THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 24, Issue of June 15, pp. 21821–21827, 2001

Printed in U.S.A.

The Pyrimidine Ring-opened Derivative of 1,N^6-Ethenoadenine Is Excised from DNA by the *Escherichia coli* Fpg and Nth Proteins*[S]

Received for publication, February 2, 2001, and in revised form, March 14, 2001
Published, JBC Papers in Press, March 20, 2001, DOI 10.1074/jbc.M100998200

Elżbieta Speina, Jarosław M. Cieśla, Jacek Wójcik, Monika Bajek, Jarosław T. Kuśmierek, and Barbara Tudek‡

From the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5A, 02-106 Warsaw, Poland

It was previously shown that 1,N^6-ethenoadenine (ϵA) in DNA rearranges into a pyrimidine ring-opened derivative of 20-fold higher mutagenic potency in *Escherichia coli* (AB1157 *lac*ΔU169) than the parental ϵA (Basu, A. K., Wood, M. L., Niedernhofer, L. J., Ramos, L. A., and Essigmann, J. M. (1993) *Biochemistry* 32, 12793–12801). We have found that at pH 7.0, the stability of the *N*-glycosidic bond in ϵdA is 20-fold lower than in dA. In alkaline conditions, but also at neutrality, ϵdA depurinates or converts into products: ϵdA → B → C → D. Compound B is a product of water molecule addition to the C(2)–N(3) bond, which is in equilibrium with a product of N(1)–C(2) bond rupture in ϵdA. Compound C is a deformylated derivative of ring-opened compound B, which further depurinates yielding compound D. Ethenoadenine degradation products are not recognized by human *N*-alkylpurine-DNA glycosylase, which repairs ϵA. Product B is excised from oligodeoxynucleotides by *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Nth). Repair by the Fpg protein is as efficient as that of 7,8-dihydro-8-oxoguanine when the excised base is paired with dT and dC but is less favorable when paired with dG and dA. Ethenoadenine rearrangement products are formed in oligodeoxynucleotides also at neutral pH at the rate of about 2–3% per week at 37 °C, and therefore they may contribute to ϵA mutations.

1,N^6-Ethenoadenine (ϵA)[1] and other exocyclic DNA adducts such as 3,N^4-ethenocytosine (ϵC) or N^2,3-ethenoguanine (ϵG) are introduced to DNA by the human carcinogen vinyl chloride and related compounds (1). These DNA lesions are also formed during interaction with DNA of the peroxidation products of ω-6-polyunsaturated fatty acids (2). Ethenoadenine has been found in the DNA of unexposed humans and rodents at highly variable levels, ranging from 0.043 to 31.2 ϵA molecules/10^8 of unmodified adenine residues (3, 4). Upon treatment of animals with vinyl chloride, the level of ϵA increased in the DNA of rat liver, lung, lymphocytes, and testis (in liver and lung several-fold) (3). The level of ϵ-DNA adducts correlates with increased oxidative stress, such as observed during the accumulation of transient metal ions in Wilson disease, a human metal storage disease, and with increased content of polyunsaturated fatty acids in the diet (5–7).

Ethenoadenine is eliminated from DNA by *N*-methylpurine-DNA glycosylases. Eukaryotic glycosylases from yeast, rat, and human excise this lesion about 500-fold more efficiently than bacterial AlkA protein (8). Molecular dosimetry experiments suggest, however, that it is a persistent lesion, because 2 weeks after exposure of rats to vinyl chloride, the ϵA level in liver DNA remains very similar to that obtained directly after treatment (9).

All known exocyclic DNA adducts are mutagenic. In bacteria, ϵA is recognized mostly as an unmodified adenine by DNA polymerases, infrequently giving rise to AT → TA substitutions (10). In mammalian cells, ϵ-DNA adducts are classified among lesions with the highest mutagenic potency. In site-directed mutagenesis either 70 (10) or 10% (11) of ϵA residues in DNA were replicated erroneously, giving rise mainly to AT → GC (10) but also to AT → TA (preferential mutation on the leading strand) and AT → CG substitutions (11). In the same studies only 0.3% of 7,8-dihydro-8-oxoguanine (8-oxoG) residues induced GC → TA transversions (11). Treatment of mammalian cells with compounds inducing etheno-DNA adducts additionally triggers chromosomal aberrations and sister chromatid exchanges (1).

Early investigations by Tsou *et al.* (12) and Basu *et al.* (13, 14) have shown that in alkali, but also under physiological conditions, ϵA is rearranged into a pyrimidine ring-opened derivative, 4-amino-5-(imidazol-2-yl)imidazole, which has about 20-fold higher mutagenic potency in *Escherichia coli* (AB1157 *lac*ΔU169) than the parental ϵA (14). Because the secondary lesions arising from ϵA might contribute significantly to its mutagenesis, we undertook a detailed study of the chemical stability of ϵA in DNA, during which we found enzymes repairing a derivative formed during ϵA chemical rearrangement and identified excised lesion.

EXPERIMENTAL PROCEDURES

Materials—1,N^6-ϵdA was synthesized using the modified procedure of Barrio *et al.* (15) described for the synthesis of its ribo-cogener. The material obtained (needles, melting point, 163–164 °C) was 99% pure (HPLC). Its identity was confirmed by UV, NMR, and electrospray MS.

Oligodeoxynucleotide (40-mer) containing a single ϵA at position 20 in the sequence 5′-d(GCT ACC TAC CTA GCG ACC T **ϵA**C GAC TGT

* This work was supported by European Commission Grant ENV4-CT97-0505 through a collaborative research agreement between the International Agency for Research on Cancer, Lyon, France, and the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (PAS) (GI/43/4), by grant C-2/VII/11 from the Polish-French Center of Plant Biotechnology, and by Grants 6 PO4A 065 18 and E-35/SPUB/P04/206/97 from the State Committee for Scientific Research (Poland). This project was realized within the framework of the activity of the Center of Excellence in Molecular Biotechnology, Institute of Biochemistry and Biophysics PAS (WP10). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] Tables 1S and 2S.

‡ To whom correspondence should be addressed. Tel.: +4822-658-47-24; Fax: +48-39121623; E-mail: tudek@ibb.waw.pl.

[1] The abbreviations used are: ϵA, 1,N^6-ethenoadenine; ϵdA, 1,N^6-ethenodeoxyadenosine; ANPG-40, human *N*-methylpurine-DNA glycosylase, truncated form; ϵC, 3,N^4-ethenocytosine; 8-oxoG, 7,8-dihydro-8-oxoguanine; HPLC, high performance liquid chromatography; Fapy, 7MeG-2,6-diamino-5*N*-methyl-formamidopyrimidine; Fpg protein, *E. coli* formamidopyrimidine-DNA glycosylase; Nth protein, *E. coli* endonuclease III; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry.

