repair of O^6-ethyldG by recombinant human O^6-methyldG transferase was not significantly influenced by the nature of bases flanking the O^6-ethyldG (Bender *et al.*, 1996). The rate of repair was found to be slightly lower for O^6-ethyldG paired with dT rather than dC, however this was not statistically significant (Bender *et al.*, 1996).

Bulky DNA adducts are repaired by the NER pathway. The rate of repair of a number of bulky adducts including, CPDs, BPDE and 1-nitrosopyrene induced adducts, has been shown to vary greatly within individual genes *in vivo* (Tornaletti

and Pfeifer, 1994; Wei *et al.*, 1995 and 1996). However, no consensus sequence was found for either the rapidly or the slowly repaired sites. It is likely that the steric hindrance caused by DNA-protein interactions may play a more significant role in modifying the activity of excision repair enzymes than DNA sequence.

4.5 PTE repair in higher eukaryotes

The repair of PTEs has previously been introduced in Section 1.8. Briefly, PTEs are considered to be resistant to repair in higher eukaryotes. Studies using rodents or human fibroblast cell lines exposed to alkylating agents have shown that methyl and ethyl phosphotriester lesions have a half life that exceeds that of any other known alkylation product *in vivo* (Shooter, 1978). Repair mechanisms have been found in lower organisms namely *E. coli* (McCarthy *et al.*, 1983) and *Aspergillus nidulans* (Baker *et al.*, 1992). The Ada protein found in *E. coli* can only repair PTEs in the S*p* configuration (approximately 50% of total PTEs) (Weinfeld *et al.*, 1985). A mechanism for the repair of alkyl PTEs has yet to be demonstrated in higher eukaryotic species.

4.6 Specific aims of study

- To measure the levels of PTEs in mouse liver DNA treated *in vivo* with NDEA and assess the value of PTEs as long term markers of cumulative exposure.
- To determine whether there is any evidence for the non-random repair of PTEs over time.

4.7 Results

The DNA samples were prepared as part of a previous study (Oreffo *et al.*, 2000). Briefly, Balb/c mice were treated with a single intraperitoneal dose of NDEA at 90 mg/kg body weight. The animals were sacrificed at 5 hours, 10 hours, 24 hours, 4 days, 7 days, 28 days and 56 days after dosing. The liver tissue was removed and immediately frozen for subsequent DNA isolation. The DNA was isolated and stored at -80°C until subsequent DNA adduct analysis.

4.7.1 Quantification of PTEs in mouse liver DNA from Balb/c mice treated in vivo with NDEA

DNA was digested and analysed according to the PTE SVPD/NP1 postlabelling protocol outlined in the Materials and Methods 2.10. The postlabelling products were separated upon a 7M urea/ 20% polyacrylamide denaturing gel. Figure 4.3 shows an autoradiograph of the postlabelling gel. The gels were analysed by autoradiography and storage phosphorimage analysis. Table 4.1 shows the results from the quantification of PTEs in the mouse liver DNA taken from mice 5 hours to 56 days following a single dose of NDEA. The number of PTEs continued to increase up to 10 hours after treatment. There was a then substantial decline in the quantity of PTEs in the mouse liver DNA between 10 hours and 4 days (Fig. 4.4). Thereafter, there was a steady decline in the quantity of PTEs over time (despite a small increase in PTEs between 4 and 7 days). The level of PTEs detected at 56 days was approximately 15% of that measured 10 hours after dosing (Figure 4.4).

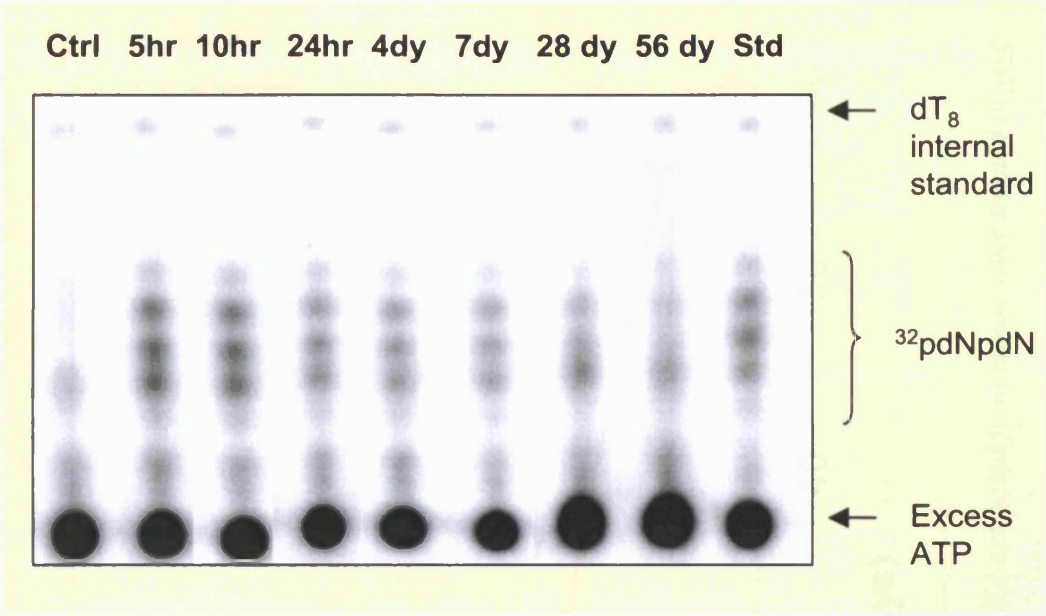


Figure 4.3: Autoradiograph of gel used for the separation of postlabelling products from mouse liver DNA extracted from Balb/c mice sacrificed 5 hours, 10 hours, 24 hours, 4 days, 7 days, 28 days and 56 days after a single intra peritoneal dose of NDEA (90 mg/ kg). Control DNA (Ctrl) taken from untreated Balb/c mice.External standard (Std) is an equimolar mixture of the 16 dinucleoside phosphates (50 fmol).

Table 4.1: Decrease in the level of PTE lesions over time in DNA extracted from mouse liver treated in vivo with NDEA (90 mg/kg).

Time	PTE (1/ 10^6 nucleotides)		Mean
0	0.00	0.00	0.00
5 hours	35.12	30.24	32.68
10 hours	36.79	36.41	36.60
24 hours	20.61	22.85	21.73
4 days	13.44	14.14	13.79
7 days	12.57	16.77	14.67
28 days	13.94	10.25	12.10
56 days	4.67	6.18	5.43

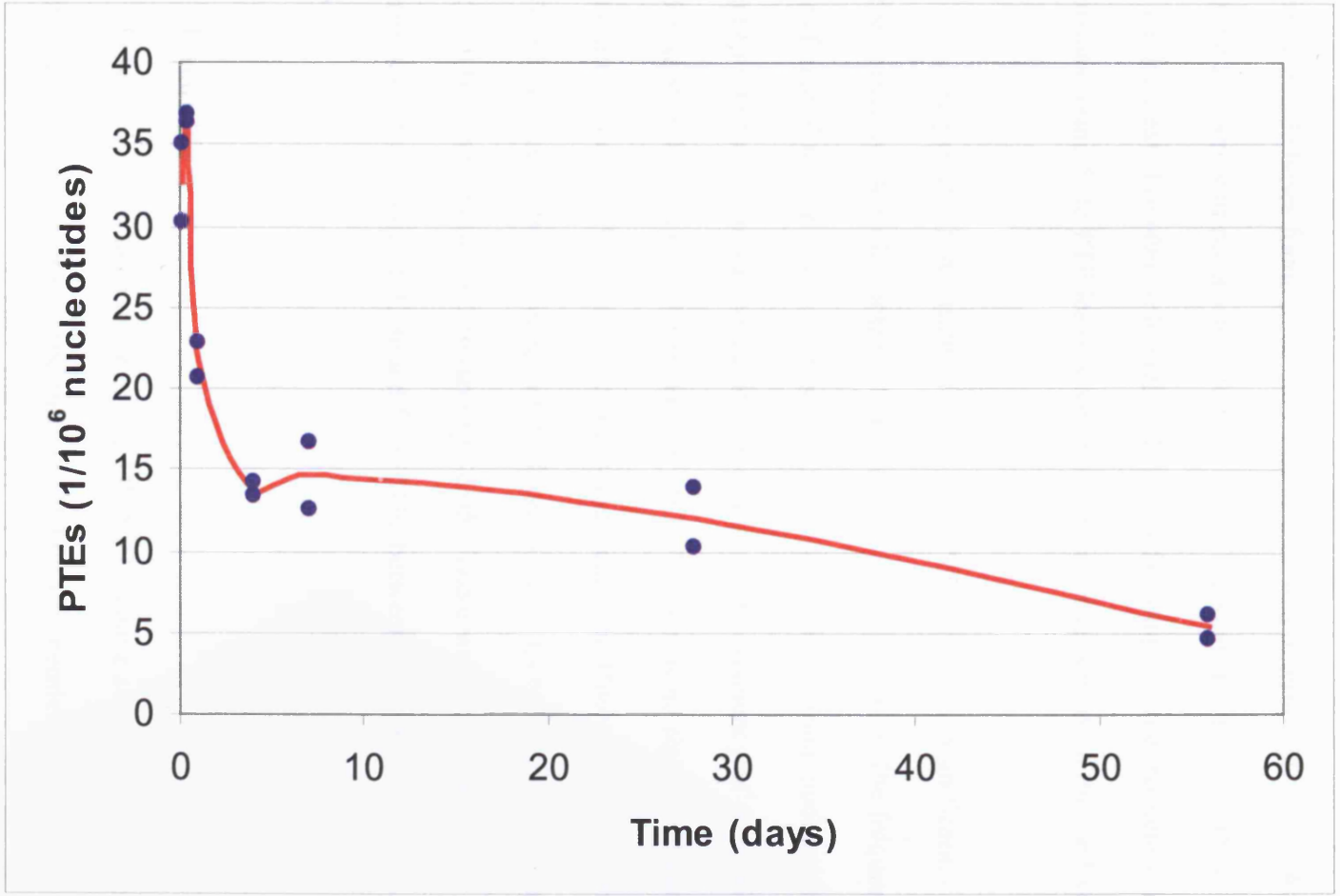


Figure 4.4: Decrease in the level of PTE lesions over time in DNA extracted from mouse liver treated in vivo with NDEA (90 mg/kg).

4.7.2 Measurement of the frequency of nucleotides found 5' to phosphotriesters over a timescale of 56 days

Using the new 'direct' 5' NNA protocol developed in Chapter 3, the frequency of the nucleoside bases found 5' to the PTEs lesions was determined. Figure 4.5a shows the frequency of nucleosides found 5' to PTE lesions in the liver DNA of the Balb/ c mice treated *in vivo* with NDEA. Figure 4.5b compares the frequency of nucleosides found 5' to PTE lesions with the normal base content of mouse liver DNA.

The frequency of pyrimidines found 5' to PTE lesion was significantly greater than the normal nucleoside content of mouse liver DNA ($p < 0.01$). The frequency of purines found 5' to PTE lesions was significantly lower than normal nucleoside content ($p < 0.05$). There was no significant change in the frequency of dC and dA found 5' to PTE lesions over the 56 days ($p > 0.2$). There was no significant change in the frequency of dG in the first 7 days following treatment. However, there was a significant increase in the frequency of dG found 5' to PTEs between 5 hours and 28 days ($p < 0.05$) and 5 hours and 56 days ($p < 0.05$). There was also a significant decrease in the frequency of dT found 5' to PTEs between 5 hours and 56 days ($p < 0.05$).

4.8 Discussion

Previously it has been reported that DNA PTEs have a half life that is greater than that of other lesions *in vivo* and that alkyl-PTEs are chemically stable and resistant to repair (Shooter and Slade, 1977; Shooter, 1978). However, in this study the initial decline in the level of PTEs over the first four days following treatment was rapid. Approximately 50% of the PTEs measured at 10 hours had been 'lost' by 48

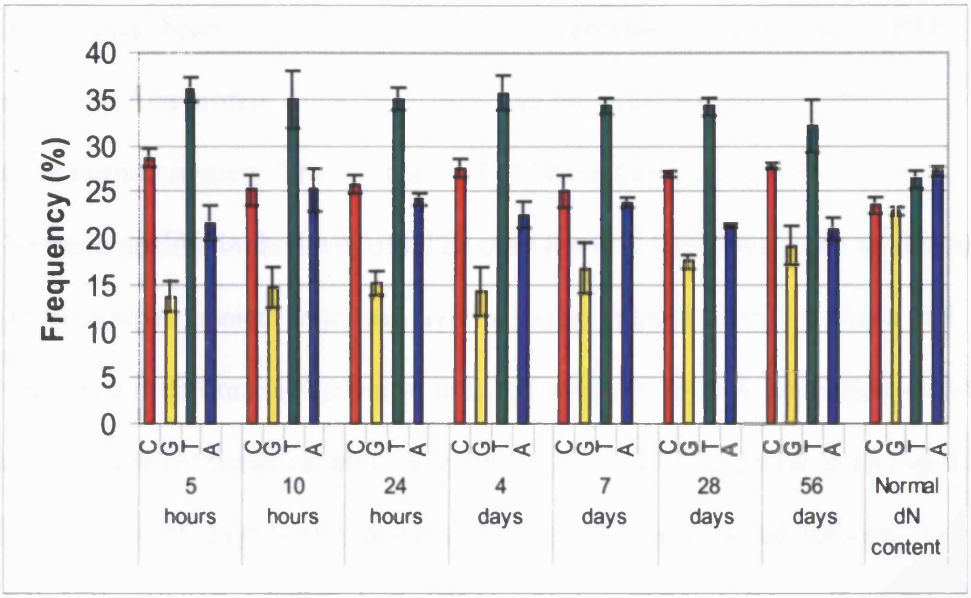


Figure 4.5a: Frequency of nucleosides found 5' to PTEs in Balb/c mouse liver DNA 5 hours to 56 days following a single dose of NDEA (90 mg/kg). Figures are the mean of 3 independent experiments. Error bars = +/- standard deviation.

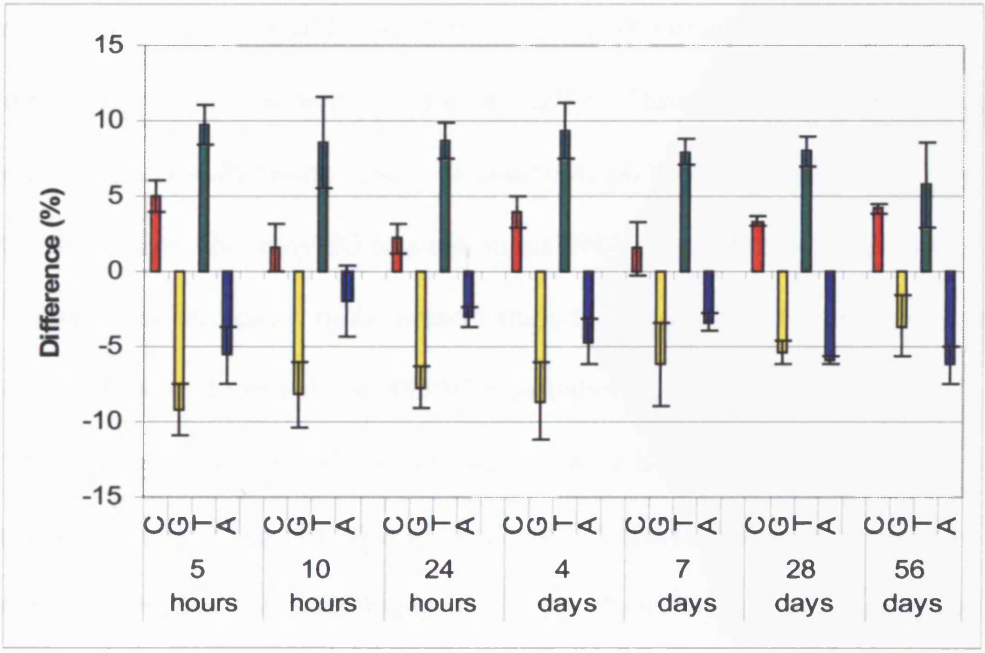


Figure 4.5b: Difference between 5' nearest neighbour results and normal base content for mouse liver in Balb/c mouse liver DNA 5 hours to 56 days following a single dose of NDEA (90 mg/kg). Figures are the mean of 3 independent experiments. Error bars = +/- standard deviation.

hours following treatment. Thereafter, the rate of decline slowed considerably with a detectable level of PTEs still present 56 days following treatment. To date no repair system for PTEs has been characterised in higher organisms. The best studied PTE repair system, the Ada protein found in *E. coli,* can only remove PTEs in the S*p* configuration (approximately 50% of total PTEs) (Weinfeld *et al.*, 1985).

The rapid decline in the level of PTEs over the first four days could be due to either: (a) active repair present in mouse liver or (b) an increase in cell division and turnover due to an inflammatory response induced by the treatment. Liver is known to be a highly proliferative tissue capable of regenerating itself in response to an insult. The level of PTEs at 56 days is only marginally above the background activity, suggesting that the dilution of PTEs in the DNA may be at least in part responsible for the apparent decline in PTE lesions. However, this explanation cannot completely account for the initial rapid decline in the level of PTEs. NDEA forms several other types adducts upon reaction with DNA. Oreffo *et al.* (2000) treated 21 Balb/c mice with a single dose of *N*-nitrosodiethylamine (90 mg/kg). Three animals were killed at each time point (5hours, 10 hours, 1, 4, 7, 28 and 56 days) following treatment. The level of O^6-ethyldG and N7-ethyldG was measured DNA extracted from liver tissue using an immunoslot blot assay. In the present study the level of phosphate alkylation has also been determined using the SVPD/NP1 postlabelling assay in the same samples. Both N7-ethyl dG and O^6-ethyldG are known to be actively repaired in higher eukaryotes. Like PTEs, the level of O^6-ethyldG adducts peaked at 10 hours in the Balb/c mice treated *in vivo* with 90 mg/ kg NDEA. There was then a rapid decline in the level of O^6-ethyldG between 10 and 24 hours (approximately 50% of the adduct load at 10 hours had been removed by 24 hours). Only 5% of the adduct load at 10 hours remained at 4 days. The level of O^6-ethyldG fell below the limit of detection at

7 days. O^6-alkyldG is strongly promutagenic and cytotoxic lesion therefore needs to be repaired rapidly before the next round of DNA replication.

The level of N7-ethyldG in the Balb/c mice treated *in vivo* with 90 mg/kg NDEA peaked at 5 hours and decreased steadily thereafter. The level of adducts fell below the limit of detection at 28 days. The half life of N7-ethyldG was found to be approximately 7 days, therefore making the half life of N7-ethyldG longer than that observed for PTEs (~ 2 days) in the mouse liver DNA. The half life of PTEs in the present study is considerably shorter than has been previously reported (Shooter and Slade, 1977; Den Englese *et al.*, 1986 and 1987; Bodell *et al.*, 1979).

Two assays have previously been used to detect PTEs in biological samples: (i) alkali hydrolysis assay (Shooter and Slade, 1977) and (ii) detection of DNA adducts following treatment with radiolabelled agent (Bodell *et al.*, 1979; Den Englese *et al.*, 1986 and 1987).

Bodell *et al.* (1979) measured the half lives in two cultured cell lines: XP-12 (*Xeroderma pigmentosum* fibroblast cell line) and GM-637 (normal, transformed fibroblast cell line). Prior to treatment the cells were prelabelled with [^{3}H]thymidine in order to enable the level of alkylation damage to be corrected for cell turnover following treatment. Cells were treated with [^{14}C] *N*-ethyl-*N*-nitrosourea for 1 hour. Following treatment the cell media was changed. GM-637 cells were harvested at 20, 48 and 72 hours following treatment. XP-12 cells were only harvested at 48 hours following treament. DNA was digested by nucleases (NP1 and potato acid phosphatase)and the products identified by HPLC. The level of PTE damage was estimated from the amount of dTp(Et)dT detected. The half lives of N7-ethyldG and ethyl-PTEs were calculated to be ~50 hours and <8 days respectively. There was no

appreciable difference between the ability of the two cell lines to remove N7-ethyldG and PTE damage.

Den Englese *et al.* (1986) studied the ability of male Wistar rats to remove ethylation and methylation damage. Eighteen rats were treated with a single dose of [^{3}H] *N*-ethyl-*N*-nitrosourea (140 mg/ kg). At each time point (2 hours, 1, 3, 6, 28 and 56 day following treatment) three rats were killed. Fifteen rats were treated with [^{3}H] *N*-nitrosodimethylamine (10 mg/kg). Three rats were killed at 2 hours, 6 hours, 1, 6 and 28 days following treatment. DNA extracted from the livers of the treated rats was digested and the products determined by HPLC as desribed by Bodell *et al.* (1979). For the methylation damage some adducts were initially removed very quickly. The initial half lives (first 24 hours following treatment) of O^6-methyldG and N7-methyldG was estimated to be 21 hours, 29 hours. After 24 hours the half lives of O^6-methyldG and N7-methyldG increased slightly to 25 hours and 58 hours, respectively (24 hours to 28 days). The half life of methyl-PTEs was calculated to be ~7 days. Ethylation products were removed more slowly from the DNA than their corresponding methylation products. The half lives of O^6-ethyldG, N7-ethyldG and ethyl-PTEs were calculated to be 11 hours, 6.1 days and 32 days respectively.

The SVPD/NP1 postlabelling assay used in the present study has a number of advantages over the more traditional methods in terms of specificity and sensitivity. The alkaline hydrolysis assay measures the extent of strand breaks induced by alkali hydrolysis. PTEs are readily hydrolysed under alkaline conditions, however they are not the only alkali labile lesion. Abasic sites may also be hydrolysed to form strand breaks, albeit at a slower rate than PTEs. However, this may lead to an over estimation of the extent of phosphate alkylation. The assay used to assess the extent of PTE damage by Bodell *et al.* (1979) and Den Englese *et al.* (1986) involved the

extrapolation of total PTE damage based upon the level of dTp(R)dT found in the DNA digest. In the present study it has been shown that the extent of phosphate alkylation varies according to the 5' nearest neighbour. The extent of PTE damage is greater when the 5' nearest neighbour is a pyrimidine than a purine. Consequently this method may also lead to an over estimation of the extent of phosphate alkylation.

The key disadvantage of the present study is that the extent of cell turnover following treatment was not determined. However, whilst cell turnover may account for some of the decline in the level of PTEs, it cannot completely account for the initial rapid decline in PTEs particularly when compared to the relatively slow decline in the level of N7-ethyldG in the same samples. Therefore the initial rapid decline in PTEs could, at least in part, be due to active PTE repair.

Overall, the frequency of pyrimidines 5' to PTEs remained significantly greater than the normal nucleoside content of mouse liver DNA throughout the timescale of the study. The frequency of purines 5' to PTEs remained significantly lower than the normal nucleoside content at all the time points throughout the timescale of the study. A significant difference was found between the frequency of dG found 5' to PTE at 28 and 56 days compared to the frequency of dG found 5' to PTEs at 5 hours following treatment ($p < 0.05$). A significant difference was also found between the frequency of dT found 5' to PTEs at 5 hour and at 56 days after treatment ($p < 0.05$). However it must be noted that there was no significant change in the frequency of nucleosides found 5' to PTEs between 5 hours and 7 days following treatment when the decline in the quantity of PTE lesions was at its greatest. It is known that when the PTE direct repair protein is not limiting, PTE repair (S*p*-stereoisomer only) is complete within a couple of hours (McCarthy *et al.*, 1983). Therefore it seems unlikely that it would take 28 days in order to register a significant

change in the frequency of nucleosides found 5' to PTEs if direct repair was involved. Furthermore, if repair was involved in the initial non-random manifestation of PTEs, it would be expected that the manifestation of PTEs would become more non-random over time. However, on the contrary, there was in fact a slight decrease in the differences between the frequency of nucleosides found 5' to PTEs and normal nucleoside content over the time course of this study. The decline in the difference between the manifestation of nucleotides 5' to PTEs and normal nucleotide content, 28 and 56 days following treatment may due to either: (i) experimental error as a result of the low level of PTEs remaining in the DNA at these time points and/ or (ii) PTEs may be more stable within some sequences than others. The frequency of nucleosides 5' to PTEs remained non-random with respect to normal nucleoside content even at 56 days following treatment. Taking all the evidence into account it seems unlikely that repair is playing a significant role in the non-random manifestation of PTEs. However, the fact that the half life determined for N7-ethyldG is greater than the half life of the ethyl-PTEs, raises the possibility of that active repair is contributing towards the decline of PTEs. It must be stressed that the above statement in based on the observations of only two independent experiments.

Chapter 5:

The manifestation of PTEs in intact cells, isolated nuclei and DNA

5.1 Introduction

Previously Guichard *et al.* (2000) reported that the frequency of nucleosides 5' to the site of PTE lesions was non-random *in vivo,* but random *in vitro.* It was found that the frequency of dT 5' to the site of PTE lesions in DNA extracted from the livers of mice treated *in vivo* with NDEA was significantly greater than would have been expected with respect to the normal nucleoside content. The frequency of dG 5' to the site of PTE lesions was found to be significantly lower than expected with respect to normal nucleoside content. In contrast, Guichard *et al.* (2000) reported that in studies using CT-DNA treated *in vitro* with either DES or DMS the frequency of nucleosides 5' to PTEs was random with respect to normal nucleoside content. These findings were suggestive of the non-random manifestation of PTEs *in vivo* being the result of some intrinsic *in vivo* factor. Since it seems unlikely that repair plays a significant role in the non-random manifestation of PTEs *in vivo* (studied in Chapter 4) it was proposed that the non-random manifestation of PTEs *in vivo* may be due to non-random formation as a result of the higher order packaging of DNA found *in vivo.*

5.2 Influence of nuclear structure upon adduct formation

It has been hypothesised that the presence or absence of DNA-protein interactions may influence the accessibility of DNA to genotoxins. There is a wealth of evidence that points to the fact that nuclei structure reduces the accessibility of sites for modification by bulky chemical carcinogens (MacLeod, 1995). Higher ordered DNA packaging may also influence the formation of damage by ultra violet radiation and oxidative stress, for example CPDs (Liu *et al.*, 2000) and 8-hydroxydG (Enright *et al.*, 1996 and 1992) have been found to be more abundant in internucleosomal DNA than in nucleosomal DNA.

The manifestation of small alkyl adducts may also be influenced by nuclear structure. The nature of the alkylating agent affects its affinity for reaction with either the nucleosomal or internucleosomal DNA. *N*-methyl-*N*-nitrosourea (MNU), an S_N1 monofunctional alkylating agent, preferentially forms adducts in the internucleosomal (Sudhakar *et al.*, 1979a and 1979b; Berkowitz and Silk, 1981) and transcribing regions of DNA (Berkowitz and Silk, 1981). However, DMS, a S_N2 monofunctional alkylating agent, induces adducts randomly in chromosomal DNA (McGhee and Felsenfeld, 1979; Berkowitz and Silk, 1981).

The presence of non-histone proteins in contact with DNA can either increase or decrease the accessibility of specific guanine bases to simple alkylating agents. Cloutier *et al.* (2001) reported the distribution of alkyl guanine adducts formed by a variety of simple, monofunctional alkylating agents along sequences of the Fragile-X Mental Retardation 1 (*FMR1*) promoter and exon 1 in human lymphoblasts *(in vivo)* and naked DNA (*in vitro)*. The *FMR1* promoter has four sites where DNA-protein interactions are observed. For each tested agent the *in vivo* and *in vitro* distribution of alkylated guanines were almost identical with the exception of the four regions known to have DNA-protein interactions. It would appear that the distribution of adducts in the regions known to have DNA-protein interactions is influenced by the chemical nature of the alkylating agent. Only hypo-reactive sites were found in DNA treated *in vivo* with S_N1 methylating agents (MNU, *N*-nitroso (acetoxymethyl) methylamine (NDMAOAc) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)) in the four areas known to have DNA-protein interactions. In contrast, in DNA treated *in vivo* with DMS, DES, MMS and EMS (S_N2 alkylating agents) both hypo and hyper-reactive sites were found in the four regions known to have DNA-protein interactions. Methyl and ethyl iodoalkane (S_N2 alkylating agents) produced only hyper-reactive sites

within the regions known to have DNA-protein interactions. Generally the degree of influence that DNA-protein interactions have upon the manifestation of DNA damage depends upon the ultimate genotoxic species and its mechanism of action. Alkylation of DNA by S_N2 alkylating agents, in particular the alkyl iodoalkanes, is facilitated at some sites by DNA-protein interactions.

