Mutagenic and Genotoxic Effects of Three Vinyl Chloride-Induced DNA Lesions: 1,N^6-Ethenoadenine, 3,N^4-Ethenocytosine, and 4-Amino-5-(imidazol-2-yl)imidazole†

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ABSTRACT: The mutagenic and genotoxic properties of 1,N^6-ethenoadenine (εAde), 3,N^4-ethenocytosine (εCyt), and 4-amino-5-(imidazol-2-yl)imidazole (β) were investigated in vivo. The former two modified bases are known DNA adducts formed by the human carcinogen vinyl chloride; β is formed by pyrimidine ring-opening of εAde. Chemically synthesized deoxyhexanucleotides containing εAde and β, d[GCT(εA)GC], and d[GCT(β)GC], respectively, were described previously [*Biochemistry* (1987) *26*, 5626–5635]. εCyt was inserted into an oligonucleotide, d[GCTAG(εC)], by a mild enzymatic synthetic procedure, which avoided exposure of the base to alkaline conditions. 3,N^4-Etheno-2′-deoxycytidine 3′,5′-bisphosphate coupled with reasonable efficiency (30–40%) to the 3′-nucleoside of an acceptor pentamer, d(GCTAG), in a reaction catalyzed by T4 RNA ligase in the presence of ATP. Each of the three modified hexanucleotides and an unmodified control were inserted into a six-base gap positioned at a known site in the genome of bacteriophage M13-*Nhe*I. A nick was placed in the DNA strand opposite that containing the single DNA lesions, enabling the formation of singly adducted single-stranded genomes by denaturation. After transfection of the adducted phage DNAs into *Escherichia coli*, each of the adducts was found to be genotoxic. The most toxic lesion was β, which reduced survival of the genome by 97%. εCyt and εAde reduced survival by 90% and 65%, respectively. An examination of the surviving phage populations revealed that each of the three adducts was mutagenic. The least mutagenic lesion was εAde (0.1% of the survivors were mutant), which showed primarily A → G transitions. The εAde rearrangement product, β, was also found to induce mutations but at a 20-fold higher frequency (~2%). In this case, however, mutagenesis was random, possibly because the hydrogen-bonding face of this lesion has been obliterated. εCyt induced mutations at a frequency of 1.5–2%; its mutations were mainly C → T transitions, although targeted C → A and −1 deletions were also detected. The possible respective roles of these three DNA lesions in the mutagenic and carcinogenic activities of vinyl chloride and related haloalkanes are discussed.

Vinyl chloride is an established human and rodent carcinogen (Singer & Grunberger, 1983). This toxin is metabolically converted to chloroethylene oxide, which nonenzymatically rearranges to chloroacetaldehyde (IARC Monographs, 1979; Guengerich et al., 1979; Singer & Grunberger, 1983), a bifunctional electrophile that forms an hydroxyethane bridge between the exocyclic amino groups and ring nitrogens of adenine, cytosine, and guanine bases in DNA (Kusmierek & Singer, 1982). The hydroxyethane intermediates are postulated to dehydrate eventually to the stable etheno products shown in Figure 1 (Kochetkov et al., 1971; Barrio et al., 1972; Sattsangi et al., 1977). This suggested mechanism was established definitively for εAde[1] and εCyt (Kusmierek & Singer, 1982). Recent studies by Guengerich and co-workers indicate that the etheno adducts are also formed by the vinyl chloride oxidation product 2-chlorooxirane (Raney & Guengerich, 1991; Guengerich, 1992). Other well-known carcinogens such as ethyl carbamate and vinyl carbamate have also been demonstrated to form the etheno adducts in nucleic acids (Leithauser et al., 1990).

Etheno adducts have been isolated from the organs of rats and mice exposed to vinyl chloride and related derivatives (Laib, 1986; Eberle et al., 1989; Swenberg et al., 1990; Park et al., 1993). The relative abundance of the etheno adducts detected in vivo depends upon the details of experimental design. For example, chloroacetaldehyde treatment of V79 cells generates 1,N^6-ethenoadenine (εAde) as the most abundant cyclic adduct, followed by 3,N^4-ethenocytosine (εCyt) and N^2,3-ethenoguanine [εGua(2,3)] (Foiles et al., 1993), whereas vinyl chloride exposure of neonatal rats produces εGua(2,3) as the major product (Swenberg et al., 1990).

The etheno ring of εAde and εCyt interferes with the formation of hydrogen bonds with complementary bases, while εGua(2,3) can form a G·T wobble pair. The attempted in vitro replication of templates containing εAde, εCyt, and εGua(2,3) reveals that all three lesions can induce mispairing, with

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[1] Abbreviations: εdAdo, 1,N^6-etheno-2′-deoxyadenosine; εAde, 1,N^6-ethenoadenine; εdCyd, 3,N^4-etheno-2′-deoxycytidine; εCyt, 3,N^4-ethenocytosine; εGua(2,3), N^2,3-ethenoguanine; εGua(1,2), 1,N^2-ethenoguanine; β, 4-amino-5-(imidazol-2-yl)imidazole; P-dCyd-P, 2′-deoxycytidine 3′,5′-bisphosphate; P-εdCyd-P, 3,N^4-etheno-2′-deoxycytidine-3′,5′-bisphosphate; bp, base pair; b, base; ds, double stranded; RF, replicative form; ss, single stranded; GHD, gapped heteroduplex DNA; M13-*Nhe*I, insertion mutant of M13mp19 containing d(GCTAGC) in the center of the *Sma*I site; IPTG, isopropyl β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; MF, mutation frequency.

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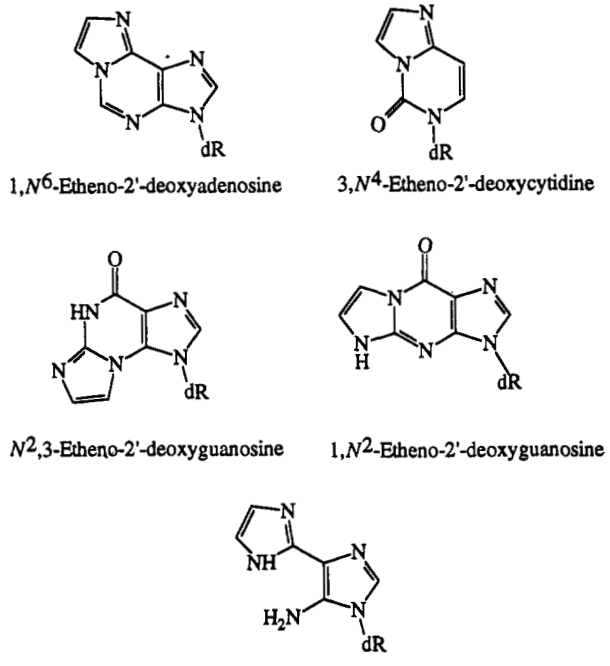


FIGURE 1: Structures of the etheno adducts and the bi-imidazole product, β.

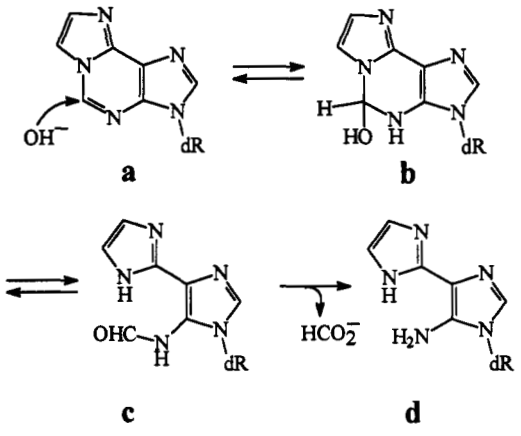
ϵGua(2,3) showing the most strongly mutagenic activity (Barbin et al., 1981; Singer et al., 1984, 1987, 1991; Singer & Spengler, 1986). The hydrated precursors to the etheno adducts, by contrast, do not appear to be mutagenic (Singer & Spengler, 1986), as inferred from the in vitro replication of ϵCyt and its hydrate. The mutational spectrum of chloroacetaldehyde in *Escherichia coli* reveals that ~80% of the mutations are at cytosines and that induction of the SOS functions of the host does not influence mutagenesis significantly (Jacobsen et al., 1989; Jacobsen & Humayun, 1990).

