Biochem. J. (1989) 262, 581–589 (Printed in Great Britain)



# Mechanism of DNA strand nicking at apurinic/apyrimidinic sites by *Escherichia coli* [formamidopyrimidine]DNA glycosylase

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*Escherichia coli* [formamidopyrimidine]DNA glycosylase catalyses the nicking of both the phosphodiester bonds 3′ and 5′ of apurinic or apyrimidinic sites in DNA so that the base-free deoxyribose is replaced by a gap limited by 3′-phosphate and 5′-phosphate ends. The two nickings are not the results of hydrolytic processes; the [formamidopyrimidine]DNA glycosylase rather catalyses a $\beta$-elimination reaction that is immediately followed by a $\delta$-elimination. The enzyme is without action on a 3′-terminal base-free deoxyribose or on a 3′-terminal base-free unsaturated sugar produced by a $\beta$-elimination reaction nicking the DNA strand 3′ to an apurinic or apyrimidinic site.

## INTRODUCTION

In DNA, the principal target of alkylating agents is the *N*-7 of guanine (Lawley *et al.*, 1975). The *N*-7-alkylguanine is not, by itself, a harmful lesion for the cell, but it can be hydrolysed away leaving an apurinic site (AP site) or undergo a rearrangement to yield a ring-opened imidazole form. The ring-opened *N*-7-methylguanine or formamidopyrimidine (Fapy) is a block to DNA synthesis *in vitro* (Boiteux & Laval, 1983); it is thus, if not repaired, a lethal lesion for the cell. The Fapy is removed in *Escherichia coli* by the [Fapy]DNA glycosylase (Chetsanga & Lindahl, 1979). The *fpg* gene, which codes for the [Fapy]DNA glycosylase in *E. coli*, has been cloned and sequenced and the enzyme has been overproduced (Boiteux *et al.*, 1987). The isolation of milligram quantities of the enzyme has facilitated the study of its physicochemical properties, and we have reported that the [Fapy]DNA glycosylase possesses an associated strand-nicking activity that recognizes specifically AP sites (O'Connor & Laval, 1989). In the present work we show that the [Fapy]DNA glycosylase nicks both phosphodiester bonds 3′ and 5′ of the AP site, leaving a gap limited by 3′-phosphate and 5′-phosphate ends, and that the cleavage mechanism is consistent with the hypothesis of an efficient $\beta\delta$-elimination reaction (Scheme 1).

## MATERIALS AND METHODS

### Enzymes

*E. coli* [formamidopyrimidine]DNA glycosylase ([Fapy]DNA glycosylase) was purified as previously described (Boiteux *et al.*, 1987). The enzyme used for the experiments reported in this paper is the homogeneous Mono S HR5/5 fraction obtained by f.p.l.c. ($479 \times 10^3$ units and 0.470 mg of protein per ml).

Endonuclease III of *E. coli* was purified from extracts of the overproducer strain BW531 (a gift from Dr. B. Weiss, Johns Hopkins University, Baltimore, MD, U.S.A.). Upon analysis by SDS/polyacrylamide-gel electrophoresis, a major band of protein at 25 kDa was detected along with two barely detectable ones at 29 and 18 kDa. The preparation contained $36 \times 10^3$ units and 0.45 mg of protein per ml. The unit of nicking activity near AP sites liberates, from *E. coli* alkylated and depurinated [$^3$H]DNA, 1 pmol of acid-soluble oligonucleotide in 1 min at 37 °C (Pierre & Laval, 1980).

The preparations of *E. coli* endonuclease IV and *E. coli* [uracil]DNA glycosylase have been described previously (Bailly & Verly, 1987). T4 polynucleotide kinase and calf thymus deoxynucleotidyl terminal transferase were purchased from Pharmacia, *E. coli* exonuclease III was from Bethesda Research Laboratories, calf spleen phosphodiesterase was from Merck and calf intestine alkaline phosphatase (VIIS) was from Sigma Chemical Co. Snake-venom phosphodiesterase (Sigma Chemical Co.) was purified by Blue Sepharose chromatography (Oka *et al.*, 1978).

### Experiments with DNA containing doubly labelled AP sites

**Preparation of the substrate.** Double-stranded DNA with apyrimidinic sites labelled with $^3$H in the 1′- and 2′-positions of the base-free deoxyribose and with $^{32}$P on the phosphate 5′ to this deoxyribose, denoted by [d(–),1′,2′-$^3$H,5′-$^{32}$P]DNA, was prepared as described by Bailly & Verly (1987). It was dissolved (30 $\mu$g/ml) in 25 mM-Hepes/KOH buffer, pH 7.5, containing 0.1 M-KCl and 1 mM-EDTA; the specific radioactivities were 47500 d.p.m. of $^3$H/$\mu$g and 17500 d.p.m. of $^{32}$P/$\mu$g. The acid-soluble radioactivities (soluble in 5% $HClO_4$ at 0 °C), negligible for the substrate DNA, were 90% for $^3$H and 4% for $^{32}$P after a strong alkaline treatment inducing a $\beta\delta$-elimination at every AP site.

**DEAE-Sephadex chromatography.** This was performed as described previously (Bailly & Verly, 1987).

Abbreviations used: Fapy, formamidopyrimidine; AP, apurinic or apyrimidinic; d(–), symbol for base-free deoxyribose (AP site); s(=), symbol for unsaturated sugar.

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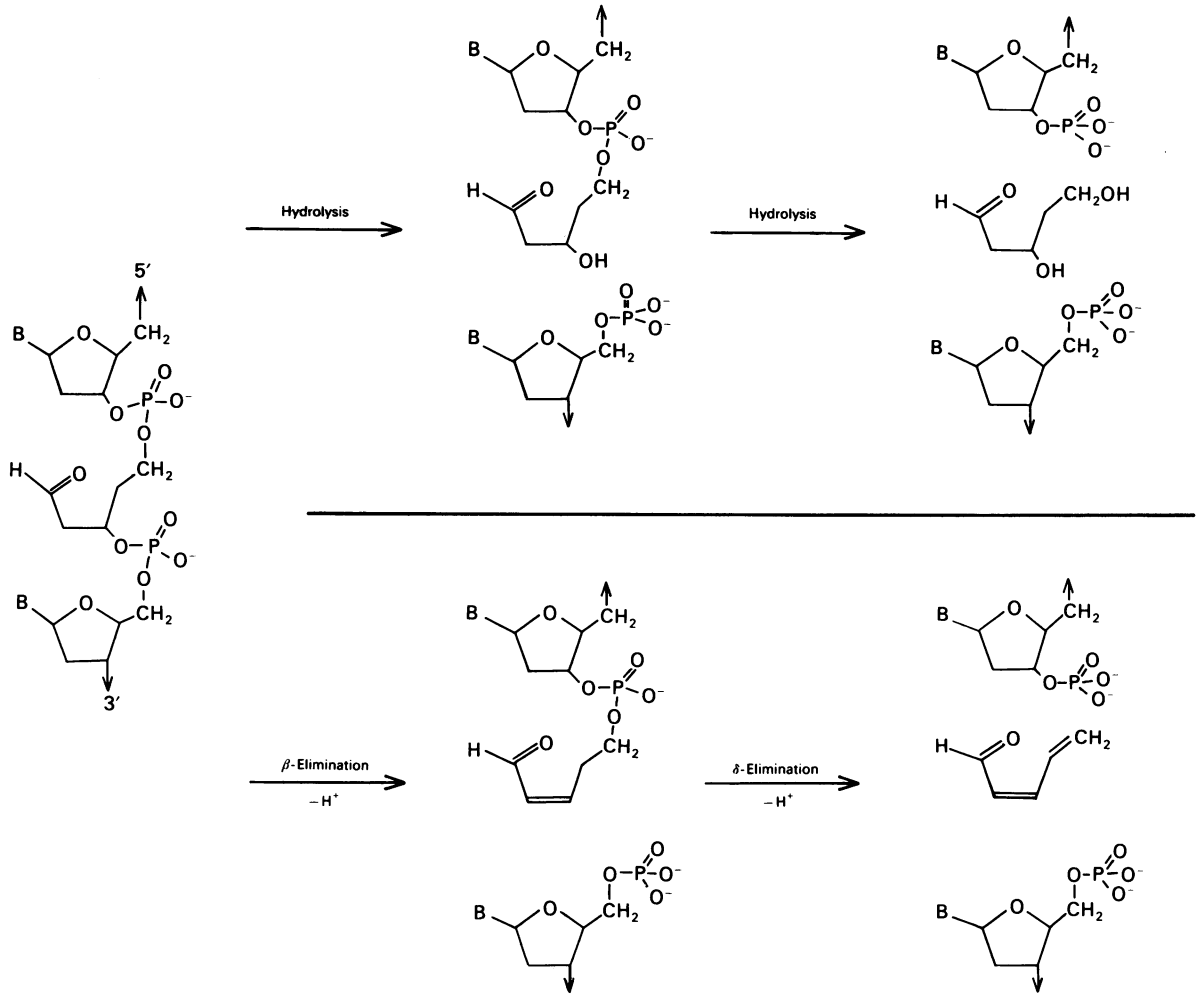