5.3 Specific aims of study

- To develop an *in vitro* model with which to study the non-random manifestation of PTEs.
- To determine whether the frequency of bases 5' to PTEs is random or non-random with respect to normal base content in isolated nuclei where cellular activity, i.e. repair, is considered to be compromised.
- To determine whether DNA-protein packaging influences the manifestation of PTEs, by comparing the manifestation of PTEs in cells, isolated nuclei and DNA treated *in vitro* with an alkylating agent.

5.4 Results

5.4.1 Manifestation of PTEs in cells treated in vitro with NDEA

The initial objective of this study was to develop an *in vitro* model with which to study the non-random manifestation of PTEs previously reported in mouse liver DNA treated *in vivo* with NDEA (Guichard *et al.*, 2000). NDEA is a proximate carcinogen requiring metabolic activation by cytochrome P450 2E1 and/ or 2A6 to form the ultimate genotoxic species. The h2E1/OR cell line was developed to constitutively express human CYP2E1. The h2E1/OR cell line was derived from a human B-lymphoblastoid cell line (AHH-1 TK +/-) that was transfected with a plasmid vector containing a human CYP2E1 cDNA. h2E1/OR cells were treated *in*

vitro with NDEA (0-10 mM) for 2 hours, 24 hours and 4 days. DNA extracted from the treated cells was assayed using the SVPD/NP1 postlabelling protocol for the detection of PTEs. Figure 5.1 depicts the autoradiograph of the PAGE separated products. There was not a detectable level of PTEs generated in any of the samples. The absence of a detectable level of PTEs being generated even in cells treated with a high concentration of NDEA over several days, led to the conclusion that the metabolic activation of NDEA was not occurring efficiently in these cells.

5.4.2 Manifestation of PTEs in cells treated in vitro with DES

h2E1/ OR and HeLa cells were treated *in vitro* with DES. HeLa cells are an established cell line derived from a human cervical carcinoma. h2E1/OR cells were treated *in vitro* with DES (0 – 10 mM) for 2 hours. DNA extracted from the treated cells was assayed using the SVPD/NP1 postlabelling protocol for the detection of PTEs. Figure 5.2 depicts an autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. An equimolar mix of 16 dNpdN standards (4.2 fmol each) was labelled and analysed under identical conditions to the DNA digests thus enabling the quantification of PTEs on the polyacrylamide gel. Table 5.1 shows the results for the quantification of PTEs in DNA isolated from h2E1/ OR cells treated *in vitro* with DES. PTEs were detected in cells treated with 5 and 10 mM DES (9 and 10.4 PTE/ 10^6 nucleotides, respectively).

The frequency of nucleosides found 5' to PTEs was determined using the new 'direct' method for PTE 5' NNA. Figure 5.3a shows the results for the 5' NNA of DNA extracted from h2E1/OR cells treated *in vitro* with 10 mM DES. Figure 5.3b

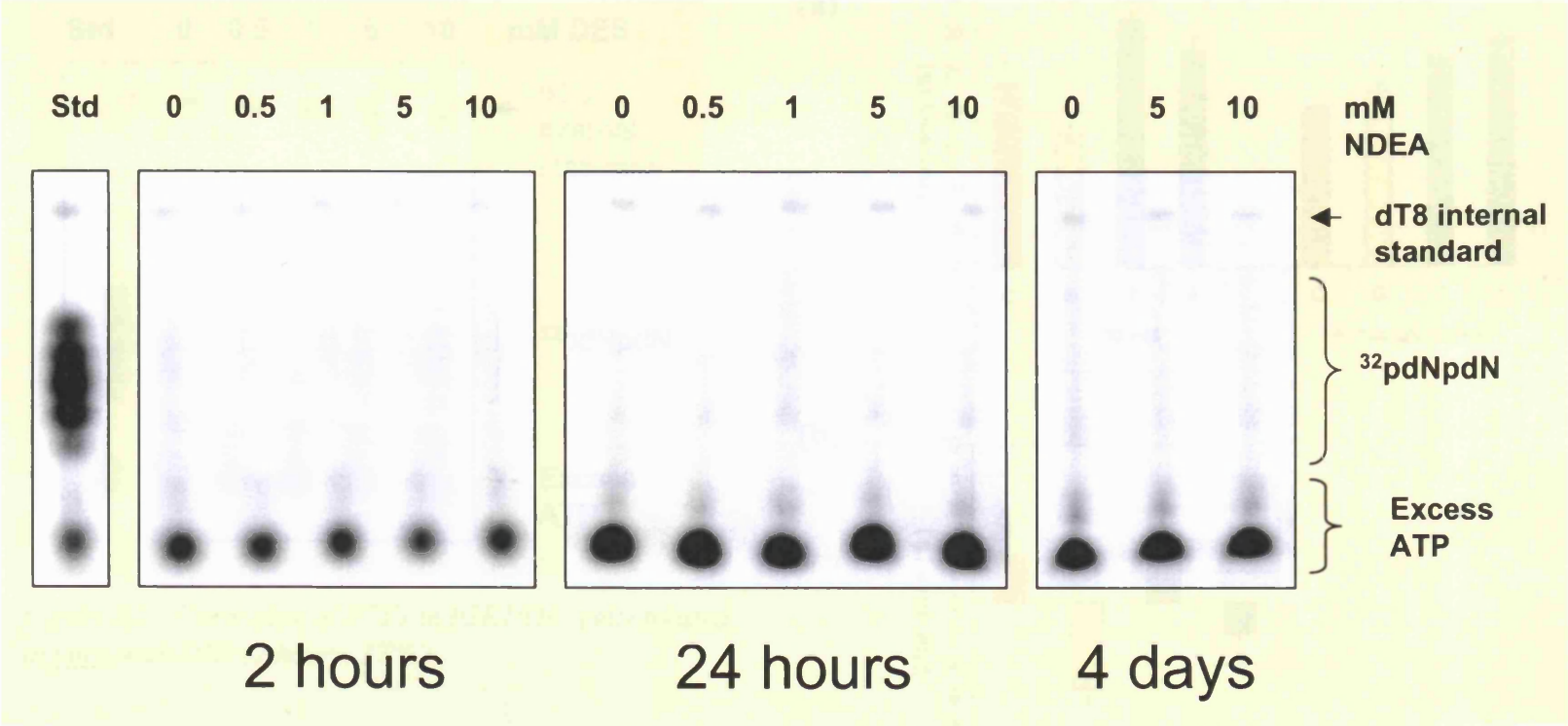


Figure 5.1: Autoradiographs of polyacrylamide gels for the SVPD/ NP1 postlabelling detection of PTEs in h2E1/ OR cells treated in vitro with NDEA (2 hours, 24 hours and 4 days at 37°C).

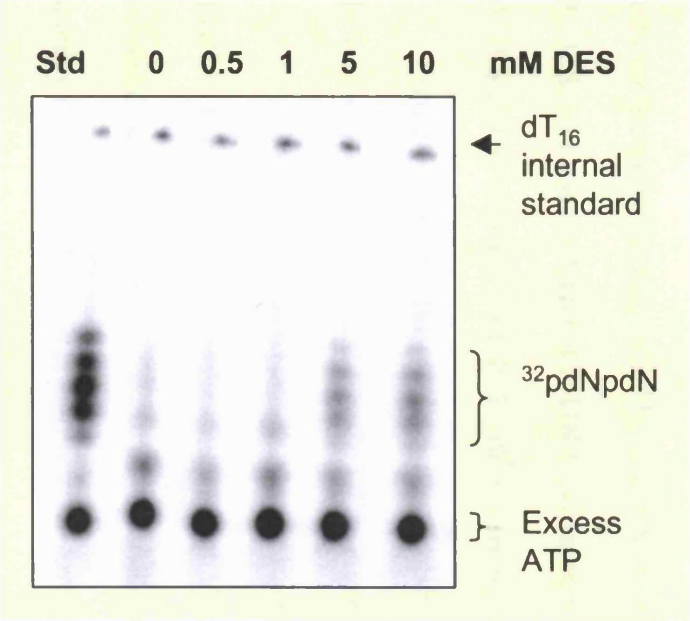


Figure 5.2: Formation of PTEs in h2E1/OR cells treated <u>in vitro</u> with DES (2 hours, 37°C).

Table 5.1: Quantification of PTEs formed in h2E1/OR cells treated <u>in vitro</u> with DES (2 hours, 37°C).

Dose (mM DES)	0	0.5	1	5	10
PTE /10^6 nucleotides	0	n.d.	n.d.	9.0	10.4

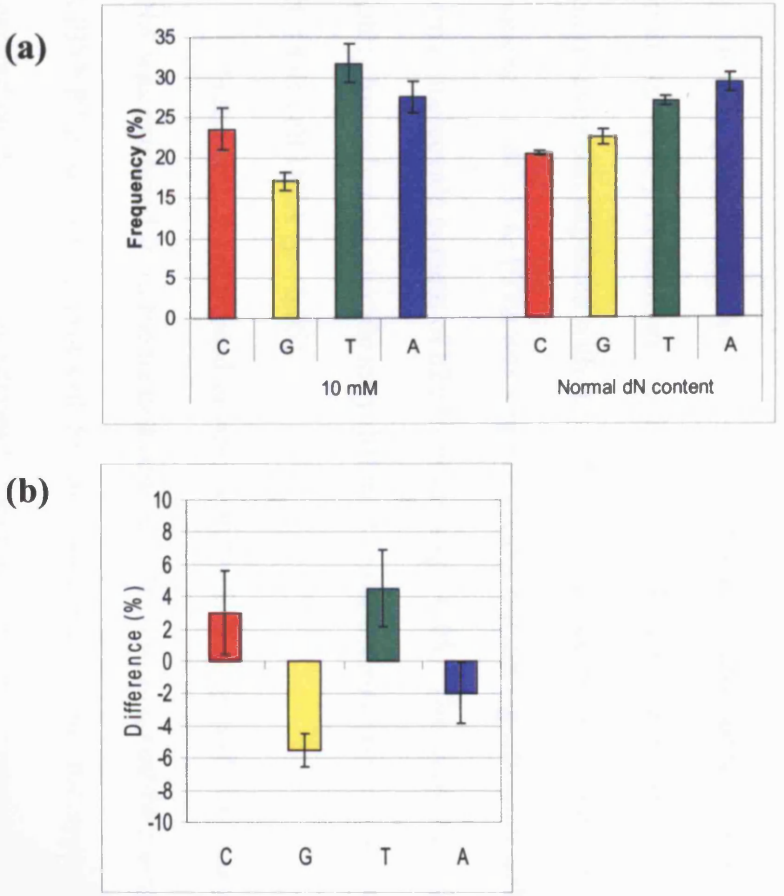


Figure 5.3: Results for the 5' NNA of PTEs in h2E1/OR cells treated with 10 mM DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

shows the difference between the frequency of bases found 5' to PTEs and normal base content of h2E1/OR cell DNA. The frequency of dT found 5' to PTEs was significantly greater than would be expected when compared to normal nucleoside content ($p < 0.05$). In addition, the frequency of dG was significantly lower than would have been expected with respect to normal nucleoside content ($p < 0.05$). The frequency of dC 5' to PTEs was slightly greater but not significantly different to the normal nucleoside content of h2E1/OR cells ($p > 0.05$). The frequency of dA was slightly lower but not significantly different to the normal nucleoside content of h2E1/OR cell DNA ($p > 0.05$).

HeLa cells were treated *in vitro* with DES (0 – 10 mM) for 3 hours at 37°C. DNA was extracted from the treated cells and PTEs were quantified using the SVPD/NP1 postlabelling protocol. Figure 5.4 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. Table 5.2 shows the results for the quantification of PTEs in DNA isolated from HeLa cells treated *in vitro* with DES. PTEs were detected in DNA extracted from cells treated with 1, 5 and 10 mM DES (19.5, 49.0 and 84.6 PTE/ 10^6 nucleotides respectively).

Figure 5.5a shows the results for the 5' NNA of DNA extracted from HeLa cells treated *in vitro* with 5 and 10 mM DES. Figure 5.5b shows the difference between the frequency of bases found 5' to PTEs and normal base content of HeLa cell DNA. The frequency of pyrimidines found 5' to PTEs was significantly greater than would be expected when compared to normal nucleoside content ($p < 0.01$). The frequency of dA 5' to PTEs was significantly lower than would have been expected with respect to normal nucleoside content ($p < 0.001$). The frequency of dG found 5' to PTEs was slightly lower, but not significantly different from normal nucleoside

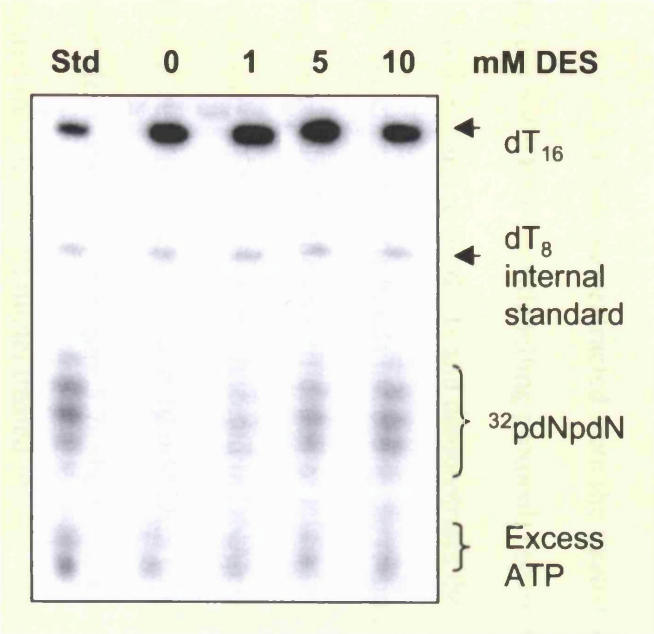


Figure 5.4: Formation of PTEs in HeLa cells treated in vitro with DES (3 hours, 37°C).

Table 5.2: Quantification of PTEs formed in HeLa cells treated in vitro with DES (3 hours, 37°C).

Dose (mM DES)	0	1	5	10
PTE /10^6 nucleotides	0	19.5	49.1	84.6

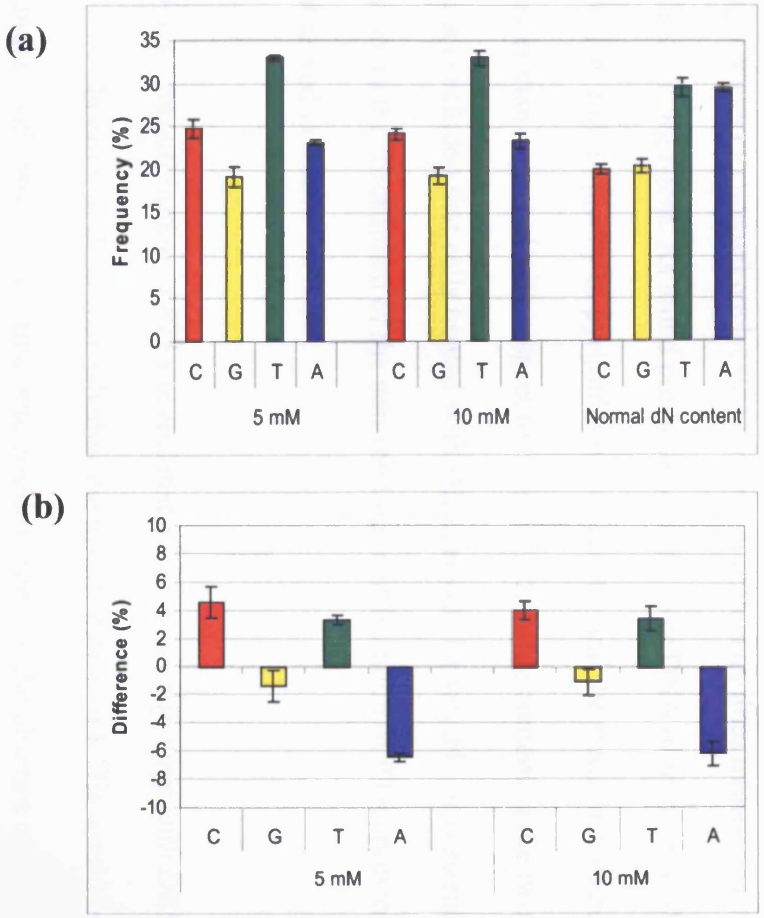


Figure 5.5: Results for the 5' NNA of PTEs in HeLa cells treated with DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

content of HeLa cell DNA ($p > 0.05$).

Overall, the frequency of nucleosides 5' to PTEs was found to be non-random with respect to normal nucleoside content in both cells lines treated *in vitro* with a direct acting agent. As found previously in the liver DNA of mice treated *in vivo* with NDEA, the frequency of pyrimidines was greater and the frequency of purines was lower than expected with respect to normal nucleoside content in the two cell lines. It was concluded that cells treated with DES provided a suitable *in vitro* model in which to study the mechanism of the non-random manifestation of PTEs previously observed *in vivo*.

5.4.3 Manifestation of PTEs in isolated nuclei treated in vitro with DES

In order to determine whether the manifestation of PTEs would remain non-random with respect to normal nucleoside content in the absence of cytoplasmic activity, nuclei isolated from HeLa and h2E1/OR cells were treated with DES *in vitro*.

Nuclei isolated from h2E1/OR cells were treated with 0 – 10 mM DES for 3 hours at 37°C. DNA was extracted from the treated nuclei and PTEs were quantified using the SVPD/NP1 postlabelling protocol. Figure 5.6 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. Table 5.3 shows the results for the quantification of PTEs in the isolated h2E1/OR nuclei treated *in vitro* with DES. PTEs were detected in DNA extracted from nuclei isolated from h2E1/OR cells treated with 5 and 10 mM DES (approximately 6 and 26.6 PTE/ 10^6 nucleotides respectively). Figure 5.7a shows the results for the 5' NNA of DNA extracted from h2E1/OR nuclei treated *in vitro* with 10 mM DES. Figure 5.7b shows the difference between the frequency of nucleosides found 5' to PTEs and normal

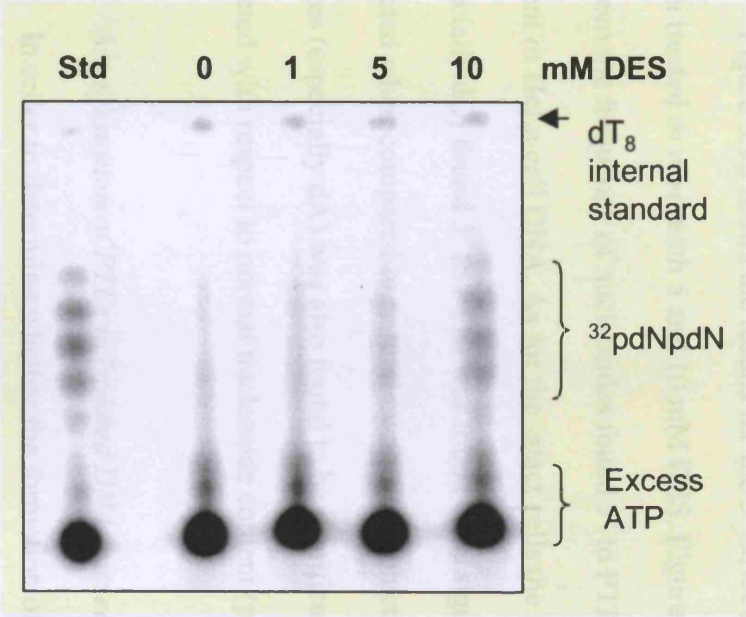


Figure 5.6: Formation of PTEs in nuclei isolated from h2E1/OR cells treated in vitro with DES (3 hours, 37°C).

Table 5.3: Quantification of PTEs formed in nuclei isolated from h2E1/ OR cells treated in vitro with DES (3 hours, 37°C).

Dose (mM DES)	0	1	5	10
PTE /10^6 nucleotides	0	3.6	6.0	26.6

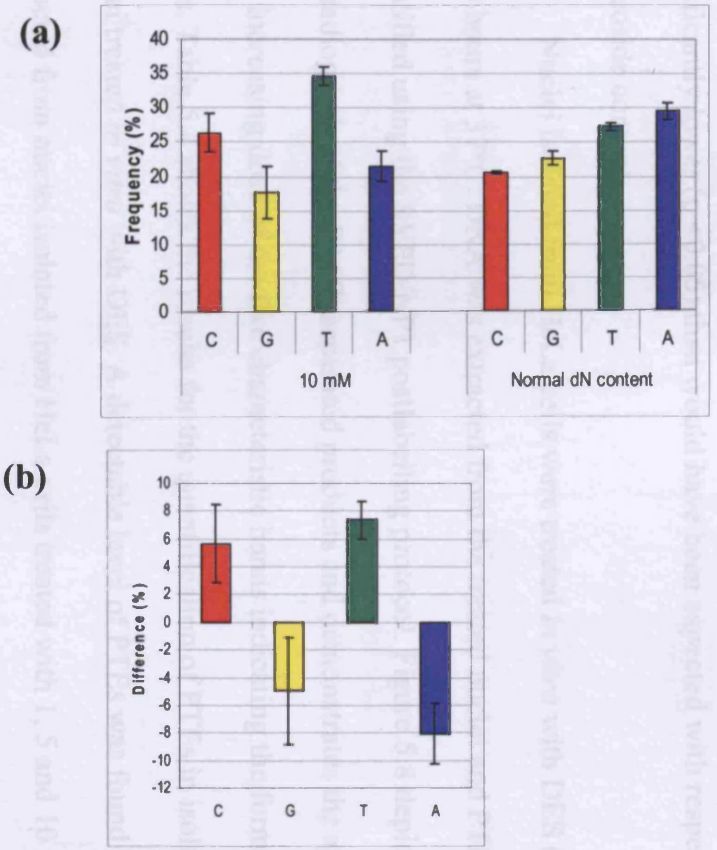


Figure 5.7: Results for the 5' NNA of PTEs in nuclei isolated from h2E1/OR cells treated with 10 mM DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

nucleoside content of h2E1/OR cell DNA. The frequency of pyrimidines found 5' to PTEs was found to be significantly greater ($p < 0.01$) and the frequency of purines significantly lower ($p < 0.05$) than would have been expected with respect to normal nucleoside content.

Nuclei isolated from HeLa cells were treated *in vitro* with DES (0 – 10 mM) for 3 hours at 37°C. DNA was extracted from the treated nuclei and PTEs were quantified using the SVPD/NP1 postlabelling protocol. Figure 5.8 depicts an autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. Table 5.4 shows the results for the quantification of PTEs in isolated HeLa nuclei treated *in vitro* with DES. A detectable level of PTEs was found in DNA extracted from nuclei isolated from HeLa cells treated with 1, 5 and 10 mM DES (approximately 30.0, 106.6 and 748.6 PTE/ 10^6 nucleotides respectively).

Figure 5.9a shows the results for the 5' NNA of DNA extracted from HeLa nuclei treated *in vitro* with 5 and 10 mM DES. Figure 5.9b shows the difference between the frequency of nucleosides found 5' to PTEs and normal nucleoside content of HeLa cell DNA. As for the intact cells the frequency of pyrimidines (especially dC) found 5' to PTEs was found to be significantly greater than would be expected when compared to normal nucleoside content ($p < 0.05$). The frequency of purines (especially dA) was also found to be significantly lower than would have been expected with respect to normal nucleoside content ($p < 0.05$).

5.4.4 Manifestation of PTEs in isolated DNA treated in vitro with DES

In order to determine whether the formation of PTEs was non-random in the absence of higher DNA structure and cellular activity, DNA isolated from HeLa and

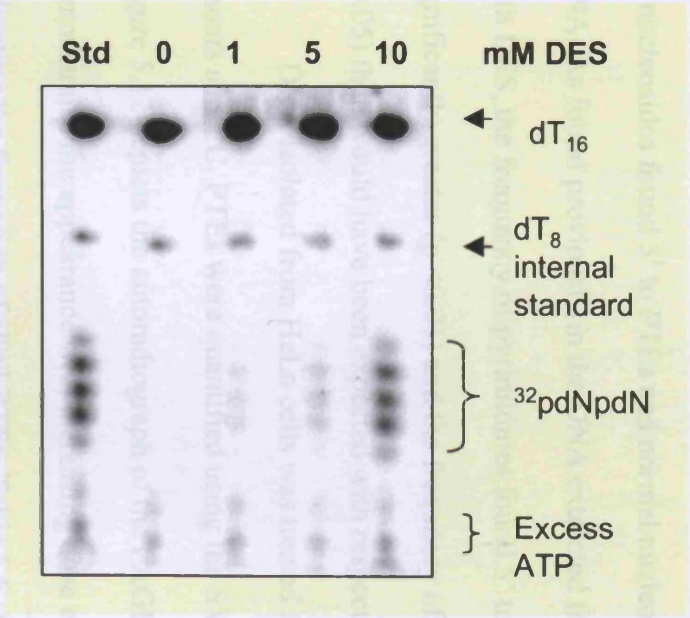


Figure 5.8: Formation of PTEs in nuclei isolated from HeLa cells treated in vitro with DES (3 hours, 37°C).

Table 5.4: Quantification of PTEs formed in nuclei isolated from HeLa cells treated in vitro with DES (3 hours, 37°C).

Dose (mM DES)	0	1	5	10
PTE /10^6 nucleotides	0	30.0	106.6	748.6

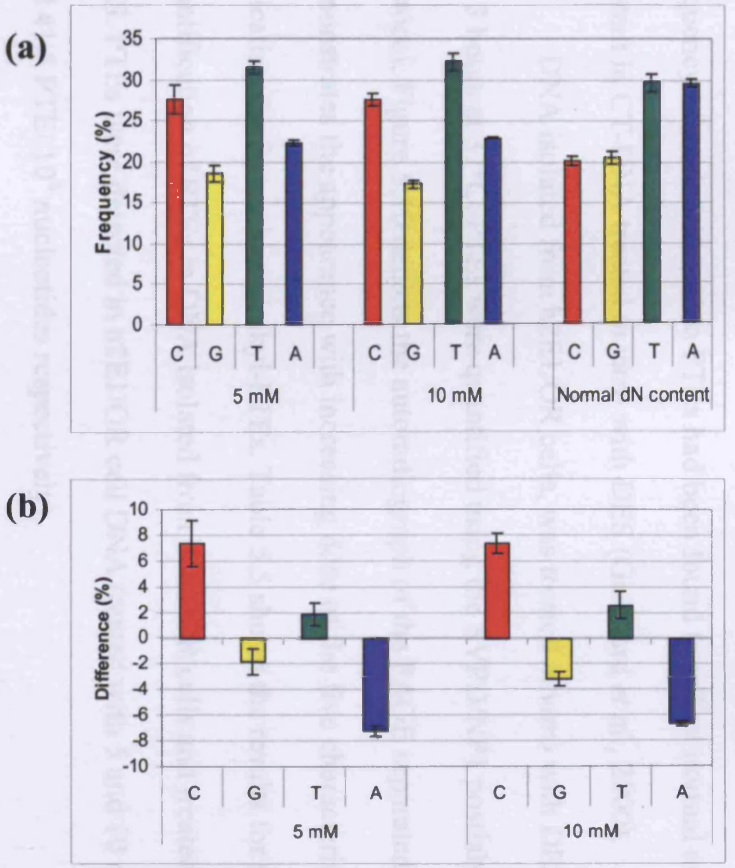


Figure 5.9: Results for the 5' NNA of PTEs nuclei isolated from HeLa cells treated with DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

h2E1/OR cells was treated *in vitro* with DES. It was hypothesised that the manifestation of PTEs in naked DNA extracted from cells would be random as the frequency of nucleosides 5' to PTEs had been found to reflect normal nucleoside content in CT-DNA treated *in vitro* with DES (Guichard *et al.*, 2000).