Little information is available on the toxic effects of the etheno adducts. An M13 genome of approximately 7 kb with a small gapped region was randomly modified to an average of three ϵAde and two ϵCyt per gapped segment; at this level of modification, survival was approximately 30% (Jacobsen & Humayun, 1990). Primer elongation in vitro on the same modified DNA by *E. coli* DNA polymerase I suggests that DNA synthesis pauses only temporarily at many adenine and cytosine sites in the single-stranded (ss) region.

Until recently, it was believed that the etheno adducts are not repaired in vivo. In recent work, however, Singer and co-workers demonstrated that human and rodent cells have DNA binding proteins that possess an ϵAde–DNA glycosylase activity (Rydberg et al., 1991, 1992). In addition, this activity also releases 3-methyladenine from methylated DNA, and likewise 3-methyladenine–DNA glycosylase acts to remove ϵAde from DNA in vitro (Singer et al., 1992). It was noted in additional studies that these proteins also recognize all of the other etheno adducts shown in Figure 1 (B. Singer, personal communication). Their function in vivo is yet to be determined.

Exocyclic adducts structurally similar to the etheno derivatives are formed by many other bifunctional reagents (Guengerich et al., 1991), and a property common to a number of these purine adducts is their chemical lability (Leonard, 1984, 1992). ϵAde, for example, converts to a bi-imidazole product, 4-amino-5-(imidazol-2-yl)imidazole (β), in alkali (Yip & Tsou, 1973). The sequence of events believed to lead to this product is shown in Scheme I. The bi-imidazole

Scheme I



derivative (structure d in Scheme I) is denoted as β.

The work described here compares the mutagenic and cytotoxic effects of ϵCyt and ϵAde in *E. coli*. The genetic effects of a third product, the bi-imidazole lesion, β, are also defined. Although β forms rapidly at alkaline pH, as indicated above, in our hands it was also a significant product formed upon storage of ϵAde containing oligonucleotides under mild conditions of neutral pH. The mutagenic activity of one of the three lesions studied here, ϵCyt, has been studied in vivo in several recent studies (Palejwala et al., 1991, 1993a,b). While our results confirm the principal type of mutation induced by ϵCyt, they differ strikingly in the quantitative features of mutagenesis. The mutations induced by ϵAde and β have not been defined previously in vivo.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains. Chloroacetaldehyde was purchased from Fluka. 2'-Deoxycytidine 3',5'-bisphosphate (P-dCyd-P) and bacteriophage T4 RNA ligase were from Pharmacia. Restriction endonucleases, bacterial alkaline phosphatase, bacteriophage T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. [γ-^{32}P]ATP was from New England Nuclear. *E. coli* strain GW5100 was as described (Wood et al., 1990); DL7 (AB1157, *lac*ΔU169, *uvr*$^+$) was constructed in this laboratory (Lasko et al., 1988). Bacteriophage M13mp19 was from Pharmacia, and the construction of the insertion mutant M13-*Nhe*I has been described (Basu et al., 1987). Restriction endonuclease digestion, gel electrophoresis, and autoradiography were done as previously described (Basu et al., 1987; Lasko et al., 1987). Denaturing 5% and 20% polyacrylamide [19:1 acrylamide: bis(acrylamide)] gel electrophoresis was carried out in the presence of 7 M urea at 300 V. DNA sequencing was performed with T7 DNA polymerase by a modified method of Sanger et al. (1977).

Chromatographic Methods. HPLC with diode array detection was essentially as described (Wood et al., 1990). Anion-exchange HPLC was carried out with a Whatman Partisil SAX column (10-μm particle size; 4.6 mm i.d. × 25 cm), and reversed-phase HPLC was performed on a Nucleosil C18 column (10 μm; 4.6 mm × 25 cm); see figure legends for details. Fluorescence spectroscopy was performed on a Farrand MK1 spectrofluorometer.

Oligonucleotide Synthesis. The oligodeoxynucleotide d(GCTAG) was synthesized by the phosphoramidite procedure on an Applied Biosystems 381A automated DNA synthesizer and purified by HPLC. The ϵAde-containing hexanucleotide d[GCT(ϵA)GC] was prepared as described (Basu, et al., 1987).

3,N^4-Etheno-2′-deoxycytidine 3′,5′-bisphosphate (P-ϵdCyd-P) was synthesized by allowing P-dCyd-P and chloroacetaldehyde to react at pH 3.5 as per Barrio et al. (1972); the product was purified on a Sephadex G-10 column, recrystallized from water, and identified by its UV, NMR, and fluorescence spectra (Barrio et al., 1972). This intermediate was enzymatically ligated to the 3′ end of a pentamer by using T4 RNA ligase. The methodology for enzymatic synthesis of DNA by this route is described by Brennan et al. (1983), by Tessier et al. (1986), and more recently by Wood et al. (1990). Briefly, d(GCTAG) (0.1 μmol) and a 5-fold molar excess of either P-ϵdCyd-P or P-dCyd-P were allowed to react in 50 mM Tris-HCl (pH 8.0), 0.4 mM ATP, 10 mM $MgCl_2$, 1 mM hexaamminecobalt chloride, 25% w/v poly(ethylene glycol) 8000, 8 mM spermine, 40 mM phosphocreatine, 20 mM dithiothreitol, bovine serum albumin (10 μg/mL), creatine phosphokinase (175 units/mL), adenylate kinase (170 units/mL), and 30 μM T4 RNA ligase in a total volume of 100 μL at 17 °C. The progress of the reaction was monitored by anion-exchange HPLC. Incubation for approximately 10 days was necessary to achieve 30–40% ligation of P-ϵdCyd-P to the pentamer, although the unmodified P-dCyd-P ligated relatively rapidly. The reaction was terminated and the oligonucleotides were purified by anion-exchange HPLC. The hexanucleotides, subsequent to desalting on a Sephadex G-10 column, were 3′-dephosphorylated with calf intestinal phosphatase (20 units) and repurified by reversed-phase HPLC, as above. After desalting, a portion of the hexanucleotides was digested to the component nucleosides and analyzed by reversed-phase HPLC.

Construction of M13 Genomes Containing ϵAde, ϵCyt, or β. Replicative form (RF) DNAs of M13mp19 and M13-*Nhe*I were digested with *Sma*I and *Bgl*II, respectively. The latter was further treated with bacterial alkaline phosphatase (or, in some experiments, calf intestinal phosphatase) to remove the 5′-phosphate. Following phenol extraction and precipitation with ethanol, equal amounts of these DNAs were mixed to create gapped heteroduplexes (GHD) possessing a six-base gap in either the (+) or (−) strand of the *Nhe*I restriction site [see Basu et al. (1987)]. In certain experiments the GHD was purified from a low-melting agarose gel by cutting the desired band, followed by phenol extraction of the agarose after melting at 70 °C. The GHDs were precipitated with ethanol and reanalyzed on an agarose gel. For genotoxicity experiments the isolated GHD (5 μg) was mixed with 250 ng of ss M13mp19 as an internal control.