CCC ACT GCT CGA A)-3′ was purchased from Eurogentec Herstal, (Herstal, Belgium). The purity and identity of this oligomer was verified by HPLC and mass spectrometry. The oligomer was digested enzymatically to deoxynucleosides, which were separated by HPLC (for the details see below and Fig. 5). The deoxynucleoside content calculated on the basis of peak areas was for dC, 16.1, dG, 7.5, dT, 8.0, dA, 7.5, and ϵdA, 0.9 residues/molecule, which is in good agreement with the previewed values of dC, 16, dG, 7, dT, 8, dA, 8, and ϵdA, 1 residue(s)/molecule. The identity of the ϵA-oligomer was confirmed by mass spectrometry using an electrospray Quadrupol-time of flight instrument (Q-TOF, Micromass). The measured molecular mass of ϵA-oligomer was 12,145.3 ± 0.4, which is consistent with the expected mass of 12,144.9. No significant traces of the compound, the molecular weight of which would correspond to the presence in the oligomer of compound **B** instead of ϵdA (M_r 12,163), were detected (less than 2%). No peak corresponding to the presence of compound **C** in this oligomer (M_r 12,134) was recorded. Both methods confirmed the identity and purity of ϵA-oligomer.

Four complementary oligodeoxynucleotides containing T, C, G, or A opposite ϵA were either purchased from Eurogentec or synthesized according to standard procedures using a Beckman Oligo 1000 M synthesizer (Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences).

E. coli Fpg and Nth proteins were purified from overproducing strains (JM105 *supE endA sbcB*15 *hsdR*4*rpsL thi*Δ(*lac-proAB*) carrying the pFPG230 plasmid and BH410 (as JM105 but fpg-1:Kn harboring the pNTH10) as previously described (16, 17). Human *N*-methylpurine-DNA glycosylase (ANPG-40) as well as bacterial strains overproducing Fpg and Nth glycosylases were a kind gift from Dr. Jacques Laval (Institut G. Roussy, Villejuif, France). T4 kinase was from TaKaRa, nuclease P1 from Amersham Pharmacia Biotech, snake venom phosphodiesterase from PL Biochemicals, and *E. coli* alkaline phosphatase from Sigma.

Instrumentation—HPLC was performed using a Waters dual pump system with a tunable UV/visible light absorbance detector managed by Millenium 2010SS (version 2.15) controller. All separations were performed on a Waters Nova-Pak® C18 reversed-phase column (60 Å, 4 μm, 4.6 × 250 mm). NMR spectra were measured on a UNITY 500plus (Varian) spectrometer equipped with a gradient generator unit Performa II, Ultrashims, and a high stability temperature unit using 5 mm $^1H\{^{13}C/^{15}N\}$ pulsed field gradient triple probe. Electrospray ionization mass spectrometry of nucleosides was performed on a Mariner apparatus with time of flight detection. UV spectra were recorded on a Cary 3E spectrophotometer.

Thin Layer Chromatography—Silica gel 60 F_{254} aluminum sheets (Merck, catalog no. 1.0554) were used for analytical purposes. For preparative purposes 20 × 20 cm plates were prepared using silica gel 60 PF_{254} (Merck, catalog no. 1.07747). The following solvent systems were used: methanol/chloroform, 15:85 (I); isopropyl alcohol/25% aqueous NH_3/water, 70:10:10 (II) and 70:5:5 (III).

Solvolytic Degradation of ϵdA—The solutions of ϵdA (1–2 mM) were incubated in pH 12 (0.02 N NaOH), pH 9.2 (0.1 M $Na_2B_4O_7$), or pH 7.5 (0.1 M phosphate buffer) at 37 °C or at room temperature (23 °C) for various periods (15 min to 30 days) and were analyzed by HPLC. The representative separations, retention times, and chromatographic conditions are given in Fig. 2. The neutral depurination of 2′-deoxyadenosine and ϵdA was studied in 0.1 M phosphate buffer, pH 7.5, at 60 °C by HPLC. The HPLC analysis of depurination was performed similarly to the analysis of alkaline degradation of ϵdA with the exception that gradient was present for 30 min (the relevant retention times under these conditions (in minutes) were: dA, 11.5; A, 9.7; ϵdA, 13.0; ϵA, 11.9; and product **B**, 8.4).

Isolation and Purification of ϵdA Degradation Products—A reaction mixture contained ~0.1 mmol of ϵdA in 1 ml of 0.05 N NaOH. After 3–7 days at 37 °C, TLC in solvent II showed the presence of product **B** (R_f = 0.52) and **C** (R_f = 0.67), some nonreacted ϵdA (R_f = 0.60), **D** (R_f = 0.38), and other products. The separation of products was done using preparative silica gel plates run 2–4 times in the same direction in solvent III. The appropriate bands were eluted by methanol, and the purity of products was verified by HPLC. The final purification was done by preparative TLC in solvent I. The products obtained were more than 95% pure, and they were used for studies by UV, NMR, and MS. UV (λ_{max}, nm): **B**, 259 (H_20), 265 (pH 12), 267 (pH 1); **C**, 274 (H_2O), 265 (pH 12), 282 (pH 1) (in conformity with spectra of ribo-cogener (18)); **D**, 247 (H_2O), 247 (pH 12), 242 (pH 1). Electrospray-MS analytical data (*m/z* assignment and relative abundance in parentheses): ϵdA, 276.1 (MH^+, 100), 160.1 (BH^+, 35); **B**, 294.1 (MH^+, 100), 178.1 (BH^+, 35); **C**, 266.1 (MH^+, 100), 150.1 (BH^+, 15); **D**, 288.3 (not assigned, 100), 316.3 (not assigned, 40). NMR data are gathered in supplemental Tables 1S and 2S.

NMR Measurements—Samples for the NMR measurements were prepared in D_2O or Me_2SO-d_6 at concentrations of ~5 mM. Spectra were measured at 25 °C using proton 1D, TOCSY (19–21), ROESY (22, 23) and $\{^1H/^{13}C\}$gHSQC (23–26) experiments under standard conditions and with standard parameters. All spectra were analyzed using VNMR 5.1A (Varian) software. Proton spectra in aqueous solution were calibrated against a water signal (27). In Me_2SO-d_6 solution the residual solvent signal was used as a reference (28) in both proton and carbon dimensions in $\{^1H/^{13}C\}$ correlation spectra.

Ethenoadenine Decomposition in Oligodeoxynucleotides—The kinetics of compounds **B** and **C** formation in oligodeoxynucleotides as well as the identification of substrates for repair glycosylases was performed by HPLC. ϵA-40-mer was incubated in 0.2 N NaOH for 1–4 h, neutralized by the addition of an equivalent amount of 1 N HCl and 1/10 volume of 1 N Tris-HCl, pH 7.0, ethanol-precipitated, and washed. Then oligomers were digested enzymatically to nucleosides. The reaction mixture (50 μl) contained 1.5 nmol of oligomer, 1.5 units of nuclease P1, 0.075 units of snake venom phosphodiesterase, 0.3 units of *E. coli* alkaline phosphatase in 20 mM Tris-HCl, pH 8.5, and 10 mM $MgCl_2$. After digestion (1 h, 37 °C), proteins were ethanol-precipitated, and the supernatant containing the nucleosides was evaporated to dryness, dissolved in water, and subjected to HPLC. The representative separations, retention times, and chromatographic conditions are given in Fig. 5.

