Bacteriophage-T4 and *Micrococcus luteus* UV endonucleases are not endonucleases but β-elimination and sometimes βδ-elimination catalysts

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Bacteriophage-T4 UV endonuclease nicks the $C_{(3')}$-O–P bond 3′ to AP (apurinic or apyrimidinic) sites by a β-elimination reaction. The breakage of this bond is sometimes followed by the nicking of the $C_{(5')}$-O–P bond 5′ to the AP site, leaving a 3′-phosphate end; δ-elimination is proposed as a mechanism to explain this second reaction. The AP site formed when this enzyme acts on a pyrimidine dimer in a polynucleotide chain undergoes the same nicking reactions. *Micrococcus luteus* UV endonuclease also nicks the $C_{(3')}$-O–P bond 3′ to AP sites by a β-elimination reaction. No subsequent δ-elimination was observed, but this might be due to the presence of 2-mercaptoethanol in the enzyme preparation.

INTRODUCTION

AP endonucleases class I (Mosbaugh & Linn, 1980) were believed to hydrolyse the phosphodiester bond 3′ to AP (apurinic or apyrimidinic) sites in DNA, leaving a base-free deoxyribose at the 3′-end of the nick. The 3′-ends left by these enzymes were, however, bad primers for *Escherichia coli* DNA polymerase I; a treatment with an AP endonuclease nicking 5′ to the AP site was needed before DNA synthesis could start (Mosbaugh & Linn, 1982).

In 1984 we showed that a 3′-terminal base-free deoxyribose was easily removed by the 3′→5′ exonuclease activity of *E. coli* DNA polymerase I so that the nicking of the $C_{(3')}$-O–P bond 3′ to AP sites by AP endonucleases class I could not result from a hydrolysis (Bailly & Verly, 1984). We suggested that it could rather be a β-elimination reaction leaving a 3′-terminal unsaturated sugar without a 3′-OH group to prime DNA synthesis. It could also be a more complicated βδ-elimination reaction releasing the unsaturated sugar and leaving a 3′-phosphate end, also unsuitable for priming DNA synthesis. Both kinds of 3′-ends can be transformed into 3′-OH groups by either *E. coli* exonuclease III or endonuclease IV, explaining the published observations.

In 1987 we demonstrated that *E. coli* endonuclease III, one of the best-characterized AP endonucleases class I, was not an endonuclease but a β-elimination catalyst (Bailly & Verly, 1987).

In the present paper we show that phage-T4 UV endonuclease and *Micrococcus luteus* UV endonuclease are not endonucleases. In addition to their DNA glycosylase activity, specific for pyrimidine dimers, they catalyse the nicking of the $C_{(3')}$-O–P bond 3′ to AP sites by a β-elimination reaction. Manoharan *et al.* (1988) and Kim & Linn (1988) have also presented data suggesting that phage-T4 UV endonuclease catalyses a β-elimination to nick 3′ to AP sites. We show here that, in addition to the β-elimination reaction, phage-T4 UV endonuclease catalyses, although more slowly, the nicking of the $C_{(5')}$-O–P bond 5′ to the AP site, leaving a gap limited by 3′-phosphate and 5′-phosphate ends.

MATERIALS AND METHODS

5′-Labelled oligonucleotides without or with an AP site

pdA_{16} and dT_8dA were from Pharmacia; $dA_{10}dT_2dA_{10}$, $dT_{10}dA_2dT_{10}$ and dT_8dGdT_7 were from Eurogentec. Labelling the 5′-end was performed with [γ-^{32}P]ATP and phage-T4 polynucleotide kinase. The labelled oligonucleotides were then purified by chromatography on a NENSORB cartridge (NEN Research Products) and eluted in ethanol/water (1:4, v/v). Depurination of [^{32}P]pdT_8dGdT_7 into [^{32}P]$pdT_8d(-)dT_7$, where the AP site is denoted by d(–), was obtained by incubation in 30 mM-HCl for 24 h at 37 °C (Tamm *et al.*, 1952).

U.v. irradiation

The u.v. irradiation was performed with a Philips 15 W germicidal lamp emitting more than 90% of its energy at 254 nm and measured with a Latarjet dosimeter. [^{32}P]$pdA_{10}dT_2dA_{10}$ (88 μM-nucleotides) in 50 mM-NaCl was irradiated for 15 min at 0 °C with a flux of 2 J/m^2 per s. The poly(dA)·poly([*Me*-1′,2′-^{3}H]dT) (75000 d.p.m./μg), prepared as described by Liuzzi *et al.* (1987), in 50 mM-NaCl (10 μg/ml) was irradiated for 1 h at 0 °C with the same flux.

Polyacrylamide-gel electrophoresis and autoradiography

The labelled oligonucleotide samples were diluted at least 2-fold with stop solution (90% formamide in 0.1 M-Tris/borate buffer, pH 8.3, containing 2 mM-EDTA, 0.05% Xylene Cyanol and 0.05% Bromophenol Blue). Samples (10 μl) were placed in the wells (12 mm diam.) of a 20% polyacrylamide denaturing gel of 0.8 mm thickness [19% acrylamide (2X Serva) and 1% bisacrylamide (2X Serva) with 8.3 M-urea (Serva, analytical grade) in 0.1 M-Tris/borate buffer, pH 8.3, containing 2 mM-EDTA]. The electrophoresis was carried out at 700 V for

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

15 h. The autoradiography was performed at −70 °C with Fuji X-ray films with the use of a Kodak X-Omatic superfast intensifying screen.

DNA containing doubly labelled AP sites

The preparation has been described by Bailly & Verly (1987). Doubly labelled dUTP was obtained by deamination of [α-^{32}P]dCTP and [1′,2′,5-^{3}H]dCTP (Amersham). The deamination mixture, together with dATP, dGTP, dCTP and dTTP, was used for the synthesis of DNA; the dTTP/dUTP ratio was 500:1. The newly synthesized radioactive DNA was incubated with *E. coli* uracil-DNA glycosylase until the [5-^{3}H]uracil was completely released. The DNA then contained AP sites labelled with ^{3}H at the 1′ and 2′ positions of the base-free deoxyribose and with ^{32}P in the phosphate 5′ to this base-free deoxyribose; it is represented by [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA. The specific radioactivities were 37500 d.p.m./μg for ^{3}H and 24000 d.p.m./μg for ^{32}P; 95% of ^{3}H and of ^{32}P were correctly placed, the remaining 5% being in dCMP residues. From the information given by Amersham on the [1′,2′,5-^{3}H]dCTP, it is deduced that 40% of the ^{3}H localized at AP sites was at the 1′-position of the base-free deoxyribose and 60% in the hydrogen atom at the 2′-position, which is *cis* relative to the labelled 1′-hydrogen atom in the sugar cyclic β-form.

For some experiments the AP-site-containing DNA was reduced with $NaBH_4$ as described by Gossard & Verly (1978).

The acid-soluble radioactivity, i.e. not precipitable in 5% (v/v) $HClO_4$ at 0 °C, was measured as described by Bailly & Verly (1987).

