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Studies on the miscoding properties of 1,N^6-ethenoadenine and 3,N^4-ethenocytosine, DNA reaction products of vinyl chloride metabolites, during in vitro DNA synthesis

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ABSTRACT

1,N^6-Ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) are formed when electrophilic vinyl chloride (VC) metabolites, chloroethylene oxide (CEO) or chloroacetaldehyde (CAA) react with adenine and cytosine residues in DNA. They were assayed for their miscoding properties in an *in vitro* system using *Escherichia coli* DNA polymerase I and synthetic templates prepared by reaction of poly(dA) and poly(dC) with increasing concentrations of CEO or CAA. Following the introduction of etheno groups, an increasing inhibition of DNA synthesis was observed. dGMP was misincorporated on CAA- or CEO-treated poly(dA) templates and dTMP was misincorporated on CAA- or CEO-treated poly(dC) templates, suggesting that εA and εC may miscode. The error rates augmented with the extent of reaction of CEO or CAA with the templates. Base-pairing models are proposed for the εA.G and εC.T pairs. The potentially miscoding properties of εA and εC may explain why metabolically-activated VC and its reactive metabolites specifically induce base-pair substitution mutations in *Salmonella typhimurium*. Promutagenic lesions may represent one of the initial steps in VC- or CEO-induced carcinogenesis.

INTRODUCTION

The biological effects of VC, a recognized carcinogen in animals and humans[1,2], appear to depend on its conversion by microsomal cytochrome P-450-dependent monooxygenases[3] into CEO[4], which can rearrange nonenzymically to form CAA[5] (Fig. 1). Although both compounds, CEO and CAA, can react with DNA bases[4,6,7] and are mutagenic to prokaryotic and eukaryotic microorganisms or mammalian cells[8-16], qualitative and quantitative differences in their biological and chemical activities have been observed. CEO has a higher chemical reactivity than CAA[4,12,17,18] and is also more mutagenic in bacteria[12,19], yeast[14], and mammalian cells[11]. CAA was not found to be carcinogenic in mice when tested by four different routes of administration[20]. In an initiation-promotion experiment, CEO but not CAA produced skin tumours in mice[21]. Following s.c. administration to mice, CEO was also a potent

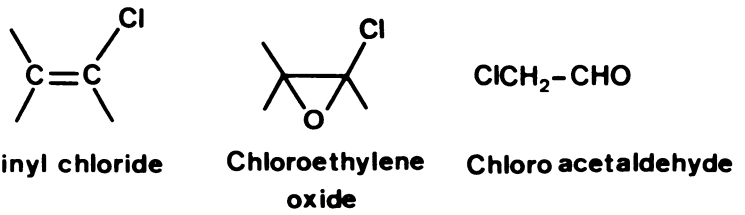

Fig. 1 - Chemical formulae for vinyl chloride, chloroethylene oxide (CEO) and chloroacetaldehyde (CAA).

tumour inducer[21].

CEO and CAA both react with nucleic acid bases: εA and εC (Fig. 2) were first described by reaction of CAA with adenine and cytosine moieties[6,7]. These etheno derivatives possess characteristic fluorescence spectra[7] and have therefore been extensively used in biochemistry[7,22-28]. Subsequent to our report on the formation of εA by reaction of CEO or VC in the presence of mouse liver microsomes with adenosine *in vitro*[4], εA and εC have been shown to be produced in RNA and DNA bases by VC *in vivo* and by metabolically activated VC *in vitro*[29-31].

Although a guanine alkylation product in DNA of VC-treated rats has been described[12,32], reaction of CAA or metabolically activated ^{14}C-VC with adenosine and cytidine yields no adducts other than εA and εC in various experimental systems, except the hydrated etheno-intermediates[6,7,26,27,30,33]. No reaction has been reported to occur between CAA and the sugar-phosphate backbone of nucleic acids[7,27,34-37]; the phosphodiester linkage is not hydrolysed during the course of the reaction[35].

As the introduction of etheno groupings into DNA bases would be expected to interfere with the normal Watson-Crick base-pairing[21], we have therefore investigated whether εA and εC are potentially promutagenic DNA lesions. We used *E. coli* DNA polymerase I and synthetic templates prepared by reaction of the homopolymers poly(dA) and poly(dC) with CEO or CAA. Such fidelity studies with DNA-polymerases *in vitro* have been applied to synthetic polydeoxyribonucleotides treated with increasing concentrations of β-propiolactone[38] and *N*-methyl-*N*-nitrosourea[39,40]. When assayed for DNA replication, an increasing error rate on the carcinogen-modified templates was observed. In the case of

N-methyl-*N*-nitrosourea, the miscoding effects have been attributed to O^6-methylguanine[40,41] and O^4-methylthymine[39], two DNA lesions thought to be involved in the induction of carcinogenesis by methylating agents[42]. Our data report for the first time that a human carcinogen, VC, could form miscoding DNA adducts. A brief account of the data was previously presented[43].