Excision of ϵA and Its Degradation Products from Oligomers by Repair Glycosylases—The 40-mer oligodeoxynucleotide containing a single ϵA or its rearrangement products obtained by oligomer incubation in 0.2 N NaOH for 1–16 h was radiolabeled with ^{32}P at the 5′-end and annealed to the complementary strand (double molar excess). The release of ϵA or its rearrangement products by glycosylases was assessed by measuring the cleavage of 40-mer at the site of lesion. The standard reaction mixture (20 μl) contained 5′ ^{32}P-labeled duplex (1 pmol), 100 mM KCl, 1 mM EDTA, and 5 mM β-mercaptoethanol in 70 mM Hepes-KOH, pH 7.6 for the Fpg protein or pH 7.8 for ANPG-40 or Nth glycosylases. The mixtures were incubated at 37 °C for a 10 min in the presence of excess of repair glycosylases (100–150 ng of each protein/sample) and then subjected to 20% PAGE in the presence of 7 M urea. To cleave the oligomer at the apurinic/apyrimidinic site remaining after excision of ϵA by ANPG-40 protein, the reaction mixture prior to PAGE was incubated in 0.2 N NaOH at 70 °C for 30 min. Gels were exposed to x-ray film, scanned in an LKB densitometer, and quantified using Microcal Origin.

Kinetic Studies with Determination of K_m and V_{max} for the Fpg Protein—Kinetic constants were established in ϵA-oligomer incubated in 0.2 N NaOH for 4 h. The concentration range of the oligomer was 0.6–48 nM when the modified base was paired with dT, 4.8–65 nM when paired with dA, 0.3–75 nM paired with dC, and 2–160 nM paired with dG. The amounts of pure Fpg protein used in the reaction were adjusted to obtain less than 50% utilization of substrate and equaled 0.4 ng when modified base was paired with dT and dG, 2 ng when paired with dA, and 0.2 ng with dC. In each experiment two control samples were set: negative without enzyme, to quantify nonspecific breakage of oligodeoxynucleotide; and positive with excess enzyme (150 ng of Fpg), to get 100% cleavage of oligomer. The reaction was performed precisely for 10 min and stopped by adding sequencing kit stop solution, and reaction products were separated by PAGE. Autoradiograms were scanned (LKB scanner), and the peaks on resulting plots, corresponding to cleavage product and nonreacted oligomer, were quantified using multi-peak Lorentzian fitting in Microcal Origin. In calculations the average substrate concentration (*i.e.* $([S]_0 + [S]_t)/2$) and average velocity (*i.e.* $([S]_0 - [S]_t)/t$) were used (29). V_{max} and K_m values were calculated by two methods: a program using the Eisenthall-Cornish-Bowden nonparametric algorithm (30) and direct fitting of the hyperbolic Michaelis-Menten equation to the data points in Microcal Origin. Values obtained by both methods differed usually by no more than 3–6%.

RESULTS

Alkali-induced Rearrangements of 1,N^6-Ethenodeoxyadenosine—1,N^6-Ethenodeoxyadenosine at pH 12 was degraded sequentially into three products: ϵdA → **B** → **C** → **D** (Fig. 1), which could be separated by HPLC (Fig. 2, A and *B*) or by TLC. The first stage, ϵdA → **B**, was the fastest ($t_{1/2}$ at 23 °C was 2.5 h). The **B** → **C** and **C** → **D** reactions at pH 12 were at least 10 times slower than the conversion ϵdA → **B** (Fig. 2). The rearrangement of ϵdA → **B** is strongly pH-dependent; a half-time of this reaction at 37 °C equals 1.5 h at pH 12, 7 days at pH 9.2, while

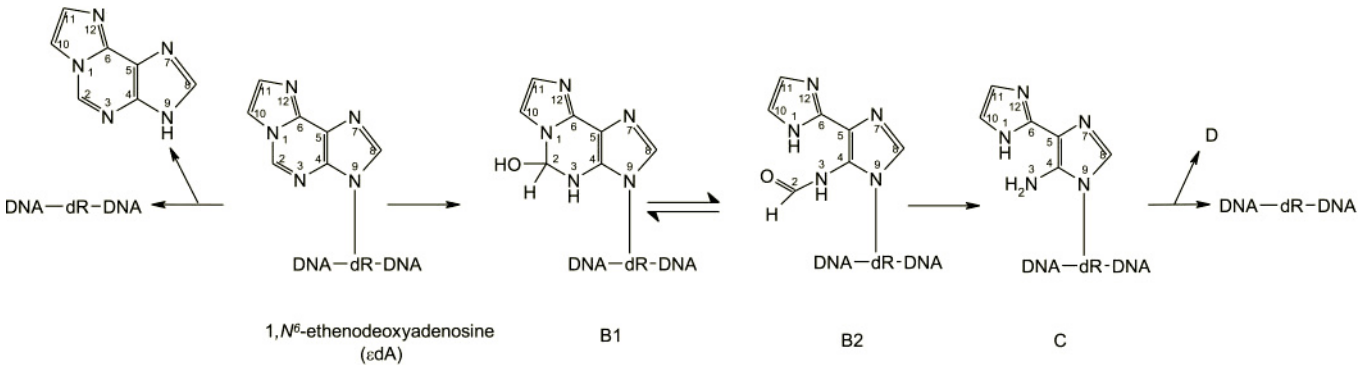

FIG. 1. **Proposed fate of 1,N^6-ethenodeoxyadenosine in DNA including structures of the parent ϵdA and products of its degradation.** The numbering of atoms as in the purine ring was retained in all structures for simplicity and convenience.

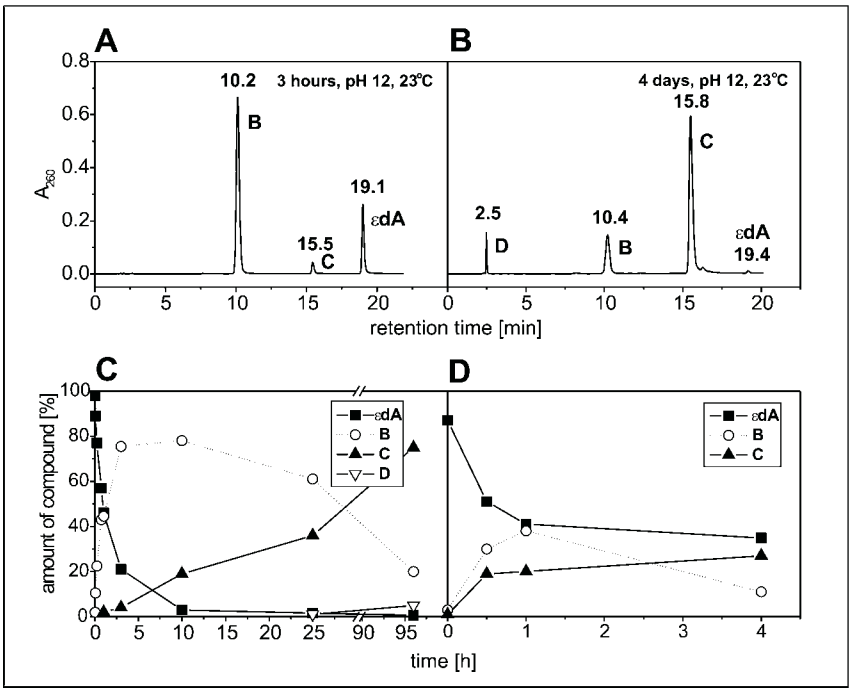

FIG. 2. **Products of 1,N^6-ethenoadenine rearrangement in alkali.** *Panels A* and *B* show HPLC separation of ϵdA and products of its degradation formed at 23 °C after 3 h (*A*) or 4 days (*B*) in 0.02 N NaOH. Chromatographic conditions were the following: linear gradient of 20 mM NH_4HCO_2, pH 9.0, 90% methanol in water over 60 min with a flow rate of 1 ml/min and UV absorbance detection at 260 nm. *Panel C*, kinetics of ϵdA rearrangement in monomer (pH 12 at 23 °C) as measured from the peak areas obtained in HPLC. *Panel D*, kinetics of ϵdA rearrangement in polymer (pH 13 at 37 °C) as measured from peak areas obtained in HPLC analysis and as described in Fig. 5.