**Scheme 1. Two nicking mechanisms at an AP site to produce a gap limited by 3′-phosphate and 5′-phosphate ends**

The upper part of the Scheme shows successive hydrolyses of the C-3′–O–P bond 3′ to the AP site and of the C-5′–O–P bond 5′ to the AP site; the released sugar is deoxyribose (this mechanism has never been observed). The lower part of the Scheme shows successive nickings of the C-3′–O–P bond 3′ to the AP site by β-elimination and of the C-5′–O–P bond 5′ to the AP site by δ-elimination; the released sugar is a 2,3- and 4,5-unsaturated derivative of deoxyribose.

**Chromatography on Whatman 3MM paper**. A solution of D-2-deoxyribose (5 μmol in 5 μl of water) was added to the sample (5 μl). Elution was carried out with a mixture of 1 M-ammonium acetate, pH 7.5, and ethanol (3:7, v/v) in an atmosphere saturated with the same mixture. The chromatography was stopped when the solvent had migrated about 40 cm past the application spots. Lanes of 2 cm width were cut into 1 cm pieces, which were soaked in 1.2 ml of water for elution during 4 h. To determine the position of the D-2-deoxyribose marker, 200 μl of each eluate was submitted to the diphenylamine reaction and the absorbance was measured at 600 nm; the remaining part (1 ml) was used for assay of $^{3}H$ and $^{32}P$ radioactivities.

### Experiments with labelled synthetic oligonucleotides

**5′-Labelling of the oligonucleotides**. The oligonucleotide (10 nmol), [γ-$^{32}$P]ATP (15 nmol; 150 μCi) and 10 units of T4 polynucleotide kinase, in 100 μl of 60 mM-Tris/HCl buffer, pH 8.0, containing 9 mM-$MgCl_2$, 0.1 M-KCl and 15 mM-2-mercaptoethanol, were incubated for 1 h at 37 °C. The reaction was stopped by the addition of EDTA.

**3′-Labelling of the oligonucleotides**. The oligonucleotide (10 nmol), [α-$^{32}$P]ddATP (15 nmol; 100 μCi) and 70 units of calf thymus deoxynucleotidyl terminal transferase, in 150 μl of 0.1 M-potassium cacodylate buffer, pH 7.2, containing 2 mM-$CoCl_2$ and 0.2 mM-dithiothreitol, were incubated for 2 h at 37 °C. The reaction was stopped by the addition of EDTA.

**Depurination**. When the oligonucleotide had an internal purine, depurination was carried out in 30 mM-HCl for 24 h at 37 °C. When the purine was at the 3′ end, depurination was performed in 10 mM-HCl for 1 h at 65 °C. The AP site is denoted by d(–).

**Preparation of [5′-$^{32}$P]pdT$_8$d(–)dT$_7$, [5′-$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ and [5′-$^{32}$P]pdT$_8$d(–)**. dT$_8$dGdT$_7$, dT$_{11}$dGdT$_{11}$ (Eurogentec) and dT$_8$dA (Pharmacia), purified by polyacrylamide-gel electrophoresis, were each 5′-labelled by phosphorylation, then depurinated.

**Preparation of dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA**. dT$_{11}$dGdT$_{11}$ (Eurogentec), purified by polyacrylamide-gel electrophoresis, was depurinated, then 3′-labelled by addition of dideoxyadenosine 5′-monophosphate.

**Digestion with snake-venom phosphodiesterase.** $[^{32}P]pdT_8d(-)dT_7$ (20 pmol) and 10 $\mu$units of purified phosphodiesterase, in 10 $\mu$l of 50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, were incubated for 1 h at 37 °C.

**Digestion with *E. coli* exonuclease III.** $[^{32}P]pdT_8d(-)dT_7$ (20 pmol), hybridized to poly(dA), and 0.1 unit of exonuclease III, in 10 $\mu$l of 50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, were incubated for 1 h at 26 °C.

**Alkaline treatments.** Mild alkaline treatment involved incubation in 0.2 M-NaOH at 37 °C for 15 min. Strong alkaline treatment involved incubation in 0.2 M-NaOH at 65 °C for 30 min. The samples were neutralized with HCl.

**Treatment with *E. coli* endonuclease IV, *E. coli* endonuclease III or *E. coli* [Fapy]DNA glycosylase.** $[^{32}P]pdT_8d(-)dT_7$ or $dT_{11}d(-)dT_{11}[^{32}P]ddA$ (20 pmol), hybridized to poly(dA), and the enzyme in 10 $\mu$l of reaction medium were incubated for 1 h at 26 °C for the first substrate and at 37 °C for the second. The reaction media were as follows: for *E. coli* endonuclease IV, 50 mM-Hepes/KOH buffer, pH 8.2, containing 0.2 M-NaCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol; for *E. coli* endonuclease III (3.6 units), 50 mM-Hepes/KOH buffer, pH 7.8, containing 0.1 M-KCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol; for [Fapy]DNA glycosylase (20 units), 50 mM-Hepes/KOH buffer, pH 7.5, containing 0.1 M-KCl and 1 mM-EDTA.