Prior to ligation into the GHD, 0.2 μg of d[GCT(ϵA)GC], d[GCTAG(ϵC)], d[GCT(β)GC], or d(GCTAGC) was phosphorylated with [γ-^{32}P]ATP (100 μCi) by using T4 polynucleotide kinase (30 units) at 37 °C for 20 min (experiments not described showed that the three modified oligonucleotides were stable, to the limit of detection by HPLC, to the conditions used for genome construction and denaturation). An excess of unlabeled ATP (final concentration 1 mM) was added, and the incubation was continued for an additional 15 min. The enzyme was inactivated by heating at 65 °C for 15 min, and 5% of the reaction mixture was electrophoresed for ~2 h on a 20% polyacrylamide gel. The specific activity of the oligonucleotide was calculated after excision of the hexanucleotide band from the gel and monitoring radioactivity. The balance of the reaction mixture was combined with GHD (1 μg) and T4 DNA ligase (800 units) and incubated overnight at 16 °C in ligase buffer in the presence of 1 mM ATP (Basu et al., 1987). The ligated genomes containing the hexamers were purified on a Sepharose CL 4B column (1.3 × 23 cm) preequilibrated with 10 mM Tris-HCl (pH 7.8), 1 mM Na_2-EDTA, and 0.1 M NaCl. The M13 DNA was collected in the void volume. After electrophoresis on an agarose gel (0.8%) for ~1.5 h, the radioactivity in the M13 DNA isolated from the gel was determined. The ligation efficiency was calculated by relating the amount of oligonucleotide covalently incorporated into the M13 genome to the amount of GHD used.

Transformation of E. coli Cells with Site-Specifically Modified Genomes. Site-specifically modified genomes were incubated with *Sma*I (40 units) to destroy any recircularized M13mp19 formed during the ligation reaction [see Wood et al. (1990)]. The restriction enzyme was removed by phenol extraction, and the DNA was precipitated with ethanol. Following dissolution in 10 μL of water, the M13 DNA was used for cellular transformation experiments in which the genetic effects of the adducts in *duplex* DNA were to be evaluated. Alternatively, to examine the same effects in *single-stranded* (ss) genomes, the DNA was boiled at 100 °C for 3 min to make it ss; the DNA was then cooled rapidly to 0 °C to inhibit renaturation.

The ds or denatured DNAs were used for transformation of *E. coli* DL7 cells (a *lac* strain prepared from AB1157). The methods of transformation were either the $CaCl_2$ protocol (Basu et al., 1987) or electroporation as described (Wood et al., 1990). Immediately after each transformation, a portion of the cells was plated in the presence of *E. coli* GW5100, IPTG, and X-gal to determine the number of independent transformants. The remainder of the cells was incubated for 1 h at 37 °C to allow for phage replication and subsequently centrifuged at 15000*g* (10 min) to isolate the progeny phage-containing supernatant.

Isolation of Mutants and Determination of Mutation Frequency (MF). The progeny phage pool following transformation of *E. coli* DL7 cells was analyzed for mutants in the following manner. For M13 genomes containing ϵAde and β, all targeted base substitutions reverted an *amber* codon in the *lacZα* fragment and thus generated dark blue plaques in the presence of IPTG and X-gal, whereas the wild-type population provided a light blue plaque phenotype. In addition to the mutants, a background of dark blue plaques was generated by the genetic engineering techniques used to construct these genomes. These dark blue plaques had the *Nhe*I site of M13-*Nhe*I deleted, and the product was M13mp19 with its intact *Sma*I site. To eliminate this subpopulation, RF DNA prepared from a mixed population of progeny phage after the first transformation (T_1) was digested with an excess of *Sma*I followed by a treatment with exonuclease III. Since linear DNA can be recircularized in vivo, exonuclease III was used to render the linearized DNA biologically inactive. The resultant DNA was used for a second *E. coli* transformation (T_2). The preparation of RF DNA, *Sma*I/exonuclease III selection, and subsequent transformation were repeated twice more (T_3 and T_4). After T_4, individual dark blue plaques were picked and DNA sequencing in the vicinity of the originally adducted site was performed to analyze the type or types of induced mutations. The ratio of dark blue to total plaques (after T_4) was determined as R_1, and the MF was determined as follows: $MF = R_1R_s(10^2)$, where R_s was the fraction of dark blue plaques after T_4 that contained a mutation at the *Nhe*I site, as determined by DNA sequencing.

In the case of ϵCyt, since the adduct was not situated within an *amber* codon, both wild-type and targeted base substitution mutants provided the light blue plaque phenotype; adduct-induced, as well as other types of small deletions primarily generated colorless plaques. The ratio ($R_{1'}$) of light blue and colorless plaques (wild-type, adduct-induced base substitutions

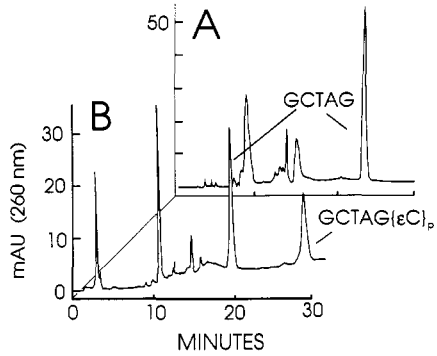


FIGURE 2: Anion-exchange HPLC profiles displaying the formation of d[GCTAG(εC)] in a T4 RNA ligase-mediated reaction between the acceptor d(GCTAG) and the adducted donor P-εdCyd-P. The incubation mixture was analyzed immediately after addition of the enzyme (time 0) (A) and after incubation at 17 °C for 240 h (B). Chromatographic conditions: 0.1–1.0 M KH_2PO_4 in 5% methanol over 40 min at a flow rate of 1.0 mL/min. No additional peaks were detected for 10 min beyond the portion of the chromatogram shown.

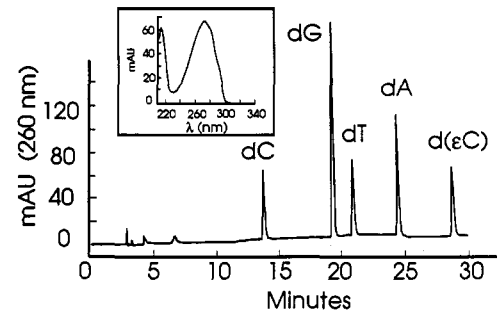


FIGURE 3: Reversed-phase HPLC profile of the snake venom phosphodiesterase and bacterial alkaline phosphatase digestion products from d[GCTAG(εC)$_p$] synthesized enzymatically. Chromatographic conditions: 0–25% acetonitrile in 0.1 M ammonium acetate over 30 min with a flow rate of 1.0 mL/min. The inset shows the absorption spectrum of the peak at 28.5 min as determined by the diode array detector.

and frameshift mutations, and deletions introduced during genetic engineering manipulations) to dark blue plaques (six-base deletions due to regeneration of M13mp19 as a side product of the genome construction) was determined. To eliminate the wild-type population of the progeny phage, RF DNA isolated after T_1 was digested with *Nhe*I and exonuclease III and subsequently transformed (T_2) into *E. coli*. After two more rounds of *Nhe*I selection (T_3 and T_4), the ratio ($R_{4'}$) of light blue and colorless plaques to dark blue plaques was determined once again. Finally, individual plaques after T_4 were picked, and the DNA in the region of the *Nhe*I site was sequenced. The MF of εCyt was determined as MF = $[(R_{4'}/R_{1'})R_{s'}](10^2)$, where $R_{s'}$ denotes the ratio of adduct-induced mutants to the total colorless plaque population after T_4.