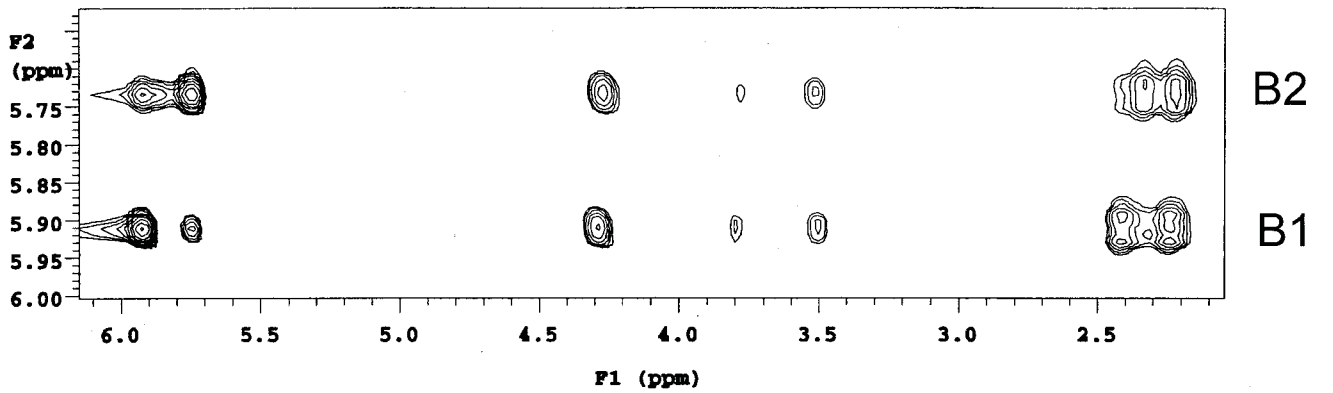

FIG. 3. **Part of the TOCSY spectrum of the product B in Me_2SO-d_6.** Two traces corresponding to the sugar moieties of both isomers **B1** and **B2** are indicated. The two cross-peaks observed at 5.92/5.74 and 5.74/5.92 ppm corresponding to $H_{1'}$ protons are due to **B1** ⇄ **B2** equilibrium.

at pH 7.5 about 1 year, as estimated on the basis of 30-day measurements (not shown). A half-time for **B** → **C** and **C** → **D** reactions at pH 7.5, 37 °C is about 12 days, as judged by the HPLC analysis of the isolated compounds **B** and **C.** Thus, at physiological pH the first step of rearrangement in nucleosides occurs very slowly, and the subsequent steps are much faster.

We were searching for other than high pH factors that could stimulate ϵdA rearrangements under physiological conditions. The degradation of ϵdA was tested in the presence of several amino acids (glycine, L-proline, L-lysine, L-serine, all at 80 mM, and 80% saturated DL-tyrosine), 50 mM glutathione (reduced form), and 80 mM mercaptoethanol, as well as 100 mM NaHS, NaN_3, KF, and KI, all at 37 °C in 0.1 M phosphate, pH 7.5. None of these compounds accelerated ϵdA → **B** conversion (not shown).