DNA isolated from h2E1/OR cells, was treated *in vitro* with DES (0 – 10 mM) for 3 hours at 37°C. PTEs were quantified using the SVPD/NP1 postlabelling protocol. Figure 5.10 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. Table 5.5 shows the results for the quantification of PTEs in DNA isolated from h2E1/OR cells and treated *in vitro* with DES. PTEs were detected in h2E1/OR cell DNA treated with 5 and 10 mM DES (38.7 and 41.5 PTE/ 10^6 nucleotides respectively).

Figure 5.11a shows the results for the 5' NNA for h2E1/OR cell DNA treated *in vitro* with 10 mM DES. Figure 5.11b shows the difference between the frequency of nucleosides found 5' to PTEs and normal nucleoside content of h2E1/OR cell DNA. As found previously in the DNA extracted from whole cells and nuclei treated with DES, the frequency of pyrimidines found 5' to PTEs was found to be significantly greater ($p < 0.01$) and the frequency of purines was significantly lower ($p < 0.05$) than would have been expected with respect to normal nucleoside content.

DNA isolated from HeLa cells was treated *in vitro* with DES (0 – 10 mM) for 3 hours at 37°C. PTEs were quantified using the SVPD/NP1 postlabelling protocol. Figure 5.12 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs. Table 5.6 shows the results for the quantification of PTEs in DNA isolated from HeLa cells and treated *in vitro* with

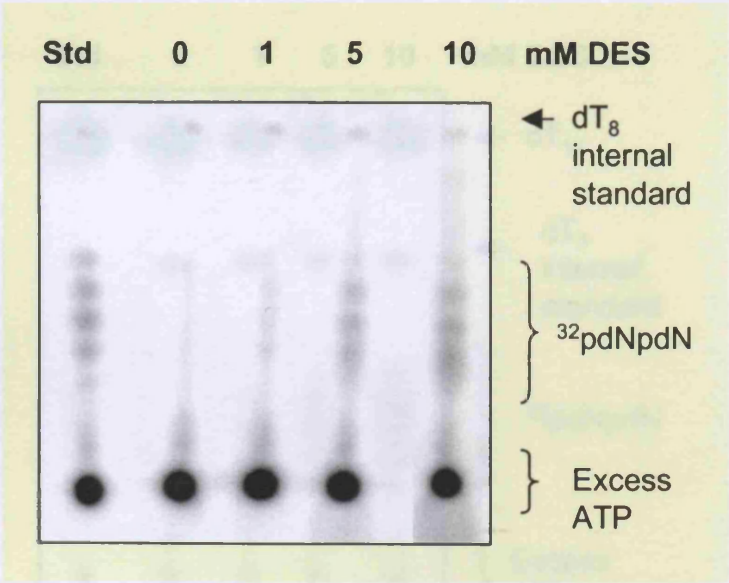


Figure 5.10: Formation of PTEs in DNA isolated from h2E1/OR cells treated in vitro with DES (3 hours, 37°C).

Table 5.5: Quantification of PTEs formed in DNA isolated from h2E1/OR cells treated in vitro with DES (3 hours, 37°C).

Dose (mM DES)	0	1	5	10
PTE /10^6 nucleotides	0	5.0	38.7	41.5

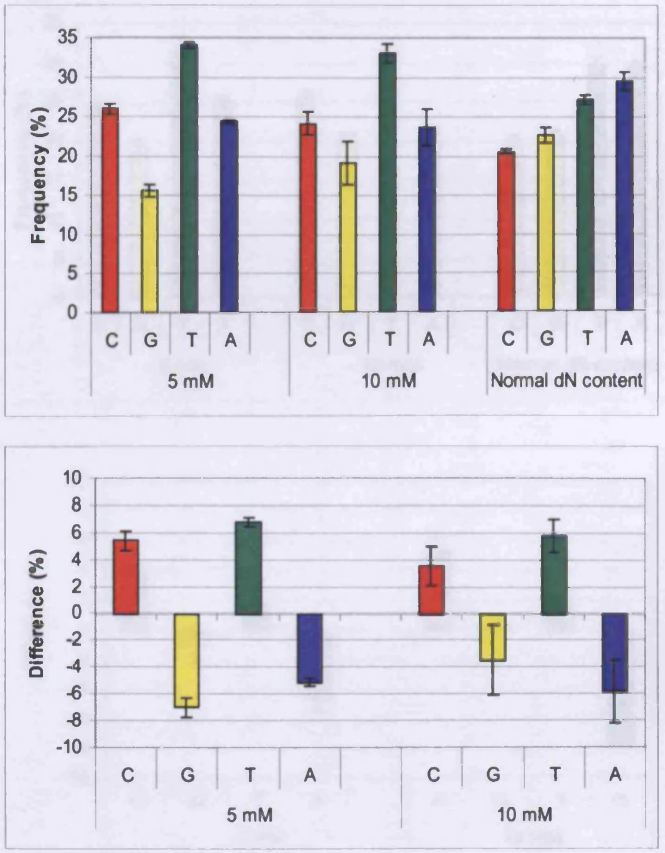


Figure 5.11: Results for the 5' NNA of PTEs in DNA isolated from h2E1/OR cells treated with DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

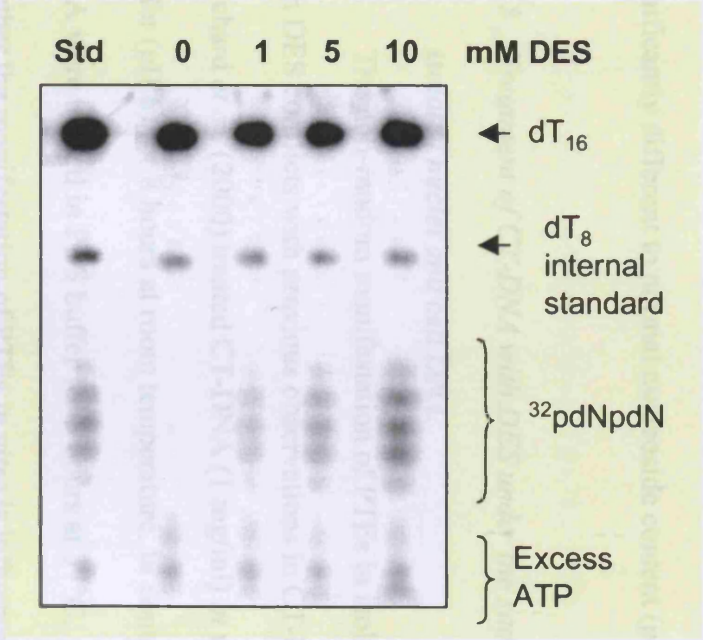


Figure 5.12: Formation of PTEs in DNA isolated from HeLa cells treated in vitro with DES (3 hours, 37°C).

Table 5.6: Quantification of PTEs formed in DNA isolated from HeLa cells treated in vitro with DES (3 hours, 37°C).

Dose (mM DES)	0	1	5	10
PTE /10^6 nucleotides	0	76.6	189.1	463.7

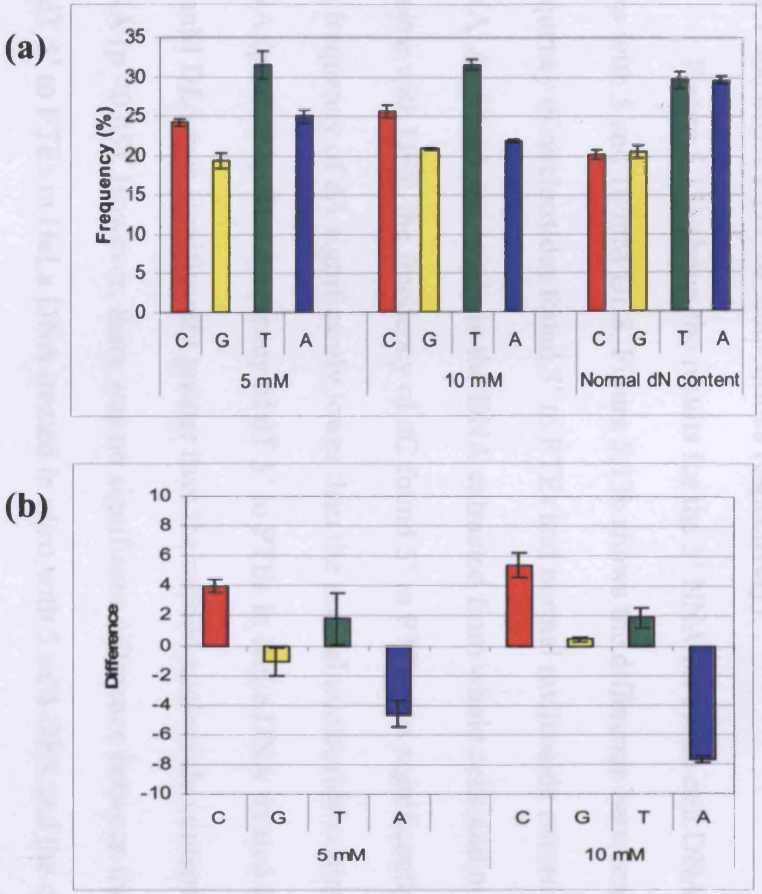


Figure 5.13: Results for the 5' NNA of PTEs in DNA isolated from HeLa cells treated with DES:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

DES. PTEs were detected in HeLa cell DNA treated with 1, 5 and 10 mM DES (76.6, 189.1 and 463.7 PTE/ 10^6 nucleotides respectively).

Figure 5.13a shows the results for the 5' NNA for HeLa cell DNA treated *in vitro* with 5 and 10 mM DES. Figure 5.13b shows the difference between the frequency of nucleosides found 5' to PTEs and normal nucleoside content of HeLa DNA. As found previously in the DNA extracted from whole cells and nuclei treated *in vitro* with DES, the frequency of dC found 5' to PTEs was significantly greater and the frequency of dA significantly lower than the normal nucleoside content of HeLa DNA ($p < 0.001$). The frequency of dT 5' to PTEs in HeLa DNA treated *in vitro* with 10 mM DES was significantly greater than the normal nucleoside content of HeLa DNA ($p < 0.05$). However, there was no significant difference between the frequency of dT 5' to PTEs in HeLa DNA treated *in vitro* with 5 mM DES and the normal nucleoside content of HeLa DNA ($p > 0.05$). As found in the HeLa cells and nuclei treated *in vitro* with DES, the frequency of dG 5' to PTE was slightly lower but not significantly different to normal nucleoside content ($p > 0.1$).

5.4.5 Treatment of CT-DNA with DES under the same conditions used for the isolated nuclei and cell DNA.

The non-random manifestation of PTEs in isolated cell DNA treated *in vitro* with DES conflicts with previous observations in CT-DNA (Guichard *et al.*, 2000). Guichard *et al.* (2000) treated CT-DNA (1 mg/ml) *in vitro* in 0.5 M sodium phosphate buffer (pH 6) for 8 hours at room temperature. In contrast, the isolated nuclei and cell DNA were treated in PBS buffer for 3 hours at 37°C. Consequently to investigate whether the manifestation of PTEs *in vitro* is influenced by reaction conditions, CT-DNA (1 mg/ml) was treated with 0 – 10 mM DES in PBS buffer for either 3 hours at

37°C or 8 hours at room temperature. PTEs were quantified using the SVPD/NP1 postlabelling protocol.

Figure 5.14 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs in CT-DNA treated *in vitro* with DES for 3 hours at 37°C in PBS buffer. Table 5.7 shows the results for the quantification of PTEs in CT-DNA (3 hours, 37°C in PBS buffer). PTEs were detected in CT-DNA treated with 5 and 10 mM DES (41.1 and 47.4 PTE/ 10^6 nucleotides respectively).

Figure 5.15 depicts the autoradiograph of the PAGE separated products and demonstrates the appearance with increasing dose of the five characteristic bands indicating the formation of alkyl-PTEs in the CT-DNA treated with DES for 8 hours at room temperature in PBS buffer. Table 5.8 shows the results for the quantification of PTEs in CT-DNA treated *in vitro* with DES (8 hours, room temperature in PBS buffer). PTEs were detected in CT-DNA treated with 5 and 10 mM DES (18.8 and 34.3 PTE/ 10^6 nucleotides respectively).

Figure 5.16a shows the results for the 5' NNA for CT-DNA treated *in vitro* with 10 mM DES for either 3 hours at 37°C or 8 hours at room temperature. Figure 5.16b shows the difference between the frequency of nucleosides found 5' to PTEs and normal nucleoside content of CT-DNA. As reported previously in the liver DNA of mice treated *in vivo* (Chapter 4) and the cell materials (whole cells, isolated nuclei and DNA) treated *in vitro* (Section 5.4.2 – 5.4.4) the frequency of pyrimidines was significantly greater ($p < 0.01$) and the frequency of purines ($p < 0.01$) was significantly lower 5' to PTEs compared to the normal nucleoside content of CT-DNA treated for either 3 hours at 37°C or 8 hours at room temperature. The relative

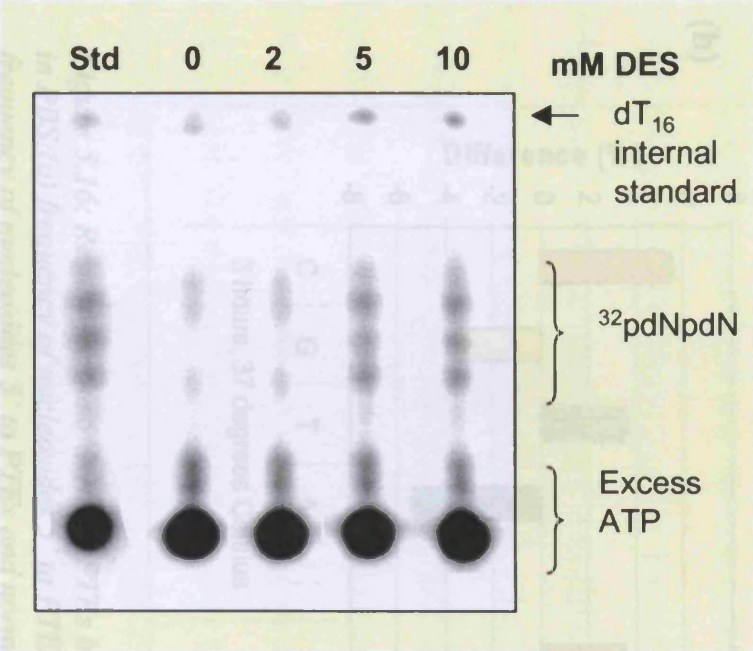


Figure 5.14: Formation of PTEs in CT-DNA treated in vitro with DES in PBS (3 hours, 37°C).

Table 5.7: Quantification of PTEs formed in CT-DNA treated in vitro with DES in PBS (3 hours, 37°C).

Dose (mM DES)	0	2	5	10
PTE /10^6 nucleotides	0	5.9	41.1	47.4

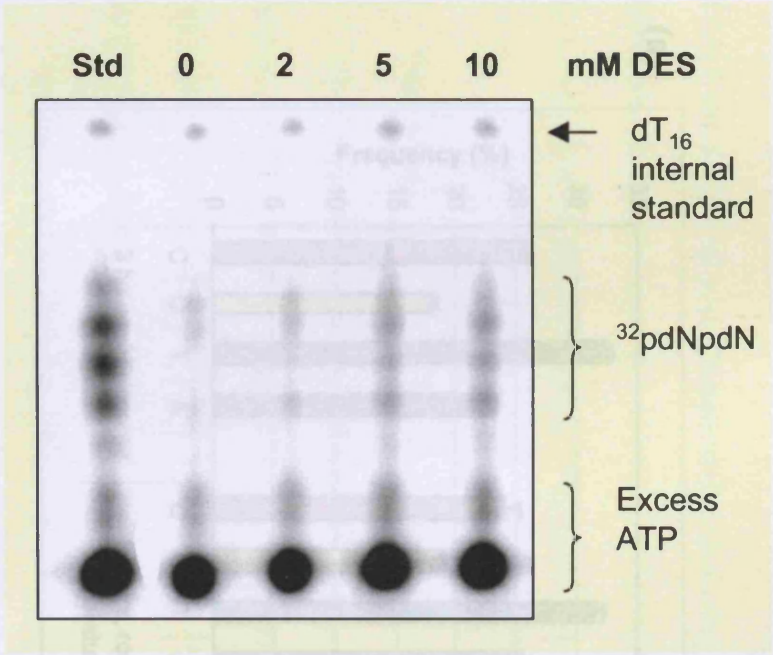


Figure 5.15: Formation of PTEs in CT-DNA treated in vitro with DES in PBS (8 hours, room temperature).

Table 5.8: Quantification of PTEs formed in CT-DNA treated in vitro with DES in PBS (8 hours, room temperature).

Dose (mM DES)	0	2	5	10
PTE /10^6 nucleotides	0	4.9	18.8	34.3

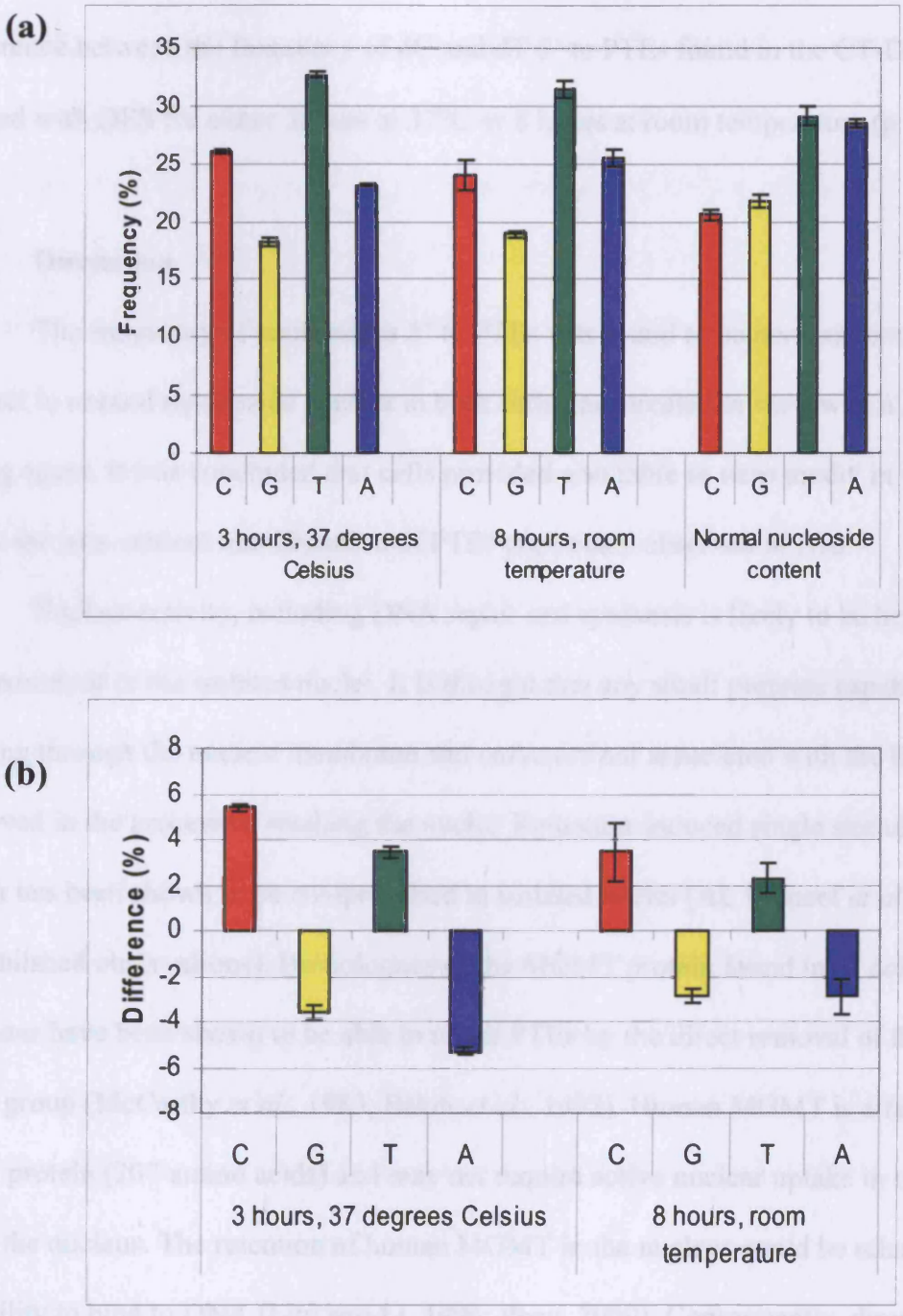


Figure 5.16: Results for the 5' NNA of PTEs in CT-DNA treated with DES in PBS:(a) frequency of nucleosides 5' to PTEs; (b) difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

frequency of dG and dA 5' to PTEs was significantly lower in the CT-DNA treated *in vitro* with DES for 3hours at 37°C ($p < 0.05$) compared to the CT-DNA treated *in vitro* with DES for 8 hours at room temperature. However, there was no significant difference between the frequency of dC and dT 5' to PTEs found in the CT-DNA treated with DES for either 3hours at 37°C or 8 hours at room temperature ($p > 0.05$).

5.5 Discussion

The frequency of nucleosides 5' to PTEs was found to be non-random with respect to normal nucleoside content in both cells lines treated *in vitro* with a direct acting agent. It was concluded that cells provided a suitable *in vitro* model in which to study the non-random manifestation of PTEs previously observed *in vivo*.

Nuclear activity, including DNA repair and synthesis is likely to be heavily compromised in the isolated nuclei. It is thought that any small proteins capable of passing through the nuclear membrane and cofactors not associated with the DNA are removed in the process of washing the nuclei. Radiation-induced single strand break repair has been shown to be compromised in isolated nuclei (AL Moneef *et al.*, unpublished observations). Homologues of the MGMT protein found in *E. coli* and *A. nidulans* have been shown to be able to repair PTEs by the direct removal of the PTE alkyl group (McCarthy *et al.*, 1983; Baker *et al.*, 1992). Human MGMT is a fairly small protein (207 amino acids) and may not require active nuclear uptake in order to enter the nucleus. The retention of human MGMT in the nucleus could be related to its ability to bind to DNA (Lim and Li, 1996; Pegg, 2000). Consequently, direct repair may not be completely compromised in the isolated nuclei. However, human MGMT does not repair PTEs (Yarosh *et al.*, 1985; Dolan *et al.*, 1984), therefore it is unlikely that direct PTE repair is occurring in the HeLa and h2E1/OR cells and isolated nuclei.

Figures 5.17 and 5.18 show the differences between 5' NNA results and normal nucleoside content for cells, nuclei and DNA treated with DES for the h2E1/OR and HeLa cells respectively. There was no significant difference between the frequency of dC, dG and dT found 5' to PTEs in h2E1/OR nuclei and whole cells ($p > 0.05$). The frequency of dA found 5' to PTEs was significantly lower in isolated nuclei than in cells ($p < 0.01$). There was no significant difference between the frequency of nucleosides 5' to PTEs in HeLa nuclei and whole cells ($p > 0.05$), with the exception of dC which was greater in nuclei treated with 10 mM DES ($p < 0.01$). Overall it was judged that there was no consistent significant difference between the frequency of nucleosides 5' to PTEs found in nuclei and whole cells treated *in vitro* with DES for the two cell lines.

There was no significant difference between the frequency of nucleosides found 5' to PTEs in nuclei and DNA isolated from h2E1/OR cells and treated *in vitro* with DES ($p > 0.05$). However, there was a significant increase in the frequency of dG and a significant decrease in the frequency of dA found 5' to PTEs in the HeLa DNA treated *in vitro* with 10 mM DES compared to the isolated HeLa nuclei treated *in vitro* with 10 mM DES ($p < 0.01$). In contrast, there was no significant difference in the frequency of dG found 5' to PTEs ($p > 0.05$) in the HeLa DNA compared to the isolated nuclei treated with 5 mM DES. However, there was a significant increase in the frequency of dA in the HeLa DNA compared to the isolated nuclei treated with 5 mM DES ($p < 0.05$).

There was no significant difference between the frequency of nucleosides found 5' to PTEs in whole cells and isolated DNA from both cell lines ($p > 0.05$), with the exception of dA, which was significantly lower in h2E1/OR DNA than in whole cells ($p = 0.01$). Overall, it was judged that there was no consistent significant

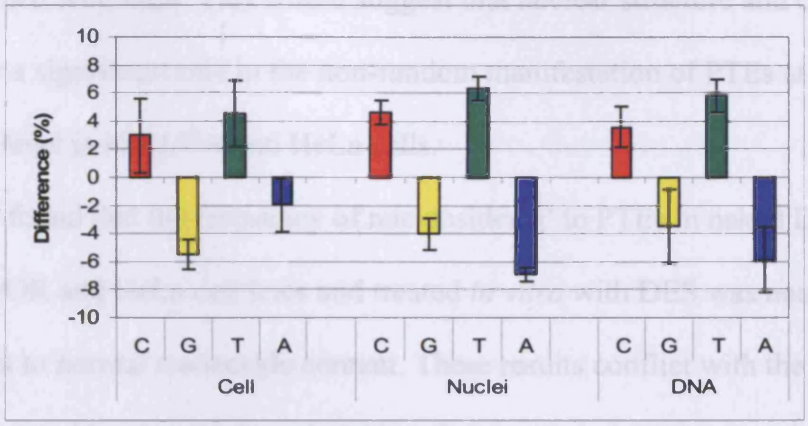


Figure 5.17: Difference between the frequency of nucleosides 5' to PTE lesions and normal nucleoside content in h2E1/ OR cells, isolated nuclei and DNA treated <u>in vitro</u> with DES. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

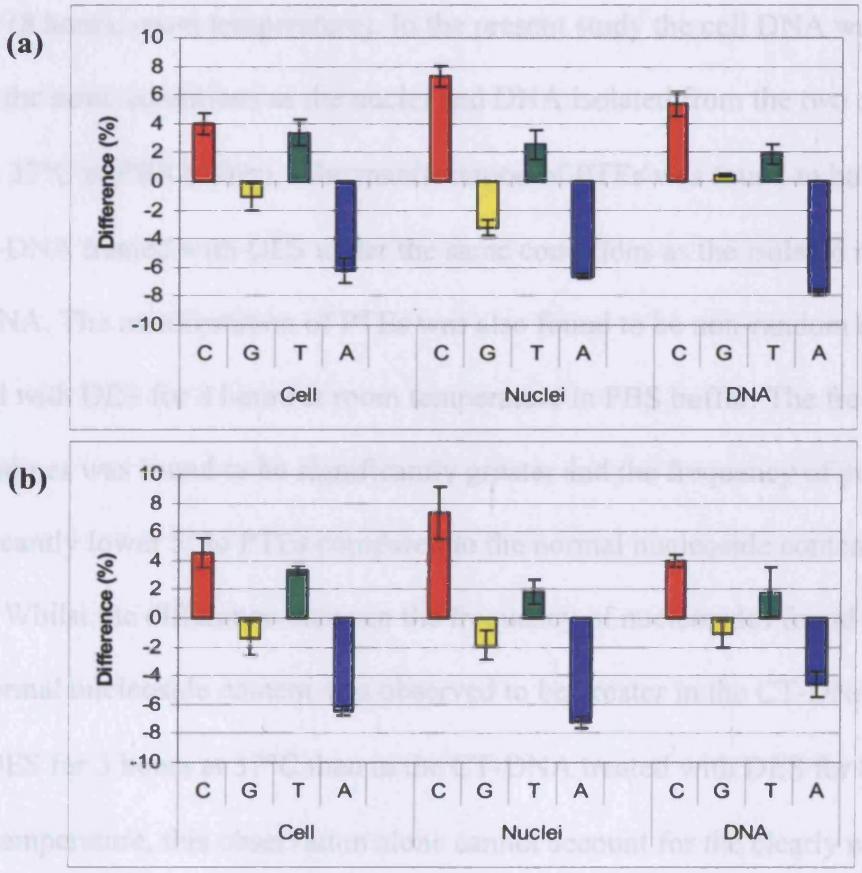


Figure 5.18: Difference between the frequency of nucleosides found 5' to PTE lesions and normal nucleoside content in HeLa cells, isolated nuclei and DNA treated <u>in vitro</u> (a) 10mM DES and (b) 5mM DES. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

difference between the 5'NNA results for the whole cells and the isolated DNA treated *in vitro* with DES. This would suggest that nuclear structure and cellular repair do not play a significant role in the non-random manifestation of PTEs at the nucleotide level in h2E1/OR and HeLa cells.