RESULTS

Synthesis of a Deoxyhexanucleotide Containing εCyt. We previously reported the total chemical synthesis of the hexanucleotide containing εAde and described its conversion to β (Basu et al., 1987). We have not previously prepared, however, oligonucleotides and genomes containing εCyt. In our hands εCyt was unstable to the conditions used in our earlier work for chemical synthesis of the εAde-containing oligonucleotide. As a result, we performed a T4 RNA ligase-mediated enzymatic synthesis, which employs mild aqueous conditions at near-neutral pH. The acceptor and donor molecules in the ligase reaction were d(GCTAG) and P-εdCyd-P, respectively. As a control, we performed a similar enzymatic ligation with the corresponding unmodified donor, P-dCyd-P, and monitored the progress of the reaction by anion-exchange HPLC. Figure 2 displays the chromatograms of the εCyt ligation at times 0 and at 240 h. The starting pentamer eluted at ~20 min whereas the product hexanucleotide eluted at ~29 min. Ligation for P-εdCyd-P was much slower than for P-dCyd-P. For example, to achieve ~35% ligation of the etheno derivative, a 240-h incubation was necessary, whereas complete ligation of the unmodified control was accomplished within approximately 100 h. Despite the sluggish reaction with the modified nucleotide, we generated ample εCyt-containing hexanucleotide (100 μg) for characterization and genetic studies. The 3′-phosphate was removed from the hexanucleotides by a treatment with alkaline phosphatase, and finally the hexamers were purified by HPLC. A portion of the hexamers was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase to the component nucleosides, which were analyzed by HPLC. Figure 3 displays the profile obtained from the εCyt-containing hexanucleotide. The relative proportions of the nucleosides were consistent with the calculated peak heights at 260 nm. The 3,N^4-etheno-2′-deoxycytidine (εdCyd) peak at ~29 min was confirmed by cochromatography with an authentic standard and by its UV absorption spectrum (see inset). As expected, this peak was absent when the control hexanucleotide d(GCTAGC) was digested and analyzed.

4-Amino-5-(imidazol-2-yl)imidazole: An Alkali-Induced Product Derived from εAde. We observed that εAde in DNA, upon storage even at −20 °C, generated the same bi-imidazole derivative β that is formed under alkaline conditions (Yip & Tsou, 1973; Basu et al., 1987). The suspected sequence of reactions leading to β in alkaline media is shown in Scheme I. We studied the genetic effects of this product for two reasons. First, it was necessary to determine the extent to which any biological effect seen from εAde could be due to contamination of the εAde oligonucleotide with one containing the degradation product β. Second, εAde can be a persistent lesion in cellular DNA, and hence β may be a biologically relevant product in cells exposed to vinyl chloride and related compounds. These points collectively provided the rationale for the study of the role of β in mutagenesis and genotoxicity. We first addressed the stability of εAde in the buffers and media used for the subsequent events of phosphorylation, ligation, and bacterial transformation. To the limit of our detection by HPLC, the 1,N^6-etheno-2′-deoxyadenosine (εdAdo)-containing hexanucleotide was stable to these conditions. We also examined the stability of d[GCT(εA)GC] at pH 13. The change in the reversed-phase HPLC profile of the hexanucleotide is shown in Figure 4. Peak A represents d[GCT(εA)GC], which generated peak B upon incubation at pH 13. Peak B gradually converted to peak C. When these peaks were collected, we found that at neutral pH both peaks A and C were stable, whereas peak B converted readily to peak A and to a lesser extent to peak C. As indicated earlier, we have detected peak C in certain batches of the εAde hexamer after prolonged storage under neutral conditions in aqueous solution. A similar pattern of interconversion was noted at the monomer level when the nucleoside εdAdo was exposed to alkali. The proposed sequence of reactions involves an attack by hydroxyl anion at C5 (originally the C2 of Ade) followed by pyrimidine ring-opening and subsequent deformylation (Scheme I). It is likely that peak C represents the deformylated bi-imidazole derivative-containing oligonucleotide (Yip & Tsou, 1973).

Construction and Characterization of M13 Bacteriophage Genomes Containing εAde, εCyt, or β. The hexanucleotides d[GCT(εA)GC], d[GCTAG(εC)], d[GCT(β)GC], and the

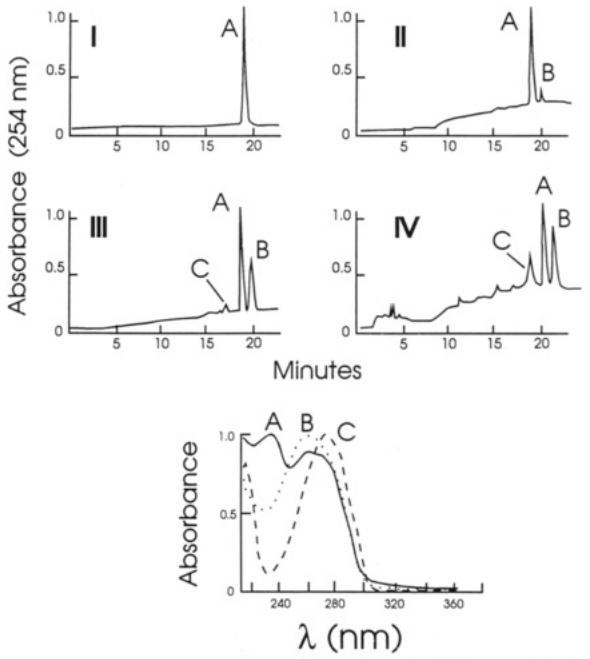


FIGURE 4: Time course of the conversion of d[GCT(ϵA)GC] to d[GCT(β)GC] at pH 13.0. The reversed-phase HPLC profile at 0, 30, 100, and 120 min are shown in I, II, III, and IV, respectively. Absorption spectra of the three peaks (A, B, and C) are displayed at the bottom. Chromatographic conditions: 0–30% acetonitrile in 0.1 M ammonium acetate over 30 min with a flow rate of 1 mL/min.

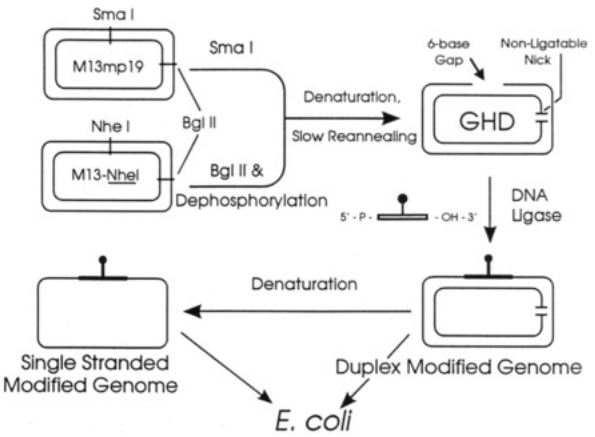


FIGURE 5: Protocol for preparation and biological evaluation of site-specifically modified M13-*Nhe*I genomes containing a single adduct in the *Nhe*I site. The gapped heteroduplex (GHD) consisted of an equal mixture of M13 genomes with six-base gaps in the (+) and (–) strands.

unmodified oligomer d(GCTAGC) were 5′ phosphorylated with [γ-^{32}P]ATP and inserted into the genome of M13-*Nhe*I (Figure 5), as described previously for ϵAde (Basu et al., 1987). Ligation efficiencies of the three modified oligonucleotides, determined by associating the amount of ^{32}P incorporated into the M13 DNA to the amount of GHD used, were between 40% and 60%.