**Treatment utilizing the 3′-phosphatase activity of T4 polynucleotide kinase.** This treatment was performed after cleaving $[^{32}P]pdT_8d(-)dT_7$ by strong alkaline treatment or by incubation with [Fapy]DNA glycosylase. To the medium of the previous reaction (10 $\mu$l) was added 1 unit of T4 polynucleotide kinase in 10 $\mu$l of 0.2 M-imidazole/HCl buffer, pH 5.0, containing 10 mM-$MgCl_2$ and 1 mM-dithiothreitol, and the mixture (20 $\mu$l) was incubated for 30 min at 37 °C.

**Treatment with calf intestine phosphatase.** This treatment was performed after cleavage of $dT_{11}d(-)dT_{11}$-$[^{32}P]ddA$ with NaOH, *E. coli* endonuclease III or *E. coli* [Fapy]DNA glycosylase. To the medium of the previous reaction (10 $\mu$l) were added 90 munits of phosphatase in 10 $\mu$l of 0.1 M-Tris/HCl buffer, pH 8.5, containing 20 mM-$MgCl_2$ and 0.1 mM-$ZnCl_2$, and the mixture was incubated for 30 min at 37 °C.

**Preparation of two reference ladders for gel electrophoresis.** Two samples of $dT_{11}dGdT_{11}[^{32}P]ddA$ (200 pmol) were incubated with different amounts (1 or 2.5 units) of calf spleen phosphodiesterase in 10 $\mu$l of 10 mM-imidazole/HCl buffer, pH 6.0, containing 0.1 mM-EDTA for 30 min at 37 °C. The reaction was stopped by a 5 min heating at 65 °C.

Each sample was divided into two. One part was left without further treatment. The oligonucleotides of the other part were 5′-phosphorylated: to this second part (5 $\mu$l) was added 1 unit of T4 polynucleotide kinase in 5 $\mu$l of 120 mM-Tris/HCl buffer, pH 8.0, containing 18 mM-$MgCl_2$, 30 mM-2-mercaptoethanol and 2 mM-ATP, and the mixture was incubated for 15 min at 37 °C.

The parts of the two samples where the oligonucleotides were not phosphorylated were mixed to yield one reference ladder. The parts of the two samples where the oligonucleotides were phosphorylated were also pooled to yield another reference ladder.

**Electrophoresis on denaturing 20% polyacrylamide gel.** This was performed as described in Bailly & Verly (1987), except that the samples were not dried but directly diluted with the loading buffer.

**Adsorption on Norit.** This was performed as described previously (Bailly & Verly, 1987).

## RESULTS

### Experiments with DNA containing doubly labelled AP sites

**Action of *E. coli* [Fapy]DNA glycosylase.** [d(−),1′,2′-$^3$H,5′-$^{32}$P]DNA was incubated with increasing amounts of [Fapy]DNA glycosylase for 30 min before measurement of the acid-soluble $^3$H and $^{32}$P radioactivities (Fig. 1). These radioactivities were nil when there was no enzyme; they increased with the amount of enzyme to reach maxima of 90% for $^3$H and 4% for $^{32}$P. These maxima are identical with those obtained after a strong alkaline treatment inducing a $\beta\delta$-elimination reaction at every AP site. It thus seems that, as in the strong alkaline treatment, [Fapy]DNA glycosylase released the radioactivity associated with the base-free deoxyribose, but not the radioactivity associated with the phosphate 5′ to

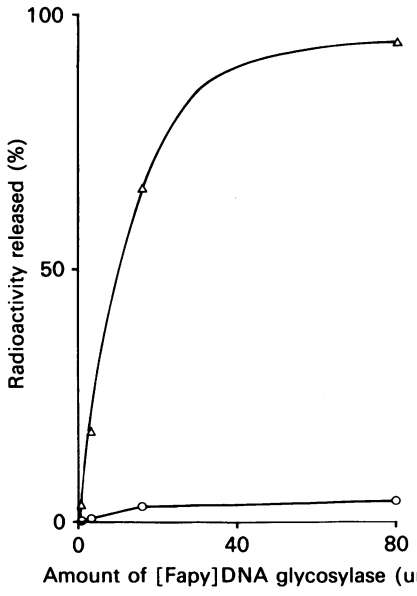


**Fig. 1. Action of [Fapy]DNA glycosylase on AP sites**

Mixtures of 2 $\mu$l of 50 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol and 50% (v/v) glycerol containing increasing amounts of [Fapy]DNA glycosylase (0.64, 3.2, 16 and 80 enzyme units) and 18 $\mu$l of [d(−)1′,2′-$^3$H,5′-$^{32}$P]DNA solution were incubated at 37 °C for 30 min. The $^3$H (△) and $^{32}$P (○) acid-soluble radioactivies were measured; each assay was carried out in duplicate and the recorded data, in percentages of the total radioactivities, are the averages of the results from these duplicates.

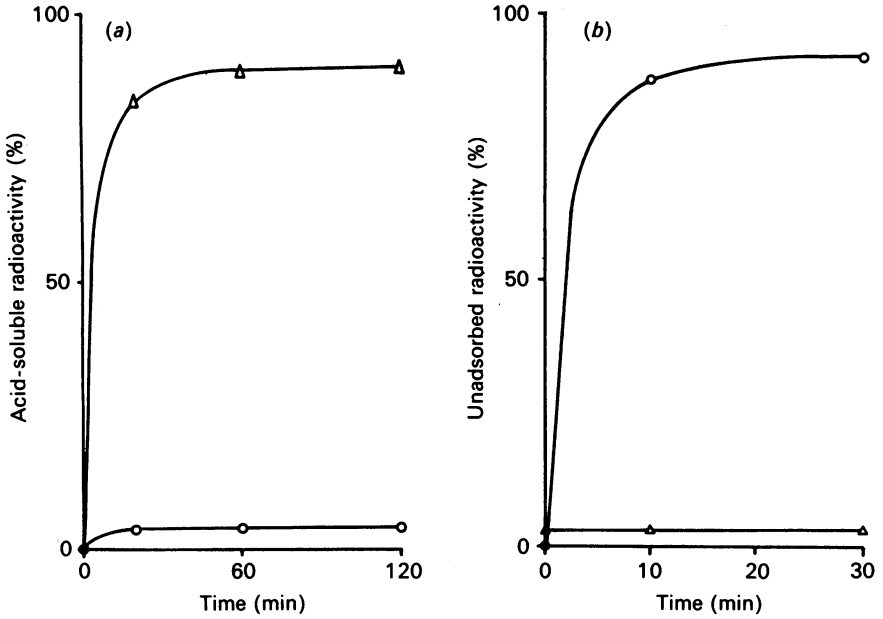


**Fig. 2. Products released by [Fapy]DNA glycosylase: acid-solubility and Norit-adsorbability**

(*a*) The [d(–)1′,2′-$^{3}$H,5′-$^{32}$P]DNA solution (1 ml) and 2 $\mu$l of [Fapy]DNA glycosylase solution (800 units) were incubated at 37 °C for 120 min. To monitor the reaction, 20 $\mu$l samples were taken at intervals for measurement of the $^{3}$H (△) and $^{32}$P (○) acid-soluble radioactivities; the results are given as percentages of total radioactivities. (*b*) At the end of the 120 min incubation, to the rest of the solution were added *E. coli* exonuclease III (0.25 unit/$\mu$g of DNA) and $MgCl_2$ to give a 10 mM final concentration. The reaction, which was carried out at 37 °C for 30 min, was monitored by taking 10 $\mu$l samples for measurement of the $^{3}$H (△) and $^{32}$P (○) radioactivities unadsorbable on Norit charcoal; the results are given as percentages of total radioactivities. The determinations at 0 min were made before addition of exonuclease III.

this sugar. Reduction of the AP sites with $NaBH_4$ made the substrate DNA resistant to the alkaline treatment, and also to the action of [Fapy]DNA glycosylase (results not shown).