DEAE-Sephadex chromatography and paper chromatography

After addition of 10 μmol of deoxyribose 5-phosphate and 1 μmol of dUMP, the sample was placed on the DEAE-Sephadex column equilibrated with 0.1 M-Tris/borate buffer, pH 8.3; the elution was carried out with a linear 0–0.25 M-NaCl gradient in the borate buffer (details in Bailly & Verly, 1987).

The ester monophosphates, purified by DEAE-Sephadex chromatography, were concentrated by retention on DEAE-Sephadex followed by elution with 0.3 M-NH_4HCO_3. The samples (8 μl) were spotted on a Whatman 3MM paper and the elution with 95% (v/v) ethanol/1 M-ammonium acetate/ammonia buffer, pH 9.0, saturated with $Na_2B_4O_7$ (7:3, v/v), was carried out in a tank saturated with the same solvent. The chromatography was stopped when the solvent front had migrated about 40 cm beyond the spot line. The paper was dried; 2 cm-wide lanes were cut into 1 cm pieces, which were placed in vials with 1 ml of water for 4 h. After addition of 10 ml of scintillation fluid, the radioactivity was determined.

Enzymes

Phage-T4 UV endonuclease (endonuclease V) was prepared from the over-producer *E. coli* AB2480 containing the plasmid ptac denV, prepared by Valerie *et al.* (1984) (Temple University School of Medicine, Philadelphia, PA, U.S.A.), which we received from Dr. Van Zeeland (University of Leiden, Leiden, The Netherlands). Two chromatographies on single-strand DNA–agarose and CM-Sephadex as described by Higgins & Lloyd (1986) enabled us to obtain an essentially pure protein as judged by SDS/polyacrylamide-gel electrophoresis (Chua, 1980). The enzyme was kept in 10 mM-potassium phosphate buffer, pH 6.5, containing 0.5 M-KCl, 10 mM-EDTA and 10% (v/v) ethylene glycol at 4 °C. The solution had a protein concentration of 0.5 mg/ml and an activity of 18000 units of pyrimidine dimer-DNA glycosylase/μl. One unit is defined as the amount of enzyme in 20 μl of 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 1 mM-EDTA, 5% (v/v) ethylene glycol, bovine serum albumin (50 μg/ml) and 100 ng of u.v.-irradiated poly(dA)·poly([*Me*-1′,2′-^{3}H]dT), which, after a 10 min incubation at 37 °C followed by a strong alkaline treatment, releases 5% of the ^{3}H in acid-soluble molecules. The preparation was without any effect on the unirradiated substrate.

M. luteus UV endonuclease was prepared by Liuzzi *et al.* (1987) by the method of Paterson *et al.* (1981). The enzyme was kept at −20 °C in 250 mM-potassium phosphate buffer, pH 7.5, containing 2 mM-2-mercaptoethanol and 30% (v/v) glycerol. The solution had an AP endonuclease activity of 425 units/ml according to Liuzzi *et al.* (1987).

E. coli endonuclease IV was prepared from *E. coli* B41 strain by the method of Ljungquist (1977). The enzyme was kept at −20 °C in 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl, 1 mM-dithiothreitol and 50% (v/v) glycerol.

E. coli endonuclease III was prepared from *E. coli* BW420 by the method of Breimer & Lindahl (1984). The enzyme solution was part of the one prepared for the work described in Bailly & Verly (1987). The enzyme was kept at −20 °C in 15 mM-potassium phosphate buffer, pH 7.4, containing 0.5 M-NaCl, 1 mM-EDTA, 7 mM-2-mercaptoethanol and 5% (v/v) glycerol.

Snake-venom phosphodiesterase (Sigma type IV) was purified by the method of Oka *et al.* (1978) to eliminate contaminating phosphatases. The enzyme was kept at −20 °C in 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 30 mM-KH_2PO_4 and 20% (v/v) glycerol; the activity was 0.23 unit/ml.

E. coli uracil-DNA glycosylase was prepared from *E. coli* B41 by the method of Lindahl *et al.* (1977). The enzyme was kept at −20 °C in 30 mM-Tris/HCl buffer, pH 7.4, containing 0.4 M-NaCl, 1 mM-EDTA, 1 mM-dithiothreitol and 5% (v/v) glycerol.

Enzyme treatments

Digestion with the 3′→5′ exonuclease activity of bacteriophage-T4 DNA polymerase. Two 20 pmol samples of [^{32}P]pdT$_8$d(–)dT$_7$ in 10 μl of HMM buffer (50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol) were incubated for 15 min at 26 °C with 0.08 or 0.4 unit of phage-T4 DNA polymerase (Pharmacia). The two samples were pooled to obtain a ladder of degradation products down to the AP site. [^{32}P]pdA$_{10}$dT$_2$dA$_{10}$ (20 pmol), u.v.-irradiated or not, in 10 μl of HMM buffer containing 50 mM-NaCl was incubated for 1 h at 37 °C with 0.5 unit (for partial digestion) or 2 units (for complete digestion) of the enzyme.

Digestion with *E. coli* endonuclease VI/exonuclease III. [^{32}P]pdT$_8$d(–)dT$_7$ (20 pmol) and pdA$_{16}$ (20 pmol), in 10 μl of 50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, were incubated for 15 min at 16 °C with 6 units of *E. coli*

exonuclease III (Bethesda Research Laboratories). The endonuclease VI/exonuclease III treatments of [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA, intact or previously nicked by *E. coli* endonuclease III or phage-T4 UV endonuclease, were made at 37 °C for 30 min in 50 mM-Hepes/KOH buffer, pH 7.5, containing 10 mM-$MgCl_2$, with 2 enzyme units/μg of DNA.

Digestion with snake-venom phosphodiesterase. [^{32}P]pdT_8d(–)dT_7 (20 pmol) in 10 μl of HMM buffer containing 2 mM ATP was incubated for 10 min at 37 °C with 33 μunits of snake-venom phosphodiesterase.

***E. coli* endonuclease III treatments.** [^{32}P]pdT_8d(–)dT_7 (20 pmol) and pdA_{16} (20 pmol) in 10 μl of 50 mM-Hepes/KOH buffer, pH 7.8, containing 0.1 M-KCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol were incubated for 2 h at 26 °C with *E. coli* endonuclease III. [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was treated with *E. coli* endonuclease III at 37 °C in 50 mM-Hepes/KOH buffer, pH 7.8, containing 0.1 M-KCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol as described by Bailly & Verly (1987).

***E. coli* endonuclease IV treatments.** [^{32}P]pdT_8d(–)dT_7 (20 pmol), previously submitted to a mild alkaline treatment or not, was incubated for 2 h at 26 °C with *E. coli* endonuclease IV in 10 μl of 50 mM-Hepes/KOH buffer, pH 8.2, containing 0.2 M-NaCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol. [^{32}P]pdA_{10}dT_2dA_{10}, u.v.-irradiated or not, hybridized to dT_{10}dA_2dT_{10}, was incubated with phage-T4 UV endonuclease, submitted to a strong alkaline treatment and then incubated 2 h at 37 °C with *E. coli* endonuclease IV.