The radiolabeled phosphodiester linkage between nucleotides 6271 and 6272 in the (+) strand or between nucleotides 6277 and 6278 in the (–) strand provided a convenient marker for characterization of the adducted genome containing the ϵCyt lesion. Physical mapping experiments to ascertain the genomic location of the radiolabel were performed by restriction endonuclease digestion followed by agarose gel electrophoresis and autoradiography. For example, in Figure 6 we have compared M13-*Nhe*I-(ϵCyt) with M13-*Nhe*I-(Cyt). As shown, electrophoretic analysis of ϵCyt-containing and unmodified [i.e., d(GCTAGC) inserted] genomes (lanes f and

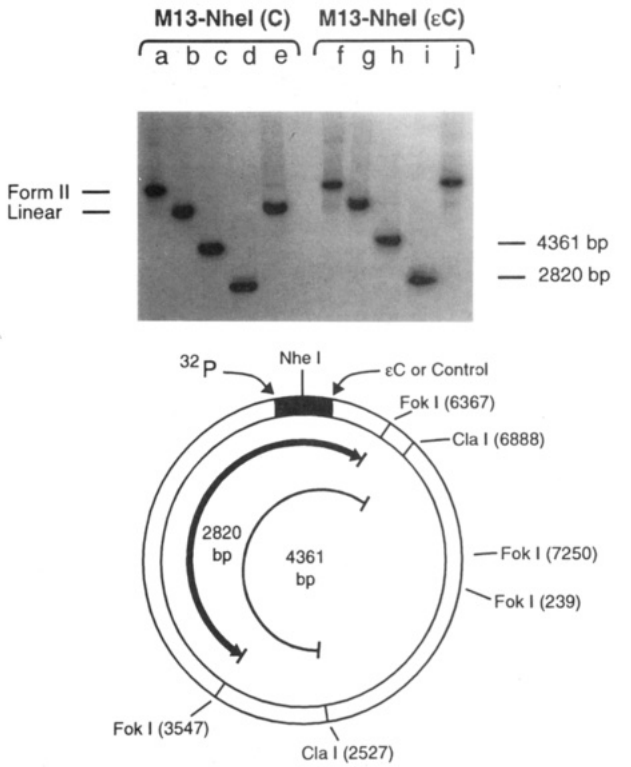


FIGURE 6: Physical mapping of M13-*Nhe*I RF molecules into which radiolabeled d(GCTAGC) and d[GCTAG(ϵC)] were introduced. A partial restriction map of the duplex genome is shown at the bottom, indicating the approximate position of the *Nhe*I site relative to the recognition sequences for the endonucleases *Eco*RI, *Cla*I, and *Fok*I; there was a nick (not shown) in the DNA strand opposite that containing the inserted hexanucleotide. An autoradiogram of an agarose gel is shown at the top in which lanes a and f correspond to d(GCTAGC) and d[GCTAG(ϵC)] ligation products of the GHD, respectively. Lanes b, c, d, and e show the material in lane a digested with *Eco*RI, *Cla*I, *Fok*I, and *Nhe*I, respectively, and lanes g-j represent the corresponding endonuclease digestion of the product in lane f.

a, respectively) indicated the presence of only nicked circular duplex (form II) DNA. When these genomes were digested with *Eco*RI, which cleaves M13 DNA only once, both formed a 7256 bp duplex linear DNA species (lanes b and g) as expected. *Cla*I cuts the genome twice, generating fragments of 4361 and 2895 bp; the hexanucleotide ligation site was expected to be on the larger fragment, and this was confirmed (lanes c and h). Likewise, *Fok*I digested the genome into four fragments, with the radiolabel specifically localized to the 2820-bp fragment (lanes d and i). Presence of ϵCyt at the *Nhe*I restriction site prevented it from being digested with this enzyme although the unmodified control was efficiently cleaved (lanes e and j). Additional experiments explored whether or not ligation occurred at both ends of the inserted oligonucleotides. To address this issue, the genomes were digested with *Pvu*II which makes two (of its three) incisions between nucleotide positions 6055 and 6056 and between 6383 and 6384 to form a duplex radiolabeled fragment of 328 nucleotides. Denaturation of this fragment should yield a 328-base ss fragment, only if the ligation occurred at *both* the 3′ and 5′ ends of the gap in the GHD. In the event of ligation on only one side, radiolabeled products of either 112 or 222 bases would be generated (Figure 7A). These ss fragments were resolved on a 5% polyacrylamide gel (run in the presence of urea). A previous study demonstrated that the ϵAde-containing oligomer ligated efficiently at both ends of the gap (Basu et al., 1987); as shown in Figure 7B, the same conclusion was reached for the ϵCyt-containing oligonucleotide, which ligated with an efficiency of ~90% at both ends (the same

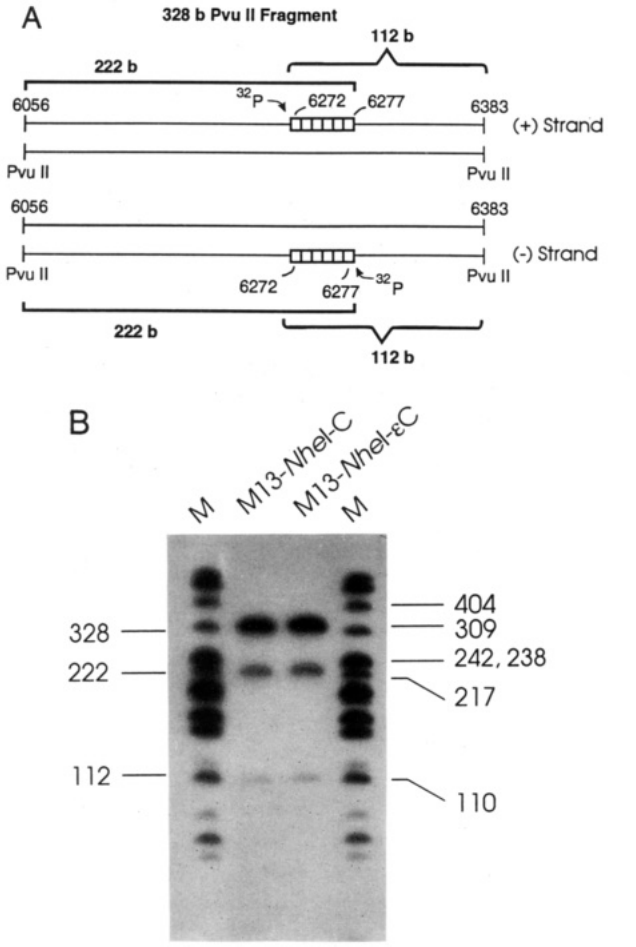


FIGURE 7: Determination of the extent of ligation of both ends of d(GCTAGC) and d[GCTAG(ϵC)] into the GHD. Frame A: Map of the 328 bp *Pvu*II restriction fragment containing the ligated hexamer in either the (+) or (–) strand. Frame B: Autoradiogram of a denaturing 5% polyacrylamide gel electrophoresed subsequent to *Pvu*II digestion and heat denaturation of the control and ϵCyt adducted genomes. The lanes denoted "M" are ss DNA size markers (an *Msp*I digest of pBR322); sizes are indicated to the right. Numbers to the left are the sizes of fragments for complete ligation (328 bases) or incomplete ligation (222, 112 bases) of hexanucleotides into the six-base gap of the GHD.

efficiency was observed for the control, C-containing oligonucleotide). Approximately 10% of the ligated material was joined to the M13 genome at only one end of the gap.

Survival of M13 Genomes Containing Etheno Adducts and β. Bacteriophage M13 genomes containing a site-specifically located adduct, and the corresponding control that was constructed by incorporation of d(GCTAGC) into the GHD, were transfected into *E. coli* cells. Two types of experiment were carried out. In one study, double-stranded (ds) M13 DNA containing a site-specifically situated adduct was transfected into *E. coli* DL7 cells. No change in the number of infective centers was noted (Table I), suggesting three possibilities: (i) that the etheno derivatives and the bi-imidazole product were nontoxic, (ii) that they were repaired efficiently, or (iii) that preferential replication of the unadducted strand masked any possible reduction in progeny attributable to the adducts. The possibility that polymerase bypass of these lesions was efficient is ruled out by experiments described below in which ss DNA was used for transformation.