MATERIALS AND METHODS

Chemicals:

Poly(dA) (mw = 2.05 x 10^5) and poly(dC) (mw = 4.85 x 10^5) homopolymers, oligo(dT)$_{12-18}$ and oligo(dG)$_{12-18}$ were purchased from P-L Biochemicals, Inc., Milwaukee, USA. CEO (purity $\geq$ 99.5%) was prepared by chlorination of ethylene oxide in the gas phase, as described in detail by Rannug et al.[19], and purified by redistillation through a Vigreux column. The compound was stored below -70°C, at which it has been shown to be stable for more than 12 months. CAA was prepared just prior to reaction with the polynucleotides, by fractional distillation of the trimer[44]; its purity determined by NMR was 98%. *E. coli* MRE 600 DNA polymerase I (grade I) was obtained from Boehringer, Mannheim, FRG. [Methyl-^{3}H] dTTP, [8-^{3}H] dGTP and the 4 deoxyribonucleosides 5'-[α-^{32}P] triphosphate, dATP, dCTP, dGTP and dTTP, were obtained from the Radiochemical Centre, Amersham, UK. Omnifluor was from NEN Chemicals, GmbH, Dreieich, FRG.

Treatment of Templates:

0.75 nmoles poly(dA) or 0.16 nmoles poly(dC) dissolved in 1.5 or 2 ml 50 mM sodium cacodylate buffer pH 7.0 were treated for 20 hrs at 37°C with up to 0.8 or 0.16 mmoles CAA, respectively. Treated and untreated polynucleotides were then purified by chromatography on a Sephadex G10 column with 50 mM sodium cacodylate buffer pH 7.0 as eluent.

1.5 nmoles poly(dA) or 0.32 nmoles poly(dC) dissolved in 1.5 or 2 ml 50 mM sodium cacodylate buffer pH 7.0 were treated for 20 min at 37°C with up to 1.53 or 0.31 mmoles CEO, respectively. In order to stop any further reaction with CAA formed (the half-life of CEO in neutral aqueous solution at 37°C is 0.9 min), the reaction mixtures were frozen in liquid nitrogen; the thawed polynucleotide solutions were rapidly chromatographed as previously described to eliminate excess CAA.

Polydeoxyribonucleotide ("DNA") Synthesis and Misincorporation Assays:

Treated and untreated poly(dA) [or poly(dC)] were annealed at a 1:1 nucleotide phosphorus ratio with oligo$(dT)_{12-18}$ [or oligo-$(dG)_{12-18}$] in a 50 mM Tris-HCl buffer pH 8.5 containing 30 mM KCl. DNA synthesis was assayed on the primed templates using *E. coli* DNA polymerase I. Each reaction mixture (150 µl) contained 2.5 nmoles treated or untreated homopolymer (nucleotide phosphorus), 4 nmoles [methyl-^{3}H]dTTP (0.67 µCi) or [8-^{3}H]dGTP (3.4 µCi), 0.2 units polymerase, 10 mM $MgCl_2$ and 50 mM Tris-HCl pH 8.5. After 1 hr incubation at 30°C, the solutions were pipetted onto Whatman GF/C glass microfibre filters, and DNA was precipitated in cold 5% trichloroacetic acid (TCA). The filters were rinsed 5 times in cold 5% TCA containing 2% (w/v) sodium pyrophosphate, twice in 5% TCA alone, once in ethanol, and the radioactivity present on the filters was determined by liquid scintillation counting using Omnifluor.

Misincorporation assays were performed using a double-labelling technique. The reaction mixtures were the same as those previously described, except for the addition of a ^{32}P-labelled non-complementary nucleotide (0.8 nmoles, 32 µCi). These assays were carried out as described for the DNA synthesis assays.