**Reaction products**. (*a*) Acid-solubility and Norit-adsorbability. After a 120 min treatment of [d(–),1′,2′-$^{3}$H,5′-$^{32}$P]DNA at 37 °C with [Fapy]DNA glycosylase, the acid-soluble $^{3}$H and $^{32}$P radioactivities had reached maximum values of 90% and 4% respectively (Fig. 2*a*). Most of the acid-soluble $^{3}$H radioactivity (96%) was adsorbed on Norit charcoal (Fig. 2*b*; 0 min). *E. coli* exonuclease III and $Mg^{2+}$ were then added and the incubation was continued for 30 min; the 3′-phosphatase activity of this second enzyme released 90% of the $^{32}$P radioactivity in Norit-unadsorbable molecules (Fig. 2*b*).

(*b*) chromatographic behaviour. [d(–),1′,2′-$^{3}$H,5′-$^{32}$P]DNA was incubated successively for 120 min with [Fapy]DNA glycosylase and for 30 min with *E. coli* exonuclease III (for details see the legend to Fig. 2); these treatments released 90% of the $^{3}$H radioactivity and 90% of the $^{32}$P radioactivity in small acid-soluble molecules. One part of the solution, after addition of D-2-deoxyribose 5-phosphate, was chromatographed on DEAE-Sephadex. The other part, after addition of D-2-deoxyribose, was deproteinized with a mixture of chloroform/isopentanol (24:1, v/v) and chromatographed on Whatman 3MM paper.

Fig. 3 shows that DEAE-Sephadex separated a $^{3}$H peak, which emerged before the application of the NaCl gradient, from a $^{32}$P peak, which was in the position of inorganic phosphate {as observed in another chromatography with [$^{32}$P]phosphate (results not shown)}, immediately after the D-2-deoxyribose 5-phosphate marker. The greater part (87%) of the $^{3}$H radioactivity collected in the first fractions of the chromatography could be adsorbed on Norit.

Fig. 4(*a*) shows that, on the paper chromatogram, $^{32}$P formed a peak in the seventh fraction, which was the position of inorganic phosphate. Most of the $^{3}$H radioactivity had disappeared; what remained formed two bumps incompletely separated in the region of the D-2-deoxyribose marker. Nearly all of the $^{3}$H radioactivity was in the chloroform/isopentanol (24:1, v/v) mixture used for the deproteinization. This solution was also chromatographed on paper together with a D-2-deoxyribose marker; Fig. 4(*b*) shows a $^{3}$H peak that migrated beyond the D-2-deoxyribose.

Taken together, these results mean that the base-free deoxyribose has been released as a rather hydrophobic Norit-adsorbable molecule.

## Experiments with labelled synthetic oligonucleotides

**3′-End of the gap produced by [Fapy]DNA glycosylase acting on an AP site**. Fig. 5 shows the results of various treatments of [$^{32}$P]pdT$_8$d(–)dT$_7$, followed by electrophoresis on denaturing 20% polyacrylamide gel and autoradiography.

The reference bands are presented first, as follows.

Lanes 1 and 2 show [$^{32}$P]pdT$_8$dGdT$_7$ and [$^{32}$P]pdT$_8$d(–)dT$_7$ respectively. The depurinated oligonucleotide (lane 2) migrated a little farther than the non-depurinated oligonucleotide (lane 1); a small non-depurinated fraction remained in the [$^{32}$P]pdT$_8$d(–)dT$_7$ preparation (lane 2).

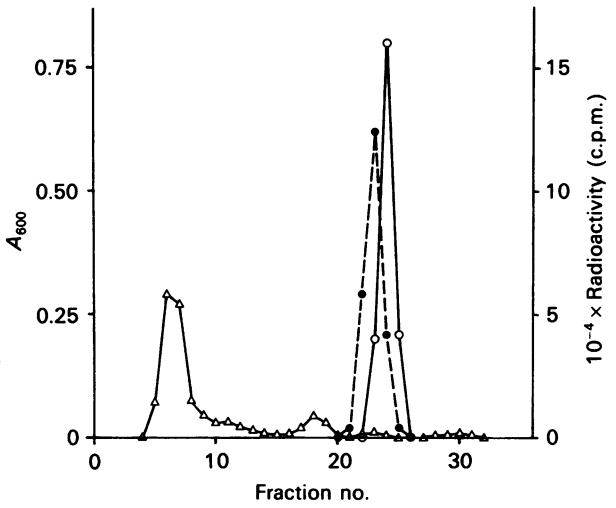


**Fig. 3. Products released by the successive actions of [Fapy]DNA glycosylase and exonuclease III: analysis on DEAE-Sephadex**

[d(–),1′,2′-$^{3}$H,5′-$^{32}$P]DNA (16 $\mu$g) was incubated at 37 °C with [Fapy]DNA glycosylase for 120 min and, after additon of $MgCl_2$ and exonuclease III, the incubation was continued for 30 min (see details in the legend to Fig. 2). To a part of the solution (600 $\mu$l), EDTA and D-2-deoxyribose 5-phosphate were added before it was applied to a column (0.9 cm × 30 cm) of DEAE-Sephadex equilibrated with 0.1 M-sodium borate/HCl buffer, pH 8.3. The column was eluted with 200 ml of a linear 0–0.25 M-NaCl gradient in the same buffer; 3 ml fractions were collected. The $^{3}$H (△) and $^{32}$P (○) radioactivities were measured, and a diphenylamine reaction followed by an absorbance measurement at 600 nm (●) was performed to determine the position of the D-2-deoxyribose 5-phosphate peak.

Lane 3 shows [$^{32}$P]pdT$_8$d(–)dT$_7$ digested with snake-venom phosphodiesterase. The enzyme activity stopped immediately before the AP site, giving [$^{32}$P]pdT$_8$d(–) (see also Bailly & Verly, 1989*a*); the weaker bands, at the level of tri-, di- and mono-nucleotides, are probably derived from the part of the substrate that had not been depurinated.

Lane 4 shows [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), partially degraded by *E. coli* exonuclease III. Most of the molecules were nicked 5′ to the AP site to yield [$^{32}$P]pdT$_8$, which was then degraded exonucleolytically; a few [$^{32}$P]pdT$_8$dGdT$_7$ and [$^{32}$P]pdT$_8$d(–)dT$_7$ molecules were neither nicked nor degraded, and other ones were degraded without having been nicked.

Lane 5 shows [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), treated with *E. coli* endonuclease IV. In addition to minor bands of [$^{32}$P]pdT$_8$dGdT$_7$ and unchanged [$^{32}$P]pdT$_8$d(–)dT$_7$, there is a major one of [$^{32}$P]pdT$_8$ resulting from the nicking of [$^{32}$P]pdT$_8$d(–)dT$_7$ 5′ to the AP site (Bailly & Verly, 1989*a*).