In a second experiment (2-ss, Table I), the biological effects of the adducts in ss DNA were evaluated. The ds DNA described above had a nick in the DNA strand opposite that containing the lesion. The nicked DNA was denatured and immediately used for transformation of *E. coli*. The results

Table I: Survival of Site-Specifically Modified M13 in *E. coli* DL7[a]

expt no.	DNA strandedness	method of transfection	rel survival (%) control[b]	ϵAde[b]	ϵCyt[b]	β[b]
1	ds	$CaCl_2$	100 (7.2×10^5)[c]	93.4	103.5	88.3
2	ss	$CaCl_2$	100 (9.2×10^4)	33	10.3	3.5
3	ss	electroporation	100 (8.4×10^6)	39.6	16.2	4.3

[a] Survival (in percent) was determined by comparing the number of infective centers from adducted DNA with that of the control genome (assumed to be 100% survival). [b] Ligation efficiencies of d(GCTAGC), d[GCT(ϵA)GC], d[GCTAG(ϵC)], and d[GCT(β)GC] were 55%, 51%, 47%, and 44%, respectively. [c] Transformation efficiencies (per μg of DNA) are shown in parentheses for the control genome.

showed that the survival values for the adducted genomes were substantially lower than for the unadducted genome.[2] Compared to the progeny from the unmodified genome, the number of infective centers was reduced by approximately 3-fold for the ss genome containing a single ϵAde, whereas it was reduced by 6- to 10-fold for the analogous genome containing ϵCyt. Genotoxicity was most evident for the genome containing the β residue; in this case survival was reduced by 30-fold below that of the control genome. The results were independent of the method of transformation (both the traditional $CaCl_2$ protocol and the more efficient method of electroporation were evaluated). We conclude from the data that, under the conditions evaluated, ϵCyt is more genotoxic than ϵAde and that β was the most toxic lesion in this group.

The Mutagenic Activity of Etheno Adducts and β. One strategy used to analyze mutants took advantage of the placement of the adducts in the solitary *Nhe*I site of M13-*Nhe*I. Mutation at any of the six bases of the *Nhe*I site renders the duplex M13 genome refractory to cleavage by *Nhe*I whereas the DNA of wild-type progeny is destroyed. This protocol of enrichment of mutants is similar to the one described originally by Loechler et al. (1984). For two of the adducts, ϵAde and β, determination of the MF was facilitated by the original placement of the adducts in an in-frame *amber* codon. The wild-type phage exhibited a light blue plaque color phenotype in a *sup*E strain whereas any targeted mutation at the second base of the TAG would restore the Lac$^+$ phenotype and generate dark blue plaques on IPTG/X-gal containing medium. The MF of ϵAde and β were determined as the relative number of blue plaques in the light blue wild-type population, after complete elimination of the M13mp19 phage generated during genetic engineering manipulations. The same strategy could not be used for the ϵCyt mutants because the adduct was not part of an *amber* codon. In this specific case, both wild-type and base substitution mutants exhibited the light blue phenotype. We, therefore, used the M13mp19 phage as an internal standard, and the MF was determined from the relative proportion of dark blue to light blue plaques before and after elimination of the wild-type (*Nhe*I sensitive) phage population.

[2] To confirm these data, an internal control was used in certain experiments. The method was based upon the observation that most (>98%) of the phage from M13-*Nhe*I (either adducted or control) exhibited nearly colorless plaques in the presence of IPTG and X-gal due to the presence of an *amber* codon in the *lacZα* fragment. We found that a reliable estimation of phage survival could be obtained by using an internal control of genomic DNA that provided only dark blue plaques. We, therefore, combined a small amount (5%) of M13mp19 ss DNA with the GHD to confirm that the efficiency for transformation remained the same in different mixtures. In addition, we examined the relative efficiencies of ligation of the hexanucleotides into the gap of the GHD to rule out the possibility that the observed diminished survival was due to inefficient ligation.

Table II: Quantitative Features of Mutagenesis of Site-Specifically Modified M13 in *E. coli* DL7[a]

expt no.	DNA strandedness	method of transfection	mutation frequency (%) control	εAde	εCyt	β
1	ds	$CaCl_2$	<0.01	<0.01	<0.01	<0.01
2	ss	$CaCl_2$	<0.01	0.12	1.4	1.6
3	ss	electroporation	<0.01	0.11	2.1	ND[b]

[a] See text for the method of calculation. [b] Not determined.

Table III: DNA Sequence Analysis of Mutants Derived from M13 Genomes Containing εAde or εCyt

εAdet

expt no. (as per Table II)	total sequenced	A→G		A→C		A→T		genetic engineering mutants[a]
		+[b]	–[b]	+	–	+	–	
2	40	8	6	4	0	3	1	18
3	8	3	0	3	1	1	0	0

εCyt

expt no. (as per Table II)	total sequenced	C→T		C→A		C deletion		genetic engineering mutants[a]
		+	–	+	–	+	–	
2	28	12	2	4	0	5	2	3
3	30	14	3	5	3	4	1	0

[a] "Genetic engineering" mutants were typically small deletions caused, we believe, by nuclease activity of some of the enzymes used to construct the site-specifically modified and control genomes (Loechler et al., 1984). They typically occurred at a frequency of ~0.3% and were equally abundant from control and adducted genomes. [b] + and – refer to the strand from which the mutation was presumed to have originated.

At the limit of our detection (~0.01%), mutations were undetectable for all of the ds genomes evaluated. In the ss control M13 genome, mutation at the *Nhe*I site was also undetectable, as expected. All three adducts studied in ss DNA, by contrast, were detectably mutagenic in *E. coli*. As shown in Table II, εAde was the weakest mutagen with an MF of ~0.1%. Both β and εCyt were more strongly mutagenic, displaying MFs of 1.5–2.0%. The type of base change induced by each adduct was distinct (Table III). εAde induced all three possible base substitutions, although A → G transition events predominated. εCyt induced C → T as the major event, but C → A transversions and a targeted C deletion also occurred at a lower frequency. The relative occurrences of the C → T, C → A, and –1 deletions were 2.4:1:1. In each case, the number of mutants derived from adducts in the (+) strand was significantly greater than the same from the (–) strand. This observation has been made with most DNA adducts and has been rationalized by the preferential replication of the (+) strand of this bacteriophage (Loechler et al., 1984; Basu et al., 1989).

Whereas the etheno adducts induced only targeted mutations, the bi-imidazole product β generated targeted as well as locally untargeted mutations of multiple types. Figure 8 displays a partial mutation spectrum obtained from transfection of *E. coli* with the M13 genome containing a β residue. Many of these events involve one targeted and one untargeted mutation at (or near) the *Nhe*I site. Since we have been able to isolate 16 different types of mutants out of 38 plaques sequenced from two independent transformations, it is likely that we only detected a sampling of all the types of mutation induced by this unusual adduct. Nevertheless, the diverse nature of the mutations induced by this adduct is very rare. We note, however, that a similar pattern is observed with UV-induced frameshifts, since these are often accompanied by an apparently random base change (Wood & Hutchinson, 1987).