UV endonuclease treatments. [^{32}P]pdA_{10}dT_2dA_{10} (20 pmol), u.v.-irradiated or not, hybridized to dT_{10}dA_2dT_{10} (20 pmol) or not, in 10 μl of HNE buffer (50 mM-Hepes/KOH buffer, pH 7.4, containing 50 mM-NaCl and 1 mM-EDTA) was incubated for 10 min at 37 °C with 550 units of phage-T4 UV endonuclease. [^{32}P]pdT_8-d(–)dT_7 (20 pmol) and pdA_{16} (20 pmol) in 10 μl of HNE buffer were incubated with 320 units of phage-T4 UV endonuclease or 1.7 units of *M. luteus* UV endonuclease. [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA in HNE buffer was incubated at 37 °C for 20 min with phage-T4 UV endonuclease (5.3 units/μg of DNA).

Alkaline treatments

The alkaline treatments were carried out in 0.2 M-NaOH for 15 min at 37 °C (mild alkaline treatment) or for 30 min at 65 °C (strong alkaline treatment).

RESULTS AND DISCUSSION

Action of bacteriophage-T4 UV endonuclease on an oligonucleotide containing a thymine dimer (Fig. 1)

[^{32}P]pdA_{10}dT_2dA_{10}, u.v.-irradiated or not, hybridized to dT_{10}dA_2dT_{10} or not, was incubated with phage-T4 UV endonuclease. The reaction products were analysed by electrophoresis on a polyacrylamide gel, which was autoradiographed. The same radioactive substrates were submitted to various other treatments and analysed on the same gel to provide markers. We first present the electrophoretograms of nine reference samples.

Lanes 1 and 8 show the [^{32}P]pdA_{10}dT_2dA_{10} substrate, u.v.-irradiated (lane 8) or not (lane 1).

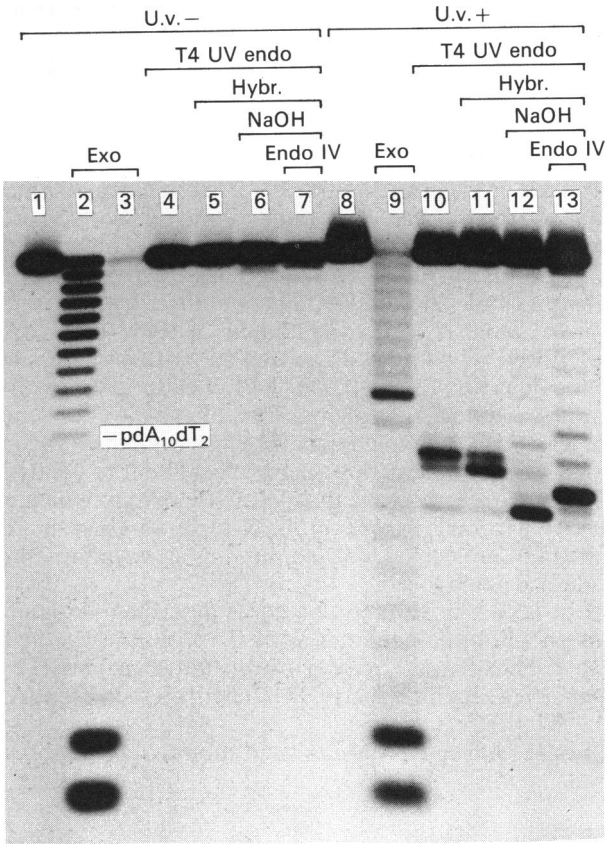

Fig. 1. Action of bacteriophage-T4 UV endonuclease on an oligonucleotide containing a thymine dimer

Lanes 1–3, non-irradiated [^{32}P]pdA_{10}dT_2dA_{10} (lane 1), partially (lane 2) or completely (lane 3) digested by the 3′→5′ exonuclease activity of phage-T4 DNA polymerase. Lanes 4–7, the labelled oligonucleotide, free (lane 4) or hybridized to dT_{10}dA_2dT_{10} (lane 5), was incubated with phage-T4 UV endonuclease; the incubation of the hybridized labelled oligonucleotide with phage-T4 UV endonuclease was followed by a strong alkaline treatment (lane 6), then by an incubation with *E. coli* endonuclease IV (lane 7). Lanes 8 and 9, u.v.-irradiated [^{32}P]pdA_{10}-dT_2dA_{10} (lane 8) was digested by the 3′→5′ exonuclease activity of phage-T4 DNA polymerase (lane 9). Lanes 10–13, the u.v.-irradiated labelled oligonucleotide, free (lane 10) or hybridized to dT_{10}dA_2dT_{10} (lane 11), was incubated with phage-T4 UV endonuclease; this incubation of the hybridized labelled substrate with phage-T4 UV endonuclease was followed by a strong alkaline treatment (lane 12), then by an incubation with *E. coli* endonuclease IV (lane 13). Key: U.v.+/–, u.v.-irradiated or not; Exo, 3′→5′ exonuclease activity of phage-T4 DNA polymerase; T4 UV endo, phage-T4 UV endonuclease; Hybr., hybridized; NaOH, strong alkaline treatment; Endo IV, *E. coli* endonuclease IV.

Lanes 2 and 3 show the non-irradiated [^{32}P]pdA_{10}-dT_2dA_{10} digested by the 3′→5′ exonuclease activity of phage-T4 DNA polymerase. In lane 2 the partial digestion of the 22-mer gave a series of steps down to [^{32}P]pdA_{10}dT_2. In lane 3 a more complete digestion left only dimers and monomers.

For lane 9 u.v.-irradiated [^{32}P]pdA_{10}dT_2dA_{10} was submitted to digestion by the phage-T4 DNA polymerase (same conditions as for lane 3). The result is complex

because not all substrate molecules contained a thymine dimer. Besides steps of partial degradation, three main bands are observed. The first one is at the level of 13-mers; the product forming this band most probably derived from the substrate molecules containing a thymine dimer, which were degraded down to [^{32}P]pdA$_{10}$-$\overline{dTdT}$dA, indicating that the 3′→5′ exonuclease of phage-T4 DNA polymerase was stopped one nucleotide before the dimer ($\overline{dTdT}$). The products in the two other bands, at the levels of dimers and monomers, derived from the substrate molecules that did not contain a thymine dimer.

For lane 12 u.v.-irradiated [^{32}P]pdA$_{10}$dT$_2$dA$_{10}$, hybridized to dT$_{10}$dA$_2$dT$_{10}$, was submitted successively to the digestion by the phage-T4 UV endonuclease and a strong alkaline treatment. The phage-T4 UV endonuclease was used to change the eleventh nucleotide into an AP site and the strong alkaline treatment to catalyse a $\beta\delta$-elimination reaction that left [^{32}P]pdA$_{10}$p, which can be seen as a major band on the electrophoretogram. The substrate molecules not containing a thymine dimer remained unchanged.