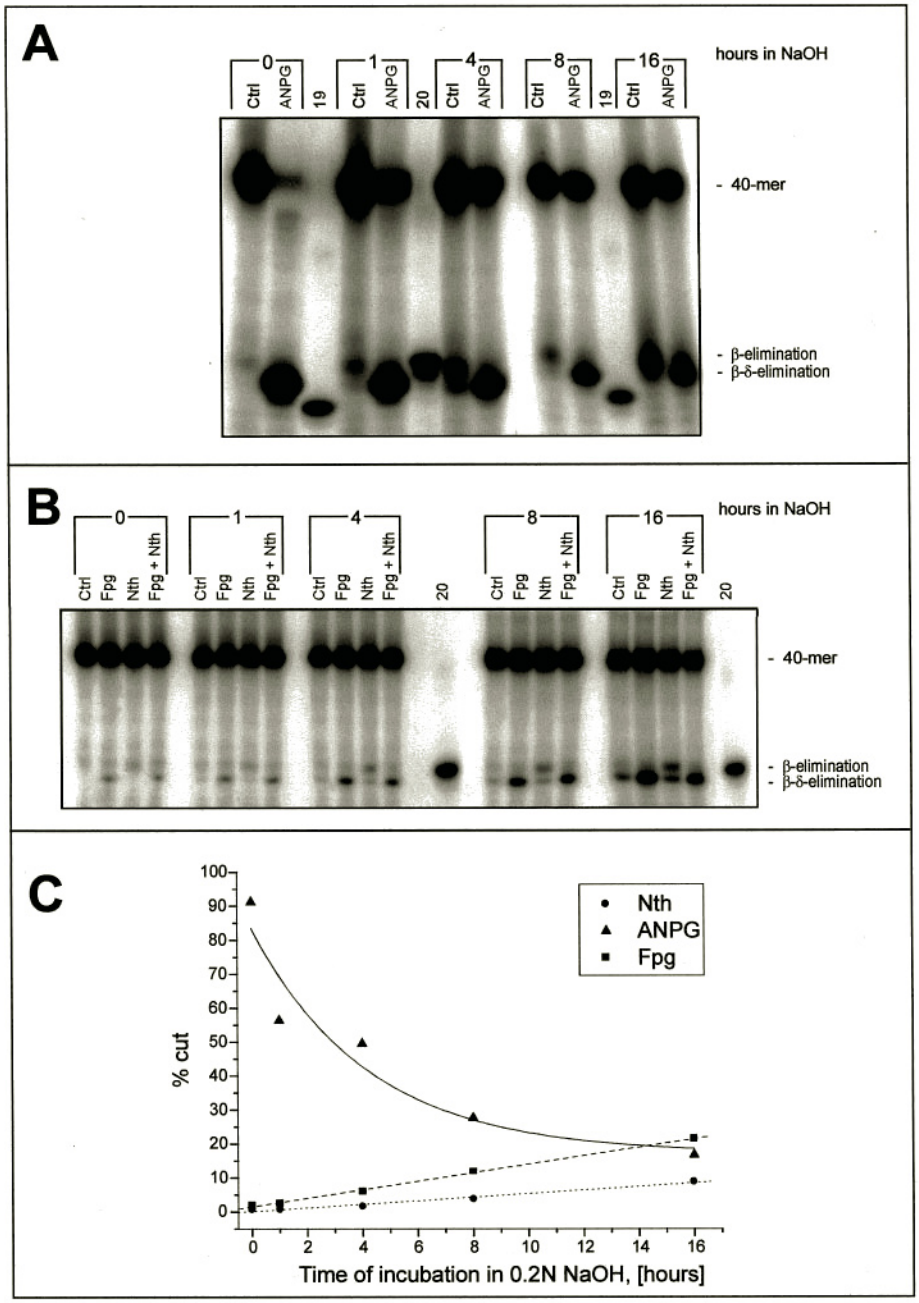

FIG. 4. Enzymatic recognition of ϵA and its degradation product(s) in ϵA-oligomer by human 3-methyladenine DNA glycosylase (ANPG) (*A*) and *E. coli* Fpg and Nth proteins (*B*). ϵA-oligomer was incubated in 0.2 N NaOH for 1–16 h and then subjected to cleavage by repair glycosylases, and products were separated by PAGE. *Lanes: Ctrl*, control ϵA-oligomer untreated with repair glycosylases; *ANPG, Fpg*, and *Nth*, oligomer digested with ANPG, Fpg, or Nth glycosylases, respectively; *19*, *20*, 19- or 20-mer standards. *Panel C*, quantification of the efficiency of oligomer digestion by repair glycosylases. The percent of oligomer cleavage was measured by phosphorimaging (Molecular Dynamics PhosphorImager) or scanning of autoradiograms. Spontaneous breaks as measured in the control lane were subtracted from ANPG and Fpg cleavage products. For the Nth glycosylase, only the β-elimination product was quantified, because it reflected the amount of excised base.

Depurination of ϵdA at Neutrality—HPLC analysis of neutral ϵdA and dA depurination at 60 °C shows that the initial rate of ϵA formation is 0.08%/h, whereas that of A is 0.004%/h. Depurination of ϵdA is concomitant with formation of product **B** with a rate of 0.2%/h (not shown); this shows that the glycosyl bond in ϵdA is 20-fold less stable than that in dA and that the pyrimidine ring opening in ϵdA is 2.5 times faster than the rupture of the glycosyl bond under neutral conditions.

Degradation of Ethenoadenine in Oligodeoxynucleotides—Composition of ϵA-oligomer incubated for 1–16 h in NaOH at 37 °C was analyzed by HPLC. Only ϵdA, products **B** and **C**, could be identified, because compound **D** was masked by components of the buffer used for enzymatic digestion of oligomer (Figs. 2*B* and 5*D*). To reach a rate of ϵdA → **B** conversion similar to that in nucleoside, oligomer was treated with NaOH at a concentration 10-fold higher than that used for nucleoside. Similarly, the fluorescence loss, because of decomposition of ϵA (the only fluorescent component of the pathway) was observed at a 10-fold higher concentration of NaOH in polymer than in monomer (not shown). Compounds **B** and **C** were already found in ϵA-oligomer not treated with NaOH (Fig. 2*D*), although their amount was negligible and differed from batch to batch; usually they constituted 2–6% of the expected ϵdA amount (not shown). After 4 h of oligomer incubation in 0.2 N NaOH, ϵA **B** and **C** were found in comparable amounts (Fig. 2*D*), whereas a 4-h incubation of monomer in 0.02 N NaOH resulted in the conversion of 80% of ϵdA into **B** (Fig. 2*C*). This suggests that in polymer the reaction is shifted toward the formation of compound **C** under conditions the same as those in the monomer rate of ϵA → **B** conversion.

Structure of Ethenodeoxyadenosine Products: NMR and MS Analysis—Proton chemical shifts of ϵdA compounds **B** and **C** were assigned using TOCSY and ROESY; the spectra are listed in supplemental Table 1S and carbon chemical shifts in supplemental Table 2S.

The NMR signals in the spectra of ϵdA have been assigned

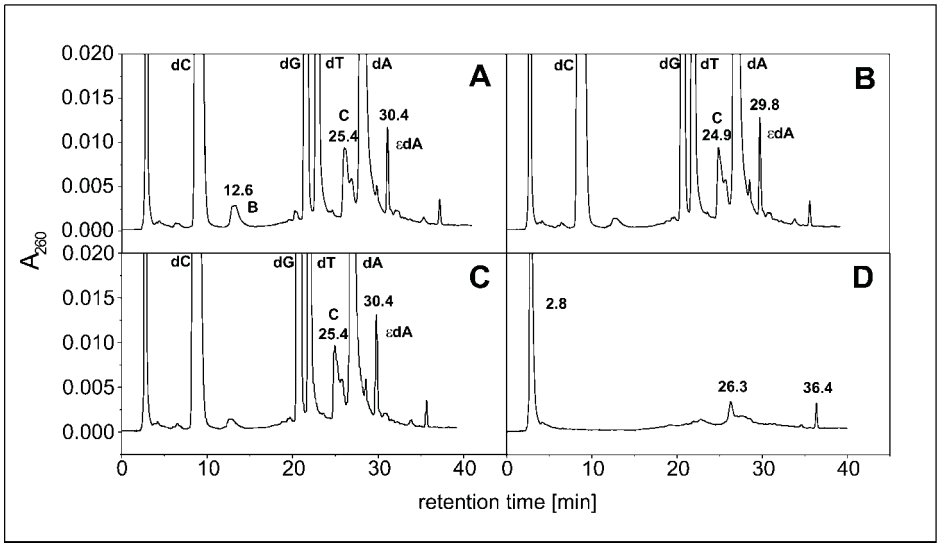

FIG. 5. **HPLC identification of ϵA derivatives excised by Fpg and Nth proteins.** ϵA-oligomer was incubated for 4 h in 0.2 N NaOH at 37 °C, treated or not with the excess of DNA repair glycosylases, and precipitated to remove excised base. Precipitated oligomer was digested to the nucleosides, which were separated subsequently by HPLC using isocratic elution with 20 mM NH_4HCO_2, pH 6.0, for 10 min and then a linear gradient of 20 mM NH_4HCO_2, pH 6.0, 30% methanol in water over 30 min at a flow rate 1 ml/min) and UV absorbance detection at 260 nm. *A*, ϵA-oligomer incubated for 4 h in 0.2 N NaOH; *B*, the same as in *A* but digested with the Fpg protein; *C*, the same as in *A* but digested with the Nth protein; *D*, background signals derived from enzymatic solutions used for preparation of samples for HPLC. The relative quantity of ϵdA (peak areas [A_{260}*s*10^4]) in ϵA-oligomer untreated and treated with Fpg and Nth glycosylases was 12.9 (*A*), 15.9 (*B*), and 15.6 (*C*), whereas the quantity of compound **B** equaled 8.1 (*A*), 3.7 (*B*), and 3.4 (*C*). The quantity of compound **C** was 19.3 (*A*), 18.3 (*B*), and 18.3 (*C*).

