Phosphotriester adducts (PTEs): DNA’s overlooked lesion

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In addition to reacting with DNA base moieties, many chemical genotoxins also react with the oxygen atoms of the internucleotidic phosphodiester linkages to form phosphotriester adducts (PTEs). In view of their stability under physiological conditions, it has been suggested that PTEs may be useful biomarkers for measuring cumulative genotoxin exposure. The methodology for their determination is varied and still not completely developed but includes determination of hydrolysis products and ³²P-postlabelling approaches. More recently, transalkylation and direct mass spectrometry techniques have been devised, which give extra chemical information on the structures of the PTEs. The proportion of DNA damage formed as PTEs is much greater with SN1 compared to SN2 alkylating agents, and it has been shown in DNA that the formation of PTEs is partially sequence dependent. PTEs have been considered to be refractory to repair in prokaryotic cells, e.g. PTEs in Escherichia coli are repaired by O⁶-methylguanine-DNA methyltransferase (O⁶-MGT or Ada protein). However, studies on in vivo persistence of PTEs in mammalian systems have not ruled out the possibility of a contribution from an active repair process for PTEs. The biological significance of PTEs is largely unstudied and unknown, although effects of PTEs on DNA polymerases, and some exo- and endonucleases have been observed. Also site-specific PTEs impair the repair processing of adjacent sites of DNA damage, which may be a biological mechanism of importance for these lesions. In this review, we will consider the analytical methods available for the determination of PTEs, their stability in vitro and in vivo, the mechanisms for their repair, their possible biological significance and their potential role as biomarkers in human molecular epidemiology studies.

Introduction

An initial key stage in genotoxic chemical carcinogenesis is the interaction of the chemical carcinogen with DNA. Consequently, numerous studies have been undertaken to characterize the products of the reactions of chemical carcinogens with DNA and to elucidate their biological significance (1–4). Since the carcinogenic and mutagenic effects of alkylating agents are generally considered to be the result of DNA base alkylation (5), the majority of studies of the interaction of chemical carcinogens with DNA have focused on the formation of adducts with the DNA bases. Furthermore, because of their biological significance, base lesions are usually subject to efficient repair by a variety of mechanisms (6) and any interference/impairment of these mechanisms could be harmful to the cell via a consequential increase in deleterious mutations.

As well as interacting with DNA base moieties, many potent chemical genotoxins can also react with the non-carbon-bound oxygen atoms of the internucleotide phosphodiester moiety to form phosphotriester adducts (PTEs) (7). The phosphorus atom of the phosphodiester moiety, linking the adjacent nucleosides of the DNA’s sugar-phosphate backbone, is at the centre of a tetrahedron configuration of four oxygen atoms. Esterification, by adduct formation, of one of the two non-carbon-bonded oxygens forms the phosphotriester configuration (Figure 1a) resulting in the neutralization of the phosphodiester moiety’s negative charge.

Depending on which oxygen is esterified, the resulting PTE can be in one of two configurations, Rp and Sp (Figure 1b). In the Rp diastereomeric configuration, the alkyl group points perpendicularly out from the helix of double-stranded DNA (dsDNA). In the Rp configuration, the alkyl group points into the major groove.

A wide range of alkylating carcinogens can form PTE adducts in DNA (Table I), including dialkylsulphates, alkyl methanesulphonates and N-alkylnitrosoureas (7). In addition, cyclophosphamide and cyanoethylene oxide have also been shown to react with the sugar-phosphate backbone in DNA (45, 47). The relative abundance of PTEs depends upon the alkylating character of the agent. Generally, the extent of PTE formation parallels that of other oxygen alkylation products, such as O⁶-alkylguanine and O⁴-alkylthymine, which are known to be highly mutagenic; thus, PTEs may serve as surrogate markers of known mutagenic lesions in DNA. Also, as several studies have shown that PTEs are chemically stable under physiological conditions (8, 31, 48) and are considered refractory to DNA repair [compare ref. (21)], they may serve as biomarkers for measuring cumulative genotoxic exposure (34).

Despite these observations, many of which were made over 20 years ago, the potential use of PTEs as biomarkers of carcinogen exposure has still not been completely explored. In this review, we will examine and discuss the analytical protocols available for the assessment of PTEs, their stability and repair and their possible biological significance, with the aim of determining their relevance and potential role as biomarkers in human molecular epidemiology studies.
One of the key reasons that so little attention has been paid to the detection of carcinogen-induced PTEs in DNA has been the lack of convenient and suitable analytical methods. Consequently, the extent of PTE formation by most carcinogens is largely unknown.

Methods using radiolabel detection
One of the earliest methods for the analysis and study of PTEs in DNA used radiolabelled alkylating agents or DNA and measured the radioactivity bound to DNA, which resisted heating to 100°C in neutral aqueous solutions (27). It was supposed that such treatment would lead to a complete loss of the alkylated purines and, as the alkylation of the pyrimidines is very low, it seemed that most of the residual radioactivity ought to be due to alkyl groups bound to phosphates. It was shown, using this method, that the majority of labelled alkyl groups on the DNA were labile on heating to 100°C but that a certain proportion remained bound even after 90 min of heating and resisted further heating (27, 31). The extent of the residual radioactivity, which presumably reflected PTE formation, differed depending on the alkylating agent used. Bannon and Verly (31) also completely hydrolysed the labelled heat-depurinated DNA in 90% formic acid at 175°C for 30 min and then analysed the volatile material and the involatile alkyl-phosphate and alkylated bases derived from the PTE present in the hydrolysis products. Comprehensive studies of products of ethylating agent attack on DNA were later carried out by Sun and Singer (22) using a variety of hydrolysis conditions, including 70% HClO₄, 100°C, 1 h. Another one of the earlier methods for detection of PTEs relied on the radiochemical detection of eluates from ion-exchange chromatography of enzymatic digests of DNA treated with alkylating agents (9, 24).

Up until recently, the methods, as described above, have not been appropriate for the detection of PTEs in vivo in human DNA owing to the requirement for a relatively high level of radioactive label in the PTE-forming agent. However, new possibilities may now emerge with the use of accelerator mass spectrometry as this has greatly improved the sensitivity for detection of ¹⁴C and ³H compared to scintillation counting, allowing human experimental studies to be carried out with trace levels of radioactivity (49).
### Table I. PTE-forming agents in vitro and in vivo