We found that the frequency of nucleosides 5' to PTEs in naked DNA isolated from h2E1/OR and HeLa cell lines and treated *in vitro* with DES was non-random with respect to normal nucleoside content. These results conflict with the previous findings reported by Guichard *et al.* (2000). Guichard *et al.* (2000) found that the manifestation of PTEs was random with respect to normal nucleoside content in naked CT-DNA treated *in vitro* with either DES or DMS in 0.5 M sodium phosphate buffer (8 hours, room temperature). In the present study the cell DNA was treated under the same conditions as the nuclei and DNA isolated from the two cell lines (3 hours, 37°C in PBS buffer). The manifestation of PTEs was found to be non-random in CT-DNA treated with DES under the same conditions as the isolated nuclei and cell DNA. The manifestation of PTEs was also found to be non-random in CT-DNA treated with DES for 8 hours at room temperature in PBS buffer. The frequency of pyrimidines was found to be significantly greater and the frequency of purines significantly lower 5' to PTEs compared to the normal nucleoside content of CT-DNA. Whilst the difference between the frequency of nucleosides found 5' to PTEs and normal nucleoside content was observed to be greater in the CT-DNA treated with DES for 3 hours at 37°C than in the CT-DNA treated with DES for 8 hours at room temperature, this observation alone cannot account for the clearly non-random manifestation of PTEs observed in both experiments. The conditions used by Guichard *et al.* (2000) to treat CT-DNA were not physiological. The 0.5 M sodium phosphate buffer contains a high concentration of free phosphate that will compete

with the various sites on the DNA for the alkylating agent. This may have resulted in the random manifestation of PTEs in CT-DNA treated *in vitro*.

Although there was no significant difference in the frequency of nucleosides found 5' to PTEs induced in materials isolated from the same cell line, there was a significant difference between the 5' NNA results for the two cell lines (Fig. 5.17 and 5.18). In the h2E1/OR cell material the relative increase in the frequency dT found 5' to PTEs with respect to normal nucleoside content was greater than for dC. In contrast the relative increase in the frequency of the pyrimidines found 5' to PTEs was greater for dC than for dT in HeLa cell material. The difference between relative frequency of dG found 5' to PTEs and the normal nucleotide content was greater in h2E1/OR cell material than in the HeLa cell material. The reasons for the differences between the 5' NNA results for the two cell lines are unclear.

One factor that could be contributing towards the differences in the 5' NNA results between the two cell lines could be DNA sequence. Some phosphates may be more susceptible to alkylation than others and consequently these will be alkylated first. However, both the h2E1/OR and HeLa cell lines are derived from human tissue, therefore it would not be expected that there would be substantial difference in overall base sequence. The relative accessibility of certain sequences due to differences in gene expression may however apply. The HeLa cell line was established from a human cervical carcinoma whilst the h2E1/OR cell line is derived from a human B-lymphoblastoid cell line that has been genetically modified to constitutively express human CYP2E1.

Another factor that may have contributed towards the observed differences in the frequency of nucleosides found 5' to PTEs between the two cell lines is that, for reasons unknown, the extent of phosphate alkylation was several fold greater in HeLa

derived material than in h2E1/OR derived material. The cells, nuclei and DNA from the two cell lines were treated according to the same methods. The fact that there was no consistent significant difference in the 5'NNA results for cells, nuclei and DNA from the same cell line, despite the fact that the reactions were carried out on separate days demonstrates that the outcome of the alkylation reaction was reproducible in material from the same cell line. However, the differences in the frequency of nucleosides found 5' to PTEs between the h2E1/OR and HeLa cell material persisted even in the naked DNA treated *in vitro,* where the absence of protein contamination had been confirmed by UV spectrometry.

We conclude that nuclear structure and cellular repair do not play a significant role in the non-random manifestation of PTEs in h2E1/ OR and HeLa cells treated with DES. The evidence is suggestive of the non-random manifestation of PTEs being a result of non-random formation. The presence of the non-random manifestation of PTEs in naked DNA treated *in vitro* would suggest that DNA sequence may play a role in the non-random manifestation of PTEs.

One consistent pattern that has been found in liver DNA of mice treated *in vivo,* cell derived material (whole cells, isolated nuclei and DNA) and CT-DNA treated in vitro in PBS, is that the frequency of pyrimidines 5' to PTEs was greater and the frequency of purines lower than would have been expected with respect to normal nucleoside content. Guanine is the most readily alkylated base accounting for between 19.6 – 83.9% of total alkylation products on DNA depending upon the alkylating agent (Beranek, 1990). Adenine is also readily alkylated with modified adenines accounting for between 6.5 – 23% of total alkylation products (Beranek, 1990). By comparison the pyrimidine bases are quite unreactive particularly with S_N2 alkylating agents. With the exception of ENU (a strong S_N1 alkylating agent)

modified cytosine and thymine products account for no more than 0.7 and 1.3% respectively of total alkylation products. Modified cytosine and thymine products induced by ENU treatment of DNA account for 2.9 - 3.4% and 9.2 – 11.1% of total alkylation products respectively (Beranek, 1990). It would seem plausible that the phosphate oxygens have to compete with other nucleophilic sites on the DNA for the alkylating electrophile. Hence a phosphodiester situated between two purines is less likely to be alkylated than a phosphodiester situated between two pyrimidines. We have consistently found that phosphodiesters 3' to a pyrimidine nucleoside are more susceptible to alkylation than phosphodiesters 3' to a purine.

Chapter 6:

The influence of DNA base sequence upon the manifestation of PTE adducts

6.1 Introduction

As discussed previously in Chapters 4 and 5 it does not appear that either nuclear structure or putative repair significantly influence the manifestation of PTEs. In Chapter 5 it was demonstrated that the manifestation of PTEs was non-random in naked DNA treated *in vitro* with DES. The non-random manifestation of PTEs appears to be due to the non-random formation of PTEs. Previous studies have demonstrated that the frequency and distribution of a variety of DNA alkylation products may be influenced by DNA base sequence (Briscoe and Cotter, 1984 and 1985; Dolan *et al.*, 1988; Mattes *et al.*, 1986; Kohn *et al.*, 1987). In this study the influence of DNA base sequence upon the manifestation of PTEs was further investigated using synthetic oligonucleotides treated with a variety of alkylating agents.

6.2 Effect of sequence upon the frequency of adduct formation and mutation

6.2.1 Influence of DNA sequence upon the manifestation of mutation hotspots

It has been established that certain sites within genes, known as hotspots, are more frequently mutated than other sites. Previous studies have reported that the manifestation of mutation hotspots is influenced by DNA sequence. Several independent studies have shown that mutations occur more frequently at sites preceded 5' by a purine in *E. coli* and yeast treated with a variety of alkylating agents including *N*-nitroso-*N*-methyl-*N*-α-acetoxybenzylamine (NMAB), MNNG, MNU and ENU (Horsfall and Glickman, 1988; Burns *et al.*, 1988, 1987a and 1987b; Richardson *et al.*, 1987; Lee *et al.*, 1992). Lee *et al.* (1992) examined the local sequence of hotspots induced in yeast treated with four different alkylating agents: MMS, EMS, MNU and ENU. Five hotspots were identified in the URA3 locus and they varied in

responsiveness to the four different alkylating agents. The base immediately 5' to the point mutation in 4 out of 5 of the hotspots noted was a guanine. In contrast, there was no clear preference for the base 3' to the hot spot.

There is evidence that suggests that the frequency of mutation may be influenced by the DNA base sequence beyond the first base found immediately 5' to a given site. Lee *et al.* (1992) noted that the sequence 5'-AGGT-3' (mutated base underlined) of the only common hotspot for all four alkylating agents is found at three other locations in the URA3 locus, yet no mutational events were found at these sites. Levy *et al.* (1996) observed that a single base substitution in the *supF* marker gene could influence the frequency of mutation up to 80 bp downstream from the site of modification upon exposure to UV radiation.

Three possible explanations that have been proposed to account for the preferential induction of mutations at certain sites include: (i) certain sites are more readily modified; (ii) a promutagenic lesion in certain sequences may miscode with a much higher frequency; and/or (iii) repair of promutagenic lesions in certain sequences may be less efficient (Section 4.3). There has been support for all three possibilities.

6.2.2 Sequence influences adduct formation

Alkylating agents have been noted to favour reaction within runs of dGs. The level of alkylation by MNU upon N7-dG and O^6-dG was found to be greater when the modified dG was preceded 5' by a purine rather than a pyrimidine in oligonucleotides treated *in vitro* with MNU (Dolan *et al.*, 1988). Briscoe and Cotter (1984 and 1985) treated a series of oligonucleotides with MNU and then determined the type and quantity of adducts formed in each oligonucleotide (Table 6.1 and 6.2). DNA

*Table 6.1: Quantities of methylated guanines in MNU treated polynucleotides (Briscoe and Cotter, 1985). Values expressed as: *µmol/mol dG and ** %.*

	Base sequence	N3-methyl dG*	N7-methyl dG*	O^6-methyl dG*	O^6-methyl dG/ N7-methyl dG**	Total adducts*
Poly(dG):Poly(dC)	GGG	20 ± 5	2397 ± 74	242 ± 8	10.1 ± 0.2	2659
Poly(dG-dC):Poly(dG-dC)	CGC	56 ± 6	1141 ± 74	239 ± 18	21.0 ± 0.5	1436
Poly(dA-dC):Poly(dG-dT)	TGT	45 ± 7	1191 ± 65	127 ± 16	10.7 ± 1.6	1363
Poly(dA-dG):Poly(dC-dT)	AGA	47 ± 6	1437 ± 83	186 ± 16	13.0 ± 0.8	1670
CT-DNA	NGN	73 ± 10	2036 ± 181	282 ± 38	13.8 ± 0.9	2391

*Table 6.2: Quantities of methylated adenines in MNU treated polynucleotides (Briscoe and Cotter, 1985). Values expressed as: * µmol/mol dA;** %; n.d. = not detectable (<8 – 10 µmol/mol).*

	Base sequence	N3-methyl dA*	N1-methyl dA + N7-methyl dA*	N1-methyl dA + N7-methyl dA / N3-methyl dA**	Total adducts*
Poly(dA):Poly(dT)	AAA	209 ± 38	42 ± 5	20	251
Poly(dA-dT):Poly(dA-dT)	TAT	68 ± 5	n.d.	-	68
Poly(dA-dC):Poly(dG-dT)	CAC	195 ± 33	33 ± 5	17	228
Poly(dA-dG):Poly(dC-dT)	GAG	178 ± 24	88 ± 11	49	266
CT-DNA	NGN	200 ± 27	65 ± 8	33	265

sequence was found to influence the frequency of DNA adducts formed relative to one another, although not the overall rank order of abundance. The three major adducts formed by MNU in double stranded DNA are N7-methyl dG, O^6-methyl dG and N3-methyl dG (in order of decreasing abundance) (Table 6.1). The frequency N7-methyl dG relative to O^6-methyl dG and N3-methyl dG was significantly lower on poly(dGdC):poly(dGdC) than on any of the other oligonucleotides or CT-DNA. Kohn *et al.* (1987) also noted that the presence of dC 5' and 3' to the target dG suppressed alkylation by most nitrogen mustards upon N7 atom of dG. In contrast, the presence of dT 5' and 3' to the target nucleotide did not influence the formation of adducts upon N7-dG indicating that this property is specific to dC and not pyrimidines in general. It was proposed that the reason alkylation may be suppressed upon the N7 atom of a dG 5' to a dC may be because the amino group of the 3' dC lies in close proximity to the 5' dG N7. The amino group of the 3' dC exerts a positive electrostatic field with its dipole in the vicinity of the dG N7, thereby countering the negative electrostatic potential of the dG N7 (Mattes *et al.*, 1986; Kohn *et al.*, 1987).

The proportion of N3-methyl dG relative to O^6-methyl dG and N7-methyl dG was significantly lower upon poly(dG):poly(dC) than on the other oligonucleotides and CT-DNA. However, the presence of dA 5' and 3' to the target nucleotide did not inhibit the formation of N3-methyl dG (Briscoe and Cotter, 1985).

MNU reacts with dA at three sites; in order of decreasing importance: N3-dA, N7-dA and N1-dA (Table 6.2). The fewest total adducts upon dA was found upon the poly(dAdT):poly(dAdT) oligonucleotide (the levels of N7-methyl dA and N1-methyl dA fell below the limit of detection of the assay). The frequency of N7- and N1-methyl dA relative to N3-methyl dA was influenced by sequence. A greater proportion of N7- and N1-methyl dA relative to N3-methyl dA was formed at sites

with a dG immediately 5' and 3' to dA. In contrast, the presence of dA 5' and 3' to the target nucleotide did not promote the formation of N7- and N1-methyl dA relative to N3-methyl dA (Briscoe and Cotter, 1985).

6.2.3 Mechanism by which sequence may influence adduct formation

All the evidence suggests that the non-random manifestation of DNA adducts is more likely to be the rule rather than the exception. The means by which neighbouring bases affect the potential of a purine residue to undergo alkylation at specific atoms is unknown. The arrangement of bases in a polynucleotide may affect the electron distribution about an individual base's atomic structure due to base stacking and other interactions, sufficient to affect its reactivity with an alkylating agent.

Cloutier *et al.* (1999) reported that the distribution profiles of piperidine-sensitive methyl purines in guanine runs differed between S_N1 and S_N2 monofunctional, alkylating agents. The S_N1 agents, MNU and NDMAOAc, and most nitrogen mustards (bifunctional, S_N2 alkylating agents) preferentially methylate the central guanine bases in guanine runs (Cloutier *et al.*, 1999; Hartley *et al.*, 1986; Kohn *et al.*, 1987; Mattes *et al.*, 1986). In contrast, the S_N2 agent, DMS, preferentially methylates guanine bases at the 5' end of guanine runs (decreasing intensity from the 5' to the 3' end).

Theoretical calculations by Pullman and Pullman (1981) on the electrostatic potential of DNA indicated that molecular electrostatic potential (MEP) is sequence dependent. In particular it was found that the N7 position of dG flanked 5' and 3' by other dGs have a considerably more negative MEP than an isolated dG. Using the MEP calculated for various 5' XGX 3' sequences, Kohn *et al.* (1987) observed a

remarkable correlation of negative MEP with the reaction intensity for most nitrogen mustards. The molecular rationale advanced for this phenomenon was that the positively charged alkylating moiety would be selectively drawn towards regions with a more negative MEP.

Small molecular weight alkylating agents in general do not possess non-covalent DNA binding capabilities, except for charge attraction. However, a detailed study of the effect of salt upon DNA alkylation suggested that the electrostatic potential might well be performing more than a collecting function (Kohn *et al.*, 1987; Wurdeman and Gold, 1988). In general, salts reduced the magnitude, but not the order of selectivity (i.e., rate differences) for different sequences. Thus bonding selectivity remained even under conditions (high ionic strength) that made ionic binding interactions extremely weak. These observations suggest that a sequence-dependent MEP may also operate on the covalent bonding step, perhaps by stabilising an incipient positive charge on an electrophile in the transition state (Warpehoski and Hurley, 1988).

MEP is a good predictor of the reaction profile of S_N1 type alkylating agents. The two sites with the most negative MEP are the phosphate oxygens on the sugar-phosphate backbone followed by the N7 atom of dG (Pullman and Pullman, 1981). However, whilst PTEs comprise a high proportion of the total alkylation products induced by agents with a strong S_N1 character, they form a relatively small proportion of alkylation products formed by S_N2 alkylating agents. It has been proposed that the π ionisation energy of a site may be better predictor of the reaction patterns of S_N2 type alkylating agents than the MEP. Base π ionisation energy is a marker of the ease with which electronic rearrangement occurs that is necessary to accommodate significant charge transfer in the transition state (Kim *et al.*, 1999). Unlike the S_N1

mechanism of nucleophilic substitution, which involves attack by a preformed electrophile, S_N2 alkylating agents form a transition state complex with the nucleophile that results in the transfer of the alkyl group to the nucleophile on the DNA. Kim *et al.* (1999) investigated the influence of base sequence upon the π ionisation energy of N7-dG in model hexamers containing stacked base pairs without the sugar phosphate backbone. It was found that guanines flanked 5' and/or 3' by a purine base have a lower ionisation potential than guanines flanked 5' and 3' by pyrimidines (Kim *et al.*, 1999) (Table 6.3). The ionisation potential was most negative in poly(dG) runs (Saito *et al.*, 2000). It was found that in double stranded oligonucleotides (with a sugar phosphate backbone) containing poly(dG) runs with 3 or 4 guanines, that the interior guanines had the lowest ionisation potential (Zhu and LeBreton, 2000).

6.2.4 Influence of local sequence upon base mispairing

There is evidence that local sequence may influence the ability of O^6-methyl dG to induce mispairing. Singer *et al.* (1989) found that the frequency of the incorporation of dT opposite O^6-methyl dG by *E. coli* DNA polymerase I was 6 to 7-fold greater than when the lesion was flanked 3' by dT as opposed to dC. Similar sequence preferences were observed for Drosophila polymerase α (Singer and Dosanjh, 1990). Similarly, Essigmann and co-workers found that there was a 10-fold increase in the frequency of misincorpation of dT base opposite O^6-methyl dG in hexamer sequence inserted into M13 DNA for *in vivo* replication when the modified base was flanked 3' by a dT rather than a dC. The frequency of the misincorporation of guanine opposite O^4-methyl dT in a hexamer sequence inserted into M13 DNA for *in vivo* replication increased approximately 10-fold when the modified base was

Table 6.3: Guanine adiabatic ionisation potentials in self complementary model oligonucleotide hexamer duplexes (adapted from Kim et al. (1999)).

Sequence		Ionisation Potential (eV)
5'-dAGGCCT-3'	AGG	6.36
5'-dACCGGT-3'	CGG	6.46
5'-dACTAGT-3'	AGT	6.78
5'-dACCGGT-3'	GGT	6.82
5'-dAGGCCT-3'	GGC	6.85
5'-dACATGT-3'	TGT	6.92
5'-dACGCGT-3'	CGC	6.94
5'-dACGCGT-3'	CGT	6.98

flanked 3' by a dG rather than dA (Basu and Essignmann, 1988).

The mechanism behind the difference in the frequency of misincorporation within different base sequences is presently unknown. In the absence of repair O^6-methyl dG and O^4-methyl dT can exhibit widely differing mutation frequencies depending upon their local DNA sequence. This variation in the frequency of misincorporation in damaged DNA in different base sequences may contribute towards the formation of mutational 'hot spots' (Singer *et al.*, 1989; Singer and Dosanjh, 1990).

6.3 Specific aims of study

- To investigate the effect of base sequence upon phosphate alkylation *in vitro*.
- To investigate whether the manifestation of PTEs varies between different alkylating agents.
- To determine whether the single stranded or double stranded nature of the oligonucleotides influences the manifestation of PTEs.

To investigate whether base sequence influences phosphate alkylation synthetic oligonucleotides were treated with a variety of alkylating agents. Two oligonucleotides were synthesised: 5'-$[dC]_{20}[dA]_{20}$-3' and 5'-$[dT]_{20}[dG]_{20}$-3'. The oligonucleotides were annealed together at a ratio of 1:0, 1:1, 1:2 and 1:5. The oligonucleotides were treated *in vitro* with four different alkylating agents. The four alkylating agents were chosen because of their differing Swain-Scott substrate constants (s): ENU(s = 0.26), MNU (s = 0.42), DES (s = 0.64), and DMS (s = 0.81).

6.4 Results

6.4.1 Formation of duplexes

It was important to ensure that duplexes were being formed by the method used to anneal the two oligonucleotides. An aliquot of 5'-$[dT]_{20}[dG]_{20}$-3' was 5' ^{32}P end labelled and annealed with unlabelled 5'-$[dC]_{20}[dA]_{20}$-3' as described in the Materials and Methods (Section 2.9.3). An aliquot of 5'-$[dC]_{20}[dA]_{20}$-3' and 5'-$[dT]_{20}[dG]_{20}$-3' were also labelled and analysed alongside the annealed oligonucleotides as markers. The products were separated upon a 30% non-denaturing gel and analysed by autoradiography and storage phosphorimage analysis. Figure 6.1a shows an autoradiograph of the gel. Figure 6.1b shows the relative intensity of the various bands upon the polyacrylamide gel. Some of the 5'-$[dT]_{20}[dG]_{20}$-3' oligonucleotides in the 1:0 oligonucleotide mix were behaving as higher molecular weight species (slow migrating bands) (35% of labelled oligonucleotides). Regions rich in dG are known to be able to adopt a poly(dG) quadruplex structure. The intensity of the slow migrating bands was appreciably reduced upon duplex formation. The vast majority of the oligonucleotides were found to be present in the duplex formation in the 1:1, 1:2 and the 1:5 oligonucleotide mixes following annealing (93 – 98% of labelled oligonucleotide). In contrast, the 5'-$[dC]_{20}[dA]_{20}$-3' oligonucleotides (CA (0:1)) did not give rise to any slow forming bands upon the polyacrylamide gel.

6.4.2 Treatment of oligonucleotides with various alkylating agents

The oligonucleotides (unlabelled, single stranded or double stranded) were treated with 0, 10 or 50 mM of alkylating agent (3 hours, 37°C) in PBS. The oligonucleotides were then precipitated using isopropanol and the pellet was washed

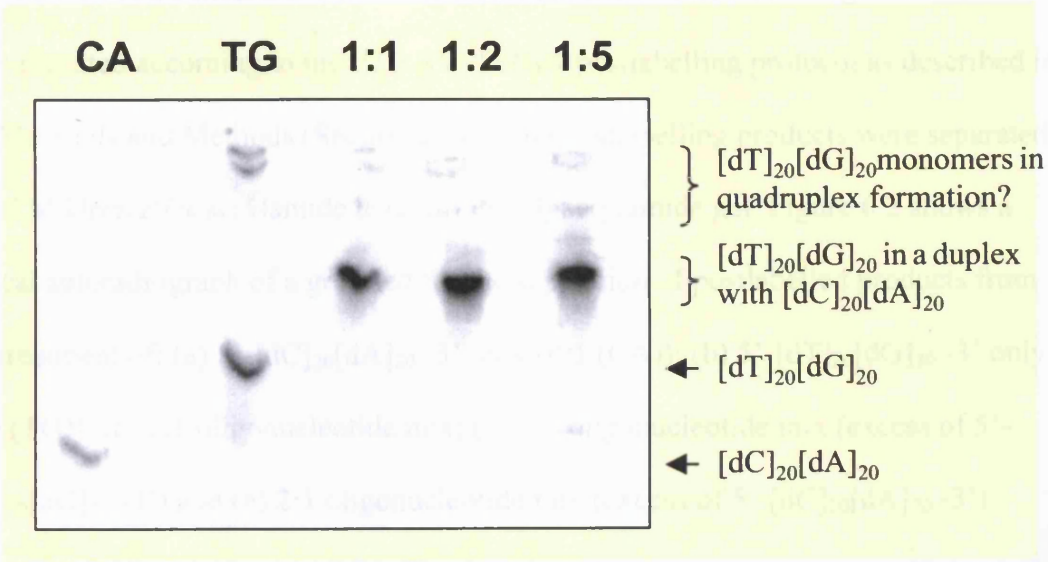


Figure 6.1a: Separation of the oligonucleotides on a 30% non-denaturing polyacrylamide gel following annealing.

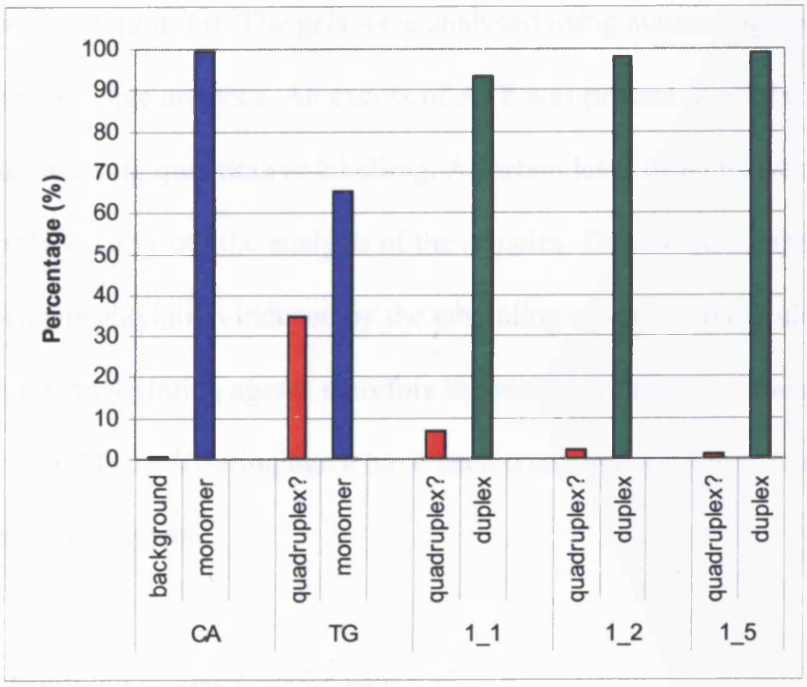


Figure 6.1b: Relative frequency of oligonucleotides in the monomer, duplex and quadruplex formation upon the polyacrylamide gel.

twice with 95% ethanol. The pellet was dried down in a centrifugal evaporator and resuspended to a concentration of 1μg/ μl in deionised water. The oligonucleotides were digested according to the PTE SVPD/NP1 postlabelling protocol as described in the Materials and Methods (Section 2.10). The postlabelling products were separated on a 7M Urea/20% acrylamide denaturing polyacrylamide gel. Figure 6.2 shows a typical autoradiograph of a gel used for the separation of postlabelled products from the treatment of: (a) 5'-$[dC]_{20}[dA]_{20}$ -3' only (0:1 (CA)); (b) 5'-$[dT]_{20}[dG]_{20}$ -3' only (1:0 (TG)); (c) 1:1 oligonucleotide mix; (d) 1:2 oligonucleotide mix (excess of 5'-$[dT]_{20}[dG]_{20}$ -3') and (e) 2:1 oligonucleotide mix (excess of 5'-$[dC]_{20}[dA]_{20}$ -3') treated with 10 and 50 mM MNU. The four dinucleotides species were sufficiently separated by PAGE to enable analysis. The order in which the dinucleotide species ran on the gel was: ^{32}pdGpdG<< ^{32}pdTpdT< ^{32}pdApdA<< ^{32}pdCpdC (in order of increasing rate of migration). The gels were analysed using autoradiography and storage phosphorimage analysis. An excess of ATP was present in all the samples analysed thus ensuring quantitative labelling. A certain level of PTEs was required in order to be able to carry out the analysis of the samples. Due to the comparatively low level of phosphate alkylation induced by the ethylating agents, in particular ENU, compared to the methylating agents therefore the results for the oligonucleotides treated with 50 mM of ethylating agent have been compared with those treated 10 mM of methylating agents.