Lane 6 shows [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), treated with *E. coli* endonuclease III. In addition to minor bands of [$^{32}$P]pdT$_8$dGdT$_7$ and unchanged [$^{32}$P]pdT$_8$d(–)dT$_7$, there are two major ones forming a doublet at the level of [$^{32}$P]pdT$_8$d(–) (compare with lane 3). This doublet results from the nicking, 3′ to the AP site, by $\beta$-elimination, leaving an unsaturated sugar at the 3′ end of the 5′ fragment (Bailly & Verly, 1987). [We do not know why $\beta$-elimination at an AP site yields two 5′ products; this question is discussed in Bailly & Verly (1987, 1988).]

Lane 7 shows [$^{32}$P]pdT$_8$d(–)dT$_7$ submitted to a mild alkaline treatment. In addition to the minor bands of [$^{32}$P]pdT$_8$dGdT$_7$ and unchanged [$^{32}$PpdT$_8$d(–)dT$_7$, there are three major ones: a doublet at the level of $^{32}$P]pdT$_8$d(–), resulting from the nicking, 3′ to the AP

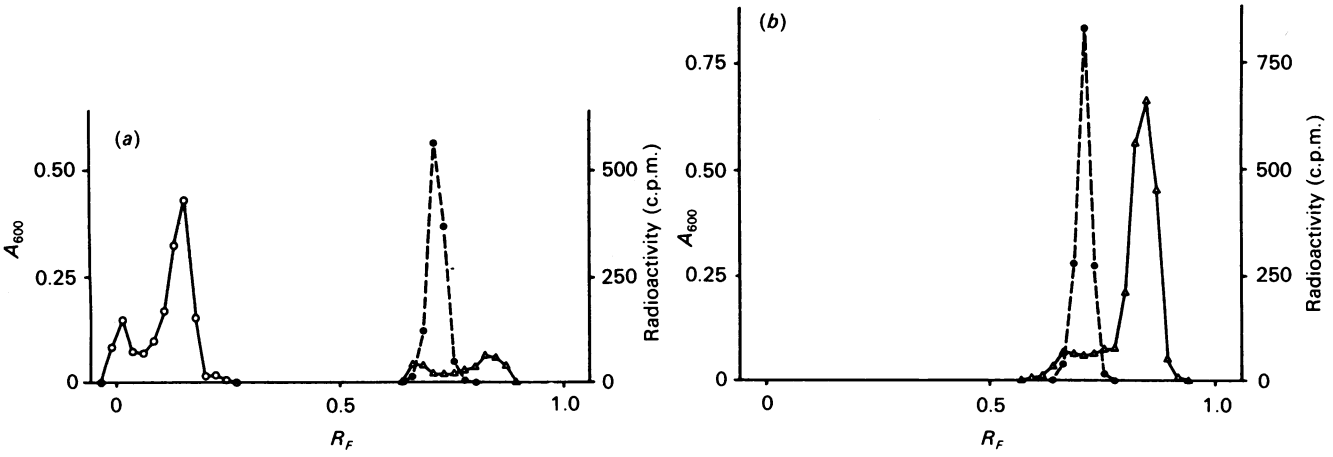


**Fig. 4. Products released by the successive actions of [Fapy]DNA glycosylase and exonuclease III: analysis by paper chromatography**

To the other part of the solution of [d(–),1′,2′-$^{3}$H,5′-$^{32}$P]DNA treated with [Fapy]DNA glycosylase and exonuclease III (see the legend to Fig. 3). EDTA and D-2-deoxyribose were added before it was deproteinized with a chloroform/isopentanol (24:1, v/v) mixture. (*a*) The aqueous phase was chromatographed on Whatman 3MM paper as described in the Materials and methods section. (*b*) The organic phase from the deproteinization step was chromatographed on Whatman 3MM paper together with D-2-deoxyribose. The $^{3}$H (△) and $^{32}$P (○) radioactivities were measured on the eluates of 1 cm pieces; a diphenylamine reaction followed by an absorbance measurement at 600 nm (●) was used to determine the position of the D-2-deoxyribose marker.

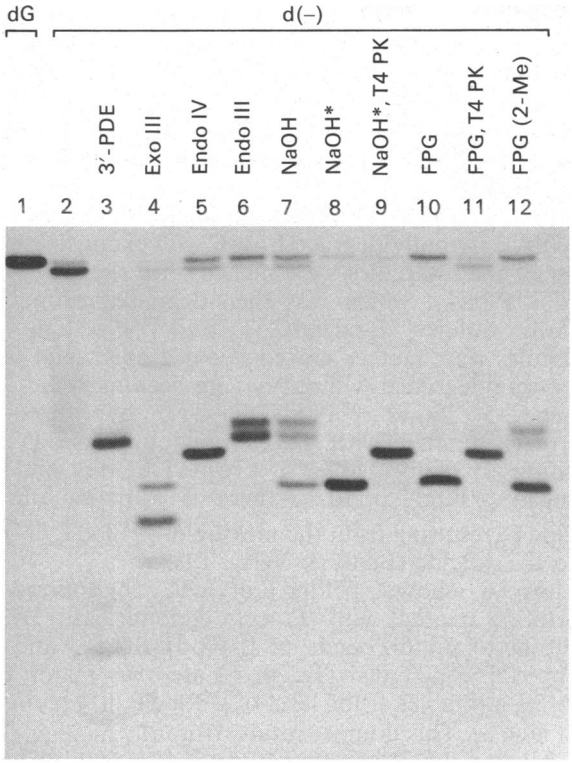


**Fig. 5. Action of [Fapy]DNA glycosylase on [$^{32}$P]pdT$_8$d(–)dT$_7$**

Lane 1, [$^{32}$P]pdT$_8$dGdT$_7$; lane 2, [$^{32}$P]pdT$_8$d(–)dT$_7$ preparation; lane 3, [$^{32}$P]pdT$_8$d(–)dT$_7$ digested with snake-venom phosphodiesterase (3′-PDE); lane 4, [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), digested with *E. coli* exonuclease III (Exo III); lane 5, [$^{32}$P]-pdT$_8$d(–)dT$_7$, hybridized to poly(dA), nicked with *E. coli* endonuclease IV (Endo IV); lane 6, [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), nicked with *E. coli* endonuclease III (Endo III); lane 7, [$^{32}$P]pdT$_8$d(–)dT$_7$ submitted to mild alkaline treatment (NaOH); lane 8, [$^{32}$P]pdT$_8$d(–)dT$_7$ submitted to strong alkaline treatment (NaOH*); lane 9, same as in lane 8, but 3′-dephosphorylated by T4 polynucleotide kinase (T4 PK); lane 10, [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), nicked with *E. coli* [Fapy]DNA glycosylase (FPG); lane 11, same as in lane 10, but 3′-dephosphorylated by T4 polynucleotide kinase; lane 12, [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), nicked by *E. coli* [Fapy]DNA glycosylase in the presence of 2-mercaptoethanol (2-Me). dG and d(–) represent [$^{32}$P]pdT$_8$dGdT$_7$ and [$^{32}$P]pdT$_8$d(–)dT$_7$ respectively.