<table>
<thead>
<tr>
<th>Agent</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNU</td>
<td>R (8, 9, 10, 11) S (12, 13, 14) T (15, 16) M (17)</td>
<td>S (18) R (19, 20)</td>
</tr>
<tr>
<td>ENU</td>
<td>R (7, 8, 11, 21, 22) S (12, 13, 14) T (23) M (23)</td>
<td>R (7, 19, 24, 25, 26) S (18)</td>
</tr>
<tr>
<td>Dimethyl sulphate</td>
<td>R (11, 27) S (12, 13) P (28) T (16)</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>R (22, 27) P (28, 29, 30)</td>
<td></td>
</tr>
<tr>
<td>EMS</td>
<td>R (8, 27, 31) S (12, 32) P (33) M (17)</td>
<td>R (19)</td>
</tr>
<tr>
<td>2-Hydroxyethyl methanesulphonate</td>
<td>S (12)</td>
<td>S (34, 35) R (24)</td>
</tr>
<tr>
<td>Dimethyl nitrosamine</td>
<td>M (37)</td>
<td>S (34) P (28, 36)</td>
</tr>
<tr>
<td>Diethyl nitrosamine</td>
<td>M (39) S (40)</td>
<td>T (38)</td>
</tr>
<tr>
<td>Phenyl glycidyl ether</td>
<td>R (41, 42, 43)</td>
<td></td>
</tr>
<tr>
<td>NNK</td>
<td>R (44)</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>P (45) M (46)</td>
<td>P (45)</td>
</tr>
<tr>
<td>1-(2-chloroethyl)-1-nitrosourea</td>
<td>S (12, 13, 14)</td>
<td></td>
</tr>
<tr>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)</td>
<td>P (10)</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide (and metabolites)</td>
<td>S (47)</td>
<td></td>
</tr>
<tr>
<td>Cyanogenlycine oxide</td>
<td>S (12, 13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S (18)</td>
<td></td>
</tr>
</tbody>
</table>

**R**, radiolabelled detection of adducts; **S**, strand breaks with or without alkali hydrolysis; **T**, transalkylation, **M**, mass spectrometry, **P**, $^{32}$P-postlabelling.

### Determination of products of alkaline hydrolysis

The earliest attempts to quantify total unlabelled PTE in DNA involved analysis of alkali-induced strand breaks at the sites of PTE lesions (12, 32, 34). This method exploits the fact that the rate of hydrolysis of PTEs is much greater than for phosphodiesters (13) and that the sedimentation coefficient of DNA increases as the number of strand breaks increases. However, it is a relatively non-specific approach, possibly confounded by the presence of other strand breaking alkali-labile lesions in the DNA, e.g. abasic sites, which may lead to the overestimation of the level of PTEs. The conditions for alkaline hydrolysis of PTEs were studied by Shooter (13), to determine if such techniques could be used to determine PTEs in bacteriophage T7 DNA, using ultracentrifuge techniques. Although hydrolysis in alkali could be used to detect and possibly assay for PTE groups, the method was thought to be most suitable for rapidly reacting alkylating agents such as alkyl nitrosoureas when loss of alkylated purines can be kept at a low level. Snyder and Regan (32) further investigated suitable conditions for alkaline hydrolysis for measuring PTEs in cellular DNA, using alkaline sucrose sedimentation analysis, and the authors concluded that PTEs could be measured accurately after a sufficient time had passed to allow repair of other DNA lesions to be completed. For methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS), this was 48 h.

Alkaline hydrolysis of PTEs will result in three products, one of which will be the alcohol and the other two will result from cleavage of a bond to a nucleoside and will yield single-strand breaks. Measurement of the amount of methanol or ethanol released from DNA alkylated with methyl nitrosourea (MNU) or ethyl nitrosourea (ENU) showed that two-thirds of the PTEs produced by MNU, and three-quarters of those produced by ENU, will hydrolyse to strand breaks (14). Weinfeld and Livingstone (50) synthesised oligonucleotides containing PTEs as the sole lesion, including dTpTpT(dE)TpCpTpApTpTpT, which was hydrolysed in 0.5 M NaOH at 37°C and the products were analysed by high-performance liquid chromatography (HPLC) and by $^{32}$P-labelling using T4-poly nucleotide kinase (T4 PNK). Twenty-seven per cent of the hydrolysis resulted in loss of the ethyl group, 30% in cleavage of the 5'-phosphate group and 43% in cleavage at the 3'-phosphate group.

### $^{32}$P-postlabelling assays

In the experiments of Weinfeld and Livingstone (50), it was of interest to note thatTp(Et)T was shown not to be a substrate for T4 PNK, indicating that the normal $^{32}$P-postlabelling conditions for detecting adducted nucleotides cannot be used for PTEs; the most likely reason for this being the loss of the negative charge on the phosphate moiety immediately 3' to the nucleoside to be 5'-phosphorylated, which is a substrate requirement for T4PNK. However, several postlabelling strategies have been developed based on the fact that the phosphotriester moiety is resistant to all nucleases tested to date, generally yielding PTE-bearing dinucleoside phosphates (dNp(R)dN). In order to permit subsequent $^{32}$P-labelling, it is necessary to remove the alkyl group (R) from the dinucleoside phosphate species by alkali hydrolysis. PTEs are labile under alkaline conditions but the character of the nucleophile affects the site of attack on the PTE. Hard nucleophiles (e.g. OH-) will preferentially attack the P atom of the PTE, and this results in a pentavalent phosphorus atom. In contrast, soft nucleophiles (e.g. NH3) preferentially attack the primary carbon in the alkyl group, which causes the cleavage of the alkyl oxygen bond. It was shown by Saris et al. (33) that using 12.5% aqueous ammonium hydroxide, the major product of ammonia hydrolysis is the unmodified dinucleoside phosphate (dNp(NH3)dN), 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 (RdpN) species accounted for ~10% of the alkali hydrolysis products (33). The resulting dNpD and dNpR species can then be labelled with [γ-$^{32}$P]ATP by T4PNK.

Singh et al. developed an early technique for analysing ethyl PTEs by postlabelling after alkaline hydrolysis (29). In this study, the phosphodiester standard 2'-deoxyguanosine 3'-mono- O-ethyl phosphate (3'Et-pdG) was synthesized from the reaction of diethyl sulphate (DES) with 2'-deoxyguanosine 3'-monophosphates and a postlabelling assay was developed for its determination. The analytical procedure for DNA ethyl PTEs involved digestion with micrococcal nuclease (MN) and calf spleen phosphodiesterase, incubation with nuclease P1 (NP1) and then alkaline hydrolysis of the PTEs in the
mixture, HPLC and 32P-postlabelling of the purified 3'Et-pdG (Figure 2).

A second postlabelling strategy used to detect PTEs is the snake venom phosphodiesterase 1 (SVPD)/NP1 postlabelling assay (Figure 2), which is a modified version of the SVPD postlabelling assay used to detect oxidative lesions, cyclobutane pyrimidine dimers and abasic sites (51–55). DNA is digested by a 'cocktail' of nucleases; DNase 1, SVPD and NP1 in the presence of shrimp alkaline phosphatase resulting in a mixture containing the PTE-bearing dinucleoside phosphates (dNp(R)dN) plus modified and unmodified nucleosides (dX and dN). Alkali hydrolysis using ammonium hydroxide yields dNpdN and dNpR species, which were then labelled with [γ-32P]ATP by T4PNK.