TABLE I
Kinetic constants for the removal of modified bases from oligonucleotides by the Fpg protein

Substrate	K_m	k_{cat}	k_{cat}/K_m
	nM	min^{-1}	
εA derivative paired with:			
dA	59.9 ± 20.5	0.49 ± 0.1	0.008
dC	6.2 ± 1.4	0.42 ± 0.02	0.068
dG	832 ± 314[a]	47.9 ± 13.1[a]	0.058
dT	5.6 ± 2.1	0.46 ± 0.07	0.082
Fapy-7MeG	10[b]	0.50[b]	0.05
8-OxoG	4[b]	0.43[b]	0.11
	134[c]	1.3[c]	0.01
	14[d]	0.13[d]	0.009
2,2,4-Triaminooxazolone	316[c]	0.73[c]	0.002
Apurinic/apyrimidinic	4[b]	2.5[b]	0.6

[a] Estimated values only.
[b] Values are given according to Ref. 37.
[c] Values are given according to Ref. 39.
[d] Values are given according to Ref. 42.

following the assignment for the ribo-cogener (31). The spectra of compound **B** at room temperature contain two sets of signals (Fig. 3) that are temperature-dependent, with the coalescence in Me_2SO-d_6 at 80 °C. Each of the signal sets probably belongs to one of the isomers of compound **B** (Fig. 1). We assigned the isomer **B1** to be that in which the adenine ring has the hydroxyl group at position 2 and the hydrogen atom at position 3. The isomer **B2** has the opened pyrimidine ring with the carbonyl group at position 2 and the proton at position 1. The ratio of **B1** and **B2** isomers in Me_2SO_{d6} solution at 25 °C is 1:1, whereas in D_2O the ratio is 13:87.

The spectra of product **C** differ from those of compound **B** in the values of chemical shifts of the H1′ and H2′ protons and lack the signal at ~8.25 ppm corresponding to the proton H2. A comparison of the signals of all other non-exchangeable protons of the sugar moiety indicates that proton and carbon chemical shifts are almost identical for both the **B** and **C** products as well as the parental compound ϵdA. This means that the sugar conformation remains very similar in all these compounds. The only differences observed are those indicating the shielding effects originated from the changes occurring within the base moiety. However, the NMR spectra of compound **D** are very different. The signals of the sugar moiety are absent, and only two very broad signals in the proton spectrum at ~3.5 and 6.9 ppm were observed. We have not assigned these signals and do not propose any structure for the compound.

The structures of the **B** and **C** compounds assigned by NMR were confirmed additionally by mass spectroscopy. The most abundant peaks in the **B** spectrum, the protonated deoxynucleoside (m/z = 294.1) and protonated base (m/z = 178.1), differ by 18 mass units from the corresponding peaks in the ϵdA spectrum (276.1 and 160.1, respectively). Then, the peaks in the **C** spectrum (266.1 and 150.1) differ by 28 units from the corresponding peaks in the **B** spectrum. These data are consistent with the following reaction scheme: ϵdA + H_2O (18) → **B** − CO (28) → **C**. The presence in the mass spectrum of **D** peaks of m/z 316.3 and 288.3, together with the NMR data showing lack of deoxyribose in this compound, would indicate that **D** is a dimeric form of base moiety of compound **C**. The conclusion can be drawn that depurination is the final step of ϵdA rearrangements.

Enzymatic Excision of Ethenoadenine and Its Degradation Products from DNA—Human glycosylase-ANPG-40 protein effectively excised ϵA from the ϵA:T pair in the 40-mer duplex. However, its capability to cleave ϵA-oligomer pretreated with NaOH decreased proportionally to the time of incubation in 0.2 N NaOH (Fig. 4*A*). The ϵA-oligomer incubated in NaOH was cleaved by *E. coli* formamidopyrimidine-DNA glycosylase (Fpg protein), leaving behind the β-δ-elimination product, and by endonuclease III (Nth protein), which worked by the β-elimination mechanism (Fig. 4*B*). At the same enzyme concentration 40-mer was cleaved to a greater extent by the Fpg than the Nth protein. When, however, both enzymes were used, only the Fpg cleavage product was found (Fig. 4*B*), which suggests that both enzymes recognized the same lesion, but/or Nth had a lower affinity to the lesion. Prolonged incubation of ϵA-oligomer in NaOH resulted also in a partial breakage of DNA at the ϵA site (Fig. 4, *A* and *B*). This could be because of alkali-triggered hydrolysis of abasic sites formed after non-enzymatic depuri-

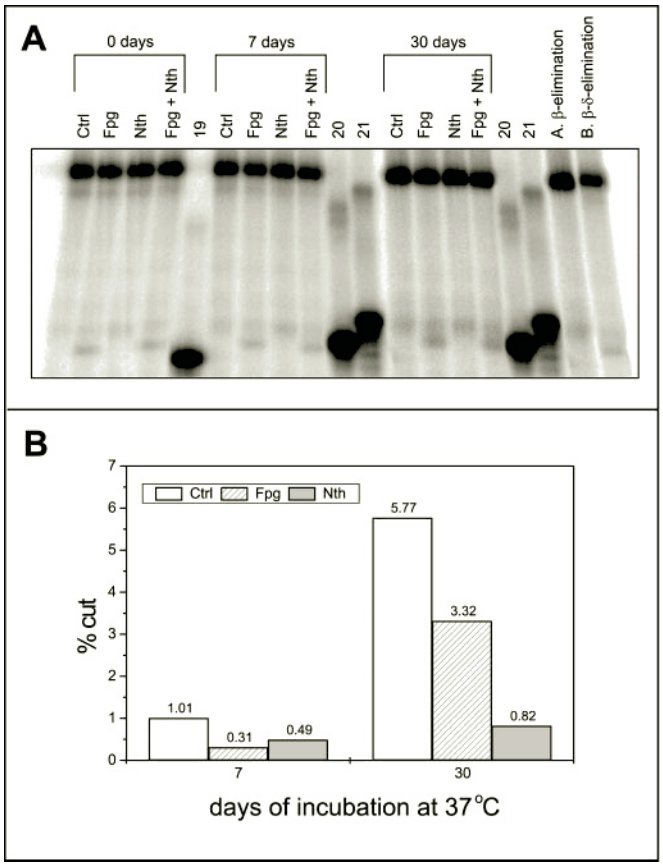

FIG. 6. *Panel A*, enzymatic recognition of ϵA degradation product(s) formed spontaneously in ϵA-oligomer after 7 or 30 days of incubation at 37 °C, pH 7.6. *Lanes: Ctrl*, control sample stored at −30 °C for several months; *Fpg,* Nth oligomer digested with repair glycosylases Fpg or Nth or both proteins; *A, β*-elimination ϵA oligomer incubated at pH 7.6, 37 °C for 30 days; spontaneous breaks are seen at the ϵA site due to *β*-elimination at the apurinic/apyrimidinic site; *B, β-δ-elimination,* the same oligomer as in *panel A* (*β-elimination*) but incubated additionally in 0.2 N NaOH at 70 °C for 30 min. A product of *β*-elimination changed the migration rate because of subsequent δ-elimination of deoxyribose. *Panel B*, quantification, performed as described in Fig. 4, of spontaneous breaks and enzymatic cleavage in ϵA-oligomer incubated up to 30 days at 37 °C, pH 7.6. For the Fpg and Nth proteins, spontaneous breaks (*Ctrl*) and enzymatic breaks observed in control oligomer (0 days of incubation at 37 °C) were subtracted and thus represent only the amount of modified base, which was formed during incubation at 37 °C.

nation of either ϵA or **D** (Fig. 1) or to other, unknown mechanism.