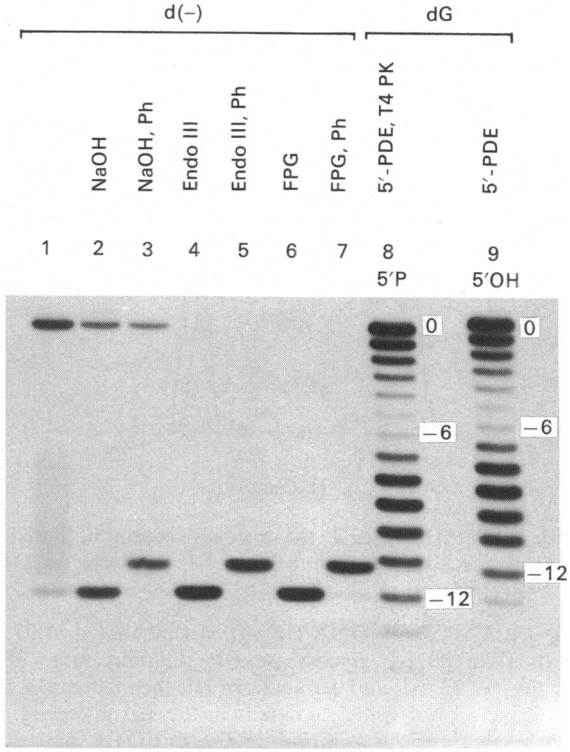


**Fig. 6. Action of [Fapy]DNA glycosylase on dT$_{11}$d(–)dT$_{11}$-[$^{32}$P]ddA**

Lane 1, dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA; lane 2, dT$_{11}$d(–)dT$_{11}$-[$^{32}$P]ddA submitted to mild alkaline treatment (NaOH); lane 3, same as in lane 2, but dephosphorylated with phosphatase from calf intestine (Ph); lane 4, dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA, hybridized to poly(dA), nicked by *E. coli* endonuclease III (Endo III); lane 5, same as in lane 4, but dephosphorylated with phosphatase from calf intestine; lane 6, dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA, hybridized to poly(dA), nicked by *E. coli* [Fapy]DNA glycosylase (FPG); lane 7, same as in lane 6, but dephosphorylated with phosphatase from calf intestine; lane 8, same as in lane 9, but 5′-phosphorylated with T4 polynucleotide kinase (T4 PK); lane 9, dT$_{11}$dGdT$_{11}$[$^{32}$P]ddA partially digested with phosphodiesterase from calf spleen (5′-PDE) (mixture of two samples). d(–) and dG represent dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA and dT$_{11}$dGdT$_{11}$[$^{32}$P]ddA respectively.

site, by $\beta$-elimination, and a band of [$^{32}$P]pdT$_8$p, when a $\delta$-elimination followed the $\beta$-elimination (see also Bailly & Verly, 1987).

Lane 8 shows [$^{32}$P]pdT$_8$d(–)dT$_7$ submitted to a strong alkaline treatment. The minor band of [$^{32}$P]pdT$_8$dGdT$_7$ is still there, but [$^{32}$P]pdT$_8$d(–)dT$_7$ has disappeared; there is a main band of [$^{32}$P]pdT$_8$p, resulting from a $\beta\delta$-elimination reaction;

For lane 9 part of the previous sample was submitted to the 3′-phosphatase activity of T4 polynucleotide kinase. [$^{32}$P]pdT$_8$p was hydrolysed into [$^{32}$P]pdT$_8$ (compare with lane 5).

We can now examine the action of [Fapy]DNA glycosylase on the same substrate, as follows.

For lane 10 [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), was incubated with [Fapy]DNA glycosylase. The main band is at the level of [$^{32}$P]pdT$_8$p (compare with lane 8).

For lane 11 part of the previous sample was submitted to the 3′-phosphatase activity of T4 polynucleotide kinase. The band at the level of [$^{32}$P]pdT$_8$p has disappeared; it has been replaced by a band at the level of [$^{32}$P]pdT$_8$ (compare with lane 9).

The conclusion is that [Fapy]DNA glycosylase breaks the C-5′–O–P bond 5′ to the AP site, as does a $\delta$-elimination that follows a $\beta$-elimination.

For lane 12 [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), was incubated with [Fapy]DNA glycosylase in the presence of 2-mercaptoethanol (10 mM), which is known to prevent the $\delta$-elimination that follows the nicking at an AP site by $\beta$-elimination (Bailly & Verly, 1988). The result is analogous to what is observed after a mild alkaline treatment (compare with lane 7): a doublet at the level of [$^{32}$P]pdT$_8$d(–) and another band at the level of [$^{32}$P]pdT$_8$p.

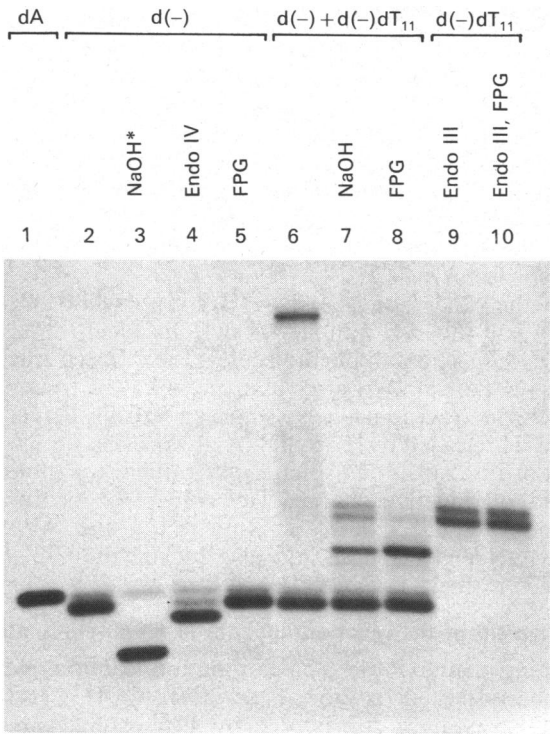


**Fig. 7. Action of [Fapy]DNA glycosylase on 3′-terminal base-free sugars**

Lane 1, [$^{32}$P]pdT$_8$dA; lane 2, [$^{32}$P]pdT$_8$d(–) preparation; lane 3, [$^{32}$P]pdT$_8$d(–) submitted to strong alkaline treatment (NaOH*); lane 4, [$^{32}$P]pdT$_8$d(–), hybridized to poly(dA), incubated with *E. coli* endonuclease IV (Endo IV); lane 5, [$^{32}$P]pdT$_8$d(–), hybridized to poly(dA), incubated with *E. coli* [Fapy]DNA glycosylase (FPG); lane 6, mixture of [$^{32}$P]pdT$_8$d(–) and [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$; lane 7, the preceding mixture submitted to mild alkaline treatment (NaOH); lane 8, same mixture, hybridized to poly(dA), incubated with *E. coli* [Fapy]DNA glycosylase; lane 9, [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$, hybridized to poly(dA), incubated with *E. coli* endonuclease III (Endo III); lane 10, same as in lane 9, but subsequently treated with *E. coli* [Fapy]DNA glycosylase. dA, d(–) and d(–)dT$_{11}$ represent [$^{32}$P]pdT$_8$dA, [$^{32}$P]pdT$_8$d(–) and [$^{32}$P]pdT$_8$d(–)dT$_{11}$ respectively.