In the original SVPD/NP1 protocol, as described by Saris et al. (33, 56), the labelled dinucleotide species (32pdNpdN) were analysed using a combination of HPLC and thin-layer chromatography (Figure 2). However, this two-step process was inappropriate for routine analysis of samples. To address this, Guichard et al. developed a method for the detection and quantification of PTEs using polyacrylamide gel electrophoresis (PAGE) (Figure 2). PAGE separates the 16 dinucleotides into five bands (28). The radiolabelled products were visualized using autoradiography and/or phosphorimaging. The protocol also allowed for the determination of the identity and frequency of the nucleosides located 5’ to the PTE lesions. The identity of the nucleotide 5’ to the phosphotriester lesion was obtained by the treatment of the gel isolated 32pdNpdN species with NP1 to release 32pdN species, which may then be analysed by HPLC with radioactivity detection (32P-HPLC) (28). In a subsequent study, oligonucleotides containing single or pairs of tetrahydrofuran moieties (representing stable apurinic sites), thymine glycols and PTEs were investigated as model substrates using the SVPD-based procedure. The detection of trinucleotide species showed that it was possible to detect tandem damage, including when two PTEs were immediately adjacent to each other (57).

Further development of the postlabelling method to improve the 5’ nearest neighbour analysis was carried out by Le Pla et al. (36). This avoided the PAGE isolation of the 32pdNpdN species and reduced the handling of the radiolabelled material. The method (termed the ‘direct’ method) involved the use of apyrase (which catalyses the breakdown of excess [γ-32P]ATP to AMP and 32Pi), followed by boiling (to remove residual 5’-dephosphorylating activity) and NP1 digestion (Figure 2), and led to >80% release of the 32pdN neighbour species with analysis by 32P-HPLC. In addition, the new direct method also permits the inclusion of the nucleoside 3’-O-alkylphosphate species (32pdNpR) in the 5’ nearest neighbour analysis; while the dNpR species are only minor products of the alkali hydrolysis reaction (see above), they were not included in the previous PTE 5’ nearest neighbour determinations (28).

Transalkylation of PTEs

An approach exploiting the transalkylation of PTE to a stronger nucleophile has been reported in attempts to identify the PTE-forming species (15, 16, 58) (Figure 3). Early studies used a strong nucleophile such as thiosulphate or cob(I)alamin to remove the PTE alkyl group from DNA, with the alkylated nucleophile being detected by HPLC. The method is fast and specific, with the alkyl group from the PTEs being transferred exclusively to cob(I)alamin within minutes; 7-methyl dG was stable for 2.5 h and 3-methyl dA and O6-methyl dG were stable for 1.3–1.5 h 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

Fig. 2. 32P-postlabelling strategies for the detection of phosphotriesters.
only detectable leaving group (16). Further studies showed that SVPD/NP1 digestion of the DNA prior to reaction with cob(I)alamin significantly improves the yield of PTEs detected (16). The detection limit for the assay was estimated to be between 0.01 and 0.05 nmol of adduct (16). More recently, the method has been developed to be used in conjunction with liquid chromatography tandem mass spectrometry (58). This method enables the sensitive detection of PTEs and the identification of the alkyl cob(I)alamins. However, this method does not offer any information on the sequence context in which the PTEs lie.

Mass spectrometry

It is also possible to analyse PTEs by mass spectrometry without any hydrolysis or transalkylation procedures of the triester. In 2004, Haglund et al. reported the complete characterization of ethyl PTEs formed by reaction of DNA with ENU. In this study, the ENU-treated DNA was enzyme digested using NP1, phosphodiesterase I and alkaline phosphatase and the enzymatic hydrolysate analysed using miniaturized LC-ESI-MS/MS and column switching (23). Ten ethyl PTEs (out of a possible 16) were identified. Interestingly, the enzymatic hydrolysate of this DNA was also subjected to transalkylation using cob(I)alamin and the ethyl cobalamin analysed. Thus, transalkylation may be a sensitive approach for monitoring total PTE levels, whereas the MS method will give more structural information on individual PTEs. Zhang et al. (17) recently described a mass spectral method for quantifying the methyl PTE of TpT [dTp(Me)dT] by LC/APCI-MS/MS (lower limit of detection 6.4 adducts/10^8 nucleotides) and used it to determine levels of this PTE in DNA from cells treated with low doses of MNU.

Formation in vitro

The suggestion that alkylating agents can react with phosphate oxygens in DNA was first made >60 years ago by Elmore et al. on the basis of electrometric titration studies of DNA treated with mustard gas [sulphur mustard, bis(2-chloroethyl)sulphide] (59). Later, more detailed studies were carried out with methylating and ethylating agents such as alkyl methanesulphonates and alkyl nitrosoureas. Bannon and Verly showed, using radiolabelled compounds and measuring the amount of label remaining in alkylated DNA after neutral thermal hydrolysis (see Analysis of PTEs in DNA), that 5.6% of the initial activity from MMS remained bound and was presumably largely PTEs, while the amount for EMS was 16.3%. By analysis of the products produced by acidic hydrolysis of the heat-depurinated DNA (see Analysis of PTEs in DNA), it was shown that 15% of the alkylation produced by EMS was on the phosphate, whereas the estimate for MMS was 1% (31).

Lawley (10) obtained evidence for the formation of PTEs in his studies of the interaction of MNU with DNA, using ion-exchange chromatography of enzymically digested DNA and radiolabelled materials. The methyl PTE of thymidyl(3′–5′)thymidine (TpT) was chemically synthesized by Swenson et al. (9) and was shown to cochromatograph with a product from DNA methylated with MNU after depurination and enzymic digestion. In these experiments, either the methyl group of MNU or the thymidine in DNA was radiolabelled. The identity of the product was confirmed by alkaline hydrolysis in 0.1 M NaOH, which yielded the same reaction products from both the synthetic product and the DNA alkylation product, i.e. TpT, thymidine and the methyl esters of 3′ thymidine monophosphate (TMP) and 5′ TMP.

The ethyl PTE was identified using ion-exchange chromatography of enzymically digested DNA by Swenson and Lawley (11) as a component in the enzymic hydrolysate from DNA treated with ENU. The relative reactivity towards ENU, EMS, dimethyl sulphate (DMS) and MNU of the non-carbon-bound phosphate oxygens in TpT phosphodiester in DNA was compared. ENU had the greatest proportion of the TpT PTE in the DNA products (4.3%) and DMS the lowest (0.09%, 48-fold lower). However, it was concluded from the comparison of results that nucleophilicity alone is not adequate to explain the relative reactivities of the phosphodiester group towards the alkylating agents.

The interaction of ENU with the alternating dAdT co-monomer [ATATAT ...] was shown to lead to 60–65% of the total of the assayed alkylation products as being on the phosphate group (60). Products were confirmed by comparison with authentic triesters dAp(Et)dT and dTp(Et)dA. Although the reaction was base sequence independent, there was a preference for one of the oxygens in the phosphate group as demonstrated by the observed differences in amounts of diastereoisomers noted by HPLC analysis.