6.4.3 Treatment of oligonucleotides with ENU

ENU (s = 0.26) predominately reacts with DNA via the S_N1 mechanism. Table 6.4 shows the results for the quantification of total PTEs formed in oligonucleotides treated *in vitro* with ENU. There was an increase in the quantity of PTEs generated by

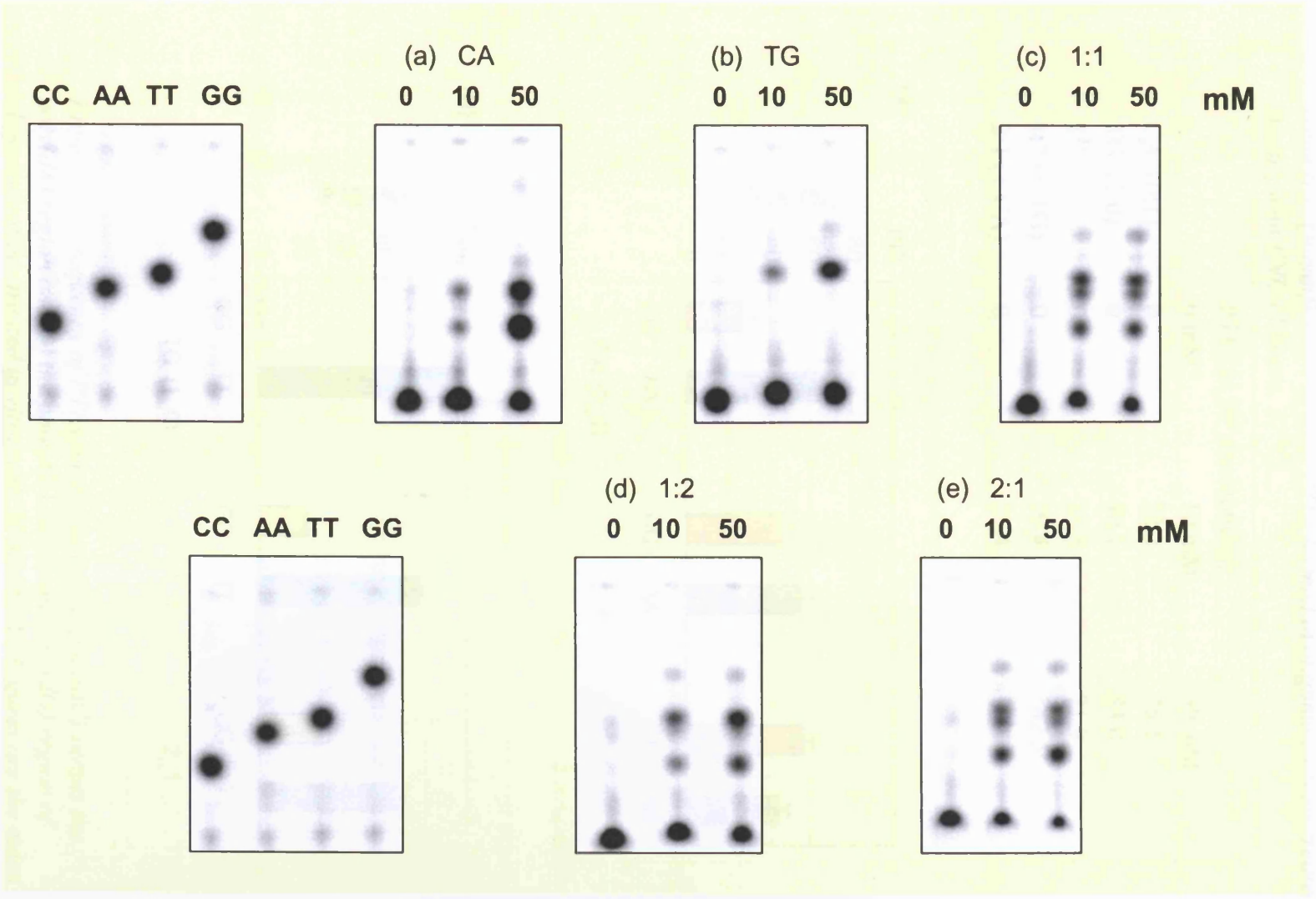


Figure 6.2: Typical autoradiographs for postlabelling gels used analyse the frequency of PTEs induced in different regions of oligonucleotides treated <u>in vitro</u> with MNU: (a) $[dC]_{20}[dA]_{20}$ only; (b) $[dT]_{20}[dG]_{20}$ only; (c) 1:1 oligonucleotide mix; (d) 1:2 oligonucleotide mix (excess of $[dT]_{20}[dG]_{20}$) and (e) 2:1 oligonucleotide mix (excess of $[dC]_{20}[dA]_{20}$).

Table 6.4: Quantification of total PTEs formed in oligonucleotides treated in vitro with ENU (3 hours, 37°C).

	PTE (1/ 10^6 nucleotides)		
	0 mM	10 mM	50 mM
CA (0:1)	0	22.3	75.1
TG (1:0)	0	40.1	83.0
1:1	0	23.5	71.9
1:2 (+ TG)	0	19.4	142.5
2:1 (+ CA)	0	25.4	130.4

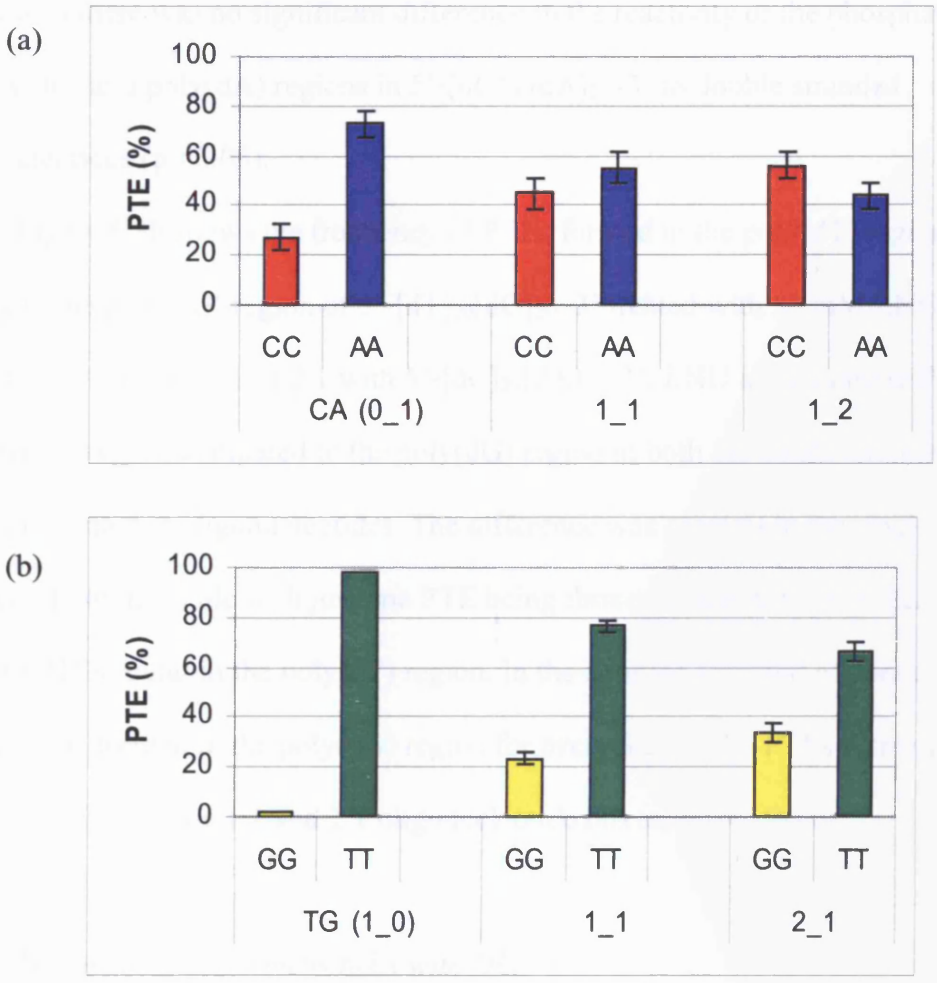


Figure 6.3: Frequency of PTEs formed in (a) the poly(dC) versus the poly(dA) region and (b) the poly(dT) versus the poly(dG) region of oligonucleotides treated in vitro with 50 mM ENU. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

ENU with an increase in dose. Figure 6.3a shows the results for the frequency of PTEs formed in the polyC region relative to the polyA region of 5'-$[dC]_{20}[dA]_{20}$-3' treated *in vitro* with 50 mM ENU when in a ratio of 0:1 (CA), 1:1 or 1:2 with 5'-$[dT]_{20}[dG]_{20}$-3'. The difference in the level of PTEs formed in the poly(dC) versus the poly(dA) region was greater in the single stranded oligonucleotides treated with ENU than in the double stranded oligonucleotides. In the single stranded oligonucleotides for every PTE formed in the poly(dC) region of the single stranded oligonucleotides approximately 2.7 PTEs were formed in the poly(dA) region. By comparison there was no significant difference in the reactivity of the phosphates in the poly(dC) and poly(dA) regions in 5'-$[dC]_{20}[dA]_{20}$-3' in double stranded oligonucleotides ($p > 0.05$).

Figure 6.3b shows the frequency of PTEs formed in the poly(dT) region relative to the poly(dG) region of 5'-$[dT]_{20}[dG]_{20}$-3' treated with 50 mM ENU when in a ratio of 1:0 (TG), 1:1 or 2:1 with 5'-$[dC]_{20}[dA]_{20}$-3'. ENU induces more PTEs in the poly(dT) region compared to the poly(dG) region in both the single stranded and the double stranded oligonucleotides. The difference was greatest in the single stranded oligonucleotide with just one PTE being formed in the poly(dG) region for every 44 PTEs found in the poly(dT) region. In the doubled stranded oligonucleotides one PTE was formed in the poly(dG) region for every 3.25 and 2 PTEs formed in the poly(dT) region for the 1:1 and 2:1 oligonucleotide mixes, respectively.

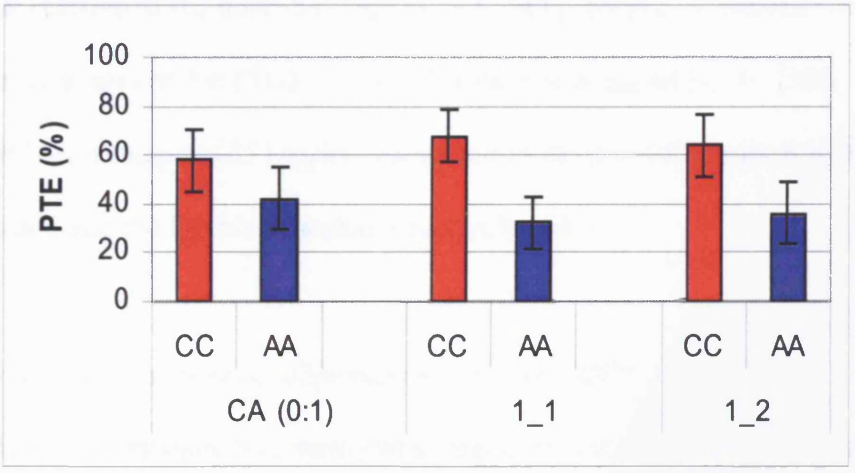
6.4.4 Treatment of oligonucleotides with DES

DES is an intermediate S_N2 ethylating agent ($s = 0.64$). Table 6.5 shows the results for the quantification of total PTEs formed in oligonucleotides treated *in vitro* with DES. There was an increase in the quantity of PTEs with an increase in dose of

Table 6.5: Quantification of total PTEs formed in oligonucleotides treated in vitro with DES (3 hours, 37°C).

	PTE (1/ 10^6 nucleotides)		
	0 mM	10 mM	50 mM
CA (0:1)	0	28.4	85.3
TG (1:0)	0	243.7	580.6
1:1	0	91.3	360.2
1:2 (+ TG)	0	99.9	318.9
2:1 (+ CA)	0	101.7	416.8

(a)



(b)

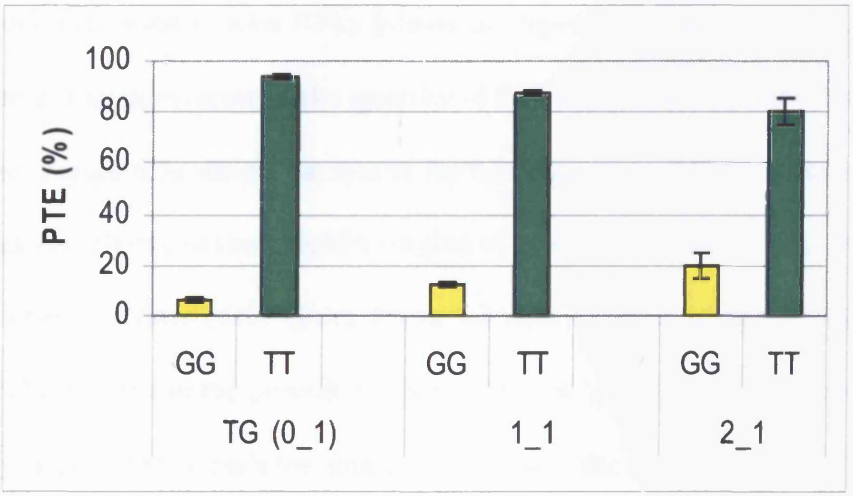


Figure 6.4: Frequency of PTEs formed in (a) the poly(dC) versus the poly(dA) region and (b) the poly(dT) versus the poly(dG) region of oligonucleotides treated in vitro with 50 mM DES. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

DES. Figure 6.4a shows the frequency of PTEs formed in the poly(dC) region relative to the poly(dA) region of 5'-$[dC]_{20}[dA]_{20}$-3' treated with 50 mM DES when in a ratio of 0:1 (CA), 1:1 or 1:2 with 5'-$[dT]_{20}[dG]_{20}$-3'. The frequency of PTEs formed in the poly(dC) region of 5'-$[dC]_{20}[dA]_{20}$-3' was greater than in the poly(dA) region in both the single stranded and the double stranded oligonucleotides. There was no significant difference between the results for the single stranded and doubled stranded oligonucleotides ($p > 0.1$).

Figure 6.4b shows the results for the frequency of PTEs formed in the poly(dT) region relative to the poly(dG) region of 5'-$[dT]_{20}[dG]_{20}$-3' treated with 50 mM DES when in a ratio of 1:0 (TG), 1:1 or 2:1 with 5'-$[dC]_{20}[dA]_{20}$-3'. DES induces more PTEs in the poly(dT) region compared to the poly(dG) region in both the single stranded and the double stranded oligonucleotides.

6.4.5 Results for the treatment of oligonucleotides with MNU

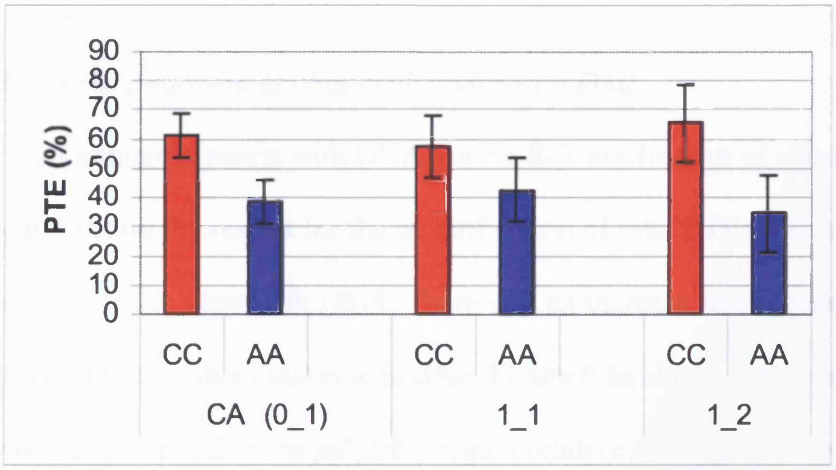
MNU is an intermediate S_N1 methylating agent ($s = 0.42$). Table 6.6 shows the results for the quantification of total PTEs formed in oligonucleotides treated *in vitro* with MNU. There was an increase in the quantity of PTEs generated by MNU with an increase in dose. Figure 6.5a shows the results for the frequency of PTEs formed in the poly(dC) region relative to the poly(dA) region of 5'-$[dC]_{20}[dA]_{20}$-3' treated with 10 mM MNU when in a ratio of 0:1 (CA), 1:1 or 1:2 with 5'-$[dT]_{20}[dG]_{20}$-3'.The frequency of PTEs formed in the poly(dC) region of 5'-$[dC]_{20}[dA]_{20}$-3' was greater than in the poly(dA) region in both the single stranded and the double stranded oligonucleotides.

Figure 6.5b shows the results for the frequency of PTEs formed in the poly(dT) region relative to the poly(dG) region of 5'-$[dT]_{20}[dG]_{20}$-3' treated with 10

Table 6.6: Quantification of total PTEs formed in oligonucleotides treated in vitro with MNU (3 hours, 37°C).

	PTE (1/ 10^6 nucleotides)		
	0 mM	10 mM	50 mM
CA only	0	2771.0	26180.5
TG only	0	599.4	3614.0
1:1	0	2553.2	3253.4
1:2 (+ TG)	0	2241.5	5065.2
2:1 (+ CA)	0	2608.2	4406.5

(a)



(b)

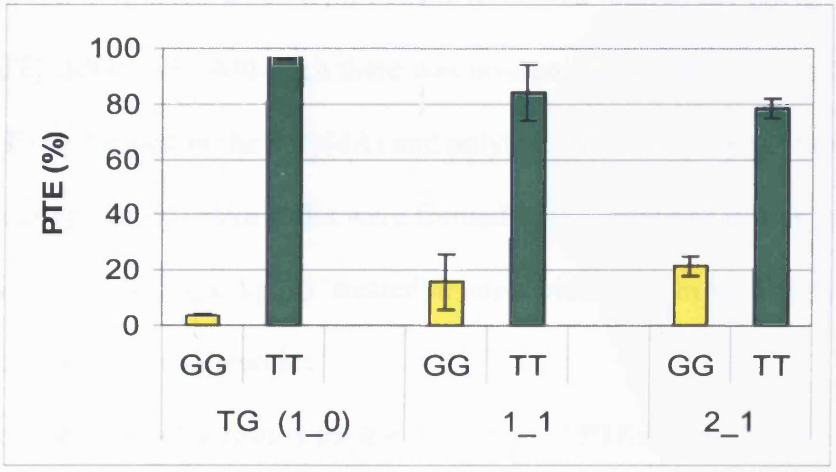


Figure 6.5: Frequency of PTEs formed in (a) the poly(dC) versus the poly(dA) region and (b) the poly(dT) versus the poly(dG) region of oligonucleotides treated in vitro with 10 mM MNU. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

mM MNU when in a ratio of 1:0 (TG), 1:1 or 2:1 with 5'-$[dC]_{20}[dA]_{20}$-3'. MNU induces more PTEs in the poly(dT) region compared to the poly(dG) region in both the single stranded and the double stranded oligonucleotides. The difference was greatest in the single stranded oligonucleotide with just one PTE being formed in the poly(dG) rich region for every 26 PTEs in the poly(dT) region. In the double stranded oligonucleotides, one PTE was formed in the poly(dG) region for every 5.3 and 3.7 PTEs formed in the poly(dT) region for the 1:1 and 2:1 oligonucleotide mixes, respectively.

6.4.6 Results for the treatment of oligonucleotides with DMS

DMS predominantly reacts with DNA via the S_N2 mechanism of alkylation (*s* = 0.81). Table 6.7 shows the results for the quantification of total PTEs formed in oligonucleotides treated *in vitro* with DMS. There was an increase in the quantity of PTEs generated by DMS with an increase in dose. Figure 6.6a shows the results for the frequency of PTEs formed in the poly(dC) region relative to the poly(dA) region of 5'-$[dC]_{20}[dA]_{20}$-3' treated with 10 mM DMS when in a ratio of 0:1 (CA), 1:1 or 1:2 with 5'-$[dT]_{20}[dG]_{20}$-3'. Although there was no significant difference in the frequency of PTEs formed in the poly(dA) and poly(dC) in the double stranded oligonucleotides ($p > 0.05$), more PTEs were formed in the poly(dA) versus the poly(dC) region of 5'-$[dC]_{20}[dA]_{20}$-3' treated *in vitro* with DMS in both the single and double stranded oligonucleotides.

Figure 6.6b shows the results for the frequency of PTEs formed in the poly(dT) region relative to the poly(dG) region of 5'-$[dT]_{20}[dG]_{20}$-3' treated with 10 mM DMS when in a ratio of 1:0 (TG), 1:1 or 2:1 with 5'-$[dC]_{20}[dA]_{20}$-3'. DMS induces more PTEs in the poly(dT) region compared to the poly(dG) region in both

Table 6.7: Quantification of total PTEs formed in oligonucleotides treated in vitro with DMS (3 hours, 37°C).

	PTE (1/ 10^6 nucleotides)		
	0 mM	10 mM	50 mM
CA (0:1)	0	104.3	367.8
TG (1:0)	0	133.7	179.0
1:1	0	171.3	1119.0
1:2 (+ TG)	0	166.3	433.6
2:1 (+ CA)	0	137.7	537.9

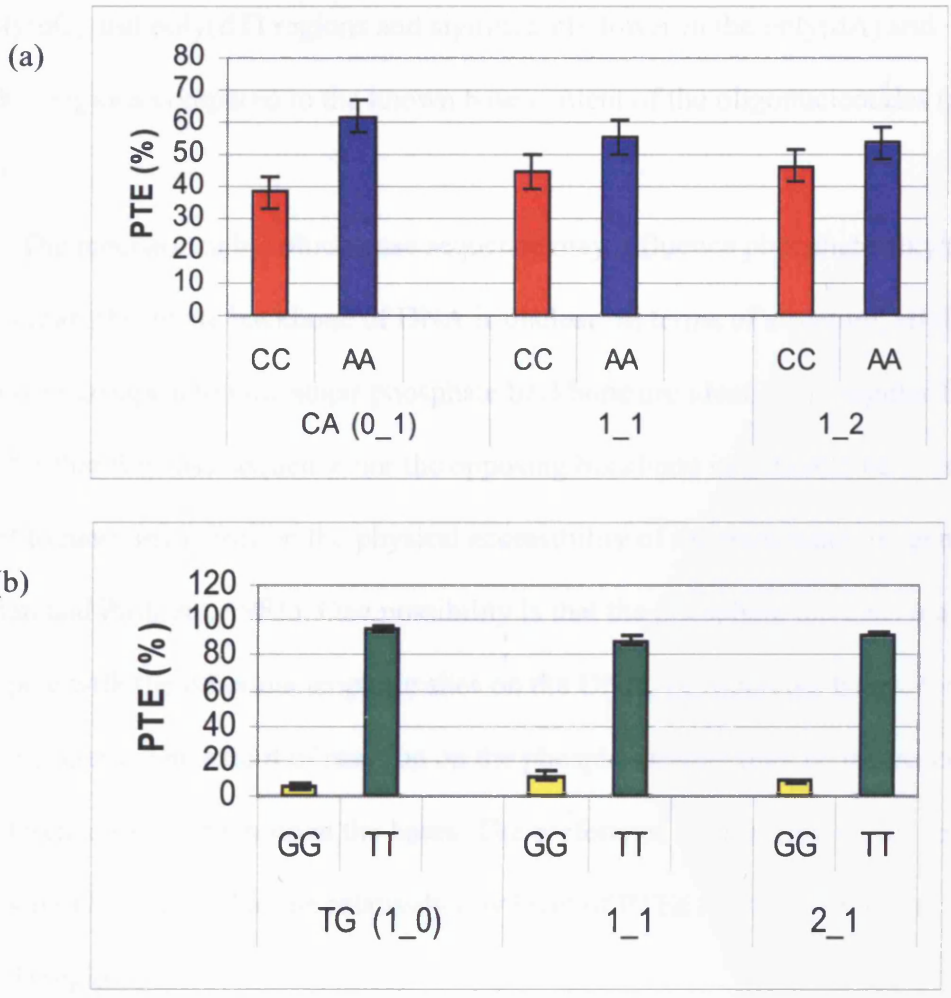


Figure 6.6: Frequency of PTEs formed in (a) the poly(dC) versus the poly(dA) region and (b) the poly(dT) versus the poly(dG) region of oligonucleotides treated in vitro with 10 mM DMS. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

the single stranded and the double stranded oligonucleotides.

6.5 Discussion

Figure 6.7 shows the frequency of PTEs formed in the different regions of the oligonucleotides that were annealed together in a ratio of 1:1. The results for the oligonucleotides treated with DES reflected the results for cell DNA and CT-DNA treated with DES *in vitro* in a PBS buffer system (Chapter 5). In the oligonucleotides treated *in vitro* with DES, the frequency of PTEs found was significantly greater in the poly(dC) and poly(dT) regions and significantly lower in the poly(dA) and poly(dG) regions compared to the known base content of the oligonucleotides ($p < 0.05$).

The mechanism by which base sequence may influence phosphate alkylation on the sugar-phosphate backbone of DNA is unclear. In terms of structure, all the phosphoryl groups upon the sugar phosphate backbone are identical in regular B-DNA. Neither the base sequence nor the opposing backbone in a double helix is thought to have any effect on the physical accessibility of the phosphate oxygens (Pullman and Pullman, 1981). One possibility is that the phosphate oxygens may have to compete with the other nucleophilic sites on the DNA, i.e. upon the bases, for the alkylating agent. The extent of reaction on the phosphodiesters may be influenced by the preference for reaction upon the bases. The preference for reaction with various sites upon dG could lead to the relatively low level of PTEs formed within the poly(dG) region.

In all instances the frequency of PTEs formed in the poly(dG) region was significantly lower than the frequency of PTEs formed in the poly(dC), poly(dA) and poly(dT) rich regions of the oligonucleotides ($p < 0.05$) (Fig. 6.7). The frequency of

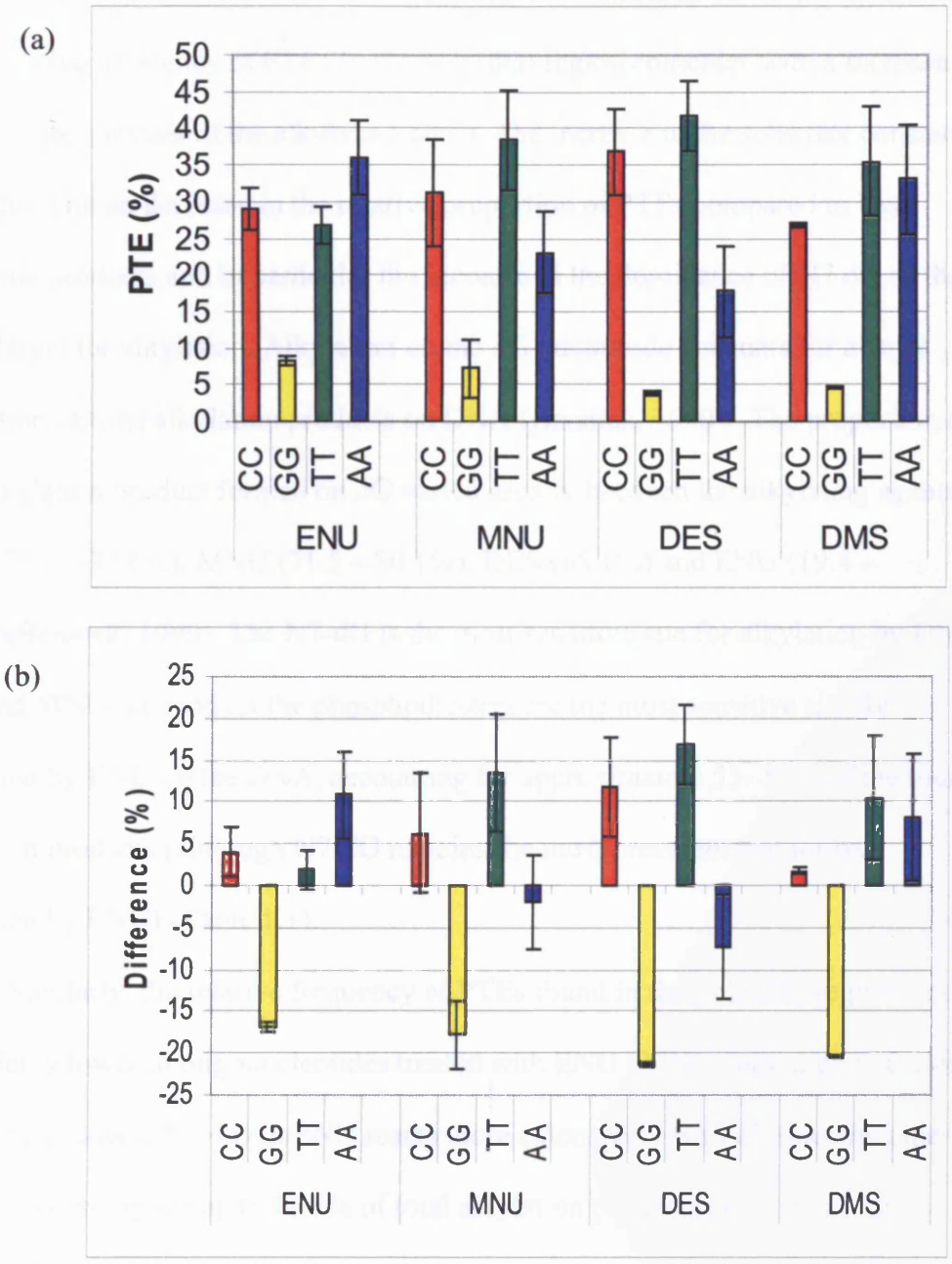


Figure 6.7: 5' NNA results for the 1:1 oligonucleotide mix treated with either 50 mM ethylating or 10mM methylating agent: (a) comparison of the frequency PTEs induced in the various regions of the oligonucleotide and (b) difference between the frequency PTEs and known base content. Figures are the mean of three independent experiments. Error bars = +/- standard deviation.