Identification of ϵA Rearrangement Product Excised by the Fpg and Nth Proteins—HPLC analysis shows that the ϵA-oligomer incubated for 4 h in 0.2 N NaOH contains ϵdA and both products of its conversion, compounds **B** and **C** (Fig. 5*A*). The amount of product **B** decreased substantially when oligonucleotide was digested with both the Fpg protein (Fig. 5*B*) and the Nth glycosylase (Fig. 5*C*), suggesting that both enzymes recognize and excise the modified base present in compound **B**. However, we were unable to discriminate between both isomers of compound **B**.

For the Fpg protein, the K_m and k_{cat} values for excision of the ϵA derivative when paired with dT and dC were very similar to the kinetic constants of known Fpg substrates 8-oxoG and Fapy-7MeG. Interestingly, the K_m for excision of the ϵdA derivative paired with dA was an order of magnitude higher (~60 nM) and when paired with dG two orders of magnitude higher than when it was paired with dT and dC (~6 nM, Table I). For the Nth protein, the K_m for excision of **B** from the **B**:T pair was 44 nM (k_{cat} =13 min^{-1}).

Decomposition of ϵA in Oligomers in Physiological pH—The ϵA-oligomer stored for several months in aqueous solutions at −30 °C was susceptible to cleavage by the Fpg and Nth proteins, suggesting that chemical rearrangement of ϵA also occurs spontaneously at neutrality. To determine the rate of this process, ϵA-40-mer was incubated up to 30 days at pH 7.4, 37 °C and digested with the Fpg and Nth proteins. Both enzymes cleaved the 40-mer in a time-dependent manner (Fig. 6). We have also observed formation of DNA breaks at the ϵA-site but not at other sites within an oligomer. The migration rate of the breakage product formed at neutrality was consistent with the *β*-elimination pattern. When, however, enzymatically nondigested oligomer was incubated additionally in 0.2 N NaOH for 30 min at 70 °C, the migration rate of the breakage product increased to that observed for *β*-δ-elimination, suggesting that spontaneous breaks at the ϵA site in neutral pH occurred by *β*-elimination at abasic sites, formed because of depurination of ϵA or compound **D** (Fig. 6*A*). The sum of enzymatic and nonenzymatic breaks of ϵA-oligomer obtained after its incubation for different periods at neutrality, 37 °C gives an estimate of the rate of ϵA rearrangement in DNA under physiological conditions. It equals 2% of ϵA in DNA/week. Because, however, in this assay we were unable to quantify product **C**, this rate is higher, approximately by 1% more per week, as judged by the rate of **B** and **C** formation in oligomer (Fig. 2*D*).

DISCUSSION

The general scheme of 1,N^6-ethenoadenine rearrangements was originally proposed by Tsou and co-workers (12). Tsou and co-workers (12), employing ^{1}H NMR and MS spectroscopy, have proven the formation of deformylated bi-imidazole nucleoside (corresponding to deoxy-cogener **C**, Fig. 1) from 1,N^6-ethenoadenosine. Here we present evidence, based on ^{1}H and ^{13}C NMR as well as on MS spectroscopy, that the first step of reaction involves formation of ϵdA derivative hydrated at the C(2)–N(3) bond (**B1**), which is in equilibrium with the pyrimidine ring-opened bi-imidazole product still retaining the formyl group (**B2**). In contrast to Basu *et al.* (13, 14), in our studies we did not observe any reversal of compound **B** to the parental ϵdA during prolonged incubation of isolated **B** at pH 7.5. Because our studies were performed at the deoxynucleoside level, whereas the former authors (13, 14) presented evidence for this reversibility at the oligodeoxynucleotide level, one cannot exclude the possibility that ϵA in monomer and in oligomer behaves differently.

We have additionally found that ϵdA depurinates with a rate 20-fold higher than that of unmodified dA. Basu and co-workers (13) claimed that they did not find differences in release of ϵA *versus* A from an oligodeoxynucleotide; however, they measured depurination at pH 2 but not at neutrality. In the rearrangement pathway, we have also shown the second depurination of compound **C**. As judged by PAGE, under physiological conditions, about 1.5% of ϵA residues per week give rise to spontaneous phosphodiester bond disruption because of ϵA or compound **D** depurination (Fig. 6). This is 1–2 orders of magnitude slower than the rate of depurination of methylated bases 7MeA, 3MeA, 7MeG (32) as well as of another exocyclic adduct, N^2,3-ethenoguanine (33). Nevertheless, we have shown for the first time that ϵA in DNA might be a source of abasic sites and spontaneous DNA strand breaks.

We have found that ϵA rearrangement products are not eliminated from DNA by *N*-methylpurine-DNA glycosylase, which participates in the repair of parental ϵA (Fig. 4). This conclusion is reached from the observation that the rate of alkali-induced ϵdA → **B** conversion in oligomer (Fig. 2*D*) was almost the same as the rate of decrease in ANPG ability to cleave 40-mer at the ϵA site (Fig. 4, *A* and *C*). One of these derivatives, compound **B**, is excised from DNA by *E. coli* DNA

glycosylases participating in the repair of the oxidized bases, Fpg and Nth proteins (34, 35). We surmise that the Fpg protein excises pyrimidine ring-opened isomer **B2**, because all known substrates of the enzyme are characterized by the presence of the carbonyl group, *e.g.* 8-oxoG, 4,6-diamino-5-formamidopyrimidine (FapyA), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), its alkylated derivatives, *e.g.* Fapy-7MeG, and 5-hydroxycytosine (36). The ϵA derivative is a novel substrate for the Fpg protein. The only other pyrimidine ring-opened base excised by the enzyme is ring-ruptured thymine (37). Interestingly, compound **B** is excised by the Fpg protein with high efficiency but only when paired with T or C but not with A or G; and thus pairs **B**:A or **B**:G might be not repaired and potentially mutagenic. The excision by Nth glycosylase of ϵA rearrangements product was not studied as thoroughly; nevertheless, it is clear that the enzyme acts only on product **B** and that K_m for this lesion is an order of magnitude higher than for the majority of oxidized pyrimidines, *e.g.* thymine glycol (17). The other purine derivative excised by this enzyme, also with a low efficiency, is FapyA but not FapyG (38). The active center cleft in the Nth protein is probably too narrow for relatively large purine residues, but when fragmented, *e.g.* oxazolone, they can be fit into it quite efficiently and excised (39).

Compound **C** is most probably not excised by the Fpg and Nth proteins, since we did not observe significant elimination of **C** in HPLC analysis (Fig. 5). Basu *et al.* (14) have shown that compound **C** in DNA is 20-fold more mutagenic than ϵA and induces a different spectrum of mutations in *E. coli* than the parental lesion. Taking into account the fact that formation of **C** under physiological conditions may be as high as 1% of ϵA residues per week and that it is not excised by three major DNA-glycosylases, ANPG, Fpg, and Nth, it cannot be excluded that this derivative is persistent in DNA and that some ϵA-induced mutations derive from its degradation products. DNA base ring fragmentation increases its flexibility and its potential to form different hydrogen bond faces. Examples of changing coding properties by DNA base ring disruption are known. Both FapyA and 4,6-diamino-5*N*-methyl-formamidopyrimidine (Fapy-7MeA), in contrast to parental bases, induce SOS-dependent A → G transitions in *E. coli* (40, 41). In fact, mutation spectra of ϵA show quite a high divergence (10, 11), which might reflect in part the proneness of ϵA for chemical decomposition and the formation of persistent mutagenic lesions.