A more comprehensive survey of the interactions of methylating and ethylating agents with nucleophilic sites in DNA PTEs—stable, persistent and significant
DNA was carried out by Beranek et al. (8), using HPLC analysis of products released after depurination and enzyme hydrolysis of alkylated DNA. The amount of PTEs as a percent of total DNA modification was as follows: MMS 0.82%, EMS 12.0%, MNU 12.1%, ENU 55.35%, in broad confirmation of the earlier results. A later compilation of the results from different workers gave values of the following: MMS 0.8%, EMS 12.0–13.0%, MNU 12.0–17.0%, ENU 55.0–57.0% and DES 16.0% (48). Thus, the proportion of PTEs in DNA treated with a strong SN1 alkylating agent such as ENU is much greater than that produced by less carcinogenic SN2 alkylating agents such as MMS (Table II).

There have been several attempts to correlate rate data for nucleophilic substitution reactions with the characteristics of the electrophilic alkylating agent, including the use of the Swain-Scott factor (61). The substrate constant(s) decreases with increasing SN1 behaviour, and electrophiles with large alkyl groups have lower s values. It has also been proposed that the hard and soft acid and base theory of Pearson (62) may better explain the outcome of DNA alkylation reactions. According to this theory, hard acids have a preference for hard bases, while soft acids prefer soft bases. In terms of hardness and softness, alkylating agents are considered to be intermediate acids. Similarly, the oxygen and nitrogen atoms in purines and pyrimidine bases are considered to be intermediate bases, although according to Pearson’s concept oxygen atoms are harder bases than nitrogen atoms. The electrophilic species formed from N-nitroso compounds (SN1) are harder acids than those generated from alkane sulphonates (SN2), and so, the N-nitroso compounds will show a preference for reaction at oxygen atoms as alkylated bases than the alkane sulphonates.

In a study of phosphate alkylation in different DNA substrates, Le Pla et al. (30) compared the in vitro formation of DES-induced PTEs in h2E1/OR human B-lymphoblastoid cells, their isolated nuclei and isolated DNA, using the 5′ nearest neighbour analysis postlabelling procedure known as the ‘direct method’ (see Analysis of PTEs in DNA). Furthermore, to determine the role of electrophile character in PTE manifestation, prepared oligonucleotides ([dT]20[dG]20,[dC]20[dA]20) were treated with three alkylating agents of differing electrophilic character (DES, MNU and ENU) and PTE manifestation was assessed by postlabelling. The formation of PTEs was determined to be non-random in the whole cells and nuclei, and DNA, with PTEs being formed to a greater extent 3′ to pyrimidine moieties than 3′ to purine moieties. Parallel studies with oligonucleotides confirmed these observations and demonstrated that the non-random formation of PTEs was primarily determined by DNA sequence and not determined by DNA packaging/chromatin factors. Furthermore, the extent of the non-random formation of PTEs was also governed by electrophile reactivity, with the more reactive electrophiles (from ENU) yielding a more random formation of PTEs. From these observations, Le Pla et al. (30) proposed a model for the non-random formation of PTEs, which was governed by (i) the phosphate oxygens having to compete with adjacent nucleophilic sites for the alkylating electrophile and (ii) the electrophile’s inherent reactivity.

Other alkylating agents which have been demonstrated to interact with the phosphate oxygens in vitro include 1-(2-chloroethyl)-1-nitrosourea (measured as PTEs in DNA) (41–43), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (PTEs in DNA) (44), cyclophosphamide active metabolites including phosphoramid mustard (diesters on 2′-deoxyguanosine-3′-monophosphate and DNA in vitro and in vivo) (45, 46), cyanoethylene oxide (diesters on 2′-deoxyadenosine-5′-monophosphates) (47), phenylglycidyl ether (PTEs on phosphates 5′ to T and A in DNA) (37) and the tobacco-specific nitrosamine 4-((N-methyl-N-nitosamino)-1-(3-pyridyl)-1-butane (NNK) (cob[alamin transalkylation) (38). The possibility that benzo[a]pyrene may produce PTEs was investigated by Gaskell et al. (39) using mass spectral determination of the reaction products of benzo[a]pyrene-7, 8-dihydrodiol-9,10-epoxide (B[a]PDE) with 2′-deoxyadenosine 3′-monophosphates. Products were purified using solid phase extraction and HPLC and were then desalted and analysed using LC-ESI-MS/MS collision-induced dissociation. In addition to the base adducts, products consistent with the structures of B[a]PDE adducts with the phosphate group of the 2′-deoxyguanosines of guanine, adenine, cytosine and thymine were detected. This is the first direct evidence for the formation of phosphodiester adducts by B[a]PDE following reaction with 2′-deoxyribonucleotides and suggests the possibility that phosphate alkylation may occur following exposure of DNA to B[a]PDE, producing PTEs (albeit maybe transiently—see later).

### Table II. Relative levels of adducts induced at different sites in DNA by a variety of common alkylating agents

<table>
<thead>
<tr>
<th>Site of alkylation</th>
<th>Percentage of total DNA alkylation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MMS</td>
</tr>
<tr>
<td>Adenine</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>1.9–3.8</td>
</tr>
<tr>
<td>N3</td>
<td>10.4–11.3</td>
</tr>
<tr>
<td>N7</td>
<td>1.8</td>
</tr>
<tr>
<td>Cytosine</td>
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<tr>
<td>O2</td>
<td>n.d.</td>
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<tr>
<td>O3</td>
<td>&lt;1.0</td>
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<tr>
<td>Guanine</td>
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<td>N3</td>
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<td>O2</td>
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<tr>
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<td>81.0–83.0</td>
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<td>Thymine</td>
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<tr>
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<td>n.d.</td>
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<td>O3</td>
<td>0.1</td>
</tr>
<tr>
<td>O4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Phosphotriester</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Adapted from Beranek (48). A dash indicates that no data have been recorded, 'n.d.' indicates that adduct levels were below the detectable limit.

### Stability in vitro

**Chemical**

Miller et al. (63) 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 alkyl PTE (64–66). In contrast, Lawrence et al. (67) 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 an isopropyl PTE. PTE containing oligonucleotides can form base-paired...
complexes with complementary oligonucleotides. These complexes have a greater stability than for similar complexes formed between normal oligonucleotides, presumably due to the removal of phosphate backbone charge repulsion (63, 64). Indeed, the melting temperatures for oligonucleotides containing an Rp ethyl PTE were found to be lower than for oligonucleotides containing an Sp ethyl PTE (64, 68).

While DNA PTEs are labile to alkali, they are very stable under neutral and acidic conditions; the alkyl group is only released by strong acid treatment (e.g. 70% HClO₄, 100°C, 1 h and 90% formic acid, 175°C, 30 min) (22, 31). Bannon and Verly (31) determined the stability of the PTEs under neutral thermal hydrolysis conditions. For DNA reacted with tritiated EMS or MMS, at least 90% of the PTEs are stable at 100°C for 90 min at pH 7.0. Thus, it may be predicted that the rate of hydrolysis at 37°C would be very low under neutral conditions.

Bodell et al. (21) found, using paper chromatographic analysis, that ethyl PTEs resulting from alkylation of DNA *in vitro* with ENU showed no loss after incubation at pH 7.4, 37°C for 10 days. Shooter and Merrifield (12) showed that by comparing the hydrolysis of an alkylated DNA in two concentrations of alkali, it was possible to distinguish between two types of lesion: apurinic sites and PTEs. Using the rapidly reacting methylating agent MNNG (t₁/₂ 7 min), the amount of PTEs appeared to be constant up to at least 48 h, whereas apurinic sites increased because of spontaneous hydrolysis. A slowly reacting methylating agent EMS (t₁/₂ for hydrolysis 11 h) showed an expected increase with time of both apurinic sites and PTEs.