Cs_2SO_4 Gradient Analysis:

Newly synthesized DNA, after incubation at 30°C for 1 hr in assays described above, was analyzed by neutral Cs_2SO_4 density gradient centrifugation. From the incubation mixture to which 10 mM EDTA had been added, DNA was extracted twice with phenol and 3 times with diethylether. It was precipitated in ethanol, redissolved in 25 mM Tris-HCl pH 8.5, 1 mM EDTA and purified from free nucleoside triphosphates by column chromatography on Sephadex G50 (eluent 25 mM Tris-HCl pH 8.5, 1 mM EDTA). Solutions of replicated polynucleotides were applied to neutral Cs_2SO_4 gradients (final density = 1.5200 g/cm^3; total volume = 5 ml). Centrifugation was carried out at 35,000 rpm in a SW 50 rotor in a Beckman L5-65 centrifuge for 72 hrs at 20°C.

Fractions of 0.1 ml were collected from the bottom of the tubes and the acid insoluble radioactivity determined. The position of the polynucleotides was monitored by UV-absorbance and the UV-spectrum of radioactive material was recorded.

RESULTS

Spectral Properties of the Modified Templates:

Poly(dA) solutions reacted with increasing concentrations of CAA or CEO (Table 1) showed the characteristic fluorescence spectrum described for 1,N^6-ethenoadenosine[7]. The intensity of fluorescence (λ_{max} = 415 nm; excitation at 310 nm) increased with increasing ratio of CAA or CEO: homopolymer (data not shown) indicating that an increasing proportion of εA residues was introduced in the polynucleotide. CAA-modified poly(dA) obtained with a 1700-fold molar excess of CAA had an ultraviolet spectrum similar to that reported for 1,N^6-ethenoadenosine[7]. The intensity of fluorescence did not increase linearly with the concentration of CAA or CEO; in accordance with previous reports, this is attributable to the quenching of fluorescence of εA residues by neigh-

Table 1 - Incorporation of complementary and non-complementary deoxyribonucleotides on CAA- or CEO-treated poly(dA) templates

Molar ratio [a] CAA(CEO) to nucleotide phosphorus	dTMP incorporation (pmoles)	Error rate x 10^5 [b] $\frac{dCMP}{dTMP}$	$\frac{dGMP}{dTMP}$
CAA-treated poly(dA)			
		(Expt I)	(Expt II)
0.0	2000	29	27
1.7	1500	28	19
17.0	600	21	46
170.0	30	43	3600
1700.0	30	40	1100
CEO-treated poly(dA)			
		(Expt III)	(Expt IV)
0.0	2000	3	2
1.7	2000	3	2
17.0	1000	4	5
170.0	200	4	26
1700.0	20	16	320

[a] Molar ratio of CAA or CEO to nucleotide phosphorus [as poly(dA)] concentrations in the reaction mixture.

[b] The error rate is expressed as the molar ratio of non-complementary to complementary nucleotides incorporated on the template.

bouring nucleic acid bases[23,26,28,45]. CAA- or CEO-treated poly(dC) solutions (Table 2) were not fluorescent; this is in accordance with previous observations[7,23,28].

Inhibition of DNA Synthesis and Misincorporation:

The introduction of etheno groupings in poly(dA) and poly(dC) templates resulted in up to a 100-fold inhibition of DNA synthesis and in up to a several hundred-fold increase in incorporation of non-complementary bases (Tables 1 and 2). The blank values (incubation of the polymerase with each of the 4 pairs of ^{3}H-labelled and ^{32}P-labelled nucleotides but without template) were 200 dpm for the ^{3}H- and 700 dpm for the ^{32}P-label per assay, respectively; the data reported in Tables 1 and 2 were calculated after subtraction of these values. In a neutral Cs_2SO_4 gradient, the radioactivity incorporated from ^{3}H-

Table 2 - Incorporation of complementary and non-complementary deoxyribonucleotides on CAA- or CEO-treated poly(dC) templates

Molar ratio [a] CAA(CEO) to nucleotide phosphorus	dGMP incorporation (pmoles)	Error rate x 10^5 [b] dAMP/dGMP	dTMP/dGMP
CAA-treated poly(dC)			
		(Expt v)	(Expt VI)
0.0	1000	200	10
0.6	1000	170	13
6.3	1000	170	12
63.0	400	150	28
630.0	60	100	78
CEO-treated poly(dC)			
		(Expt VII)	(Expt VIII)
0.0	1200	200	9
0.6	1200	180	6
6.3	1200	180	6
63.0	1000	180	11
630.0	120	430	140

[a] Molar ratio of CAA or CEO to nucleotide phosphorus [as poly(dC)] concentrations in the reaction mixture.