Acknowledgments—We thank Dr. J. Laval (URA 147 CNRS, Institut G. Roussy, Villejuif, France) for the kind gift of ANPG protein. We acknowledge the use of the NMR facility of the Laboratory of Biological NMR, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (supported in part by the State Committee for Scientific Research). We also thank Dr. A. Wysłouch and Dr. M. Dadlez for the confirmation of ϵA-oligomer identity by mass spectrometry.

REFERENCES

1. Bartsch, H., Barbin, A., Marion, M.-J., Nair, J., and Guichard, Y. (1994) *Drug Metab. Rev.* **26,** 349–371
2. Chung, F.-L., Chen, H.-J. C., and Nath, R. G. (1996) *Carcinogenesis* **17,** 2105–2111
3. Barbin, A. (1999) in *Exocyclic DNA adducts in Mutagenesis and Carcinogenesis* (Singer, B., and Bartsch, H., eds) International Agency for Research on Cancer Scientific Publication No. 150, pp. 303–313, IARC, Lyon, France
4. Nair, J. (1999) in *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis* (Singer B., and Bartsch, H. eds) IARC Scientific Publication No. 150, pp. 55–61, International Agency for Research on Cancer, Lyon, France
5. Nair, J., Sone, H., Nagao, M., Barbin, A., and Bartsch, H. (1996) *Cancer Res.* **56,** 1267–1271
6. Nair, J., Carmichael, P. L., Fernando, R. C., Phillips, D. H., Strain, A. J., and Bartsch, H. (1998) *Cancer Epidemiol. Biomarkers Prev.* **7,** 435–440
7. Bartsch, H., Nair, J., and Owen, R. W. (1999) *Carcinogenesis* **20,** 2209–2218
8. Saparbaev, M., Kleibl, K., and Laval, J. (1995) *Nucleic Acids Res.* **23,** 3750–3755
9. Swenberg, J. A., Bogdanffy, M. S., Ham, A., Holt, S., Kim, A., Morinello, E. J., Ranasinghe, A., Scheller, N., and Upton, P. B. (1999) in *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis* (Singer B., and Bartsch, H. eds) IARC Scientific Publication No. 150, pp. 29–43, International Agency for Research on Cancer, Lyon, France
10. Moriya, M., Pandya, F., Johnson, F., and Grollman, A. P. (1999) in *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis* (Singer B., and Bartsch, H., eds) International Agency for Research on Cancer Scientific Publication No. 150, pp. 263–270, IARC, Lyon, France
11. Levine, R. L., Yang, I.-Y., Hossain, M., Pandya, G. A., Grollman, A. P., and Moriya, M. (2000) *Cancer Res.* **60,** 4098–4104
12. Tsou, K. C., Yip, K. F., Miller, E. E., and Lo, K. W. (1974) *Nucleic Acids Res.* **1,** 531–547
13. Basu, A. K., Niedernhofer, L. J., and Essigmann, J. M. (1987) *Biochemistry* **26,** 5626–5635
14. Basu, A. K., Wood, M. L., Niedernhofer, L. J., Ramos, L. A., and Essigmann, J. M. (1993) *Biochemistry* **32,** 12793–12801
15. Barrio, J. R., Secrist III, J. A., and Leonard, R. J. (1972) *Biochem. Biophys. Res. Commun.* **46,** 597–604
16. Boiteux, S., O'Connor, T. R., and Laval, J. (1987) *EMBO J.* **6,** 3177–3183
17. Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) *Biochemistry* **32,** 12105–12111
18. Yip, K. F., and Tsou, K. C. (1973) *Tetrahedron Lett.* **33,** 3087–3090
19. Aue, W. P., Bartholdi, E., and Ernst, R. R. (1976) *J. Chem. Phys.* **64,** 2229–2246
20. Bax, A., and Freeman, R. (1985) *J. Magn. Reson.* **65,** 355–360
21. Braunschweiler, L., and Ernst, R. R. (1983) *J. Magn. Reson.* **53,** 521–528
22. Bothner-By, A. A., Stephens, R. L., Lee, J.-M., Warren, C. D., and Jeanloz, R. W. (1984) *J. Am. Chem. Soc.* **106,** 811–813
23. Bax, A., and Davis, D. G. (1985) *J. Magn. Reson.* **63,** 207–213
24. Kay, L. E., Keifer, P., and Saarinen, T. (1992) *J. Am. Chem. Soc.* **114,** 10663–10665
25. Palmer III, A. G., Cavanagh, J., Wright, P. E., and Rance, M. (1991) *J. Magn. Reson.* **93,** 151–170
26. Kontaxis, G., Stonehouse, J., Laue, E. D., and Keeler, J. (1994) *J. Magn. Reson. Ser. A* **111,** 70–76
27. Gottlieb, H. E., and Kotlyar, V. (1997) *J. Org. Chem.* **62,** 7512–7515
28. Hoffman, R. E., and Davies, D. B. (1988) *Magn. Reson. Chem.* **26,** 523–525
29. Lee, H.-J., and Wilson, I. B. (1971) *Biochim. Biophys. Acta* **242,** 519–522
30. Kamiński, Z. W., and Domino, E. F. (1987) *Comput. Methods Programs Biomed.* **24,** 41–45
31. Kronberg, L., Sjöholm, R., and Karlsson, S. (1992) *Chem. Res. Toxicol.* **5,** 852–855
32. Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, pp. 15–77, Plenum Press, New York
33. Kuśmierek, J. T., Folkman, W., and Singer, B. (1989) *Chem. Res. Toxicol.* **2,** 230–233
34. Hatahet, Z., Kow, Y. W., Purmal, A. A., Cunningham, R. P., and Wallace, S. S. (1994) *J. Biol. Chem.* **269,** 18814–18820
35. Laval, J., Boiteux, S., and O'Connor, T. R. (1990) *Mutat. Res.* **233,** 73–79
36. Sugahara, M., Mikawa, T., Kumasaka, T., Yamamoto, M., Kato, R., Fukuyama, K., Inoue, Y., and Kuramitsu, S. (2000) *EMBO J.* **19,** 3857–3869
37. Jurado, J., Saparbaev, M., Matray, T. J., Greenberg, M. M., and Laval, J. (1998) *Biochemistry* **37,** 7757–7763
38. Dizdaroglu, M., Bauche, C., Rodriguez, H., and Laval, J. (2000) *Biochemistry* **39,** 5586–5592
39. Duarte, V., Gasparutto, D., Jaquinod, M., and Cadet, J. (2000) *Nucleic Acids Res.* **28,** 1555–1563
40. Grąziewicz, M.-A., Zastawny, T. H., Oliński, R., and Tudek, B. (1999) *Mutat. Res.* **434,** 41–52
41. Tudek, B., Grąziewicz, M.-A., Kazanova, O., Zastawny, T. H., Obtułowicz, T., and Laval, J. (1999) *Acta Biochim. Pol.* **46,** 785–799
42. Tchou, J., Bodepudi, V., Shibutani, S., Antoshechkin, I., Miller, J., Grollman, A. P., and Johnson, F. (1994) *J. Biol. Chem.* **269,** 15318–15324