**5′-End of the gap produced by [Fapy]DNA glycosylase acting on AP sites.** Fig. 6 shows the results of various treatments of dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA, followed by electrophoresis on a denaturing 20% polyacrylamide gel and autoradiography.

The reference bands are presented first, as follows.

Lane 1 shows the substrate, dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA.

Lanes 9 and 8 show ladders used to estimate the oligonucleotide length. dT$_{11}$dGdT$_{11}$[$^{32}$P]ddA was partially digested with calf spleen phosphodiesterase (mixture of two samples) (lane 9). The oligonucleotides of this previous digestion were 5′-phosphorylated with ATP and T4 polynucleotide kinase (mixture of two samples; see the Materials and methods section) (lane 8).

Lane 2 shows dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA submitted to mild alkaline treatment. In addition to some unchanged substrate, there is a band of pdT$_{11}$[$^{32}$P]ddA resulting from the $\beta$-elimination.

For lane 3 part of the previous sample was treated with alkaline phosphatase from calf intestine. The pdT$_{11}$-[$^{32}$P]ddA band was replaced by another band of dT$_{11}$[$^{32}$P]ddA.

Lane 4 shows dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA, hybridized to poly(dA), incubated with *E. coli* endonuclease III. Nearly all of the substrate has been consumed, yielding a band of pdT$_{11}$[$^{32}$P]ddA resulting from the $\beta$-elimination.

For lane 5 part of the previous sample was treated with alkaline phosphatase from calf intestine. pdT$_{11}$[$^{32}$P]ddA was hydrolysed to dT$_{11}$[$^{32}$P]ddA.

We can now examine the action of [Fapy]DNA glycosylase on the same substrate as follows.

Lane 6 shows dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA incubated with *E. coli* [Fapy]DNA glycosylase. The greater part of the substrate has been consumed; there is a main band at the level of pdT$_{11}$[$^{32}$P]ddA (compare with lanes 2 and 4).

For lane 7 part of the previous sample was treated with alkaline phosphatase from calf intestine. The band at the level of pdT$_{11}$[$^{32}$P]ddA has almost completely disappeared; it has been replaced by another band at the level of dT$_{11}$[$^{32}$P]ddA (compare with lanes 3 and 5).

The conclusion is that [Fapy]DNA glycosylase breaks the C-3′–O–P bond 3′ to the AP site.

***E. coli* [Fapy]DNA glycosylase is inactive on a 3′-terminal base-free deoxyribose.** [$^{32}$P]pdT$_8$d(–) or a mixture of [$^{32}$P]pdT$_8$d(–) and [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ was submitted to various treatments; the reaction medium was then electrophoresed on a denaturing 20% polyacrylamide gel, which was autoradiographed (Fig. 7).

The reference bands are presented first, as follows.

Lanes 1 and 2 show [$^{32}$P]pdT$_8$dA and [$^{32}$P]pdT$_8$d(–) respectively. The depurinated oligonucleotide (lane 2) migrated a little farther than the non-depurinated one (lane 1); a small non-depurinated fraction remained in the [$^{32}$P]pdT$_8$d(–) preparation (lane 2).

Lane 6 shows a mixture of [$^{32}$P]pdT$_8$d(–) and [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$.

Lane 3 shows [$^{32}$P]pdT$_8$d(–) submitted to a strong alkaline treatment. The minor band of [$^{32}$P]pdT$_8$dA persists, but the major band is [$^{32}$P]pdT$_8$p, resulting from the $\beta\delta$-elimination reaction.

Lane 7 shows a mixture of [$^{32}$P]pdT$_8$d(–) and [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ submitted to a mild alkaline treatment. [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ has disappeared; it is replaced by a doublet resulting from the $\beta$-elimination reaction producing [$^{32}$P]pdT$_{11}$s(=) with a 3′-terminal unsaturated sugar, and [$^{32}$P]pdT$_{11}$p resulting from a $\beta\delta$-elimination. On the other hand, it is difficult to know whether much of [$^{32}$P]pdT$_8$d(–) has been modified, although, on the original autoradiogram, there is a weak band of [$^{32}$P]pdT$_8$p, indicating that some $\beta\delta$-elimination had occurred.

Lane 4 shows [$^{32}$P]pdT$_8$d(–), hybridized to poly(dA), incubated at 12 °C for 120 min with *E. coli* endonuclease IV. Two minor bands of [$^{32}$P]pdT$_8$dA and unchanged [$^{32}$P]pdT$_8$d(–) are seen; the major band is [$^{32}$P]pdT$_8$. This result shows that endonuclease IV is capable of removing a 3′-terminal base-free deoxyribose 5-phosphate.

We can now examine the action of *E. coli* [Fapy]DNA glycosylase, as follows.

Lane 5 shows [$^{32}$P]pdT$_8$d(–), hybridized to poly(dA), incubated at 12 °C for 120 min with [Fapy]DNA glycosylase. The substrate remained essentially unchanged.

Lane 8 shows a mixture of [$^{32}$P]pdT$_8$d(–) and

[$^{32}$P]pdT$_{11}$d(–)dT$_{11}$, hybridized to poly(dA), incubated at 12 °C for 120 min with [Fapy]DNA glycosylase. The [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ band has almost completely disappeared, yielding a product that migrated at the level of [$^{32}$P]pdT$_{11}$p. On the other hand, [$^{32}$P]pdT$_8$d(–) remained unchanged.

**[Fapy]DNA glycosylase is inactive on a 3′-terminal base-free unsaturated sugar produced by the action of *E. coli* endonuclease III on an internal AP site.** The buffer in which *E. coli* endonuclease III was conserved was replaced by the [Fapy]DNA glycosylase buffer so that no thiol was present in the reaction mixtures.

The results are also shown in Fig. 7. The reference bands are in lanes 6 and 7 described in the previous subsection. We then examined the successive actions of *E. coli* endonuclease III and *E. coli* [Fapy]DNA glycosylase on [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ hybridized to poly(dA); the reactions were carried out at 12 °C so that no 'melting' of the hybrid could occur during the reactions.

For lane 9 the substrate was incubated for 60 min with *E. coli* endonuclease III. [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ has nearly completely disappeared; it is replaced by [$^{32}$P]pdT$_{11}$s(=), forming a doublet.

For lane 10 part of the previous sample was incubated with *E. coli* [Fapy]DNA glycosylase for 120 min. There is no change, although, in the same conditions, [Fapy]DNA glycosylase acted on [$^{32}$P]pdT$_{11}$d(–)dT$_{11}$ to produce [$^{32}$P]pdT$_{11}$p (see lane 8).

## DISCUSSION

After treatment of DNA with alkylating agents, guanine *N*-7 adducts are spontaneously converted into 5-*N*-alkylformamido-2,6-diamino-4-hydroxy pyrimidines by opening of the imidazole ring (Haines *et al.*, 1962). In *E. coli* this lesion is recognized by the [Fapy]DNA glycosylase which releases the modified base, forming an AP site (Chetsanga & Lindahl, 1979). In addition to its DNA glycosylase activity, the [Fapy]DNA glycosylase of *E. coli* is endowed with an associated strand-nicking activity that cleaves the sugar–phosphate DNA backbone specifically at AP sites (O'Connor & Laval, 1989). In the present work we have studied the mechanism of this nicking activity using the pure enzyme prepared by Boîteux *et al.* (1987), and we have shown that it is consistent with the hypothesis that it is a $\beta\delta$-elimination reaction.