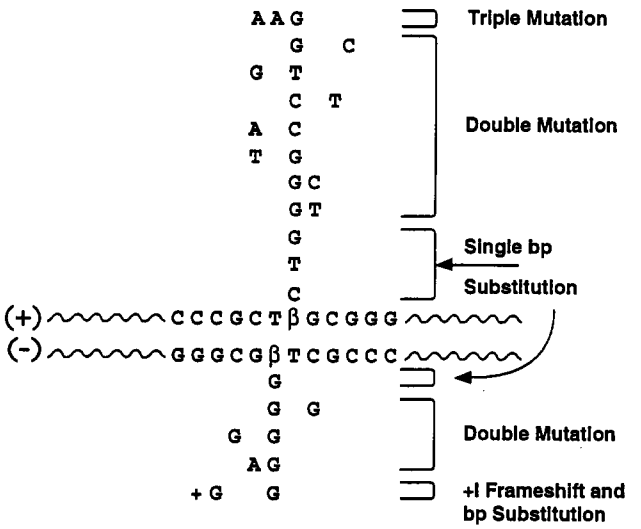


FIGURE 8: DNA sequence analysis of mutants derived from M13 genomes containing β. The genomes containing β were constructed in such a manner that they contained the lesion in either of the two strands of M13-*Nhe*I, at the positions indicated; for details see Green et al. (1984). The abundance of complex mutations induced by β made precise assignment of the strand of origin ambiguous. In the figure, for these complex mutations, we have assumed that at least one of the mutations was targeted to the original position of the lesion. The data in the figure are interpreted as described in the following examples. The C immediately above β in the (+) strand is presumed to be a β → C transversion that arose from an initial pairing event between β and G. Similarly, the β → G transversion immediately below the β in the (–) strand is presumed to have arisen from a β·C pairing.

DISCUSSION

Site-specifically modified genomes are convenient tools for the determination of the mutagenic impact of DNA lesions induced by chemical carcinogens and radiation (Basu & Essigmann, 1988; Singer & Essigmann, 1992). This technology has not been used extensively, however, to rank order the relative lethal potentials of the various DNA lesions formed by DNA-damaging agents. The present work had the dual goals of defining the mutagenic and genotoxic impacts of several vinyl chloride-induced DNA damages. Both of the etheno adducts studied have a sterically obstructed hydrogen-bonding face (Figure 1), and β has its base pairing region completely obliterated. It was anticipated on this basis that all three adducts would block DNA polymerase. Indeed, a very substantial genotoxic effect was observed for all three lesions. This result is in agreement with an in vitro primer elongation study by DNA polymerase I on a chloroacetaldehyde-modified template performed by Jacobsen and Humayun (1990). These workers found that the etheno adducts cause the polymerase to pause, but the lesions do not induce an absolute block to DNA synthesis. Using randomly modified partially duplex M13 DNA, these workers obtained a phage population reduced by 27–49% as compared to the control when the modified genome contained an average of 2.1 εCyt and 3.4 εAde, respectively, per genome.

In the present study, the reduction in phage survival was most pronounced in the case of the genome that contained β. This bi-imidazole product possesses neither a purine nor a pyrimidine ring structure, and therefore, this result was not surprising. It is difficult to rationalize why εCyt was more than twice as toxic as εAde. Modeling studies suggest that, in their normal anti orientation in a B-DNA helix, neither base would be able to form hydrogen bonds with guanine and thymine, respectively, except for the very weak hydrogen bonds involving hydrogens attached to carbons (Kouchakdjian et

al., 1991; Loechler, submitted for publication). It is noteworthy that Jacobsen and Humayun (1990) found that certain sites induce more severe blocks than others in their in vitro study of the ability of DNA polymerase to bypass lesions formed at random. These data suggest that the effect of sequence context on lesion-induced lethality may be significant.

The mutagenic specificities of the three adducts under study were remarkably different. The observation that εAde and its derivative, β, have different mutagenic specificities strongly suggests that the mutation observed for εAde cannot be attributed to its partial breakdown to β, despite the fact that β was shown to be a more potent mutagen. εCyt was demonstrated to be a reasonably potent mutagen (MF = ~2%), and it is likely that its mutation would be evident in the mutational spectrum of chloroacetaldehyde-treated DNA. The mutational spectrum induced by chloroacetaldehyde in M13 is consistent with the notion that εCyt-induced mutagenesis is a frequent occurrence (Jacobsen et al., 1989; Jacobsen & Humayun, 1990). The predominant base change observed is C → T, the major type of mutation induced by εCyt in our site-specific studies and in the recent work of Palejwala et al. (1991, 1993a,b). The chloroacetaldehyde-induced mutation spectrum also generated C → A transversions, a mutation induced at a significant frequency by εCyt in the present work. Our data also suggest that a targeted C deletion should be a frequent event, but surprisingly, this mutation was not observed in the mutational spectrum analysis. Finally, we found that the highly versatile mutagen β was just as mutagenic as εCyt; whether β forms in appreciable quantities in vivo is unknown at this time. We note, however, that another product, εGua(2,3), is also potently mutagenic in vivo (Cheng et al., 1991), and in this specific case, recent data show that the adduct is frequently formed in vivo (Park et al., 1993).

It is noteworthy that DNA polymerases can bypass the etheno adducts at a high frequency (to the extent of ~30% in the case of εAde) even though no Watson–Crick-type hydrogen bonding can occur. Indeed, we have collaborated with Patel and co-workers in the use of 2D NMR spectroscopy to confirm the lack of hydrogen bonding when thymine is located opposite εAde in the sequence context used in this genetic study (Kouchakdjian et al., 1991). We also noted, however, that the potential steric clash between the two bases can be relieved by the assumption of a non-coplanar alignment. In the site-specific mutagenic study reported here, even more remarkable is our observation that the error rate during such a bypass is low (<2%) despite the evidence provided by the NMR study that in certain energy-minimized conformations more than one hydrogen bond could be formed when a wrong base is inserted opposite the etheno adduct (de los Santos et al., 1991). DNA polymerases seem to possess an extraordinary capability to maintain the high fidelity of DNA synthesis, possibly by virtue of their ability to discriminate among specific types of hydrogen bonding between the nucleic acid bases. The present study, however, strongly suggests that the DNA polymerases might also possess an extra sensor to recognize the bases even when they are structurally modified and unable to form the hydrogen bonds with complementary bases.

Both the genotoxicity and the mutagenicity of these adducts were almost completely eliminated in ds DNA. The most reasonable explanation is the rapid repair of lesions from ds DNA. Recent work by Singer and co-workers indicates clearly that mammalian cells possess at least one repair system that recognizes and excises the etheno adducts, and it is reasonable to surmise that *E. coli* may have a similar system. Most repair systems are more efficient on ds DNA than on ss substrates, which would explain the higher mutagenicity of adducts in the latter. An alternative but not mutually exclusive explanation suggests a "strand-bias" pathway by which the unadducted strand serves as a preferential substrate for replication. Such a system has been proposed by Fuchs and co-workers (Koffel-Schwanz et al., 1987).

Several papers have been published recently on the mutagenicity of εCyt (Palejwala et al., 1991, 1993a,b). Qualitatively, most of the data in our work agree with those of Palejwala et al. although we note that their earlier work (1991) failed to detect the targeted C deletion well represented in our study and in their most recent work (1993a,b). It is unclear why they failed to detect such an important feature of the mutational specificity of εCyt in the earlier work. Admittedly, however, the full details of the method used for genome characterization have not been published for the unusual mutational system they used, and until such results are reported, it will be difficult to compare the two studies directly. There was also a major difference between the two studies in the quantitative features of εCyt mutagenesis: while we detected an MF of 1.5–2.0%, Palejwala et al. (1991) observed mutagenesis at more than 10 times that value. Such a large quantitative difference between studies is difficult to reconcile, but may be owed to any or all of the following points. The lesion was situated in different contexts in the two studies. In addition, the two studies employed different methods of cell transformation and different cell strains were used. The most significant difference between the studies, however, was the use in our work of an adducted ss genome, whereas Palejwala et al. used a gapped genome in which the adduct was located in the ss region of the gap. DNA polymerase III, the natural replicative enzyme of *E. coli*, was the probable enzyme involved in fixation of mutation in our study. Mutations were likely to have been fixed by DNA polymerase I, which is most commonly associated with DNA repair synthesis, in the study of Palejwala et al. (1991). This latter point may also explain why Palejwala et al. failed to detect εCyt-induced genotoxicity. In an attempt to address some of the discrepancies between the two studies, recently we have repeated our study of εCyt mutagenesis in the same sequence context [5′-TT(εC)TT-3′] used by Palejwala et al. (1991, 1993a,b). To rule out the possibility that the differences were specific to our mutational analysis system, the system and vector developed by Lawrence, LeClerc, and co-workers, M13mp7L2 (Banerjee et al., 1988), were used (L. A. Ramos, J. M. Essigmann, and A. K. Basu, unpublished results). Using this different system, we found that the mutation frequency of εCyt is <4%, in accord with the results reported in the present paper.