However, for 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, for example in the case of 2-hydroxyethyl PTE formed by ethylene oxide (31, 69). Thus, in a study of the stability of a variety of synthetic PTEs in neutral and alkaline conditions by Conrad et al. (69) using HPLC, the half-life of dTp(2-hydroxyethyl)dT was found to be 60 min at pH 7.0 and ≤ 1 min at pH 12.5, whereas dTp(Me)dT was stable for >3 days at pH 7.0 and had a half-life of 165 min at pH 12.5. Thus, 2-hydroxyethyl PTE (a product from alkylation with 1-(2-hydroxyethyl)-1-nitrosourea or from 2-chloroethylnitrosoureas) may readily induce single-strand breaks in DNA. However, it was also shown that DNA alkylated with 2-hydroxyethyl methanesulphonate showed no decline of PTE content up to 4 h before a decrease was observed (12). RNA phosphotriesters are similarly unstable as 2-hydroxyethyl DNA PTEs because of the presence of a beta-hydroxy group on the C₂⁺ position of the ribose.

The hypothesis that PTE adducts might stimulate DNA strand breaks because of the interaction of a β-hydroxy group was also proposed for adducts formed by the active metabolite of benzo[a]pyrene, B[a]PDE (40). These authors showed nicking of superhelical Col E1 DNA by syn- and anti-B[a]PDE using gel electrophoresis and electron microscopy. The kinetics of the nicking supported the involvement of PTEs with facilitation of the hydrolysis yielding strand scission by the C-9 hydroxyl group on the hydrocarbon.

**Cellular systems**

Studies using 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 vitro*. The stability of ethyl PTEs in cultured cell DNA was examined by Bodell et al. (21). Cultured human fibroblasts and Xeroderma pigmentosum fibroblasts, pre-la

**Formation and persistence in vivo**

Most DNA PTEs are stable under physiological conditions. Following intraperitoneal treatment of male mice with MNU (80 mg/kg) or ENU (160 mg/kg), the extent of PTE formation in DNA in various tissues was estimated by sedimentation after alkaline hydrolysis (see Analysis of PTEs in DNA) (12, 13, 18). The concentration of PTEs following MNU treatment decreased with a half-life of ~7 days in lung, liver and kidney, whereas the decrease was faster in the brain (t₁/₂ 2–3 days).

Multiple injections of MNU led to an accumulation of PTEs in lung, liver and kidney, the observed t₁/₂ being ~16 days; PTEs in brain disappeared at an intermediate rate, slower than spleen and thymus but faster than lung liver and kidney. Rapidly dividing tissues (spleen and thymus) showed a rapid loss of PTEs, presumably because of cell dilution. ENU produced more stable PTEs, the t₁/₂ in lung, liver, kidney and brain being 10–15 weeks. The accumulation of PTEs in lung, liver and brain was about the same regardless of whether a dose of ENU was delivered over 5 or 40 weeks, indicating stability of the PTEs *in vivo*. The authors postulated that the more rapid loss of methyl PTEs than ethyl PTEs from tissues was not due to a repair process but may be due to a greater chemical instability towards hydrolysis of the methyl PTEs.

PTEs derived from dimethylnitrosamine (DMN) given by continuous administration to rats via the drinking water were compared with those obtained following a single intraperitoneal dose (34, 35). The single dose yielded the highest amount of PTEs in the liver, the major site of metabolism, but the kidney and lung also contained lower amounts. Continuous administration gave detectable PTEs at doses down to 0.1 p.p.m. w/v in the liver but lung DNA PTEs were only evident at a dose of 5 p.p.m. and kidney PTE formation could not be confirmed consistently. Continuous administration of diethylnitrosamine (DEN), however, produced PTEs in all the three tissues at doses down to 0.1 p.p.m., supporting the hypothesis that ethyl PTEs are more resistant to repair and/or chemical hydrolysis than methyl PTEs.

In mice treated with MNU, ENU and EMS, PTEs were measured by HPLC of digested liver DNA (19). The relative proportions of the TpT PTEs compared to N7-alkylguanine were similar to those previously found *in vitro* (MNU 0.016, EMS 0.013, ENU 0.27), and there was no evidence for marked enzymic removal of PTEs. Singer (7) and Singer et al. (25) reported that ethyl PTEs in DNA of rats treated with a single injection of ENU showed only a very small decrease over time (over ~3 days) and that in mouse liver after MNU treatment the t₁/₂ of methyl PTE was ~1 week and after ENU treatment the t₁/₂ of ethyl PTE was ~7 weeks.

Using a modification of the HPLC method of Beranek (8), a detailed survey of alkylation patterns in DNA isolated from rat liver after treatment with ENU or DMN was made by den Engelse et al. (24). The relative amounts of PTEs compared to N7-ethyl- or methylguanine were 3.0 and 0.13, respectively.
Fig. 4. Timescale of PTE manifestation in vitro and in vivo. (a) Relative amounts of [3H]thymidine and [14C]ethyl groups in GM 637 human and Xeroderma pigmentosum fibroblasts (XP-12) as functions of time of cell culture after treatment with [14C]ethylnitrosourea. GM 637 fibroblasts; [14C] open squares, [3H] open triangles, [14C] corrected for decrease in [3H] specific activity, closed squares. Xeroderma pigmentosum fibroblasts; [14C] open circles, [3H] crosses, [14C] corrected for decrease in [3H] specific activity, closed circles. All radioactivity is expressed as per cent of specific activity (cpm/mg DNA) at 0 time. The shaded area represents the absolute loss of [14C]ethyl groups. This was determined by correcting the [14C] specific activity for the reduction in [3H] specific activity due to replication. From [21] W. J. Bodell et al., Evidence for the removal at different rates of O-ethyl pyrimidines and ethylphosphotriesters in two human fibroblast cell lines (1979) Nucleic Acids Research, 8, 2819–2829 by permission of Oxford University Press. (b) Liver Et-PTE levels at the various times after dosing, relative to the maximum level. BALB/c mice were treated with a single intraperitoneal dose of DEN (90 mg/kg) and sacrificed at various times after dosing. The DNA was then isolated and Et-PTE levels were determined by the postlabeling protocol with PAGE analysis. The level of Et-PTEs was noted to reach a maximum (~38 PTE adducts/10^9 DNA-P) 10 h after dosing. Reprinted with permission from (36) R. C. Le Pla, et al., Further development of P-32-postlabeling for the detection of alkylphosphotriesters: Evidence for the long-term nonrandom persistence of ethylphosphotriester adducts in vivo. (2004) Chemical Research in Toxicology, 17, 1491–1500. Copyright 2004 American Chemical Society.