### [Fapy]DNA glycosylase replaces the base-free sugar by a gap limited by 3′-phosphate and 5′-phosphate ends

The experiments with synthetic oligonucleotides containing an AP site [represented by d(–)], labelled either at the 5′- or at the 3′-end, [$^{32}$P]pdT$_8$d(–)dT$_7$ or dT$_{11}$d(–)dT$_{11}$[$^{32}$P]ddA hybridized to poly(dA), show that [Fapy]DNA glycosylase cleaves both phosphodiester bonds 5′ and 3′ to the AP site. The 5′ cleavage produces a 3′-phosphate end (Fig. 5, lane 10); the 3′ cleavage produces a 5′-phosphate end (Fig. 6, lane 6).

The same conclusions are drawn from the experiments with a DNA with doubly labelled AP sites, [d(–),1′,2′-$^3$H,5′-$^{32}$P]DNA in which the base-free deoxyribose is $^3$H-labelled in the 1′- and 2′-positions, and $^{32}$P-labelled 5′ to this base-free deoxyribose. When this substrate is incubated with *E. coli* [Fapy]DNA glycosylase, only a small part (4%) of the $^{32}$P radioactivity becomes acid-soluble. The enzyme has fragmented the DNA, but $^{32}$P is still in oligonucleotides and only the shorter of them are acid-soluble. But a subsequent treatment with *E. coli* exonuclease III releases the $^{32}$P radioactivity as inorganic phosphate. This shows that [Fapy]DNA glycosylase has nicked the phosphodiester bond immediately neighbouring the AP site on its 5′ side, leaving a 3′-phosphate end that was hydrolyzed by the 3′-phosphatase activity of *E. coli* exonuclease III.

On the other hand, [Fapy]DNA glycosylase releases nearly all the $^3$H radioactivity from [d(–),1′2′-$^3$H,5′-$^{32}$P]DNA in acid-soluble molecules; the released fraction (90%) is the same as after a strong alkaline treatment. The chromatographic behaviour, on DEAE-Sephadex, of the $^3$H released by [Fapy]DNA glycosylase shows that it is not in a sugar phosphate, but rather in a molecule devoid of negative charge. The conclusion is that, in addition to the C-5′–O–P bond 5′ to the AP site, [Fapy]DNA glycosylase had also nicked the C-3′–O–P bond 3′ to the AP site.

### Mechanism of the reaction appears to be $\beta\delta$-elimination

Acting on an AP site, a $\beta\delta$-elimination reaction releases the base-free deoxyribose as $CH_2$=COH–CH=CH–CH=O, creating a gap limited by 3′-phosphate and 5′-phosphate ends (Grossman & Grafstrom, 1982). This result can be obtained by a strong alkaline treatment.

In the previous section, we have seen that *E. coli* [Fapy]DNA glycosylase also replaces the base-free deoxyribose by a gap limited by 3′-phosphate and 5′-phosphate ends. Moreover, [Fapy]DNA glycosylase does not hydrolyse the phosphodiester bonds on both sides of the AP site, since the released sugar is not deoxyribose: its chromatographic behaviour on paper is different from that of deoxyribose (Fig. 4*b*). Moreover, the released sugar is adsorbed on Norit, which indicates that it might contain several double bonds, and it is soluble in organic solvents, which suggests that it has lost most of the hydroxy groups of deoxyribose; these two properties support the hypothesis that the released sugar might be $CH_2$=COH–CH=CH–CH=O.

Reduction of the AP sites with $NaBH_4$ makes [d(–),1′,2′-$^3$H,5′-$^{32}$P]DNA resistant to alkaline treatments, and also to [Fapy]DNA glycosylase. This check was necessary to support the hypothesis that the enzyme catalyses a $\beta$-elimination (followed by a $\delta$-elimination), but it was not sufficient since reduction of AP sites also prevents the activity of some 5′ AP endonucleases (César & Verly, 1983).

Thiol compounds react with the 3′-terminal unsaturated base-free sugar resulting from the nicking near an AP site by $\beta$-elimination (Manoharan *et al.*, 1988), and we have shown that this addition prevents the $\delta$-elimination (Bailly & Verly, 1988). When [$^{32}$P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), is treated with [Fapy]DNA glycosylase in the presence of 2-mercaptoethanol, some molecules are not cut 5′ to the AP site, but are cut only on the 3′ side; in the electrophoretogram, in addition to the band of [$^{32}$P]pdT$_8$p, a doublet appears at the level of [$^{32}$P]pdT$_8$d(–) (Fig. 5, lane 12), which is characteristic of a $\beta$-elimination process (Bailly & Verly, 1987). This result strongly suggests that the nicking by *E. coli* [Fapy]DNA glycosylase begins with the nicking 3′ to the AP site by $\beta$-elimination and continues with the 5′ nicking by $\delta$-elimination. In absence of thiol, the $\delta$-

elimination immediately follows the $\beta$-elimination, so that one observes only the product of the $\beta\delta$-elimination. In the presence of 2-mercaptoethanol, there seems to be a competition between the enzyme and the thiol: after nicking 3′ to the AP site by $\beta$-elimination, the enzyme would most often stay on the intermediate product, preventing the addition of the thiol, so that $\delta$-elimination would immediately follow the $\beta$-elimination as in the absence of thiol; sometimes, however, the thiol would succeed in reacting with the 3′-terminal unsaturated sugar, preventing the $\delta$-elimination.

In conclusion, *E. coli* [Fapy]DNA glycosylase acts on an AP site as a $\beta\delta$-elimination catalyst; the enzyme is thus not only a DNA glycosylase removing Fapy, but also an AP lyase (Bailly & Verly, 1989b).

### Action on a 3′-terminal base-free sugar

*E. coli* [Fapy]DNA glycosylase is without action on a 3′-terminal base-free deoxyribose (Fig. 7, lanes 5 and 8). We also investigated its action on a 3′-terminal base-free unsaturated sugar produced by a $\beta$-elimination reaction acting on an AP site. To prepare the substrate, $[^{32}P]pdT_{11}d(-)dT_{11}$, hybridized to poly(dA), was incubated with *E. coli* endonuclease III, which had been shown to nick 3′ to AP sites by a $\beta$-elimination mechanism (Bailly & Verly, 1987). The reaction was carried out in absence of thiol compounds, since their addition on the double bond of the 3′-terminal sugar resulting from the $\beta$-elimination would have prevented the $\delta$-elimination. Fig. 7, lane 9, indicates that such a treatment yielded the requested $\beta$-elimination products, and lane 10 shows that *E. coli* [Fapy]DNA glycosylase was unable to perform the $\delta$-elimination reaction on this substrate. This result strongly suggests that [Fapy]DNA glycosylase remains on the base-free site all the time necessary to catalyse the two steps needed to eliminate the sugar and create the gap limited by 3′-phosphate and 5′-phosphate ends.

We thank Dr. Bernard Weiss, from the Johns Hopkins University, who supplied the *E. coli* overproducer of endonuclease III. This work was supported by grants from the Fonds Cancérologique de la C.G.E.R. (Belgium) and the Fonds de la Recherche Scientifique Médicale (Belgium).

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Received 29 January 1989/10 April 1989; accepted 14 April 1989