For lane 13 a sample treated as described for lane 12 was, in addition, incubated with *E. coli* endonuclease IV. The 3′-phosphatase activity of this latter enzyme (Levin *et al.*, 1988; Bailly & Verly, 1989) hydrolysed [^{32}P]pdA$_{10}$p to [^{32}P]pdA$_{10}$.

For lanes 6 and 7 samples were subjected to treatments identical with those indicated for lanes 12 and 13 respectively except that [^{32}P]pdA$_{10}$dT$_2$dA$_{10}$ had not been u.v.-irradiated. The substrate molecules were not degraded.

We can now present the data obtained when the oligonucleotide was submitted to digestion by the phage-T4 UV endonuclease.

For lanes 4, 5, 10 and 11 [^{32}P]pdA$_{10}$dT$_2$dA$_{10}$, u.v.-irradiated (lanes 10 and 11) or not (lanes 4 and 5), hybridized to dT$_{10}$dA$_2$dT$_{10}$ (lanes 5 and 11) or not (lanes 4 and 10), was incubated with phage-T4 UV endonuclease. The non-irradiated oligonucleotide was not cleaved by the enzyme (lanes 4 and 5). For the u.v.-irradiated substrate, some of the molecules, probably those that did not contain a thymine dimer, were not affected; the others were cleaved and the radioactive products are distributed into three bands. The two major ones form a doublet at the level of [^{32}P]pdA$_{10}$dT, confirming that the enzyme nicks the C$_{(3')}$-O–P bond 3′ to the AP site resulting from its DNA glycosylase activity (Warner *et al.*, 1980; Nakatsu *et al.*, 1982); but the cleavage yields two 5′ fragments, which is the signature of a β-elimination reaction (see the next subsection). There is also a very light band at the level of [^{32}P]pdA$_{10}$p (compare with lane 12), indicating that, in some cases, the C$_{(5')}$-O–P bond 5′ to the AP site was cleaved. The results are nearly the same whether there was a comp-

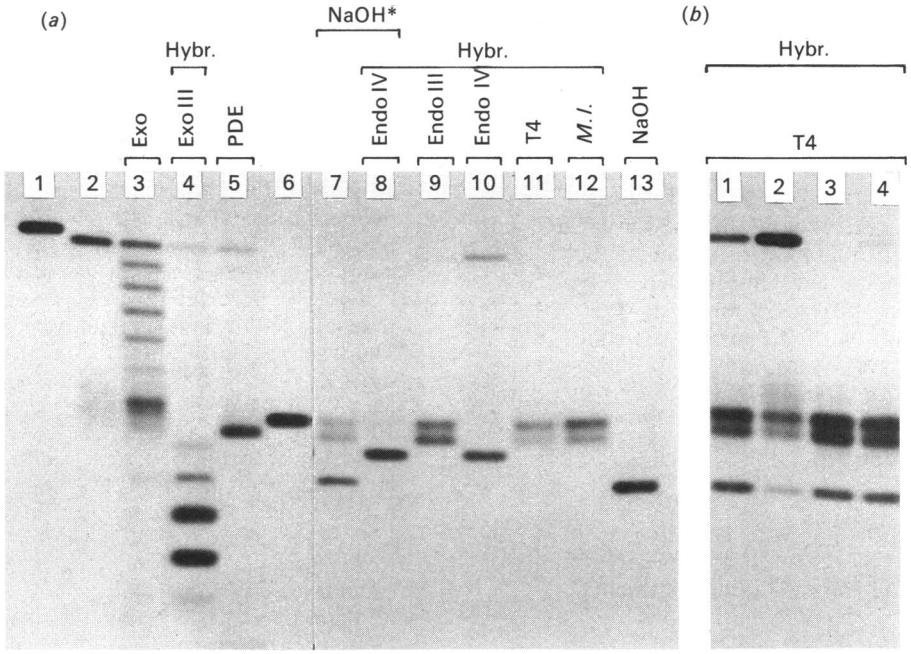

Fig. 2. Action of bacteriophage-T4 and *M. luteus* UV endonucleases on an oligonucleotide containing an AP site

(*a*) Lanes 1 and 2, [^{32}P]pdT$_8$dGdT$_7$ (lane 1) and [^{32}P]pdT$_8$d(–)dT$_7$ (lane 2). Lanes 3–5, [^{32}P]pdT$_8$d(–)dT$_7$ partially digested by the 3′→5′ exonuclease activity of phage-T4 DNA polymerase (lane 3), or partially digested with *E. coli* endonuclease VI/exonuclease III (lane 4), or digested with snake-venom phosphodiesterase (lane 5). Lane 6, [^{32}P]pdT$_8$dA. Lanes 7 and 8, [^{32}P]pdT$_8$d(–)dT$_7$ submitted to a mild alkaline treatment (lane 7) and, after hybridization with poly(dA), incubated with *E. coli* endonuclease IV (lane 8). Lanes 9–12, [^{32}P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), submitted to digestion by *E. coli* endonuclease III (lane 9), or *E. coli* endonuclease IV (lane 10), or phage-T4 UV endonuclease (lane 11), or *M. luteus* UV endonuclease (lane 12). Lane 13, [^{32}P]pdT$_8$d(–)dT$_7$ submitted to a strong alkaline treatment. (*b*) Lanes 1–4, [^{32}P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), was incubated either with 18 units of phage-T4 UV endonuclease/pmol of oligonucleotide for 60 min (lane 1) or 10 min (lane 2), or with 90 units of the enzyme/pmol of oligonucleotide for 60 min (lane 3) or 10 min (lane 4). Key: Exo, 3′→5′ exonuclease activity of phage-T4 DNA polymerase; Exo III, *E. coli* endonuclease VI/exonuclease III; PDE, snake-venom phosphodiesterase; Endo IV, *E. coli* endonuclease IV; Endo III, *E. coli* endonuclease III; T4, phage-T4 UV endonuclease; *M. l.*, *M. luteus* UV endonuclease; Hybr., hybridized; NaOH, strong alkaline treatment; NaOH*, mild alkaline treatment.

lementary strand (lane 11) or not (lane 10); we thus confirm that the complementary strand is not necessary for the enzyme action (Minton *et al.*, 1975). However, the two bands of the β-elimination doublet have not the same relative intensities: the upper band is more important when there is no complementary strand (lane 10), whereas it is the lower one when the hybrid has formed (lane 11).

Action of bacteriophage-T4 UV endonuclease and *M. luteus* UV endonuclease on an oligonucleotide containing an AP site

$[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, was incubated with phage-T4 UV endonuclease or *M. luteus* UV endonuclease. The reaction products were analysed by electrophoresis on a polyacrylamide gel, which was autoradiographed (Fig. 2*a*). The same radioactive substrate was submitted to various other treatments and analysed on the same gel to provide markers. We first present the reference samples.

Lanes 1, 2 and 6 show $[^{32}P]pdT_8dGdT_7$, $[^{32}P]pdT_8d(-)dT_7$ and $[^{32}P]pdT_8dA$ respectively.