[b] The error rate is expressed as the molar ratio of non-complementary to complementary nucleotides incorporated on the template.

labelled complementary nucleotide and from ^{32}P-labelled non-complementary nucleotide into acid-insoluble material were shown to band at the same density coinciding with the absorbance of polynucleotides. Both inhibition and misincorporation were dependent on the extents of reaction, i.e., the molar ratio of CAA or CEO to homopolymer nucleotide phosphorus.

Misincorporation of dGMP into newly-synthesized DNA was observed in the presence of *E. coli* DNA polymerase I when CAA- or CEO-treated poly(dA) was used as a template: the increases in error rates were from 19 to 3600 x 10^{-5} and from 2 to 320 x 10^{-5}, respectively (Expts II and IV, Table 1). The ^{32}P-incorporation (dpm/assay) ranged from 24,300 to 95,600 (Expt II) and from 3,700 to 5,700 (Expt IV); this 10-fold difference between 2 series of modified poly(dA) templates argues against the idea that dGMP is non-specifically adsorbed on the template. Furthermore, the error rate for dGMP incorporation on untreated poly(dA) in Expt IV is close to that reported by other investigators[46,47]. That misincorporation of dGMP was mainly a consequence of depurination of modified poly(dA) template during the assay could be excluded, since the predicted dCMP incorporation which, on partially depurinated poly-(dA), should be about 4 times higher than the incorporation of dGMP[47] was not observed; the error rates (dCMP/dTMP) were (21-43) x 10^{-5} for CAA-treated poly(dA) (Expt I, Table 1; the ^{32}P-incorporation was in the range of 1,100 to 51,400 dpm/assay) and (3-16) x 10^{-5} for CEO-treated poly(dA) (Expt III, Table 1; the ^{32}P-incorporation ranged from 300 to 5,900 dpm/assay). The increase in error rate for dCMP incorporation (Expts I and III) could be explained by depurination of the templates when incubated at 37°C; this observation corroborates the report that εA is released from CAA-modified DNA *in vitro*[30].

Misincorporation of dTMP was observed on modified poly(dC) templates; the increases in error rates were from 10 to 78 x 10^{-5} (^{32}P-incorporation: range from 4,100 to 11,500 dpm/assay, Expt VI, Table 2) after CAA- and from 6 to 140 x 10^{-5} (^{32}P-incorporation: range from 5,900 to 15,200 dpm/assay, Expt VIII, Table 2) after CEO-treatment of poly(dC). The misincorporation frequencies found for dAMP and dTMP on untreated poly(dC) (Expts V-VIII) are close to those reported by Agarwal et al.[46] and Boiteux[48]. When one of the newly synthesized products, i.e., poly(dC) template treated with a 630-fold molar excess of CAA and replicated in the presence of ^{3}H-dGTP and ^{32}P-dTTP, was sub-

mitted to enzymatic hydrolysis by DNase I from bovine pancreas, the ^{32}P-label was released into the medium demonstrating that the non-complementary deoxyribonucleoside monophosphate was incorporated by phosphodiester linkage. Untreated poly(dC) incorporated 20 times more dAMP than dTMP; this difference was previously shown for heat-denatured poly(dC) templates[48]. Therefore our findings may possibly be explained by deamination of cytosine residues prior to the assay to give uracil, which codes for adenine. As indicated by a decrease in the misincorporation of dAMP (Expts V and VII, Table 2; the ^{32}P-incorporation ranges were from 5,400 to 177,900 dpm/assay, and from 46,300 to 216,700 dpm/assay, respectively), the rate of deamination in the template was reduced after CAA or CEO treatment because of reaction with the N^4 amino group of cytosine. Only when poly(dC) was treated with CEO at the highest concentration (Expt VII, Table 2) was an exception noticed: the error rate for dAMP increased. Further experiments are required to investigate the possible role as a miscoding base of 2,3-dihydro-2-hydroxy-imidazo[1,2-c]pyrimidin-5(6H)-one, which is the precursor of εC in the reaction of CAA with cytosine residues[27].

In a double-logarithmic plot of the misincorporation rates of dGMP on modified poly(dA) templates *versus* the respective values for inhibition of DNA replication (expressed as 1/dTMP incorporated), straight lines with identical slopes were obtained for templates containing increasing amounts of εA (graphs not shown). Similar plots of the data for modified poly(dC) templates also gave linear relationships, but the slopes of the lines differed slightly. These results are consistent with the idea that a single DNA adduct, εA, is produced when either CAA or CEO reacts with poly(dA) and that this is solely responsible for the observed phenomena of DNA inhibition and misincorporation. Similar considerations may apply to εC.