PTEs formed in the poly(dG) region was greatest in the oligonucleotides treated with ENU (8.3%) followed by MNU (7.2%), DMS (4.7%) and DES (3.7%). The decrease in the relative frequency of PTEs in the poly(dG) region coincides with a increase in the substrate constant of the alkylating agent. The increase in the substrate constant coincides with an decrease in the relative proportion of PTEs compared to base alkylation products and in particular the increase in the dominance of N7 dG as the major target for alkylation. Alkylation on the dG nucleoside accounts for a large proportion of total alkylation products on DNA (Beranek, 1990). The proportion of total alkylation product formed on dG varied greatly between the alkylating agents: DMS (72.3 – 77.6%), MNU (71.5 – 80.1%), DES (68.1%) and ENU (19.4 – 22.9%)(Beranek, 1990). The N7-dG is the most sensitive site for alkylation by DMS, DES and MNU. In contrast the phosphodiesters are the most sensitive site for alkylation by ENU on the DNA, accounting for approximately 55- 57% of the total alkylation products (although N7-dG remains the most prevalent site for base alkylation by ENU) (Table 1.1).

Similarly, the relative frequency of PTEs found in the poly(dT) region was appreciably lower in oligonucleotides treated with ENU (27%), compared to the other alkylating agents (35 – 42%). ENU reacts more extensively with dT than the other agents, accounting for up to 11.1% of total alkylation products compared to 0.1 - 1.3% for MNU, DES and DMS (Beranek, 1990). However, no relationship could be established between the relative frequency of PTEs found in the poly(dC) and poly(dA) region of the oligonucleotides and the known alkylation profile of the various alkylating agents upon the dC and dA bases.

Alternatively, local sequence may act by influencing the electronic characteristics of the sugar-phosphate backbone. As mentioned previously, work has

been carried out that shows that local sequence influences the electronic characteristics of nucleophilic sites on DNA bases (Pullman and Pullman, 1981; Kim *et al.*, 1999). Changes in the electronic characteristics of the various nucleophiles on the DNA bases have been correlated with changes in the extent of alkylation at different sites. It would be very interesting to find out whether local base sequence influences either the MEP or π ionisation energies of the DNA phosphate oxygens.

The ratio of PTEs found in the four regions of the oligonucleotides differed between the various agents (Table 6.8). The ratio of PTEs formed in the different regions was greater for the S_N2 alkylating agents (DES and DMS) than for the S_N1 agents (ENU and MNU). The rate-limiting step for S_N1 alkylation is the hydrolysis of the alkylating agent to generate the electrophile. Once formed the electrophile will react with the first suitable nucleophile. The rate limiting step for S_N2 alkylation is the formation of a complex between the alkylating agent and the nucleophile (on the DNA). S_N1 type alkylating agents are considered to be less selective with respect to nucleophilic strength than S_N2 type alkylating agents. This is reflected in the more even distribution of PTEs in the oligonucleotides treated with the two S_N1 alkylating agents.

It may be concluded that base sequence influences the formation of PTEs. This is the first time that it has been demonstrated that extent of phosphate alkylation is influenced by DNA base sequence. The mechanism by which base sequence influences the manifestation of PTEs could be the focus of future studies.

Table 6.8: Ratio of PTEs induced in 1:1 oligonucleotide duplex mix treated with either 50 mM ethylating or 10mM methylating agent.

	ENU (s = 0.26)	MNU (s = 0.42)	DES (s = 0.64)	DMS (s = 0.81)
CC	3.5	4.3	9.8	5.8
GG	1	1	1	1
TT	3.3	5.3	11.16	7.6
AA	4.3	3.2	4.7	7.1

Chapter 7:

Summary and Conclusions

7.1 Introduction

Previously it has been reported that the frequency of nucleosides 5' to the site of PTE lesions was random *in vitro,* but non-random *in vivo*. It was found that in liver DNA of mice treated *in vivo* with NDEA the frequency of dT was significantly greater and the frequency of dG was significantly lower 5' to the site of PTE lesions with respect to normal nucleoside content. In contrast, in studies using CT-DNA treated *in vitro* with either DES or DMS the frequency of nucleosides 5' to PTEs was found to reflect normal nucleoside content (Guichard *et al.*, 2000).

Guichard *et al.* (2000) established the identity and frequency of nucleosides located 5' to the PTE lesions by NP1 digestion of the PAGE isolated ^{32}pdNpdN species and analysis of the released mononucleotides (^{32}pdN) by HPLC with radioactivity detection. The advantages of separating and isolating the radiolabelled species by PAGE were two fold: (i) it allowed the quantification of PTEs prior to 5' NNA and (ii) it effectively removed excess [γ^{32}P]ATP that would otherwise have interfered with the HPLC analysis. However, the key drawback of the original 5' NNA protocol was that it was both time consuming and highly manipulative, involving the direct handling of radiolabelled material of high specific activity. Furthermore, the original 5' NNA protocol frequently resulted in the loss of labelled material (particularly during the desalting of the gel isolated material prior to NP1 digestion). Consequently it was desirable to develop and establish an alternative protocol for PTE 5' NNA, ideally avoiding the gel isolation of the ^{32}pdNpdN species.

7.2 Development of the 'direct' 5'NNA protocol

As part of the present study (Chapter 3) an alternative method, namely the 'direct' 5'NNA protocol, was developed for the preparation of samples for PTE

5'NNA. Using prepared ^{32}pdNpdN species it has been demonstrated that the sequential use of apyrase, boiling and NP1 digestion led to the >80% release of ^{32}pdN species. The advantage of the 'direct' method is that it is a simpler and less time consuming method of preparing samples for 5' NNA. It avoids the direct handling of radioactive material and allows for all of the reactions to be carried out within a single tube therefore lessening the likelihood of material loss. The direct protocol also allows for the inclusion of the dNpR species in the 5'NNA assessment. These species generated by the alkali treatment of dNp(R)dN, are radiolabelled by incubation with [γ^{32}P]ATP/ T4PNK, but were separated from the ^{32}pdNpdN species by PAGE and so therefore were not included in the original 5' NNA.

We sought to establish the new 'direct' 5'NNA protocol through the analysis of samples previously studied. Analysis of liver DNA from mouse treated *in vivo* with NDEA revealed a significantly higher frequency of dT and a significantly lower frequency of dG manifest 5' to PTEs. These observations confirmed the non-random manifestation of PTEs *in vivo* reported by Guichard *et al.* (2000). However the analysis of CT-DNA treated *in vitro* with DES revealed significant differences between the frequency of nucleosides 5' to PTEs and the normal nucleoside content. There was a significantly greater frequency of dC in CT-DNA treated with 10 and 5 mM DES and a significantly lower frequency of dG manifest 5' to PTEs in the CT-DNA treated with 5 mM DES compared to the normal nucleoside content of CT-DNA. This is contrary to the previous findings, as reported by Guichard *et al.* (2000), that the frequency of nucleosides found 5' to PTEs was not significantly different to normal nucleoside content in CT-DNA treated *in vitro* with DES. There are a number of factors that could account for the contrary nature of these results. Firstly, there is the inclusion of the dNpR species in the 'direct' 5' NNA assessment, however dNpR

species only account for 0 – 10% of the products generated by alkali hydrolysis of the dNp(R)dN species (Saris *et al.*, 1993 and 1995). Secondly, there were differences in the normal nucleotide content between the two respective studies, with the values obtained by the present study being almost identical to literature values (Fasman, 1975). Perhaps most significant is the fact that in the present study the data generated is the result of eight independent determinations allowing for rigorous statistical analysis. In the previous study the data was presented as two single determinations, thus not being suitable for any statistical analysis (Guichard *et al.*, 2000).

7.3 Influence of multiply damaged sites upon the SVPD/NP1 postlabelling assay

The SVPD/NP1 postlabelling assay can detect tandem damage. However where two lesions are separated by one or more normal nucleotides they will behave as two separate independent lesions with respect to the SVPD/NP1 postlabelling assay. This has been demonstrated for a variety of adducts including phosphoglycolates, PTEs and abasic sites (Bowman *et al.*, 2001).

7.4 Change in the frequency of nucleosides found 5' to PTEs in mouse liver DNA over a timescale of 56 days

In further studies (Chapter 4), the SVPD/NP1 postlabelling assay was used to measure the level of PTEs in liver DNA of Balb/c mice up to 56 days following a single intra peritoneal dose of NDEA. The level of PTEs continued to increase up to 10 hours after treatment. Thereafter there was a rapid decline in the level of PTEs between 10 hours and 4 days, followed by a steady decline in the quantity of PTEs between 7 and 56 days. The level of PTEs detected at 56 days was approximately

15% of that measured at 10 hours after dosing. PTEs had an initial half life of ~2 days in the liver DNA extracted from mice.

As part of a previous study, the level of O^6-ethyldG and N7-ethyldG has been measured in these samples (Oreffo *et al.*, 2000). Both O^6-ethyldG and N7-ethyldG are known to be actively repaired in higher eukaryotes. Previous studies using these samples reported that the level of O^6-ethyldG and N7-ethyldG fell below the limits of detection at 4 and 28 days respectively. The half life of O^6-ethyldG and N7-ethyldG in these samples was found to be approximately 24 hours and 7 days, respectively (Oreffo *et al.*, 2000). In the present study, a detectable level of PTEs was found even 56 days following a single dose of NDEA. It has been shown in previous studies that PTEs are relatively persistent and resistant to repair *in vivo*, with a half life that exceeds that of all other DNA alkylation products (Shooter and Slade, 1977; Bodell *et al.*, 1979; Den Englese *et al.*, 1986 and 1987). Consequently, PTEs were proposed to be good long term markers of genotoxic exposure and cumulative genotoxic damage (Shooter, 1978).

Using the new 'direct' 5'NNA protocol we investigated whether the manifestation of PTEs in Balb/c mouse liver DNA changed over time following treatment with a single intra peritoneal dose of NDEA (90 mg/ kg). We analysed the frequency of nucleosides found 5' to PTEs in liver DNA from mice over a timescale ranging from 5 hours to 56 days after treatment. Initially there was no significant change in the frequency of bases found 5' to PTE lesions, when the rate of decline in the level of PTEs was at its greatest (10 hours - 4 days). However, a significant difference was found between the frequency of nucleosides found 5' to PTE at 5 hours after treatment and the frequency of dG at 28 and 56 days and dT at 56 days after treatment.

However, considering that when the level of the PTE alkyltransferase 'suicide' protein is unlimited repair is complete within hours (McCarthy *et al.*, 1983), it seems unlikely that it would take 28 days to register a repair-mediated change in the frequency of nucleosides 5' to PTEs. The frequency of nucleosides 5' to PTEs remained non-random with respect to normal nucleoside content even at 56 days following treatment. If repair was a major contributing factor towards the initial non-random manifestation of PTEs it would be expected that any preference for repair would become increasingly non-random over time. In contrast, the difference between the frequency of nucleosides 5' to PTEs and normal nucleoside content decreased over the duration of the experiment. Therefore, it seems unlikely that repair plays a significant role in the development of the non-random manifestation of PTEs. A number of factors other than repair could be contributing towards the change in the frequency of nucleosides 5' to PTEs and normal nucleoside content for example: (i) experimental error due to the low level of PTEs present at these time points and/or (ii) PTEs may be more stable within certain sequences.

Since to date no PTE repair mechanism has been demonstrated in higher eukaryotes, it was proposed that the apparent rapid decline in the level of PTEs observed between 10 hours and 4 days, could due to an increase in cell proliferation by the liver tissue in response to an insult (regenerative hyperplasia). However, the initial decline in the level of PTEs appears to exceed the rate of decline of N7-ethyldG, an adduct known to be subject to repair, in the same samples (Oreffo *et al.*, 2000). These results were suggestive of there being some form of active repair of PTEs in liver tissue of Balb/c mice. The initial half life of the ethyl PTEs in the present study was considerably shorter than has been previously reported, with a half life of ~2 days compared to 10 – 15 weeks as reported by Shooter and Slade (1977).

7.5 The manifestation of PTEs in whole cells, isolated nuclei and DNA treated *in vitro* with DES

It was important to develop an *in vitro* model with which to study the non-random manifestation of PTE lesions. It was found that the frequency of nucleosides 5' to PTE lesions was non-random with respect to normal base content in two cell lines treated *in vitro* with a direct alkylating agent, DES (Chapter 5). In order to investigate the mechanism behind the non-random manifestation of PTEs in cells, nuclei and DNA were isolated from the cells and treated *in vitro* with DES. The same non-random manifestation of PTEs was found isolated nuclei and DNA as observed in the whole cells treated *in vitro* with DES. There was no consistent significant difference in the frequency of nucelosides found 5' to PTEs between the whole cells, isolated nuclei and DNA from the same cell line treated *in vitro* with DES ($p < 0.05$). In all instances the frequency of pyrimidines was found to be greater and the frequency of purines lower 5' to PTEs than expected compared to the normal nucleoside content of the cell DNA. It was concluded that nuclear structure and putative PTE repair do not significantly influence the non-random manifestation of PTEs at the nucleotide level in cells treated *in vitro* with DES.

7.6 The manifestation of PTEs in CT-DNA treated *in vitro* with DES in PBS

The observation that the manifestation of PTEs was non random in DNA isolated from cells treated *in vitro* with DES, conflicts with the findings of the previous study (Guichard *et al.*, 2000). The difference in the manifestation of PTEs might, at least in part, be due to the different reaction conditions. Guichard *et al.* (2000) treated CT-DNA in 0.5M sodium phosphate buffer (pH 6) for 8 hours at room temperature. In the present study, cell DNA and isolated nuclei were both treated in

PBS buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.2) for 3 hours at 37°C. An experiment was conducted with CT-DNA treated *in vitro* with DES in PBS buffer for either 3 hours at 37°C or 8 hours at room temperature. As found in the previous studies using isolated cell DNA (section 5.4.4), manifestation of PTEs in CT-DNA treated in PBS buffer for either 3 hours at 37°C or 8 hours at room temperature was non-random. The frequency of pyrimidines was significantly lower and the frequency of purines significantly greater 5' to PTEs compared to normal nucleoside content of CT-DNA. The 0.5 M sodium phosphate buffer, as used by Guichard *et al.* (2000) contains a very high concentration of free phosphate that will compete with the various nucleophiles on the DNA for the alkylating agent and could therefore influence the manifestation of PTEs.

7.7 The influence of DNA base sequence upon the manifestation of PTEs

To investigate the effect of sequence and the nature of the alkylating agent upon the manifestation of PTEs, prepared homopyrimidine-homopurine oligonucleotides were treated *in vitro* with a variety of alkylating agents, including: ENU, MNU, DES and DMS, chosen because of their different alkylating character (Chapter 6). As found previously in cells, nuclei and isolated DNA treated *in vitro* with DES, the frequency of purines was found to be lower and the frequency of pyrimidines greater 5' to PTE lesions in the oligonucleotides treated *in vitro* with DES compared to the known nucleoside content (Fig. 6.7b). The frequency of PTEs formed in the poly(dG) region was significantly lower compared to the base content in the double stranded oligonucleotides treated with all four alkylating agents. However, the relative frequency of PTEs formed in the poly(dC), poly(dT) and poly(dA) varied greatly between the four alkylating agents.

As mentioned previously, the frequency of PTEs was significantly lower in the poly(dG) region than in any other region of the oligonucleotides for all the alkylating agents tested. However, the overall difference between the frequency of nucleosides found 5' to PTEs and nucleoside content was greater for the S_N2 type agents (DES and DMS) than the S_N1 type agents (ENU and MNU) (Figure 6.7b). This reflects what is known about the mechanism of action of the two classes of agents. The rate-limiting step for S_N1 alkylation is the hydrolysis of the alkylating agent to generate the electrophilic species. Once formed the electrophile will react with the first suitable nucleophile. The rate-limiting step for S_N2 alkylation is the formation of a complex between the electrophile and the nucleophile (on the DNA). S_N2 type alkylating agents are considered to be more selective with respect to nucleophilic strength than S_N1 type alkylating agents. The various nucleophiles on the DNA must compete for the S_N2 type alkylating agent, which may account for the fact that the manifestation of PTEs induced by the S_N2 alkylating agents is more non-random than for the S_N1 type alkylating agents.

Local sequence may also act by influencing the electronic characteristics of the sugar-phosphate backbone. Work has been carried out that shows that local sequence influences the electronic characteristics of nucleophilic sites on DNA bases (Pullman and Pullman, 1981; Kohn *et al.*, 1987; Kim *et al.*, 1999; Zhu and LeBreton, 2000). Changes in the electronic characteristics of the various nucleophiles on the DNA bases has been correlated with changes in the extent of alkylation at different sites. However, the influence of base sequence upon the electronic characteristics of the DNA phosphate has yet to be studied. It would be very interesting to investigate whether local base sequence influences either the MEP or π ionisation energies of the DNA phosphate oxygens.

It may be concluded that base sequence influences the formation of PTEs. This is the first time that it has been demonstrated that extent of phosphate alkylation is influenced by DNA base sequence. The mechanism by which base sequence influences the manifestation of PTEs could be the focus of future studies. The present study was limited by our current inability to separate the 16 dinucleotide species by PAGE or HPLC to enable each dinucleotide species to be individually analysed. Future experiments will most likely require the development of a method capable of adequately separating the 16 dinucleotide species to permit individual analysis, e.g., capillary electrophoresis. Alternatively the influence of sequence upon the frequency of alkylation upon the sugar-phosphate could be investigated using mass spectrometry sequencing of oligonucleotides treated with various alkylating agents.

Further studies should also investigate the mechanism behind the rapid loss of PTEs observed in the liver tissue of the Balb/c mice. It also would be interesting to investigate the rate of long-term accumulation of PTEs in non-proliferative tissue as a marker of cumulative exposure.

7.8 Summary of conclusions

- A new 'direct' method has been developed for the preparation of samples for 5'NNA. The new 'direct' 5'NNA protocol eliminated the need for PAGE purification of ^{32}pdNpdN species prior to HPLC analysis, enabling the quick and easy preparation of samples for 5'NNA in a single tube.
- The SVPD/NP1 postlabelling assay can detect tandem damage. However, where two adducts are separated by one or more normal nucleotides they will behave as two separate independent lesions with respect to the SVPD/NP1 assay.

- The decline of PTEs in mouse liver DNA initially exceeded that of N7-ethyl dG, an adduct known to be subject to repair as determined in the same samples, in a previous study (Oreffo *et al.*, 2000). This would suggest that the PTEs may be being actively repaired in the liver tissue of Balb/c mice.
- The manifestation of PTEs remained non-random in the mouse liver DNA up to 56 days after the mice were treated with a single dose of NDEA. There is no evidence that putative repair influences the manifestation of PTEs.
- The manifestation of PTEs was non-random in whole cells, isolated nuclei and DNA treated *in vitro* with DES. There was no consistent significant difference between the 5'NNA results for the whole cells, nuclei and DNA isolated from the same cell line. Consequently, it was concluded that nuclear structure does not influence the manifestation of PTEs induced by DES
- It would appear that reaction conditions influence the manifestation of PTEs *in vitro*. The high concentration of free phosphate in the 0.5M sodium phosphate buffer used in the previous study (Guichard *et al.*, 2000), may compete with the various nucleophiles on the DNA and could thereby influence the manifestation of adducts in DNA.
- DNA base sequence influences the manifestation of PTEs in synthetic oligonucleotides. The relative frequency of PTEs in the poly(dG) region of the double stranded oligonucleotide is lower than in the other regions. The difference in the frequency of PTEs in the poly(dG) region compared to the other regions increased with increasing S_N2 behaviour. Increasing S_N2 behaviour is associated with an increase in the relative proportion of alkylation products being formed upon the dG base and a decrease in the relative proportion of alkylation products being formed upon the sugar-phosphate

backbone. Other factors, namely electronic factors, may also be influencing the manifestation of PTEs.

Appendix

Papers

1. **K. J. Bowman, R. Le Pla, Y. Guichard, P. B. Farmer and G. D. D. Jones** (2001) Evaluation of Phosphodiesterase 1 Based Protocols for the Detection of Multiply Damaged Sites in DNA: The Detection of Abasic, Oxidative and Alkylative Tandem Damage in DNA Oligonucleotides, Nucleic Acids Research, Vol. 29, No. 20, e101.

2. **R. C. Le Pla, Y. Guichard, K. Bowman, M. Gaskell, P. B. Farmer and G. D. D. Jones** (2003) Further development of the postlabelling method for detecting alkyl-phosphotriesters: Investigation into the non-random manifestation of phosphotriesters lesions *in vivo. Manuscript in preparation.*

3. **R. C. Le Pla, Y. Guichard, K. Bowman, P. B. Farmer and G. D. D. Jones** (2003) Investigation into the possible mechanism behind the non-random manifestation of PTEs. *Manuscript in preparation.*

Communications

1. **Le Pla R. C., Guichard Y., Farmer P. B., and Jones G. D. D.** (2002) The molecular basis for the non-random manifestation of DNA phosphotriester adducts *in vivo*. Proceedings of the American Association for Cancer Research. *pp.* 348.

2. **Le Pla R. C., Guichard Y., Farmer P. B., and Jones G. D. D.** (2002) The molecular basis for the non-random manifestation of DNA phosphotriester adducts *in vivo*. Mutagenesis, *in press.*

3. **Bowman K. J., Le Pla R. C., Guichard Y., Farmer P. B., and Jones G. D. D.** (2002) The direct detection of oxidative, alkylative and abasic tandem damage in DNA using phosphodiesterase I based protocols. Proceedings of the American Association for Cancer Research. *pp.* 345.

4. **Bowman K. J., Le Pla R. C., Guichard Y., Farmer P. B., and Jones G. D. D.** (2002) The direct detection of oxidative, alkylative and abasic tandem damage in DNA using phosphodiesterase I based protocols. British Journal of Cancer, Vol. 87, Supplement 1, S112-113.

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Evaluation of phosphodiesterase I-based protocols for the detection of multiply damaged sites in DNA: the detection of abasic, oxidative and alkylative tandem damage in DNA oligonucleotides

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ABSTRACT

It has been proposed that DNA multiply damaged sites (MDS), where more than one moiety in a local region (~1 helical turn, 10 bp) of the DNA is damaged, are lesions of enhanced biological significance. However, other than indirect measures, there are few analytical techniques that allow direct detection of MDS in DNA. In the present study we demonstrate the potential of protocols incorporating an exonucleolytic snake venom phosphodiesterase (SVPD) digestion stage to permit the direct detection of certain tandem damage, in which two lesions are immediately adjacent to each other on the same DNA strand. A series of prepared oligonucleotides containing either single or pairs of tetrahydrofuran moieties (F), thymine glycol lesions (T^g) or methylphosphotriester adducts (Me-PTE) were digested with SVPD and the digests examined by either ^{32}P-end-labelling or electrospray mass spectrometry. The unambiguous observation of SVPD-resistant 'trimer' species in the digests of oligonucleotides containing adjacent F, T^g and Me-PTE demonstrates that the SVPD digestion strategy is capable of allowing direct detection of certain tandem damage. Furthermore, in studies to determine the specificity of SVPD in dealing with pairs of lesions on the same strand, it was found mandatory to have the two lesions immediately adjacent to each other in order to generate the trimer species; pairs of lesions separated by as few as one or two normal nucleotides behave principally as single lesions towards SVPD.

INTRODUCTION

Numerous cytotoxic agents are proposed to exert their deleterious effects via formation of lesions and adducts in genomic DNA. As well as producing 'simple' individual lesions/adducts, a number of agents, such as ionising radiation and certain chemotherapeutic drugs, are proposed to generate 'complex' multiply damaged sites (MDS) where several moieties in a local region of the DNA (~10 bp) are damaged (1–4). These complex lesions are proposed to be of heightened biological significance due to the greater challenge they present repair systems (5).

Given the proposed significance of MDS, it is desirable to have analytical techniques that will allow their direct detection. To date, the most frequently studied DNA lesions which are taken as indices of MDS formation are immediate and latent DNA double-strand breaks (DSBs), the latter becoming apparent upon post-damaging treatment. Such treatment has included the use of damage-recognising endonucleases to convert base lesions into strand breaks, with noted increases in DSB formation being taken as indicative of the presence of base damage-containing MDS (6). However, these measures are somewhat indirect and are probable underestimates, as certain MDS have been shown to be refractory to endonuclease action (7,8) and, furthermore, base lesions that comprise an immediate DSB-MDS will go undetected/unmeasured. In the present study we demonstrate the potential of protocols incorporating a phosphodiesterase I (snake venom phosphodiesterase, SVPD) digestion stage to allow direct detection of a subset/constituent of MDS, namely 'tandem' lesions, whereby two lesions are immediately adjacent to each other on the same DNA strand.