Finally, we have recently become aware of an additional study in which the effects of one of the three lesions investigated in our work (εCyt) has been studied (M. Moriya and A. Grollman, personal communication); their results on mutagenesis of εCyt in non-SOS-induced cells agree well with those reported here.

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REFERENCES

Banerjee, S. K., Christensen, R. B., Lawrence, C. W., & LeClerk, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8141–8145.

Barbin, A., Bartsch, H., Leconte, P., & Radman, M. (1981) *Nucleic Acids Res. 9*, 375–387.
Barrio, J. R., Secrist, J. A., III, & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun. 46*, 597–604.
Basu, A. K., & Essigmann, J. M. (1988) *Chem. Res. Toxicol. 1*, 1–18.
Basu, A. K., Niedernhofer, L. J., & Essigmann, J. M. (1987) *Biochemistry 26*, 5626–5635.
Basu, A. K., Loechler, E. L., Leadon, S. A., & Essigmann, J. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7677–7681.
Brennan, C. A., Manthey, A. E., & Gumport, R. I. (1983) *Methods Enzymol. 100*, 38–52.
Cheng, K. C., Preston, B. D., Cahill, D. S., Dosanjh, M. K., Singer, B., & Loeb, L. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9974–9978.
de los Santos, C., Kouchakdjian, M., Yarema, K., Basu, A., Essigmann, J. M., & Patel, D. J. (1991) *Biochemistry 30*, 1828–1835.
Eberle, G., Barbin, A., Laib, R. J., Ciroussel, F., Thomale, J., Bartsch, H., & Rajewsky, M. (1989) *Carcinogenesis 10*, 209–212.
Foiles, P. G., Miglietta, L. M., Nishikawa, A., Kusmierek, J. T., Singer, B., & Chung, F.-L. (1993) *Carcinogenesis 14*, 113–116.
Green, C. L., Loechler, E. L., Fowler, K. W., & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 13–17.
Guengerich, F. P. (1992) *Chem. Res. Toxicol. 5*, 2–6.
Guengerich, F. P., & Raney, V. M. (1991) *J. Am. Chem. Soc. 114*, 1074–1080.
Guengerich, F. P., Crawford, W. M., Jr., & Watanabe, P. G. (1979) *Biochemistry 18*, 5177–5182.
Guengerich, F. P., Kim, D.-H., & Iwasaki, M. (1991) *Chem. Res. Toxicol. 4*, 168–179.
IARC Monographs (1979) in *Evaluation of the Carcinogenic Risk of Chemicals to Humans 19*, pp 377–438, IARC, Lyon, France.
Jacobsen, J. S., & Humayun, M. Z. (1990) *Biochemistry 29*, 496–504.
Jacobsen, J. S., Perkins, C. P., Callahan, J. T., Sambamurti, K., & Humayun, M. Z. (1989) *Genetics 121*, 213–222.
Kochetkov, N. K., Shibaev, V. N., & Kost, A. A. (1971) *Tetrahedron Lett. 22*, 1993–1996.
Koffel-Schwartz, N., Maenhaut-Michel, G., & Fuchs, R. P. P. (1987) *J. Mol. Biol. 193*, 651–659.
Kouchakdjian, M., Eisenberg, M., Yarema, K., Basu, A., Essigmann, J. M., & Patel, D. J. (1991) *Biochemistry 30*, 1820–1828.
Kusmierek, J. T., & Singer, B. (1982) *Biochemistry 21*, 5723–5728.
Laib, R. J. (1986) in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B., & Bartsch, G., Eds.) IARC Scientific Publications No. 7, pp 101–108, Oxford University Press, London.
Lasko, D. D., Basu, A. K., Kadlubar, F. F., Evans, F. E., Lay, J. O., Jr., & Essigmann, J. M. (1987) *Biochemistry 26*, 3072–3081.
Lasko, D. D., Harvey, S. C., Malaikal, S. B., Kadlubar, F. F., & Essigmann, J. M. (1988) *J. Biol. Chem. 263*, 15429–15435.
Leithauser, M. T., Liem, A., Stewart, B. C., Miller, E. C., & Miller, J. A. (1990) *Carcinogenesis 11*, 463–470.
Leonard, N. J. (1984) *CRC Crit. Rev. Biochem. 15*, 125–199.
Leonard, N. J. (1992) *ChemTracts, Biochem. Mol. Biol. 3*, 273–297.
Loechler, E. L., Green, C. L., & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6271–6275.
Palejwala, V. A., Simha, D., & Humayun, M. Z. (1991) *Biochemistry 30*, 8736–8743.
Palejwala, V. A., Rzepka, R. W., Simha, D., & Humayun, M. Z. (1993a) *Biochemistry 32*, 4108–4111.
Palejwala, V. A., Rzepka, R. W., & Humayun, M. Z. (1993b) *Biochemistry 32*, 4112–4120.
Park, K.-K., Liem, A., Stewart, B. C., & Miller, J. A. (1993) *Carcinogenesis 14*, 441–450.
Rydberg, B., Dosanjh, M. K., & Singer, B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6839–6842.
Rydberg, B., Qiu, Z.-H., Dosanjh, M. K., & Singer, B. (1992) *Cancer Res. 52*, 1377–1379.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sattsangi, P. D., Leonard, N. J., & Frihart, C. R. (1977) *J. Org. Chem. 42*, 3292–3296.
Singer, B., & Grunberger, D. (1983) in *Molecular Biology of Mutagens & Carcinogens*, pp 87–94, Plenum Press, New York.
Singer, B., & Spengler, S. J. (1986) in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B., & Bartsch, G., Eds.), IARC Scientific Publications No. 7, pp 359–371, Oxford University Press, London.
Singer, B., & Essigmann, J. M. (1992) *Carcinogenesis 12*, 949–955.
Singer, B., Abbott, L. G., & Spengler, S. J. (1984) *Carcinogenesis 5*, 1165–1171.
Singer, B., Spengler, S. J., Chavez, F., & Kusmierek, J. T. (1987) *Carcinogenesis 8*, 745–747.
Singer, B., Kusmierek, J. T., Folkman, W., Chavez, F., & Dosanjh, M. K. (1991) *Carcinogenesis 12*, 745–747.
Singer, B., Antoccia, A., Basu, A. K., Dosanjh, M. K., Fraenkel-Conrat, H., Gallagher, P. E., Kusmierek, J. T., Qiu, Z.-H., & Rydberg, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9386–9390.
Swenberg, J. A., Fedtke, T. R., Fennel, T. R., & Walker, V. E. (1990) in *Progress in Predictive Toxicology* (Clayson, D. B., Munro, I. C., Shubik, P., & Swenberg, J. A., Eds.) 1st ed., pp 161–184, Elsevier, Amsterdam.
Tessier, D. L., Brousseau, R., & Vernet, T. (1986) *Anal. Biochem. 158*, 171–178.
Wood, M. L., Dizdaroglu, M., Gajewski, E., & Essigmann, J. M. (1990) *Biochemistry 29*, 7024–7032.
Wood, R. D., & Hutchinson, F. (1987) *J. Mol. Biol. 193*, 637–641.
Yip, K. E., & Tsou, K. C. (1973) *Tetrahedron Lett. 33*, 3087–3090.