The relative amounts of dTp(alkyl)dT were 0.251 (ENU) and 0.011 (DMN), respectively. Most of the ethylated products disappeared from liver DNA but the most stable product was the ethyl PTE with a t_{1/2} of 32 days. At 56 days after injection, the only products that could be detected were ethyl PTE, O^2-EtT and N7-EtG. After the DMN treatment, the t_{1/2} of methyl PTE was 7 days. The analysis was extended to other tissues (26), which showed that O^2-EtT, O^2-EtT and dTp(Et)dT had very slow rates of loss from liver, lung, kidney, brain and testis. The t_{1/2} values for dTp(Et)dT ranged from 19 to 65 days. At 56 days, the ethyl PTEs were by far the most abundant lesions in these tissues. Loss of these adducts was faster in intestine and spleen, possibly because of cell turnover. In urothelial DNA from rats treated with MNU (0.5 mg) by urethral catheter, the level of methyl PTEs calculated from the measured Tp(Me)T level was 49 adducts/10^6 nucleotides at 2 h but decreased steadily reaching 31 adducts/10^6 nucleotides at 2 days and 2.7 adducts/10^5 nucleotides at 21 days (20).

Using the SVPD/NP1 protocol (see Analysis of PTEs in DNA), Guichard et al. reported that the frequency of nucleosides 5' to the site of PTE lesions in DNA from mice that had been treated with DEN (90 mg/kg, intraperitoneally) was non-random in vivo (28). Results indicated that the frequency of thymidine and 2'-deoxyguanosine 5' to the ethyl PTE was significantly different from the corresponding normal nucleoside content with the frequency of T 5' to the site of PTE lesions in the liver DNA of these mice being significantly greater and the frequency of dG 5' to the site of PTE lesions being significantly lower. These results were indicative of the non-random formation of ethyl PTE in vivo and/or base sequence-specific ethyl PTE repair.

In further studies using the same technique, the persistence of ethyl PTEs was determined (36). There was an initial decline in ethyl PTE levels down to 37% of the original value after 4 days (t_{1/2} < 24 h) but then the level fell more slowly reaching 15% of the maximum adduct burden at 56 days (Figure 4b). The measurement of ethyl PTEs thus seemed to have potential as a long-term biomarker of genotoxic exposure. The non-randomness of the PTEs was maintained up to 56 days indicating that the cause for this was unlikely to be DNA repair. The initial rapid loss of the PTEs could be due to liver tissue proliferation, although it was speculated that there may be a contribution from active repair processes (see later).

Phosphotriester repair

With the long-term persistence of PTEs evident from animal studies (see above), PTEs are considered to be refractory to repair in mammalian cells; indeed, with one exception, no PTE repair mechanism has been defined in any eukaryotic systems. However, PTE repair mechanisms have been found in numerous prokaryotic organisms, most notably, Escherichia coli (70).

Repair in lower organisms

In E. coli, methyl PTEs are removed by O^6-methylguanine-DNA methyltransferase (O^6-MGT), which is also known as the Ada protein (71) [due to its key regulatory role in the adaptive response to alkylative damage (see below)] and also as O^6-alkylguanine-DNA alkyltransferase (O^6-AGT). O^6-MGT has a broad substrate specificity that includes O^6-methylguanine, O^4-methylthymine and methylphosphotriesters as well as some larger alkyl groups (72) though the rate at which O^6-MGT deals with the larger lesions may be as much as 10–500 slower compared to its removal of methyl moieties (73, 74). In dealing with methylation damage (including PTEs), O^6-MGT acts as
a direct repair methyltransferase in that it both removes the methyl group from the DNA and irreversibly transfers the alkyl moiety to one of its own cysteine residues, generating 3-methylcysteine in the protein. Effectively, this is a direct, error-free reversal of the DNA alkylation damaging step and leads to the restoration of the original DNA structure, which in the case of PTEs is the restoration of the intact phosphodiester moiety. Direct reversal of $O^6$-methylguanine by $O^6$-MGT is rapid, occurring in $<1$ s at 37°C (75).

$O^6$-MGT has been purified to physical homogeneity from E. coli mutants (76). The purified E. coli protein has a molecular mass of $\sim 39$ kDa but is rapidly and specifically cleaved at a single Lys–Gln bond in the protein to generate an $\sim 19$-kDa fragment (the C-terminal half of the protein), which retains the ability to act on $O^6$-methylguanine and $O^6$-methylthymine (75–78), and an $\sim 20$-kDa fragment (the N-terminal half of the protein, N-Ada), which retains the ability to act on PTEs (see later). Studies on N-Ada fragment reveal it to possess two component domains, N-Ada N (residues 1–75) and N-Ada C (residues 78–178), connected by a flexible linker.

Use of DNA substrates containing predominantly methylphosphotriesters, prepared by heating alkylated DNA (at 80°C for 18 h in neutral buffer) to release 7-methyguanine, 7-methyladenine and 3-methyladenine by depurination, established that the 19-kDa fragment of $O^6$-MGT is inactive against methylphosphotriesters (71). This in turn enabled the exploitation of the 19-kDa fragment as a reagent to prepare alkylated DNA substrates free of $O^6$-methylguanine and $O^6$-methylthymine lesions, containing almost solely methylphosphotriesters. When these substrates were treated with the intact 39-kDa form of the native enzyme, around half of the methyl PTE moieties were transferred in a stoichiometric reaction to the protein’s cysteine residues, suggesting that only one of the isomers is recognized and transferred (70, 71). Quantitative analysis of each isomer, by reverse-phase HPLC, phosphorus NMR and circular dichroism, indicated that it is only the Sp-isomer that is repaired (79).

Different cysteine residues in the $O^6$-MGT protein are used as acceptors in the repair of methylphosphotriesters and methyl base adducts (Figure 5a). Incubation of the purified native (39 kDa) form of $O^6$-MGT with DNA containing mainly either $O^6$-alkylguanine or methylphosphotriesters showed that each $O^6$-MGT enzyme molecule can accept only one methyl group per molecule from each type of substrate, with the 19-kDa fragment exclusively accepting methyl groups from $O^6$-methylguanine. When the 39-kDa form of the enzyme is incubated with an excess of a mixture of the two different DNA substrates, containing $O^6$-methylguanine and methylphosphotriesters, the protein contained approximately two methyl groups per molecule (71). Moreover, pre-incubation of the protein with an excess of $O^6$-methylguanine-containing DNA substrate completely saturated its ability to repair $O^6$-methylguanine but the protein still retained most of its ability to repair methylphosphotriesters. This indicates that the active sites for the repair $O^6$-methylguanine and methylphosphotriesters are distinct. Further specific studies have shown that $O^6$-alkylguanine and $O^6$-methylthymine are repaired by the alkyl group being irreversibly transferred to Cys-321, a cysteine located on the carboxyl terminal domain (83–85), while the alkyl group from PTE lesions are transferred to Cys-38 (82) (formerly thought to be Cys-69) (84, 85, 86), a cysteine residue found on the amino terminal domain of $O^6$-MGT (Figure 5a). The latter methyltransferase appears to be autocatalysed by coordination of the acceptor Cys-38 residue to a single zinc ion, which is also bound by three other cysteine residues Cys-24, -69 and -72. Methyl iodide has been shown to specifically alkylate Cys-38 (87), showing that this ligand is electronically activated relative to the other three. The studies of He et al. (82) have shed light on this selectivity. The sulphur atoms of Cys-42, -69 and -72 are hydrogen bonded to amide protons of the protein main chain, which suppresses their reactivity but stabilizes the protein structure. The S atom of Cys-38, in contrast, is devoid of hydrogen-bonding interactions, maintaining an intrinsically high nucleophilicity for Cys-38. Interestingly, further studies from this group have shown that mutation of the active-site residue Cys-38 of N-Ada can convert it from a sacrificial/suicide DNA repair protein (see below) to an enzyme that uses methanethiol as an external sacrificial reagent and so be able to repair DNA methylphosphotriesters catalytically (88).