The choice of an SVPD digestion strategy is based on the observation that certain DNA lesions (including apurinic sites and thymine glycols) prevent SVPD-mediated hydrolysis of the adjacent 5′-internucleotide phosphodiester linkage (9–13). Therefore, digestion of damage-containing DNA with this enzyme [in the presence of DNase I and alkaline phosphatase (AP)] yields single damage as lesion-bearing dimer species, dNpX, where the damage (X) is 3′ to a normal nucleoside 3′-phosphate moiety (dNp-) (Scheme 1A). These species are ideal substrates for 5′-^{32}P-end-labelling, via incubation with T4 polynucleotide kinase (T4 PNK) plus [γ-^{32}P]ATP, and this allows their ready detection [via sequencing PAGE and/or HPLC with radioactivity detection (^{32}P-HPLC)] as the corresponding 5′-^{32}P-end-labelled dimers, [^{32}P]dNpX (Scheme 1A). This protocol is the SVPD-based post-labelling assay described by Weinfeld and co-workers (14,15). The assay has been used to detect radiation-induced thymine glycols (T^g) and

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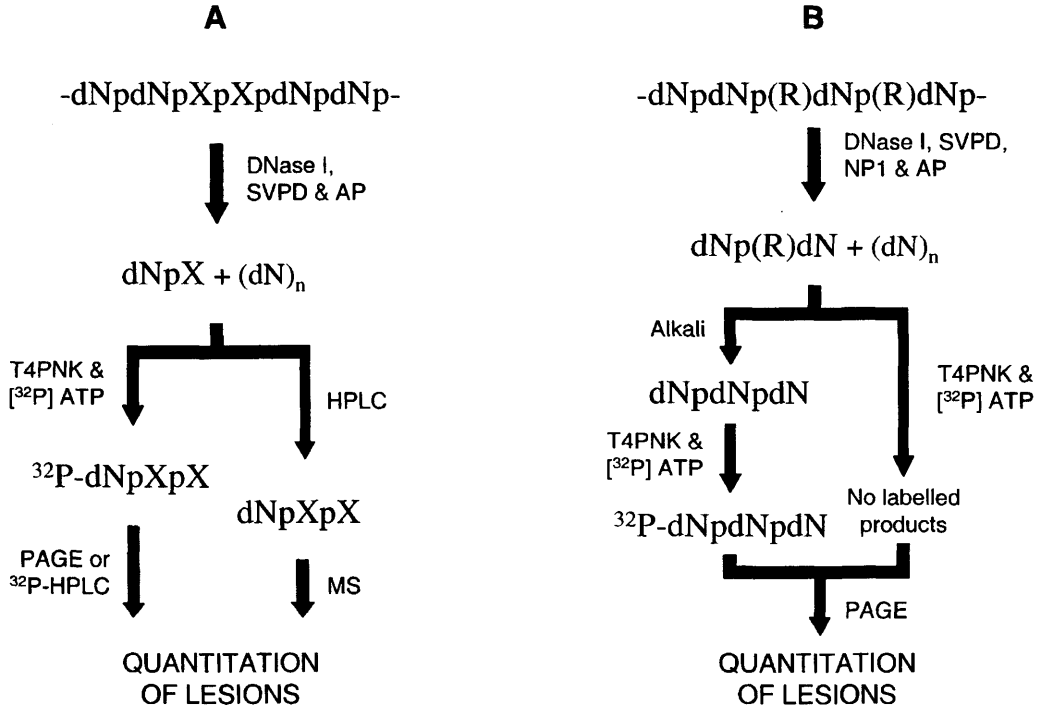

Scheme 2.

MATERIALS AND METHODS

Materials

The tetrahydrofuran-containing oligonucleotides **1–8**, the thymidine-containing precursors to oligonucleotides **9–16** and the methylphosphotriester-containing oligonucleotides **17–22** were prepared via routine automated phosphoramidite DNA oligonucleotide synthesis by the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester, on the 1 μmol scale using an Applied Biosystems model 394 DNA/RNA synthesiser (PE Applied Biosystems, Warrington, UK). Oligonucleotides **1–8** and **17–22** were prepared using 5′-*O*-dimethoxytrityl-1′,2′-dideoxyribose-3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (dSpacer CE phosphoramidite) and 5′-*O*-dimethoxytrityl-2′-deoxythymidine-3′-[(*O*-methyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (dT-Me phosphoramidite), respectively, according to the supplier's instructions (Glen Research, Sterling, VA). Phosphodiesterase I (Type IV from *Crotalus atrox*) (SVPD), DNase I and OsO_4 were purchased from Sigma (Poole, UK), whilst T4 PNK and shrimp alkaline phosphatase (SAP) were purchased from US Biochemical (Cleveland, OH). [γ-^{32}P]ATP (Redivue, >3000 Ci/mmol) and poly(dT)$_{16}$ were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

OsO_4 oxidation

Samples of 50 nmol oligonucleotides **9**, **10** and **13–16** and 25 nmol samples of oligonucleotides **11** and **12** were incubated in 250 μl of 50 μM NH_4OH and 1% OsO_4 at room temperature for 0, 6 or 48 h. The oxidation reaction was stopped by extraction of the OsO_4 into 3 vol of ether and the extraction repeated five times.

Digestion

Samples of 10 μg oligonucleotides **1–8** and **9–16** were incubated with SVPD (0.04 U), DNase I (0.4 U) and SAP (0.4 U) in 30 μl of digestion buffer (10 mM Tris, 1 mM EDTA, 4 mM $MgCl_2$, pH 7.5) at 37°C overnight (~16 h). The proteins were precipitated by addition of 3 vol of ethanol, pelleted by centrifugation and aliquots of the ethanol supernatants dried by vacuum lyophilisation. The resulting dried residues were resuspended in distilled water (0.1 μg/μl) and then boiled (10 min) to inactivate residual nuclease and phosphatase activity, then stored at –20°C.

Similarly, 10 μg samples of oligonucleotides **17–22** were incubated with SVPD (0.04 U), DNase I (0.4 U) NP1 (0.5 U) and SAP (0.4 U) in 30 μl of digestion buffer (10 mM Tris, 1 mM EDTA, 4 mM $MgCl_2$, 2 mM $ZnCl_2$, pH 7.5) at 37°C overnight (~16 h). Again the proteins were precipitated by addition of 3 vol of ethanol, pelleted by centrifugation and aliquots of the ethanol supernatants dried by vacuum lyophilisation. The resulting dried residues were resuspended in distilled water (0.1 μg/μl) and then boiled (10 min) to inactivate residual nuclease and phosphatase activity, then stored at –20°C.

Alkali hydrolysis of PTE

Alkali hydrolysis of the digested PTE-containing samples was carried out using aqueous ammonia. To an aliquot (50 μl) of digest solution was added an equal volume of aqueous ammonia (25%) and the reaction incubated in a tightly capped vial at 70°C for 24 h. The reaction mixture was evaporated

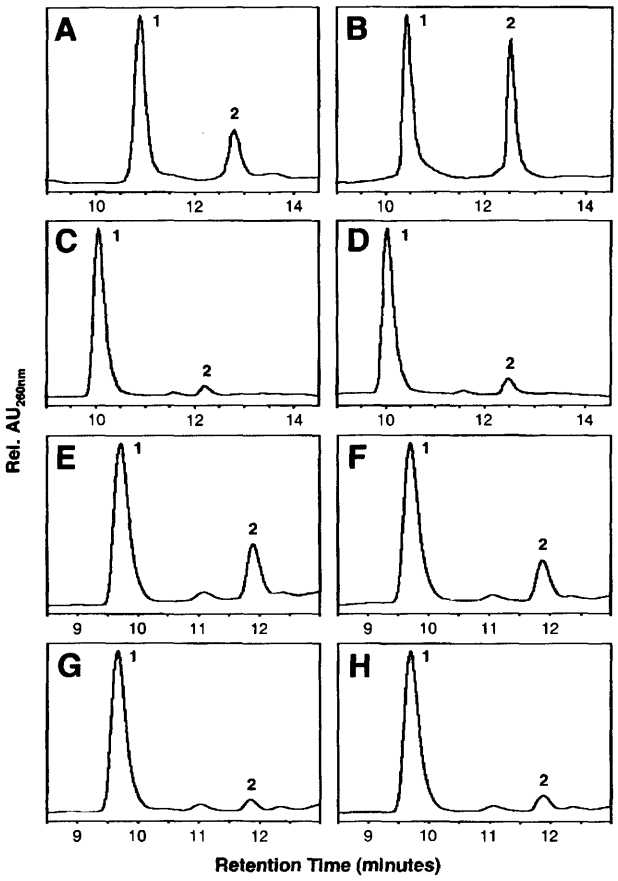

Figure 1. HPLC chromatographs showing digestion of the thymidylyl tetrahydrofuran-containing oligonucleotides **1–8** (**A–H**, respectively). The oligonucleotides were incubated with SVPD, DNase I and SAP at 37°C overnight and the isolated digests analysed by reverse phase HPLC as described in Materials and Methods.

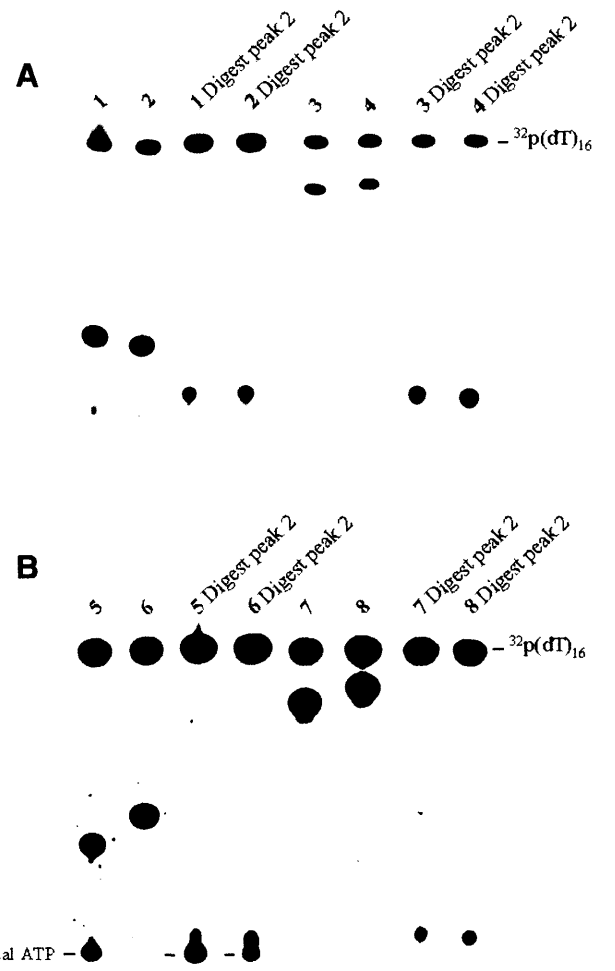

Figure 2. ^{32}P-end-labelling analysis of the HPLC isolated material in peak 2 of the digests of oligonucleotides **1–8**. The material contained in the HPLC isolated peaks was collected, aliquots incubated with [γ-^{32}P]ATP + T4 PNK and the radiolabelled products analysed by PAGE as described in Materials and Methods. (**A**) The radiolabelled products from **1–4**; (**B**) the radiolabelled products from **5–8**. Poly(dT)$_{16}$ was used to consume the bulk of the excess [^{32}P]ATP; the radiolabelled product, [^{32}P](dT)$_{16}$, remained near the top of the gel.

RESULTS

Tetrahydrofuran moieties

The tetrahydrofuran-containing oligonucleotides **1–8** were treated with SVPD, DNase I and SAP and the isolated digests subjected to semi-preparative reverse phase HPLC as described in Materials and Methods. Figure 1A–D shows the HPLC chromatograms for the digests of oligonucleotides **1–4** (containing single and adjacent F) whilst Figure 1E–H shows the HPLC chromatograms for the digests of oligonucleotides **5–8** (containing pairs of separated F). In each instance, two principle peaks were resolved, peaks 1 and 2. Co-migration of the single peak of an authentic sample of thymidine (T) with peak 1 (data not presented) indicates that this peak contains T. For **2** (Fig. 1B), peak 1 was of a similar size to peak 2, however, for **1**, **3** and **4** (Fig. 1A, C and D) peak 1 was 3.8-, 20- and 11-fold greater, respectively, compared to peak 2, whilst for **5–8** (Fig. 1E–H) peak 1 was 2.7-, 3.75-, 15.6- and 12.5-fold greater, respectively, compared to peak 2.

The material contained in peak 2 of **1–8** was collected and aliquots analysed by ^{32}P-end-labelling and by electrospray mass spectrometry. Figure 2A shows the outcome of the end-labelling analysis for oligonucleotides **1–4** (containing single and adjacent F), while Figure 2B shows the outcome of the end-labelling analysis for oligonucleotides **5–8** (containing pairs of separated F). From Figure 2A and B it is clear that for each oligonucleotide examined, peak 2 contains a species that is a substrate for T4 PNK-mediated ^{32}P-end-labelling and that each labelled product migrates to approximately the same position under the conditions of electrophoresis used. End-labelling analysis of material from peak 1 of **1–8** revealed no labellable substrate present in this peak (data not presented). Figure 3A–D shows the electrospray ionisation mass spectra (negative mode) of the material contained in isolated peak 2 for oligonucleotides **1–4**. This data, along with the electrospray mass spectrometry data for the material present in peak 2 of oligonucleotides **5–8**, is presented in Table 2. For oligonucleotides **1** and **3** (containing a single F) peak 2 contained prominent negative ions $(M-H)^-$ of *m/z* 421.6 and 421.1, respectively (Fig. 3A and C), corresponding to TpF [calculated $(M-H)^-$ *m/z* 421.3], while peak 2 from oligonucleotides **2** and **4** (containing

Table 2. Mass spectrometry data of the material contained in HPLC isolated peak 2 of oligonucleotides **1–8**

Sample	Peak number	Retention time (min)	$(M-H)^-$ (*m/z*)	Proposed structure
1	2	12.8	421.6	TpF
2	2	12.5	601.8	TpFpF
3	2	12.3	421.1	TpF
4	2	12.5	601.0	TpFpF
5	2	11.9	421.6	TpF
6	2	11.9	421.6	TpF
7	2	11.9	421.6	TpF
8	2	11.9	421.6	TpF

mass spectrometry. Two samples were collected for peak 1 of **12**; sample I between 12.5 and 13.0 min and sample II between 13 and 13.5 min. Figure 5A shows the outcome of the end-labelling analysis for oligonucleotides **9–12** (containing single and adjacent T^g; end-labelling analysis of untreated **9** and **10** is also shown), while Figure 5B shows the outcome of the end-labelling analysis for oligonucleotides **13–16** (containing pairs of separated T^g); these particular gels were electrophoresed for 8–9 h in an attempt to improve the resolution of the labelled products. Interestingly, every HPLC peak was found to contain at least one species which was a substrate for T4 PNK-mediated ^{32}P-end-labelling. The labelled species in the peaks for oligonucleotides **9–11** and **13–16** migrate as either one or two bands, whereas several labellable substrates were present for each peak for oligonucleotide **12**. A different pattern of products was observed between samples I and II of peak 1 of oligonucleotide **12**, suggesting that this peak contained several species. The species isolated from oligonucleotides **10** and **12** (containing adjacent T^g) were clearly poor substrates for T4 PNK-mediated ^{32}P-end-labelling, as the band intensities are considerably lower than the band intensities for substrates isolated from the other oligonucleotides.

The electrospray ionisation mass spectra (negative mode) of the material contained in isolated peak 2 for oligonucleotides **9–12** (containing single and adjacent T^g) are shown in Figure 6A–D and this data, along with the electrospray mass spectrometry data for the material present in all of the denoted peaks of oligonucleotides **9–16**, is presented in Table 3. For oligonucleotides **9** and **11** (containing a single T^g) all peaks contained prominent negative ions $(M-H)^-$ of *m/z* 588.0–588.5 (see Fig. 6A and C), corresponding to $dApT^g$ [calculated $(M-H)^-$ *m/z* 588.5], while for oligonucleotides **10** and **12** (containing adjacent T^g) all peaks contained prominent negative ions $(M-H)^-$ of *m/z* 926.0–926.2 (see Fig. 6B and D), corresponding to $dApT^gpT^g$ [calculated $(M-H)^-$ *m/z* 926.7]. However, each peak from oligonucleotides **13–16** (containing pairs of separated T^g) contained a major ion $(M-H)^-$ of *m/z* 588.0–588.2 (Table 3), corresponding to $dApT^g$.

Phosphotriester adducts

The methylphosphotriester-containing oligonucleotides **17–22** were treated with SVPD, DNase I, NP1 and SAP and the digests, either untreated or treated with alkali, analysed by

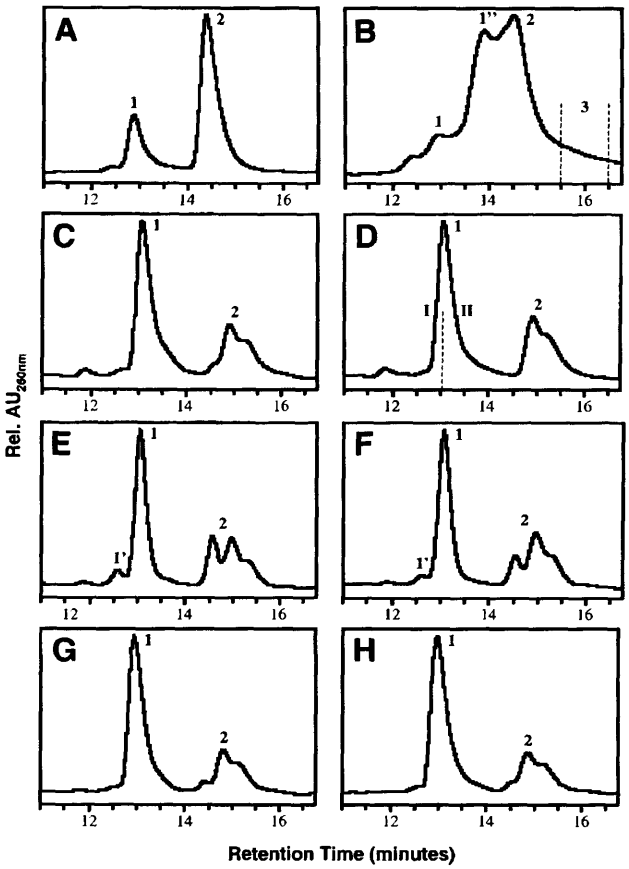

Figure 4. HPLC chromatographs showing digestion of the thymine glycol-containing oligonucleotides **9–16** (**A–H**, respectively). The oligonucleotides were incubated with SVPD, DNase I and SAP at 37°C overnight and the isolated digests analysed by reverse phase HPLC as described in Materials and Methods.

^{32}P-end-labelling as described in Materials and Methods. Figure 7A–D shows the outcome of the analysis of oligonucleotides **17–20** (containing single and adjacent Me-PTE) whilst Figure 7E and F shows the outcome of the end-labelling analysis of **21** and **22** (containing pairs of separated Me-PTE). Digestion, alkali treatment and end-labelling of **17** and **19** (containing single Me-PTE) leads to the formation of dimer [^{32}P]TpT (2T) (Fig. 7A and C). However, identical treatment of **18** and **20** (containing adjacent Me-PTE) leads to the formation of trimer [^{32}P]TpTpT (3T) plus some 2T (Fig. 7B and D). Finally, digestion, alkali treatment and end-labelling of **21** and **22** (containing pairs of separated Me-PTE) leads to the formation of solely 2T (Fig. 7E and F). Analysis of the normal (non-PTE-containing) oligonucleotides leads to no labellable substrate (data not presented).

DISCUSSION

One type of MDS that has been the focus of extensive study is DNA DSB (5,23). In DSB-MDS at least two single-strand breaks occur in close proximity (~10 bp) on opposite strands of the DNA duplex. However, many other types of MDS are possible, including those consisting of pairs of lesions near or immediately adjacent (tandem) to each other on the same

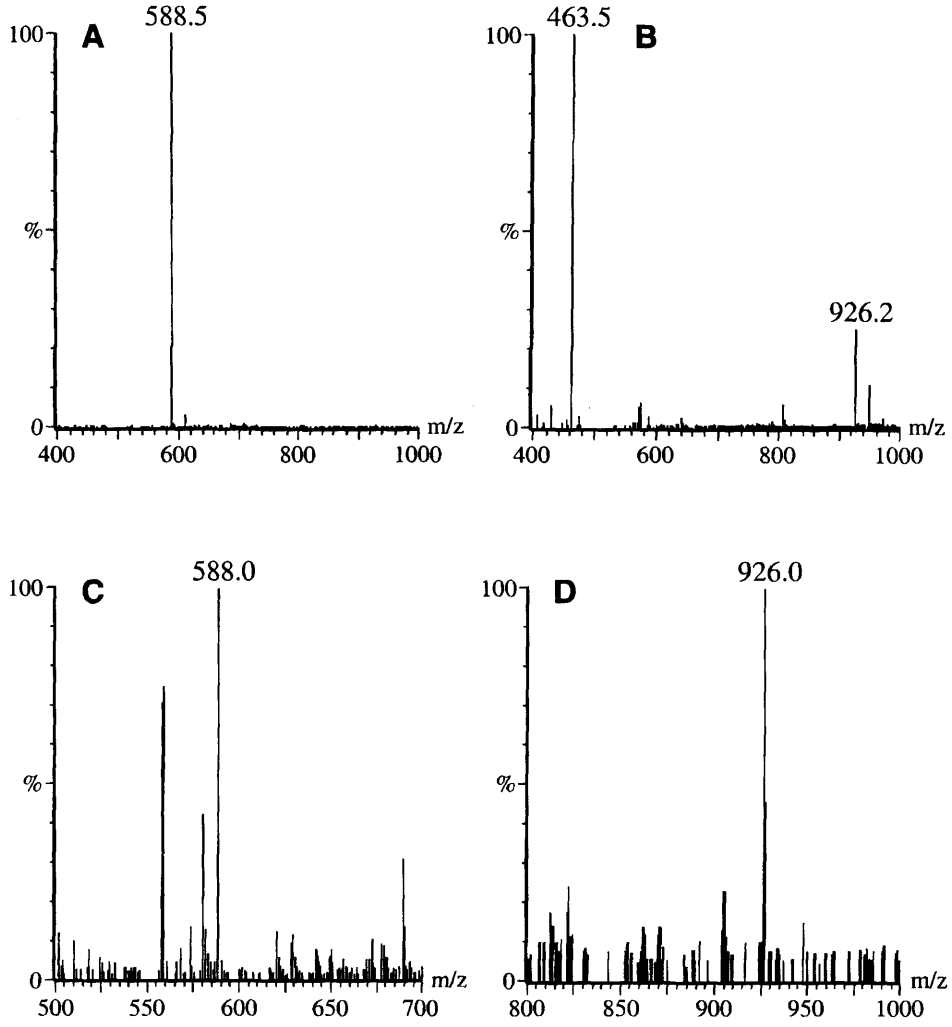

Figure 6. The electrospray mass spectra of the material contained in HPLC isolated peak 2 of oligonucleotides **9–12** (**A–D**, respectively).

species of *m/z* ~926, corresponding to dApT^{g}pT^{g}. Equally, digestion, alkali treatment and labelling of **18** and **20**, containing tandem Me-PTE, yielded the labelled trinucleotide 3T. Again, the starting material was partially demethylated prior to analysis [leading to a slower migrating species, presumably [^{32}P]TpTp(Me)T, being present in undigested/alkali non-treated **18** (Fig. 7B), which was completely removed by digestion alone] and this in turn leads to the presence of some 2T in the digested + alkali-treated lanes of both **18** and **20** (Fig. 7D). The higher ratio of recovered TpFpF versus released T for **2** and **4**, compared to the ratio of recovered TpF versus released T for **1** and **3** (see above), indicates that the presence of tandem damage renders the phosphodiester bond immediately 5′ to the tandem furan entity further refractory to digestion by SVPD (compared to the lower degree of resistance afforded by a single F), to the extent that only ~23% of these bonds are hydrolysed in **2** and ~17% in **4**.

By itself, ^{32}P-end-labelling coupled with PAGE analysis was unable to distinguish between single and tandem F-containing species; the labelled species [^{32}P]TpF and [^{32}P]TpFpF migrate to approximately the same extent on PAGE analysis (see Fig. 2A) due to their comparable charge to mass ratios. Similarly, ^{32}P-end-labelling coupled with PAGE was unable to clearly distinguish between the single and the tandem T^{g}-containing species (Fig. 5A); furthermore, the tandem T^{g}-containing species, dApT^{g}pT^{g}, were poor substrates for labelling compared to the single damage-containing species dApT^{g}. In order to distinguish between single and tandem F- and T^{g}-containing species it was necessary to exploit HPLC combined with 'off-line' MS analysis (HPLC+MS). Successful HPLC+MS-mediated detection of the TpFpF species in the digests of **2** and **4** (see Fig. 3B and D) and of the dApT^{g}pT^{g} species in the digests of **10** and **12** (see Fig. 6B and D) plus successful detection of 3T in the digests of **18** and **20** by ^{32}P-end-labelling/PAGE (see Fig. 7B and 7D) demonstrates that the SVPD digestion strategy is indeed capable of selectively retaining certain tandem damage and so allowing their direct detection.

Oligonucleotides **5–8**, containing pairs of F separated by either one or two thymidine nucleotides, oligonucleotides **13–16**,

Table 3. Mass spectrometry data of the material contained in HPLC isolated peaks of oligonucleotides **9–12**

Sample	Peak number	Retention time (min)	$(M-H)^-$ (*m/z*)	Proposed structure
9	1	12.9	588.5	dApT^g
	2	14.4	588.5	dApT^g
10	1 + 1″	12.75–13.9	926.0	dApT^{g}pT^g
	2	14.5	926.2	dApT^{g}pT^g
	3	15.5–16.5	926.2	dApT^{g}pT^g
11	1	13.1	588.0	dApT^g
	2	14.9	588.3	dApT^g
12	1 (I)	13.1	926.1	dApT^{g}pT^g
	1 (II)	13.1	926.1	dApT^{g}pT^g
	2	14.9	926.1	dApT^{g}pT^g
13	1′	12.6	588.1	dApT^g
	1	13.05	588.1	dApT^g
	2	14.5–15.5	588.0	dApT^g
14	1′	12.6	588.1	dApT^g
	1	13.1	588.2	dApT^g
	2	14.5–15.5	588.1	dApT^g
15	1	12.9	588.2	dApT^g
	2	14.5–15.5	588.1	dApT^g
16	1	13.0	588.1	dApT^g
	2	14.5–15.5	588.2	dApT^g

technology. A similar strategy has recently been reported by Cadet and co-workers for detection of dβF-8-oxodGuo tandem damage in γ-irradiated DNA (31).

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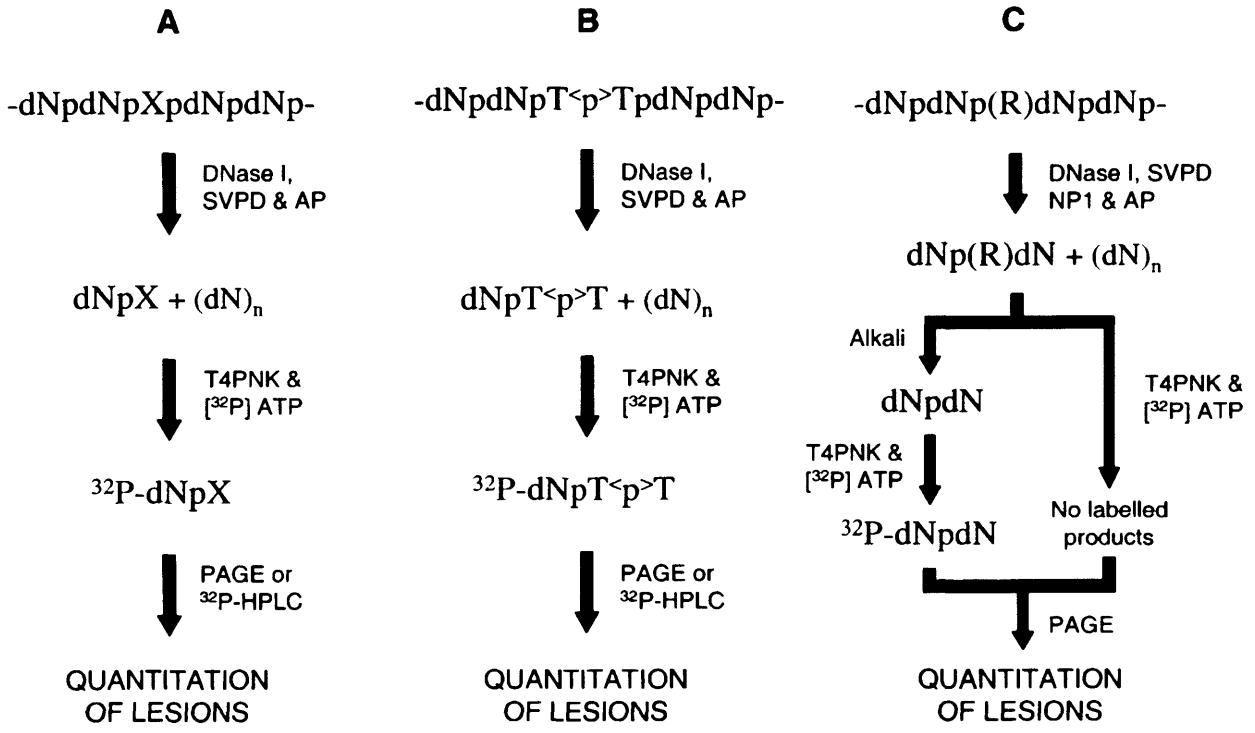


Scheme 1.