Lane 3 shows $[^{32}P]pdT_8d(-)dT_7$ subjected to the 3′→5′ exonuclease activity of phage-T4 DNA polymerase. A ladder of partial degradation products can be seen; a prominent band appears at the level of 10-mers, indicating that the exonuclease was slowed down one nucleotide before the AP site. We have shown that the degradation can, however, be complete: after pausing one nucleotide before the AP site, the enzyme can resume its activity.

For lane 4 $[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, was submitted to digestion by *E. coli* endonuclease VI/exonuclease III. This AP endonuclease hydrolysed the phosphodiester bond 5′ to the AP site to yield $[^{32}P]pdT_8$, which was subsequently degraded by the 3′→5′ exonuclease activity of the enzyme; the 8-mer and its degradation products form a ladder of a few bands on the autoradiogram.

Lane 5 shows that the degradation of $[^{32}P]pdT_8d(-)dT_7$ by snake-venom phosphodiesterase gave a unique product $[^{32}P]pdT_8d(-)$. This enzyme is thus stopped immediately before the AP site, whereas the 3′→5′ exonuclease activity of phage-T4 DNA polymerase paused one nucleotide before the AP site (see lane 3).

Lane 10 shows that *E. coli* endonuclease IV has cleaved $[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, 5′ to the AP site to yield $[^{32}P]pdT_8$.

For lanes 7 and 13 $[^{32}P]pdT_8d(-)dT_7$ was submitted to a mild alkaline treatment (lane 7): the β-elimination reaction yields two 5′ products with a 3′-terminal unsaturated sugar, which we represent by $[^{32}P]pdT_8s(=)$, forming a doublet at the level of $[^{32}P]pdT_8d(-)$ (compare with lane 5); another band of $[^{32}P]pdT_8p$ results from the δ-elimination that often followed the β-elimination (see also Bailly & Verly, 1987). When submitted to a strong alkaline treatment (lane 13), the substrate gave only the $\beta\delta$-elimination product $[^{32}P]pdT_8p$.

For lane 8 the mild alkaline treatment of $[^{32}P]pdT_8d(-)dT_7$ was followed by an incubation with *E. coli* endonuclease IV. The three bands resulting from the mild alkaline treatment (see lane 7) are replaced by a single one, $[^{32}P]pdT_8$ (compare with lane 10). This result means that the AP endonuclease activity of *E. coli* endonuclease IV is capable of acting on the β-elimination products $[^{32}P]pdT_8s(=)$ to remove the 3′-terminal base-free unsaturated sugar 5-phosphate, and that, in addition to its AP endonuclease activity, the enzyme has also a 3′-phosphatase activity, which hydrolysed $[^{32}P]pdT_8p$ into $[^{32}P]pdT_8$.

For lane 9 $[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, was incubated with *E. coli* endonuclease III. The two radioactive products of the reaction migrated as a doublet in the same position as the doublet of β-elimination observed after a mild alkaline treatment (see lane 7). *E. coli* endonuclease III is known to nick 3′ to AP sites by catalysing a β-elimination reaction (Bailly & Verly, 1987).

We can now present the results obtained when the oligonucleotide was submitted to digestion by T4-phage UV endonuclease or *M. luteus* UV endonuclease.

For lanes 11 and 12 $[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, was incubated with T4-phage UV endonuclease (lane 11) or *M. luteus* UV endonuclease (lane 12). All the molecules have been cleaved, in both cases yielding two close bands forming a doublet at the level of $[^{32}P]pdT_8d(-)$ (compare with lane 5). The upper band is more intense than the lower one. These results indicate that it is the $C_{(3')}$-O–P bond 3′ to the AP site that was broken, confirming the results already obtained with the oligonucleotide containing a thymine dimer (see the preceding subsection). But this nicking is not the result of a hydrolysis. Indeed, hydrolysis of this bond yields a unique 5′ product, as shown in lane 5 when $[^{32}P]pdT_8d(-)dT_7$ was degraded with snake-venom phosphodiesterase. With the UV endonucleases, on the other hand, there are two 5′ products forming a doublet, which rather suggests that the nicking resulted from a β-elimination reaction: the doublet is exactly in the same position as the doublet produced by β-elimination during a mild alkaline treatment (lane 7) or an incubation with *E. coli* endonuclease III (lane 9).

In Fig. 2(*b*) we present a similar experiment but where larger amounts of phage-T4 UV endonuclease and longer times of treatment were used. $[^{32}P]pdT_8d(-)dT_7$, hybridized to pdA_{16}, was incubated for 60 min (lanes 1 and 3) or for 10 min (lanes 2 and 4) at 25 °C with 18 units (lanes 1 and 2) or 90 units (lanes 3 and 4) of phage-T4 UV endonuclease/pmol of oligonucleotide. The four lanes, but especially lane 3 corresponding to the higher UV endonuclease concentration and the longer time of treatment, show, in addition to the doublet of β-elimination, a $[^{32}P]pdT_8p$ band. We thus confirm what we have already observed with the oligonucleotide containing a thymine dimer (see the preceding subsection): the enzyme can sometimes nick the $C_{(5')}$-O–P bond 5′ to the AP site.

Mechanisms of the nickings 3′ and 5′ to AP sites catalysed by bacteriophage-T4 and *M. luteus* endonucleases

The substrate used in this subsection is a DNA containing doubly labelled AP sites. As mentioned in the Materials and methods section, 95% of ^3H and 95% of ^{32}P were located at the AP sites, the rest being in dCMP residues. The ^3H label was in the stable hydrogen atom at the 1′-position and one of the two hydrogen atoms at the 2′-position of the base-free deoxyribose, whereas the ^{32}P was located in the phosphodiester bond 5′ to the base-free deoxyribose. This substrate is called [d(–)-1′,2′-^3H,5′-^{32}P]DNA.

Products of the nicking reactions. A strong alkaline treatment of [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA released 95% of the ^{3}H and 4% of the ^{32}P in acid-soluble molecules. The small amount of ^{32}P that became acid-soluble is explained by the fragmentation of the DNA strands at AP sites, which gave some oligonucleotides small enough to be acid-soluble; but ^{32}P remained adsorbable on Norit since it was in the 3′-phosphate ends of these oligonucleotides. On the other hand, the part of ^{3}H that became acid-soluble because of the fragmentation of the DNA strands was negligible (4% of the 5% in dCMP residues = 0.2%); the release of ^{3}H depended on the β- and δ-elimination reactions. The β-elimination released as H^+ (becoming volatile water) one hydrogen atom of the 2′-position. Since 60% of the ^{3}H labelling the AP sites was in one of the two hydrogen atoms at the 2′-position, if OH^--catalysed β-elimination sets free any one of them then β-elimination should release as H^+ 30% of the ^{3}H ($30 \times 0.95 = 28.5\%$ of the total ^{3}H), and δ-elimination should release, as an unsaturated aldehyde, the rest, i.e. 70% ($70 \times 0.95 = 66.5\%$ of the total ^{3}H). This conclusion cannot be extrapolated to an enzyme catalysing a β-elimination reaction. If, in this case, the H^+ loss is totally asymmetrical, the fraction of ^{3}H released could be either 0% or 60% ($60 \times 0.95 = 57\%$ of the total ^{3}H); if partially asymmetrical, it can be anything between 0% and 60%. *E. coli* endonuclease III, which is only a β-elimination catalyst, acting on [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA, released 43% of the ^{3}H (Bailly & Verly, 1987).