Earlier studies using multidimensional heteronuclear magnetic resonance techniques to reveal the solution structure of an N-terminal 10-kDa fragment of $O^6$-MGT, which retains the zinc binding and phosphotriester activity, show the Cys-38 residues at a solvent-exposed surface of the protein, readily accessible from the outside (89). However, it should be noted that steric accessibility alone does not appear to be responsible for the selection of Cys-38 because Cys-69 is also surface exposed yet is not methylated by methyl iodide (87).

As mentioned above, in B-form DNA, the Sp-isomers of methylphosphotriesters project into the solution from the

![Fig. 5. DNA repair and transcriptional activation by Escherichia coli Ada: (a) domain organization of E. coli Ada and DNA repair reactions performed by each domain. Whereas both the N-Ada and the N-Ada domains are required for the sequence-specific DNA binding of Ada, N-Ada alone is fully capable of repairing PTEs (80, 81). (b) Overview of transcriptional activation. Methylation of Cys38 increases the strength of sequence-specific DNA binding by Ada by 102- to 104-fold, thereby enabling the protein to bind the promoters of the ada regulon and activate four genes: ada, alkB, alka and aidB. Adapted from (82) Molecular Cell, 20, He et al., A Methylation- Dependent Electrostatic Switch Controls DNA Repair and Transcriptional Activation by E. coli Ada. 117–129 (2005), with permission of Elsevier.](attachment:image-url)
phosphodiester backbone, while the Rp isomers project inwards into the major groove with greater steric crowding. Thus, the Sp-isomer is expected to be more easily reached by the Cys-38 site of O^6^-MGT (89). Indeed, the stereo-specificity of Ada to repair only Sp methyl PTEs can be rationalized on the basis of the structure. In the crystal structure of Cys-38 methylated N-Ada bound to DNA, the sulphur atom of S(Me)Cys38 projects directly towards the pro-S oxygen atom of the phosphated site located only \( \sim 3.5 \text{ Å} \) away, such that it is readily in-line to achieve S_2 transfer of a methyl group from the pro-S oxygen atom to the sulphur atom of Cys-38. On the other hand, the pro-R oxygen is too far away from Cys38 (5.0 Å) and is pointed in the wrong direction to allow in-line methyl transfer (82).

**Suicide proteins and the adaptive response**

In reactions where O^6^-MGT levels are limiting, efficient transfer of some of the available methyl groups occurs, and then in essence, the reactions cease (71, 75). This indicates that the methylated acceptors cannot regenerate and that the protein’s activity is spent in the reaction. Consequently, O^6^-MGT is known as a ‘suicide’ protein, a sacrificial stoichiometric reagent, meaning that it cannot regenerate itself once it has removed an alkyl group. Consequently, a single O^6^-MGT protein molecule can at most repair only two lesions; an alkyl base and/or the Sp diastereoisomer of an alkylphosphotriester of DNA; the inactivated protein is degraded via the ubiquitin proteolytic pathway. This repair mechanism is a metabolically expensive route for repairing alkylative damage and is a particularly inefficient means of protection, as the repair capacity may be saturated if large numbers of lesions are formed. However, with respect to the latter issue, *E.coli* and some other prokaryotes have evolved mechanisms for rapidly increasing the amount of O^6^-MGT activity in cells continually exposed to alkylaation.

It is well established that *E.coli* exposed to very low levels of N-methyl-N’-nitro-N-nitrosoguanidine and subsequently challenged with a much higher dose exhibit marked resistance to both the lethal and the mutagenic effects of the chemical in the ‘adapted’ cells compared to the non-adapted controls. The resistance was dependent on active protein synthesis prior to the second challenge dose (90). This phenomenon was called the ‘adaptive response to alkylating damage’. Indeed, the O^6^-MGT protein (now referred to as Ada during this discussion of its regulatory function) plays a central role in the up-regulation of repair in *E.coli* in response to alkylating agents (70, 84, 91). Methylation at the Cys-38 residue, located on the amino terminal domain, converts the Ada protein into an efficient transcripational activator of several genes including the ada gene itself. This is the foundation for inducing ada expression and considerably increasing the amount of O^6^-MGT in adapted cells. Transfer of a methyl group to Cys-38 increases dramatically the affinity of the protein for DNA (87) enabling the protein to stably bind to the ada promoter sequence, known as the ‘Ada box’, with both the N-Ada\_N and the N-Ada\_C domains being required for this sequence-specific DNA binding (82). The Ada box is immediately upstream of the putative RNA polymerase-binding site and serves as a positive regulator of the ada gene by facilitating the binding of RNA polymerase (92–96) (Figure 5b). The extended sequence of the Ada box is shared by other genes regulated by activated Ada including alkA (which encodes for a particular glycosylase, AlkA, involved in the repair of several other types of alkylation damage), alkB [AlkB repairs 1-methyladenine and 3-methyl-cytosine lesions by an oxidative demethylation mechanism (97, 98)] and aidB (77, 93, 99, 100). Methylation of the Cys-321 residue does not activate the Ada protein for transcriptional regulation of *ada*.

**Repair in higher organisms**

Numerous prokaryotes have been examined for an adaptive response to alkylating agents and for DNA alkyltransferase activity [compare ref. (84)] with many showing an adaptive response and activity towards methylphosphothriesters. However, *Aspergillus nidulans* is the only known eukaryote to possess an O^6^-MGT activity (a 19.5-kDa species that is inducible), which recognizes primarily methylphosphothriesters (101).

In humans, the O^6^-MGT protein (21.7 kDa) is smaller than the *E.coli* O^6^-MGT but is reportedly unable to remove alkylphosphothriesters in DNA (102, 103). However, the findings of Le Pla et al. (36) raise the possibility of active repair of PTEs of mammalian cells. Using a postlabelling protocol (the direct method; see ‘Analysis’ section), this group investigated the long-term manifestation of PTEs in vivo, demonstrating their persistent and non-random manifestation up to 56 days after dosing, so demonstrating the enduring character of PTEs as long-term markers of genotoxicity. Quantitation of total liver Et-PTEs indicated an initial rapid decline in the level of these lesions over the 10–96 h period following dosing. This accounted for an \( \sim 65\% \) reduction in the level of Et-PTEs, with an apparent half-life of \( < 24 \text{ h} \) [this being considerably shorter than those previously reported (18, 21, 24, 26)]. However, in a parallel study that used the same samples (104), the measured levels of N-7EtG decreased with a half-life of \( \sim 7 \) days. The shorter half-life of Et-PTEs compared to that reported for N-7EtG in the same samples raises the possibility of active repair contributing to the early removal of PTEs; but this remains to be substantiated.