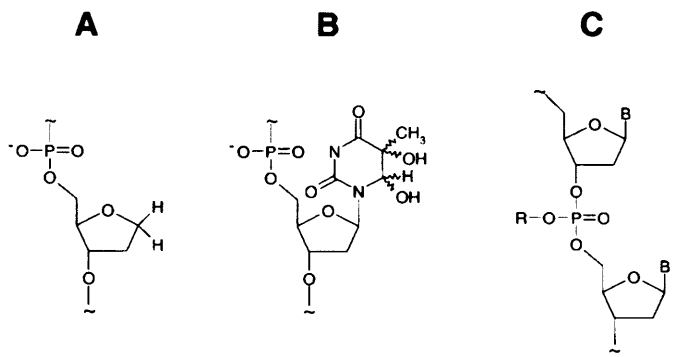
phosphoglycolate termini (14), to compare oxidative lesions produced by Fenton chemistry and ionising radiation (15) and to detect phosphoglycolate lesions produced in cellular DNA by oxidative stress (16). The assay has also been used to measure apurinic site formation in DNA (17) and to detect UV light-induced cyclobutane pyrimidine dimer (T<p>T) formation (18). Intriguingly, the pyrimidine dimer lesions furnish the lesion-bearing trimer species, dNpT<p>T, after SVPD digestion and then the corresponding labelled trimers, [^{32}P]dNpdT<p>T, after phosphorylation with ^{32}P (18; Scheme 1B).

The SVPD-based post-labelling assay has been further developed for the detection of simple alkylphosphotriesters (19). The protocol is based on the inability of all known nucleolytic enzymes to cleave the internucleotide PTE bond (20,21). Consequently, complete digestion of alkylated DNA with SVPD plus nuclease P1 (NP1), in the presence of DNase I and AP, yields PTE-dinucleoside phosphate species [dNp(R)dN] (Scheme 1C) (NP1 is included in the digestion to allow for the specific detection of alkylphosphotriesters, by hydrolysing any dNpX species that are generated as a result of base alkylation). These species are then converted to corresponding dinucleoside phosphates (dNpdN), by treatment with alkali, to permit subsequent ^{32}P-labelling (in the absence of alkali treatment, no labellable substrate is produced). The resulting labelled dinucleotides ([^{32}P]dNpdN) are then analysed by PAGE (Scheme 1C).

In a scenario analogous to that of the pyrimidine dimer lesions (see above), the presence of *two* SVPD-resistant lesions immediately adjacent to each other [i.e. -dNpXpXpdN- or -dNp(R)dNp(R)dN-] should yield the corresponding tandem damage-containing trimer species, dNpXpX or dNp(R)dNp(R)dN, after SVPD digestion (Scheme 2A and B). Thus, it should be practicable to exploit SVPD digestion to 'retain' certain tandem damage as dNpXpX or dNp(R)dNp(R)dN and then to use either ^{32}P-end-labelling or an alternative form of analysis (i.e. semi-preparative HPLC combined with MS analysis) to effect their detection (Scheme 2A and B). To investigate this, a series of oligonucleotides **1–22** (Table 1) containing either single, tandem or pairs of separated tetrahydrofuran moieties (F) (oligonucleotides **1–8**) (see Scheme 3A for tetrahydrofuran structure), thymine glycol lesions (T^{g}) (oligonucleotides **9–16**) (Scheme 3B) or methyl phosphotriester adducts [Me-PTE, denoted by p(Me) in Table 1] (oligonucleotides **17–22**) (Scheme 3C) were prepared and exploited as model substrates to evaluate the SVPD-based digestion strategy as a means of detecting tandem damage. All of the oligonucleotides were prepared via routine automated phosphoramidite DNA oligonucleotide synthesis; the F- and Me-PTE-containing oligonucleotides using the appropriate phosphoramidite precursors, whilst the T^{g}-containing oligonucleotides were prepared from the corresponding thymidine-containing oligonucleotides via OsO_4-mediated oxidation (22). The F- and T^{g}-containing oligonucleotides were digested with SVPD, DNase I and AP, the digests were then subjected to semi-preparative HPLC and the isolated components examined by either ^{32}P-end-labelling/PAGE or electrospray mass spectrometry. The Me-PTE-containing oligonucleotides were digested with SVPD, NP1, DNase I and AP and the digests, either untreated or treated with alkali, directly examined by ^{32}P-end-labelling/PAGE.

Table 1.

Designation	Sequence
Tetrahydrofuran (**F**)-containing oligonucleotides	
1	TpFpT
2	TpFpFpT
3	$(Tp)_5F(pT)_5$
4	$(Tp)_5FpF(pT)_5$
5	TpFpTpFpT
6	TpFpTpTpFpT
7	$(Tp)_5FpTpF(pT)_5$
8	$(Tp)_5FpTpTpF(pT)_5$
Thymine glycol (T^g)-containing oligonucleotides	
9	$dApT^g$
10	$dApT^gpT^g$
11	$(dAp)_5T^g(pdA)_5$
12	$(dAp)_5T^gpT^g(pdA)_5$
13	$dApT^gpdApT^g$
14	$dApT^gpdApdApT^g$
15	$(dAp)_5T^gpdApT^g(pdA)_5$
16	$(dAp)_5T^gpdApdApT^g(pdA)_5$
Methyl phosphotriester [**p(Me)**]-containing oligonucleotides	
17	Tp(Me)T
18	Tp(Me)Tp(Me)T
19	$(Tp)_2Tp(Me)T(pT)_7$
20	$(Tp)_2Tp(Me)Tp(Me)(pT)_7$
21	Tp(Me)TpTp(Me)T
22	$(Tp)_2Tp(Me)TpTp(Me)(pT)_6$



Scheme 3.

to dryness using a DNA centrifugal evaporator and the residue dissolved in an appropriate volume of water, then stored at –20°C.

HPLC

Reverse phase HPLC was performed on a Phase Sep Partisil 10 µm ODS-2 column (250 × 4.6 mm i.d.) (Phase Separations Ltd, Deeside, UK). The HPLC instrumentation consisted of a Waters Alliance 2690 Separations Module coupled to a 484 Tuneable Absorbance Detector, equipped with Millennium 2010 Chromatography Manager software (Waters, Milford, MA). Aliquots of the digested oligonucleotides (10 µg) were loaded onto the column and eluted using the following HPLC gradient. Solvent A: 0.1 M triethylamine, 1% methanol, pH 5.0; solvent B: 100% methanol; gradient: 0 min, 0% B; 10 min, 30% B; 15 min, 30% B; 20 min, 70% B; 22.5 min, 70% B; 25 min, 0% B. The flow rate was 1 ml/min, with detection at 260 nm. To isolate the material contained in each of the major peaks resolved, fractions of the eluant were collected at the appropriate times and dried by vacuum lyophilisation. The material in the peaks anticipated to contain the tetrahydrofuran-bearing 'dimer' (TpF) (i.e. peak 2 from oligos **1**, **3** and **5–8**) or the corresponding 'trimer' (TpFpF) (i.e. peak 2 from oligos **2** and **4**) (see below) and material in all of the peaks for **9–16** were dissolved in distilled water to a calculated concentration of 100 µM (based on quantitative recovery of TpF, TpFpF, $dApT^g$ or $dApT^gpT^g$ in any one fraction).

Mass spectroscopy

Aliquots of the HPLC isolated materials (~1 nmol) were diluted 10-fold with 70% acetonitrile:30% imidazole buffer (50 mM) and the molecular weights assigned by negative ion electrospray mass spectrometry using a Micromass Platform (Micromass UK Ltd, Wythenshawe, UK).

^{32}P Radiolabelling

Aliquots of the HPLC isolated materials of **1–16** and aliquots of either the untreated or alkali-treated digests of **17–22** were diluted 1000-fold with distilled water and further aliquots containing ~500 fmol of potentially labellable substrate were incubated with T4 PNK (7.5 U), [γ-^{32}P]ATP (3000 Ci/mM, 1.65 pmol) and ~100 fmol $poly(dT)_{16}$ (when used as a labelling reference) in 10 µl of kinase reaction buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol) at 37°C for 1 h. When $poly(dT)_{16}$ was used to consume excess [γ-^{32}P]ATP, the samples were incubated for an additional 30 min at 37°C with 40 pmol $poly(dT)_{16}$ plus a further quantity of T4 PNK (3.75 U). Following addition of an equal volume of formamide loading buffer [90% formamide, 0.02% bromophenol blue and 0.02% xylene cyanol in 1× TBE (89 mM Tris–borate, pH 8.3, 2 mM EDTA)], the radiolabelled products in a quarter of the mixture were separated by 20% denaturing PAGE using a Model S2 sequencing gel electrophoresis apparatus with either 1.6 or 0.8 mm spacers (Life Technologies, Paisley, UK). Electrophoresis was conducted at 1100–1200 V until the bromophenol blue had migrated either 11–12 cm (3–4 h) (oligonuceotides **1–8** and **17–22**) or 25–26 cm (8–9 h) (oligonucleotides **9–16**) and then, with the gel wrapped in cling-film, the radiolabelled products were visualised by contact autoradiography using Kodak X-Omat XAR-5 film (30 × 40 cm).

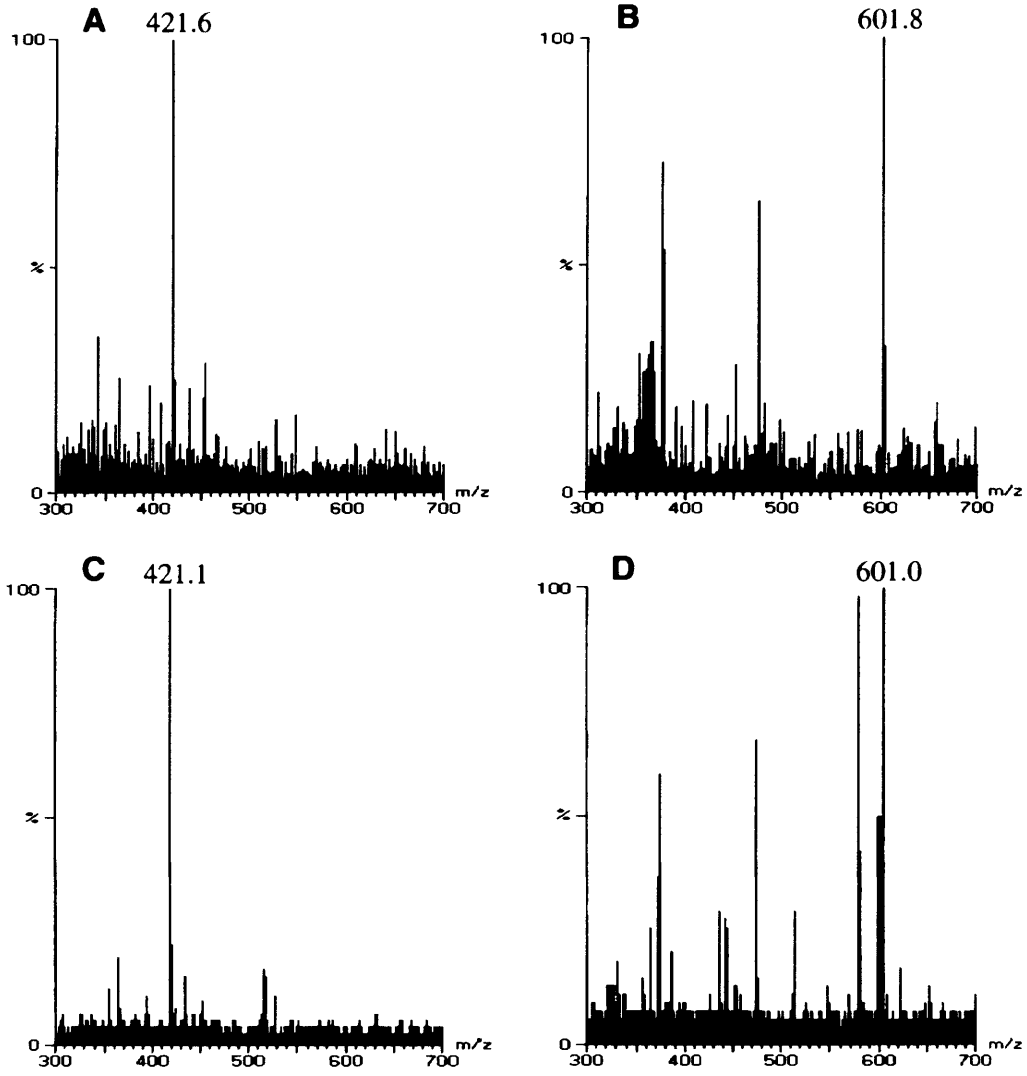


Figure 3. Electrospray mass spectra of the material contained in HPLC isolated peak 2 for oligonucleotides **1–4** (**A–D**, respectively).

adjacent F) contained prominent negative ions $(M-H)^-$ of *m/z* 601.8 and 601.0, respectively (Fig. 3B and D), corresponding to TpFpF [calculated $(M-H)^-$ *m/z* 601.4]. For oligonucleotides **5–8** (containing pairs of separated F), each peak 2 contained prominent negative ions $(M-H)^-$ of *m/z* 421.6 (Table 2), corresponding to TpF.

Thymine glycol lesions

In preliminary experiments, the thymidine-containing precursors to oligonucleotides **9–16** were exposed to OsO_4 for various times to determine the time required for stoichiometric oxidation of the thymidines in each oligonucleotide (data not presented). Consequently, oligonucleotides **9**, **10**, **13** and **14** (the shorter oligonucleotides) were prepared by exposure to OsO_4 for 6 h, while oligonucleotides **11**, **12**, **15** and **16** (the longer oligonucleotides) were prepared by exposure to OsO_4 for 48 h, as described in Materials and Methods. The stoichiometric production of the thymine glycol-containing oligonucleotides was confirmed by electrospray ionisation mass spectrometry (negative mode).

The thymine glycol-containing oligonucleotides **9–16** were treated with SVPD, DNase I and SAP and the digests subjected to semi-preparative reverse phase HPLC as described in Materials and Methods. Figure 4A–D shows the HPLC chromatograms for the digests of oligonucleotides **9–12** (containing single and adjacent T^g), while Figure 4E–H shows the HPLC chromatograms for the digests of oligonucleotides **13–16** (containing pairs of separated T^g). Generally, two principle peaks (or collection of peaks) were resolved by HPLC; peak 1 of retention time 12.9 min and peak(s) 2 of retention times ~14–15.5 min. An exception was **10**, for which there was a prominent peak at 13.8 min, denoted peak 1″ (collected along with peak 1), and a significant 'tail' over 15.5–16.5 min, denoted 'peak' 3; for **13** and **14** a minor peak, peak 1′, that eluted before peak 1 was also collected and analysed. Co-migration of the single peak of an authentic sample of deoxyadenosine (dA) with peak 1 (data not presented) indicates that this peak contains dA.

Material from all of the denoted peaks was collected and aliquots analysed by ^{32}P-end-labelling and by electrospray

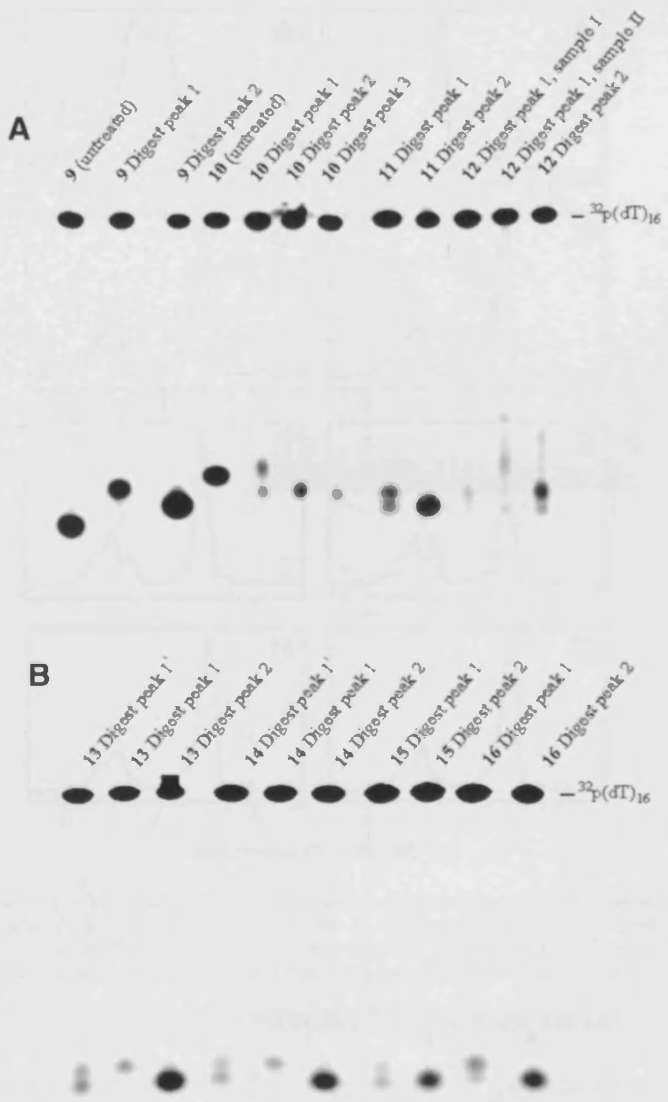


Figure 5. ^{32}P-end-labelling analysis of HPLC isolated material from the digests of oligonucleotides **9**–**16**. The material contained in HPLC isolated peaks was collected, aliquots incubated with [γ-^{32}P]ATP + T4 PNK and the radiolabelled products analysed by PAGE as described in Materials and Methods. (**A**) The radiolabelled products from **9**–**12**; (**B**) the radiolabelled products from **13**–**16**. Poly(dT)$_{16}$ was used to consume the bulk of the excess [^{32}P]ATP; the radiolabelled product, [^{32}P](dT)$_{16}$, remained near the top of the gel.

strand (5,24). In the present study, oligodeoxyribonucleotides containing either single, tandem or pairs of separated tetrahydrofuran moieties, thymine glycol lesions and methylphosphotriester adducts have been prepared as model substrates to examine protocols incorporating an SVPD digestion stage as potential methods to allow the analysis of certain tandem damage in DNA.

The tetrahydrofuran moieties (Scheme 3A) were initially chosen as model lesions with which to test the SVPD-based protocol as they represent stable abasic sites (stable to β-elimination) and as such they should confer resistance to SVPD hydrolysis on the 5′ phosphodiester bond (compare with 17). (For the sake of simplicity, when the tetrahydrofuran moieties, thymine glycol lesions and methylphosphotriester adducts are collectively discussed, they will be referred to as lesions, even though the tetrahydrofuran moieties are not considered true bona fide lesions.) These two properties, together with the ease of preparing oligonucleotides containing the tetrahydrofuran moieties at nominated positions by routine phosphoramidite DNA oligonucleotide synthesis procedures, provided us with ideal robust model oligonucleotide substrates with which to examine the SVPD digestion strategy as a means of detecting tandem damage. However, the tetrahydrofuran moieties are not 'true' lesions. Consequently, two other bona fide lesions/adducts were included in the investigation. These were thymine glycol lesions (Scheme 3B), which are the major pyrimidine oxidative lesions produced by both ionising radiation and oxidative stress (25,26), and alkylphosphotriester adducts (Scheme 3C), which are highly abundant and persistent adducts produced by potent alkylating agents (27,28); for example, phosphotriesters constitute >50% of the adducts generated by ethyl nitrosourea (28). Thymine glycols render the phosphodiester bond immediately 5′ to the glycol-bearing nucleoside refractory to SVPD-mediated hydrolysis (11,14), whilst alkylphosphotriester formation renders the adducted phosphate moiety resistant to nuclease cleavage (20,21).

SVPD is a 3′→5′ exonuclease that digests both DNA and RNA. It requires a 3′-OH terminus for exonuclease activity and hydrolyses nucleic acids to release 5′-mononucleotides (29). SVPD interacts with the base 3′ to a phosphodiester linkage and certain modified bases (including thymine glycols) or a missing base (an abasic site) inhibit cleavage of the phosphodiester bond (9–13). Also, SVPD has an associated single strand-specific endonuclease activity (12). Consequently, prolonged digestion of damage-containing DNA with SVPD, in the presence of DNase I (to generate the required 3′-OH termini) and an alkaline phosphatase (to hydrolyse phosphate monoesters), ultimately yields particular single damage as lesion-bearing dimer species, dNpX (12,15; Scheme 1A). Accordingly, digestion of oligonucleotides **1** and **3**, containing a single F, yielded labellable species of *m/z* ~421, corresponding to TpF, while SVPD digestion of oligonucleotides **9** and **11**, containing single T^{g}, yielded labellable species of *m/z* ~588, corresponding to dApT^{g}. Likewise, digestion, alkali treatment and labelling of **17** and **19**, containing single Me-PTE, yielded the labelled dinucleotide 2T. However, the presence of some 2T in the undigested/alkali non-treated control lane of **17** (Fig. 7A) indicated that a proportion of the starting material was demethylated; this unmethylated material was completely removed by digestion alone. For **1** and **3** the greater than predicted relative yield of released thymidine compared to recovered TpF, as determined by HPLC analysis, indicates that the phosphodiester bond immediately 5′ to the F is indeed partially hydrolysed, ~58% for **1** and ~52% for **3**. Similar observations have been made previously for prepared T^{g}-containing dimers dApT^{g}, dCpT^{g} and dGpT^{g} whereby prolonged digestion with SVPD resulted in ~50% loss of these dimer species as compared to digestion at shorter times (30). As mentioned above, it is proposed that phosphotriester lesions are refractory to nuclease digestion (20,21).

SVPD digestion of oligonucleotides **2** and **4**, containing adjacent F, yielded a labellable species of *m/z* ~601, corresponding to TpFpF, while SVPD digestion of oligonucleotides **10** and **12**, containing adjacent T^{g}, yielded (poorly) labellable

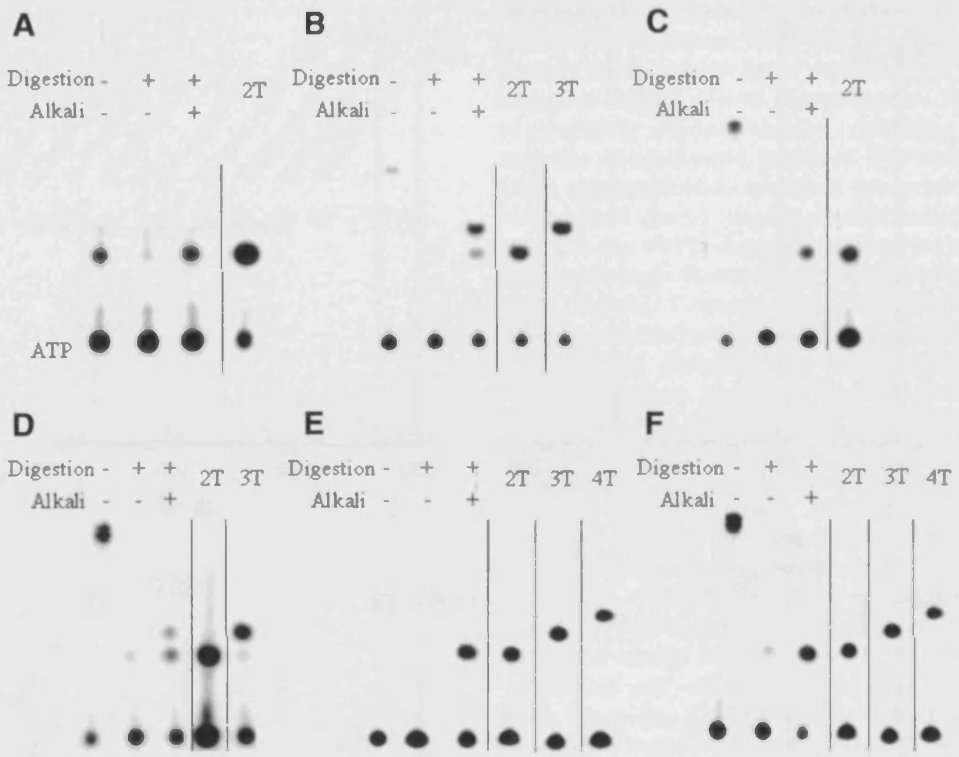


Figure 7. ^{32}P-end-labelling analysis of the digests of oligonucleotides **17**–**22** (**A**–**F**, respectively). The oligonucleotides were incubated with SVPD, DNase I, NP1 and SAP at 37°C overnight, aliquots of the digest (either untreated or treated with alkali) incubated with [γ-^{32}P]ATP + T4 PNK and the radiolabelled products analysed by PAGE as described in Materials and Methods. A limiting amount of poly(dT)$_{16}$ was used as a labelling reference; the radiolabelled product, [^{32}P](dT)$_{16}$, remained near the top of the gel (not shown).

containing pairs of T^{g} separated by either one or two deoxyadenosine nucleotides, and **21** and **22**, containing pairs of Me-PTE separated by a single thymidylyl dinucleoside phosphate, were prepared to establish the specificity of SVPD in dealing with pairs of lesions on the same strand. In particular, these oligonucleotides were prepared to determine whether it was mandatory to have two lesions at adjacent positions in order to generate SVPD-resistant trimer fragments or whether pairs of lesions separated by normal nucleotides would also lead to longer SVPD-resistant fragments (i.e. TpFpTpF from **5** and **7** or TpFpTpTpF from **6** and **8**). In every instance, SVPD digestion of oligonucleotides **5**–**8** yielded a labellable species of *m/z* ~421, corresponding to TpF, with no evidence for larger SVPD-resistant fragments apparent. Similarly, SVPD digestion of oligonucleotides **13**–**16** yielded a labellable species of *m/z* ~588, corresponding to dApT^{g}, with no evidence for larger SVPD-resistant fragments apparent. For **5**–**8**, the greater than anticipated relative yield of released thymidine compared to recovered TpF, as determined by HPLC analysis, indicates that the phosphodiester bonds immediately 5′ to the individual separated tetrahydrofurans were hydrolysed to ~55–65%. This is similar to the extent of hydrolysis determined for the corresponding 5′ phosphodiester bond in the single F-containing oligonucleotides **1** and **3** (see above). Finally, digestion, alkali treatment and labelling of **21** and **22** exclusively yielded the labelled dinucleotide 2T, with no evidence for any longer fragments (i.e. 3T or [^{32}P]TpTpTpT[4T]) apparent.

CONCLUSIONS

In the present study prepared oligodeoxyribonucleotides containing single, tandem or pairs of separated abasic, oxidative and alkylative damage were exploited as model substrates to examine protocols incorporating an SVPD digestion step as potential methods to permit the detection of tandem lesions in DNA. The successful HPLC+MS detection of tandem tetrahydrofuran- and thymine glycol-containing SVPD-resistant fragments in the digests of oligonucleotides containing these adjacent lesions, plus successful PAGE detection of the ^{32}P-end-labelled trinucleotide species in the digests of oligonucleotides containing tandem methylphosphotriester adducts, demonstrates that the SVPD digestion strategy is indeed capable of retaining certain tandem damage and allowing their direct detection. In studies to determine the specificity of SVPD in allowing the detection of pairs of lesions on the same strand, it was found requisite to have the two lesions at immediately adjacent positions in order to generate the larger SVPD-resistant fragments; pairs of lesions separated by only one or two normal nucleotides behaved principally as single lesions towards SVPD digestion. Our future investigations will include studies to detect tandem lesions in DNA, as revealed by SVPD-based digestion, in combination with LC-MS

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