[d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was incubated with increasing amounts of phage-T4 UV endonuclease, and the acid-soluble ^{3}H and ^{32}P radioactivities were monitored (Fig. 3). For the largest amounts of enzyme, these radioactivities reached 4% of the total ^{32}P and 65% of the total ^{3}H. As discussed above, the 4% acid-soluble ^{32}P was due to nicking of the DNA near each AP site. If the nicking were due to hydrolysis of the $C_{(3')}$-O–P bond 3′ to the AP sites, the acid-soluble ^{3}H should not be greater than the acid-soluble ^{32}P. Since it was much greater, the DNA fragmentation could result from a β-elimination partially followed by nicking of the $C_{(5')}$-O–P bond 5′ to AP sites. We do not know what fraction of ^{3}H a complete β-elimination should have released (see above), but if it were 43% as found with *E. coli* endonuclease III, and since the acid-soluble ^{3}H reached 65%, the conclusion should be that very often the β-elimination was followed by a nicking 5′ to the AP site, confirming the results obtained with the labelled oligonucleotides.

When [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was incubated with a large amount of *M. luteus* UV endonuclease, the acid-soluble ^{32}P also reached 4% of the total, indicating a complete fragmentation of the DNA at AP sites, and the acid-soluble ^{3}H reached 44% of the total. Here, also, the data contradict the hypothesis of a hydrolysis of the $C_{(3')}$-O–P bond 3′ to the AP sites. The best explanation is that the β-elimination at the AP sites was complete, but was not followed by a δ-elimination; *E. coli* endonuclease III, which also catalyses a β-elimination but no δ-elimination, gives a maximum of 43% acid-soluble ^{3}H from the same substrate (see above). The absence of δ-elimination, in contrast with what occurs with phage-T4 UV endonuclease, is probably due to the presence of 2-mercaptoethanol in the enzyme preparation. We have shown that thiols, which react with the β-elimination products (Manoharan *et al.*, 1988), prevent the δ-elimination reaction (Bailly & Verly, 1988*b*).

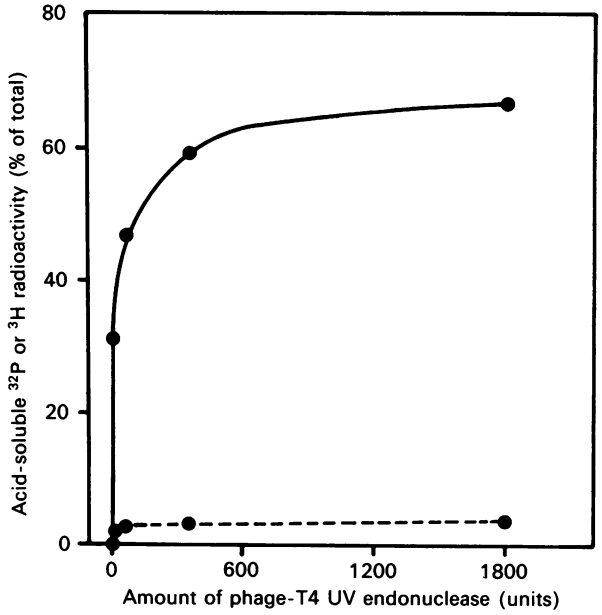

Fig. 3. Action of bacteriophage-T4 UV endonuclease on AP sites

[d(–)-1′,2′-^{3}H,5′-^{32}P]DNA (0.25 μg) was incubated for 60 min with different amounts of phage-T4 UV endonuclease in 10 μl of reaction medium before measurement of the acid-soluble radioactivities. The acid-soluble ^{32}P (----) and ^{3}H (——) radioactivities are expressed as percentages of their respective total radioactivities.

Reduction of the AP-site aldehyde group prevents the β- and δ-elimination reactions. When [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was reduced with $NaBH_4$ before it was incubated with phage-T4 UV endonuclease or *M. luteus* UV endonuclease, no acid-soluble ^{3}H or ^{32}P appeared. This observation, necessary to support the hypothesis that the UV endonuclease nicked the DNA strands by first catalysing a β-elimination reaction, is not, however, sufficient to prove that such is the reaction mechanism: indeed, rat liver AP endonuclease, which hydrolyses the $C_{(3')}$-O–P bond 5′ to AP sites, is unable to nick the DNA strands near reduced AP sites (César & Verly, 1983).

Chromatographic analyses of the 3′-terminal base-free sugar 5-phosphate. To study only the result of the first step of β-elimination and thus avoid the second step of 5′ nicking, one must use little enzyme and a short incubation time. This was done in the following experiment.

[d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was incubated with phage-T4 UV endonuclease until the acid-soluble ^{32}P reached 1% of the total; at that time, the acid-soluble ^{3}H was only 12% of the total. The acid-soluble ^{32}P was adsorbable on Norit, but not the acid-soluble ^{3}H. *E. coli* endonuclease VI/exonuclease III and $MgCl_2$ were then added to hydrolyse the $C_{(3')}$-O–P bond 5′ to the AP site and release the 3′-terminal base-free sugar 5-phosphate. The reaction was monitored by measuring the Norit-unadsorbable ^{3}H and ^{32}P and, when they reached a maximum, the reaction was stopped with EDTA. The sample was then divided into two parts. One part, after addition of D-2-deoxyribose 5′phosphate and dUMP, was analysed by chromatography on DEAE-Sephadex. The sugar phosphate of the other part, after purification on a short DEAE-Sephadex column, was analysed by chromatography on Whatman 3MM paper.