**Biological significance of DNA phosphotriesters**

While numerous studies have investigated factors governing PTE formation and in vivo manifestation (see above), the biological significance of PTEs remains largely unstudied and unknown. Mutation resulting from PTE formation has not been fully studied, although, to date, there is no evidence to suggest that alkylphosphothriesters are themselves pre-mutagenic lesions. However, with PTE formation ‘masking’/negating the negative charge of the internucleotide phosphodiester moiety, such adducts may influence cellular function by interfering with the binding of proteins that use DNA as a template or substrate. Indeed, Siebenlist and Gilbert (105) developed an interference assay, which utilizes the formation of ethyl PTEs, as a general approach to determine the sites of interaction of DNA substrates with DNA-binding proteins. DNA-binding proteins sensitive to methylation or ethylation of internucleotide phosphate groups have been shown to include: T4 PNK (50), MutS (106), MutY (107), RNA polymerase (105, 108) and human apurinic/apyrimidinic (AP) endonuclease (109). Furthermore, PTEs have been found to inhibit the elongation of modified oligonucleotides by a variety of DNA polymerases (110, 111) and digestion of DNA containing PTEs has been found to be blocked either at or near the site of a PTE lesion by all exo- and endonucleases tested to date (33, 57, 60, 112, 113).

Miller et al. (111) used oligonucleotides containing a single ethyl PTE modification as polymerase chain reaction template
to investigate the effect on DNA polymerase I activity. The rate and extent of polymerization 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 polymerization 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. However, Miller et al. did not determine which isomers correspond to the Rp and the Sp PTE stereoisomers.

Yashiki et al. (114) reported that the presence of an isopropyl PTE in the template inhibited DNA chain elongation by E. 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 an 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 was 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. (113) reported the results of a study of the effects of DNA phosphate alkylation upon the activity of several nucleases including the following: (i) 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 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 Rp or the Sp 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. Indeed, with many DNA damage repair proteins relying upon specific interactions with the DNA sugar-phosphate backbone, the presence of long-lived PTE adducts adjacent to other repairable mutagenic lesions could impede the latter’s repair, leading to their persistence and a consequential increase in mutation. The observation of PTEs impairing the repair processing of adjacent lesions, and this leading to an increase in mutation, would substantiate a key deleterious biological mechanism for these lesions and could define a contributing role for PTEs in the damage-based models of ageing and, consequently, in the pathogenesis of age-related diseases, including cancer.

Recently, Le Pla et al. (115) reported preliminary data demonstrating that site-specific PTEs do impede the in vitro repair processing of adjacent oxidized methylene blue/white light (MB/WL)-induced purine lesions by formamidopyrimidine-DNA glycosylase (Fpg). With 8-oxo-dG phosphoromimetics being incompatible with the UltraMild synthesis protocols used to synthesize PTE-containing oligos, oligonucleotides containing a site-specific PTE plus a site-specific oxidized MB/WL-induced purine lesion were prepared by treating substrate PTE oligonucleotides containing a single G with MB/WL. These were then 32P end labelled, annealed to a counter strand, treated with Fpg and the radiolabelled products resolved by 20% denaturing PAGE. The extent to which PTE adducts inhibit the repair processing of incision at MB/WL-induced purine lesions was assessed by comparing the relative amounts of the incised 5’ labelled products generated from the PTE-containing oligonucleotide versus an oxidized control, containing no PTE. PTEs located one nucleotide 5’ to the MB/WL-induced purine lesions or two nucleotides 3’ the MB/WL-induced purine lesions did not appreciably alter the overall extent of Fpg incision compared an identically treated non-PTE-containing control. However, a PTE immediately 5’ or one nucleotide 3’ to the MB/WL-induced purine lesions did inhibit Fpg incision by ~65 and 25%, respectively, compared to the control. PTEs positioned on the counter strand did not inhibit the Fpg processing of oxidized purine lesions. These data indicate that site-specific PTE’s are capable of impairing the repair processing of an adjacent lesion, highlighting a possible important biological mechanism for these lesions.

Conclusions

The applicability of the measurement of a DNA damage product as a biomarker of exposure to a genotoxic agent is partially governed by the chemical and physiological stability of the product. Thus measurement of an unstable compound, or one with a short life-time in vivo, may only allow one to monitor recent exposures, whereas a stable compound in vivo may be used to determine a longer history of exposure. Extensive experimental evidence indicates that the life-times of PTEs are among the longest of all DNA genotoxic lesions, and this review has illustrated the potential application of PTEs as biomarkers for assessing long-term exposure to some genotoxic compounds. The analytical methods that have been developed to date for their determination are often complex and many of the earlier methods do not reflect the structure of the nucleotides neighbouring the PTE lesion. However, 32P-postlabelling techniques have been developed, which permit the identification of the nucleotide 5’ to the PTE lesion, and both 3’- and 5’-nucleotides should be identifiable using recently developed mass spectral methods. To date, there are no high-throughput analytical methods, although the measurement of transalkylation products or of intact PTEs by mass spectrometry holds promise for the development of such approaches. It is well known that PTEs are relatively abundant lesions in DNA following exposure to many genotoxins, and their extent of formation has been found to be dependent upon the SN1 character of the alkylation process. It may therefore be speculated that the amount of PTEs will reflect the amount of the potentially mutagenic alkylation products on base oxygen atoms (e.g. O6-alkylguanine, O2-alkylthymine) and thus be a potentially valuable marker, as a surrogate of mutagenic risk.

Although repair systems for PTEs are present in prokaryotic systems, and has been noted in one low eukaryotic system, their existence in higher eukaryotic systems is unproved. Although many studies have indicated relative little decrease of PTE levels over time (and the effect of cell dilution may be a contributor to this), evidence has also been produced to indicate that there is a rapid initial decrease in PTE levels before they stabilize, which may be caused by an active repair process. Further studies of PTE repair seem warranted. The demonstration of such an active repair process would clearly have implications for the proposed use of PTEs as long-term biomarkers of exposure.

Despite the large amount of work that has been carried out determining these adducts and their life-times, there is still
Conflict of interest statement: Discussions.

Safety’ (contract 513943). The authors thank Dr Rob Britton for helpful comments. Studies on background levels of these adducts in DNA are needed together with a further understanding of their biological significance in vivo.

In summary, the measurement of PTE levels in DNA (even in the absence of sequence information) should be a valuable biomarker of exposure to some alkylating agents in human studies. The quantity of DNA required is similar to that used in biomarker studies using determination of added DNA bases, suggesting that lymphocyte DNA, as used commonly in the latter studies, would be suitable for studies of PTEs in human populations. Studies on background levels of these adducts in vivo are needed together with a further understanding of their biological significance.

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References