DISCUSSION

There is now sufficient evidence to demonstrate that CAA and CEO, both electrophilic VC metabolites, react with adenine and cytosine residues in nucleic acids. Apart from the hydrated etheno-intermediates, εA and εC are the only reaction products reported so far (see Introduction). When CAA- or CEO-treated poly(dA) and poly(dC) were assayed as templates for *E. coli* polymerase I, misincorporation of non-complementary nucleotides was observed; the misincorporation increased

with the degree of modification and was specific, dGMP being incorporated on modified poly(dA) and dTMP on modified poly(dC). In a collaborative study carried out by Spengler and Singer[49] on synthetic templates containing predetermined amounts of εA and εC and with a DNA-dependent RNA polymerase system, similar results were obtained.

Therefore, the high misincorporation rate of dGMP observed on CAA- and CEO-treated poly(dA) templates infers possible miscoding properties for εA. Such εA.G base-pairing could be explained by the formation of hydrogen bonds between the two bases in the *syn* and *anti* conformations, respectively (Fig. 2A). The proposed base-pairing scheme is similar to that of $A_{syn}.G_{anti\ (enol,\ imino)}$[50]. εA may thus represent a potential promutagenic lesion which can be expected to lead to A.T→C.G transversions.

Our data also suggest that εC and/or its precursor could miscode for T and thus possibly induce C.G→A.T transversions. Comple-

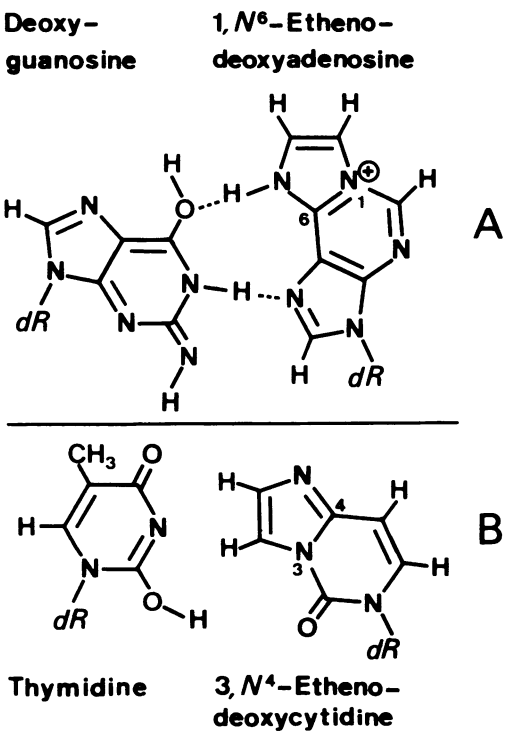

Fig. 2 - Base-pairing schemes for the miscoding bases 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC). *dR*: deoxyribose.

mentary base-pairing models[50,51] fail to explain the observed εC.T base-pairing through hydrogen bonding (Fig. 2B) as has been discussed for other mispairing bases[52]. Misincorporation of dTMP in our studies may therefore be attributable to vertical stacking forces, which have been proposed as an alternative mechanism for base-pair stabilization[53,54].

The induction of A.T→C.G and C.G→A.T transversions possibly resulting from εA and εC may explain the fact that metabolically activated VC, CAA or CEO induce base-pair substitution mutations, but not frameshift mutations in *Salmonella typhimurium*[8-10]. CAA has recently been reported to specifically induce base-pair substitution mutations in a *Bacillus subtilis* transformation assay[16]. Further experiments in this laboratory are in progress to determine whether CAA and CEO specifically induce A.T→C.G and/or C.G→A.T transversions. εA and εC may also be responsible for the mutagenic effects of CEO found in all the genetic indicator organisms tested so far[2]. The formation of such potentially promutagenic lesions in DNA, suggested for the first time with a human carcinogen, may well represent a critical step in VC- or CEO-induced carcinogenesis[21]. This type of lesion, i.e., adenine and cytosine with an additional imidazole ring between the exo-nitrogen and the adjacent endo-nitrogen, may have relevance for other carcinogens and mutagens, such as vinyl bromide, which produces εA and εC residues in RNA[55] or haloethylnitrosoureas and glycidaldehyde, which give rise to structurally--related ethano and etheno derivatives of nucleic acid bases, respectively[56-58].

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independent studies: misincorporation of dGMP on CAA-modified poly-(dA,dT) co-polymers and of dAMP, dTMP on modified poly(dG,dC) templates was observed. The error rates were dependent on the amount of εA and εC that were analyzed in the respective templates. These data lend further support that εA and εC may have miscoding properties.

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