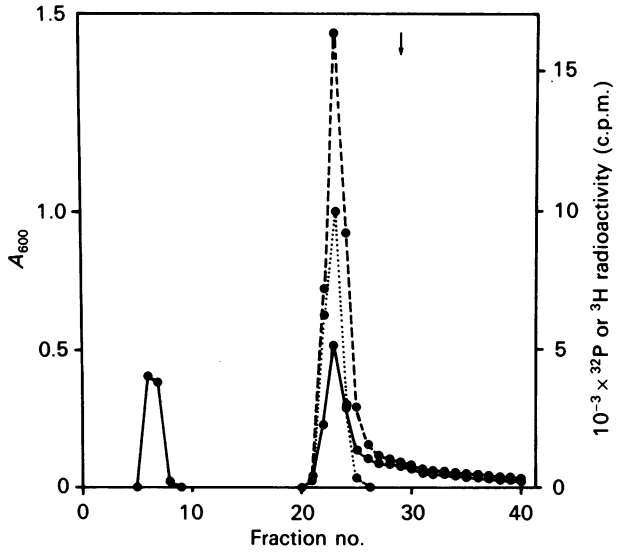

Fig. 4. DEAE-Sephadex chromatography of the radioactive products of the successive nickings of [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA by bacteriophage-T4 UV endonuclease and *E. coli* endonuclease VI/exonuclease III

To avoid breakage of the $C_{(5')}$-O–P bond 5′ to the AP sites, the DNA containing doubly labelled AP sites was only partially nicked with phage-T4 UV endonuclease (5.3 units/μg of DNA, 20 min at 37 °C). It was then submitted to digestion by *E. coli* endonuclease VI/exonuclease III (2 units/μg of DNA, 10 min at 37 °C). After addition of dUMP and deoxyribose 5-phosphate as elution markers, the reaction mixture was chromatographed on DEAE-Sephadex. The ^{3}H (——) and ^{32}P (– – – –) radioactivities were measured on the collected fractions. The dUMP peak is indicated by an arrow; the deoxyribose 5-phosphate was measured at A_{600} (· · · · ·) after reaction with diphenylamine.

The DEAE-Sephadex chromatography (Fig. 4) shows two ^{3}H peaks and a single ^{32}P peak. The first ^{3}H peak was due to molecules not retained by the column; 98% of this ^{3}H was volatile. This latter observation is a strong indication that the nicking by the UV endonuclease was the result of a β-elimination reaction, which removed as H^{+} a labelled hydrogen atom from the 2′-position of the base-free deoxyribose; it also indicates that the treatment with phage-T4 UV endonuclease that we used was light enough so that the β-elimination was not followed by any significant 5′-nicking, which would have released a non-volatile ^{3}H-labelled unsaturated sugar not retained by the column. The second ^{3}H peak is coincident with the unique ^{32}P peak; it has the position of the D-2-deoxyribose 5-phosphate marker. This peak contains the base-free sugar 5-phosphate released from the 3′-end of the nick produced by the UV endonuclease; the ^{3}H/^{32}P ratio measured in this peak is about 40% of that in the substrate DNA. We have previously shown (Bailly & Verly, 1987) that, when [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was completely degraded with snake-venom phosphodiesterase and *E. coli* endonuclease VI/exonuclease III, the ^{3}H/^{32}P ratio in the D-2-deoxyribose 5-phosphate, purified by DEAE-Sephadex chromatography, was identical with that in the substrate DNA. Thus the decreased ratio presently observed indicates that phage-T4 UV endonuclease did not hydrolyse the $C_{(3')}$-O–P

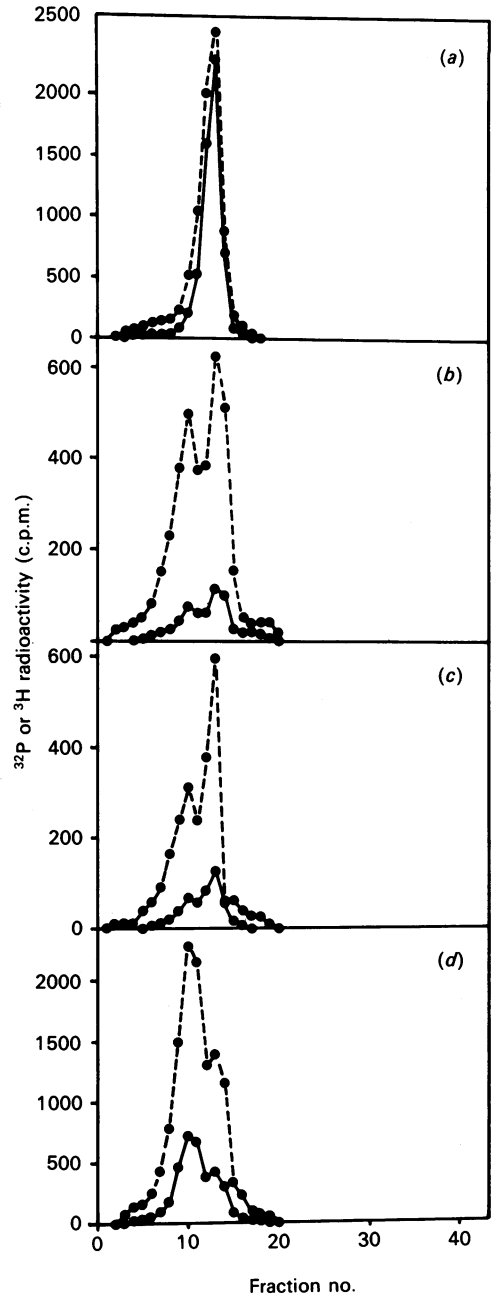

Fig. 5. Paper chromatography of doubly labelled sugar phosphates released from [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA

In every case the sugar phosphate was purified on DEAE-Sephadex before it was chromatographed on Whatman 3MM paper. The lanes corresponding to each sugar phosphate chromatography were cut into 1 cm pieces, which were eluted for measurement of the ^{3}H (——) and ^{32}P (– – – –) radioactivities. (*a*) [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA digested with *E. coli* endonuclease VI/exonuclease III and snake-venom phosphodiesterase (190 fmol of sugar phosphate); (*b*) incubation with *E. coli* endonuclease VI/exonuclease III followed by mild alkaline treatment (150 fmol of sugar phosphate); (*c*) successive incubations with *E. coli* endonuclease III and *E. coli* endonuclease VI/exonuclease III (80 fmol of sugar phosphate); (*d*) light treatment with phage-T4 UV endonuclease followed by incubation with *E. coli* endonuclease VI/exonuclease III (280 fmol of sugar phosphate).

bond 3′ to the AP site, but that the nicking was rather due to a β-elimination, leaving a base-free unsaturated sugar at the 3′-end.

The sugar 5-phosphate, resulting from the consecutive actions of phage-T4 UV endonuclease and *E. coli* endonuclease VI/exonuclease III, after purification on another DEAE-Sephadex column and concentration, was analysed by chromatography on Whatman 3MM paper. Its behaviour was compared with that of sugar 5-phosphates obtained from [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA by three other treatments and also purified on DEAE-Sephadex (Fig. 5).

In Fig. 5(*a*) one sees D-2-[^{3}H]deoxyribose 5-[^{32}P]phosphate obtained after a complete degradation of the substrate DNA with snake-venom phosphodiesterase and *E. coli* endonuclease VI/exonuclease III; the unique ^{3}H and ^{32}P peaks are coincident and centred on fraction 13. In Fig. 5(*b*) incubation of [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA with *E. coli* endonuclease VI/exonuclease III was followed by a mild alkaline treatment; both ^{3}H and ^{32}P profiles show two peaks: the first ^{3}H peak, coincident with the first ^{32}P peak, is centred on fraction 9, whereas the second ^{3}H peak, coincident with the second ^{32}P peak, is centred on fraction 13. We previously observed (Fig. 2*a*, lane 7) that nicking 3′ to an AP site by β-elimination leaves two 5′ fragments; it was thus not unexpected that a subsequent hydrolysis of the $C_{(3')}$-O–P bond 5′ to the AP site released two different sugar 5-phosphates. Although one of them migrated in the same position as D-2-deoxyribose 5-phosphate on the Whatman 3MM paper, there is no reason to think that it is D-2-deoxyribose 5-phosphate; it is rather a primary product of β-elimination or a derivative of it. In Fig. 5(*c*) [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was submitted successively to *E. coli* endonuclease III and *E. coli* endonuclease VI/exonuclease III; the same two peaks, both containing ^{3}H and ^{32}P, as in Fig. 5(*b*) can be seen, which is in agreement with the already published conclusion that *E. coli* endonuclease III nicks DNA strands containing AP sites by catalysing a β-elimination reaction (Bailly & Verly, 1987).

What was the released sugar 5-phosphate when [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA was first lightly treated with phage-T4 UV endonuclease, then incubated with *E. coli* endonuclease VI/exonuclease III, can be seen in Fig. 5(*d*) and compared with the results previously documented (Figs. 5*a*, 5*b* and 5*c*). Exactly as when the nicking 3′ to the AP sites was the result of a β-elimination reaction, two peaks, both of ^{3}H and ^{32}P, are observed located in the same positions. We note that the first peak is larger than the second one, but when the 5′-labelled oligonucleotide containing an AP site was cut with phage-T4 UV endonuclease one of the two 5′ fragments, which formed the upper band of the doublet seen on the autoradiogram, was more intense than the other (Fig. 2*a*, lane 11). The two sugar 5-phosphates were not separated by the DEAE-Sephadex chromatography (see Fig. 4).

FINAL REMARKS AND CONCLUSIONS

The phage-T4 UV endonuclease nicks the $C_{(3')}$-O–P bond 3′ to AP sites in DNA, but it can also subsequently nick the $C_{(5')}$-O–P bond 5′ to AP sites. *M. luteus* UV endonuclease nicks the $C_{(3')}$-O–P bond 3′ to AP sites.

The 3′ nicking is not the result of the hydrolysis of the phosphoester bond, but rather the result of a β-elimination reaction, for the following reasons. (1) The nicking produces two 5′ products, as does the β-elimination reaction, whereas hydrolysis gives only one. (2) When [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA is lightly treated with the UV endonuclease so that only $C_{(3')}$-O–P bonds 3′ to AP sites are cleaved, the percentage of ^{3}H released in the acid-soluble fraction far exceeds the percentage of ^{32}P; this is what is observed when the nicking is by β-elimination, whereas the two percentages remain identical when the cleavage is by hydrolysis. Moreover, the released ^{3}H is volatile, as should be the labelled 2′-hydrogen atom liberated as an H^{+} by the β-elimination reaction. (3) When, after a light treatment with the UV endonuclease, the [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA is treated with *E. coli* endonuclease VI/exonuclease III, the ^{3}H/^{32}P ratio of the released sugar 5-phosphate is lower than that of the substrate DNA, as is the case when the 3′ nicking is by β-elimination; by contrast, the ratio is the same when the 3′ nicking is by hydrolysis. (4) Strand nicking by the UV endonuclease is prevented by reduction of the AP sites in [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA.

Manoharan *et al.* (1988), using an oligonucleotide labelled with ^{13}C on the base-free deoxyribose of an AP site and n.m.r. spectroscopy, also reached the conclusion that phage-T4 UV endonuclease catalyses a β-elimination reaction. Kim & Linn (1988) drew the same conclusion, although it was based only on the technique of paper chromatography.

Nicking of the $C_{(5')}$-O–P bond 5′ to AP sites by the phage-T4 UV endonuclease was shown with a 5′-labelled oligonucleotide containing an AP site and corroborated by an experiment with [d(–)-1′,2′-^{3}H,5′-^{32}P]DNA. A heavy treatment with phage-T4 UV endonuclease released most of the ^{3}H in the acid-soluble fraction; this indicates that this ^{3}H cannot come only from the β-elimination reaction, but that a nicking 5′ to the AP site occurred in addition to the nicking 3′ to the AP site. This 5′ nicking could result from a δ-elimination following the β-elimination; but this proposal is more speculative than the mechanism of β-elimination suggested for the 3′ nicking.

The arguments are as follows. (1) δ-Elimination nicks the $C_{(5')}$-O–P bond 5′ to AP sites. (2) The δ-elimination that follows the β-elimination is a slower reaction; a light UV endonuclease treatment gives only β-elimination products, and a heavier treatment is needed to observe the breakage of the $C_{(5')}$-O–P bond 5′ to the AP site. (3) The δ-elimination depends on a previous β-elimination reaction, and reduction of the AP sites prevents the nicking, not only on the 3′ side, but also on the 5′ side of the AP site.

With the *M. luteus* UV endonuclease preparation, which contained 2-mercaptoethanol, the β-elimination reaction was not followed by the rupture of the $C_{(5')}$-O–P bond 5′ to the AP sites, but we have shown that thiols, by reacting with the β-elimination products (Manoharan *et al.*, 1988), prevent the δ-elimination (Bailly & Verly, 1988*b*).

An absolute proof for the δ-elimination reaction should await the preparation of a DNA containing an AP site labelled with ^{3}H in the 4′-position of the base-free deoxyribose, or the identification of the sugar derivative released by the successive 3′ and 5′ nickings.

When the phage-T4 UV endonuclease acts on an oligonucleotide containing a pyrimidine dimer, nicking on both sides of the AP site formed by the first DNA

glycosylase step is also observed; the 3′ nicking by β-elimination is always prevalent, but some 5′ nicking is also performed.

The sequence of the 138 amino acid residues of phage-T4 UV endonuclease is known; the active sites for the DNA glycosylase and the nicking activity seem to be independent. The nicking activity depends on Trp-128 in the sequence of Trp-Tyr-Lys-Tyr-Tyr (Valerie *et al.*, 1984). The analogy of this sequence with the tripeptide Lys-Trp-Lys or Lys-Tyr-Lys, which also nick AP-site-containing DNA, has been underlined. Several authors have suggested that the tripeptide nicking is the result of a β-elimination reaction, and we have adduced evidence supporting their hypothesis (Bailly & Verly, 1987, 1988*a*).

Since an endonuclease is a phosphoric diester hydrolase classified as EC 3.1.4.–, phage-T4 UV endonuclease and *M. luteus* UV endonuclease are not endonucleases. Since these enzymes have two activities, pyrimidine dimer-DNA glycosylase and strand nicking, they should rather be named by the first activity: bacteriophage-T4 or *M. luteus* pyrimidine dimer-DNA glycosylase. As for the activity on AP sites, it cannot be classified as AP endonuclease; it should be called AP lyase (phosphoric monoester lyase to be placed in EC class 4) even if the β-elimination reaction can be followed by δ-elimination. Such expressions like 'lyase-type phosphodiesterase' (Kim & Linn, 1988) are self-contradictory.

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