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Properties of the Main Endonuclease Specific for Apurinic Sites of *Escherichia coli* (Endonuclease VI)

Mechanism of Apurinic Site Excision from DNA

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The main endonuclease for apurinic sites of *Escherichia coli* (endonuclease VI) has no action on normal strands, either in double-stranded or single-stranded DNA, or on alkylated sites. The enzyme has an optimum pH at 8.5, is inhibited by EDTA and needs Mg^{2+} for its activity; it has a half-life of 7 min at 40 °C. A purified preparation of endonuclease VI, free of endonuclease II activity, contained exonuclease III; the two activities (endonuclease VI and exonuclease III) copurified and were inactivated with the same half-lives at 40 °C. Endonuclease VI cuts the DNA strands on the 5′ side of the apurinic sites giving a 3′-OH and a 5′-phosphate, and exonuclease III, working afterwards, leaves the apurinic site in the DNA molecule; this apurinic site can subsequently be removed by DNA polymerase I. The details of the excision of apurinic sites *in vitro* from DNA by endonuclease VI/exonuclease III, DNA polymerase I and ligase, are described; it is suggested that exonuclease III works as an antiligase to facilitate the DNA repair.

An enzyme which hydrolyzes a phosphoester bond near apurinic sites was found in *Escherichia coli* by Verly and Paquette [1]. This enzyme, which has no action on normal DNA strands or on alkylated sites [2], has been completely purified by Verly and Rassart [3]; it is a monomeric protein of $M_r = 32000$. Depurinated DNA has been repaired *in vitro* by Verly *et al.* [4] with three enzymes: the endonuclease specific for apurinic sites from *E. coli*, DNA polymerase I and the four deoxynucleoside triphosphates, ligase and its coenzyme. Mutants of *E. coli* lacking DNA polymerase I are very sensitive to methylmethanesulfonate and it is known that the lethal action of this alkylating agent is due mostly to the depurination of DNA [5]. On the other hand, however, mutations in gene *xthA*, which codes for the endonuclease specific for apurinic sites, only slightly increase the sensitivity of *E. coli* to methylmethanesulfonate [6]; this is most likely due to the fact that *E. coli* possesses two enzymes active on apurinic sites: the main enzyme which we have isolated is responsible for 90%, whereas another enzyme, more thermoresistant, called endonuclease IV, is responsible for the rest of the activity [6].

The main endonuclease specific for apurinic sites of *E. coli* is not the endonuclease II of Goldthwait *et al.* [7,8]. In contrast with the main endonuclease specific for apurinic sites, endonuclease II has *N*-glycosidase activity removing N^3-methyladenine and O^6-methylguanine from alkylated DNA [9]. Recently Kirtikar and Goldthwait [10] resolved endonuclease II into two enzymes: one specific for apurinic sites and another working on alkylated and arylalkylated DNA, which has *N*-glycosidase activity; the authors kept the name of endonuclease II for the enzyme active on the alkylated sites. Lindahl [11] has also shown that the *N*-glycosidase, removing N^3-alkyladenine from DNA, and the main endonuclease specific for apurinic sites are different enzymes.

Yajko and Weiss [12] and Weiss [13] have presented genetic and biochemical data from which they concluded that exonuclease III and endonuclease II were the same protein. Since their assay on alkylated DNA was not specific for alkylated sites (alkylated DNA always contains apurinic sites), we have investigated the possibility that exonuclease III might be another

Enzymes. Pancreatic deoxyribonuclease (EC 3.1.4.5); micrococcal nuclease (EC 3.1.4.7); *E. coli* DNA polymerase I (EC 2.7.7.7); *E. coli* alkaline phosphatase (EC 3.1.3.1); snake venom phosphodiesterase (EC 3.1.4.1); spleen phosphodiesterase (EC 3.1.4.18); polynucleotide 5′-hydroxyl-kinase (EC 2.7.1.78).

activity of the main endonuclease for apurinic sites. Ljungquist *et al.* [6] have shown that *xthA* mutants lose simultaneously the main endonuclease for apurinic sites and exonuclease III.

The main objective of this paper is a description of the molecular mechanism of the repair of depurinated DNA. We show how the apurinic site is excised when DNA is exposed *in vitro* to the main endonuclease for apurinic sites of *E. coli* and DNA polymerase I. The action in this repair of exonuclease III, which was present in our preparation, is described; it might be important especially if the exonucleolytic activity belongs to the endonuclease for apurinic sites.

In addition to the experiments on the nature of the hydrolysis products, our work also deals with other properties of the main endonuclease for apurinic sites of *E. coli*: specificity, optimum pH, thermosensitivity, divalent cation requirements. To avoid any further confusion with endonuclease II and the new endonuclease IV, we suggest to give the name of endonuclease VI to the main endonuclease for apurinic sites of *E. coli*.

MATERIALS AND METHODS

The Substrates

Labelled Alkylated-Depurinated DNA. Labelled DNA was prepared from *E. coli* B41 as previously described by Verly and Rassart [3]; the culture medium contained [^{3}H]thymidine (2 μCi/ml; New England Nuclear) or monopotassium [^{32}P]phosphate (1 μCi/ml; New England Nuclear). The DNA labelled with ^{3}H (50000 dis. min^{-1} μg^{-1}) or with ^{32}P (about 30000 dis. min^{-1} μg^{-1}) was alkylated with 0.3 M methylmethanesulfonate in a 0.5 M sodium phosphate buffer, pH 7.0, for 1 h at 37 °C; after removal of the alkylating agent, the solution was heated at 50 °C during 6 h, then dialyzed against 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 (NaCl/Cit) containing 0.01 M $MgCl_2$. The double-stranded DNA contained approximately 1 alkylated site/7 nucleotides, and 1 apurinic site/20 nucleotides [2]; treatment with NaOH yielded an acid-soluble fraction of 35%.

Labelled Acid-Depurinated DNA. To one volume of *E. coli* [^{32}P]DNA in NaCl/Cit (200 μg/ml) was added one volume of 0.5 M sodium acetate buffer, pH 4.0; the mixture was incubated at 37 °C for 120 h, then dialyzed at 4 °C against NaCl/Cit, 0.01 M $MgCl_2$. The double-stranded DNA contained an average of about 1 apurinic site/100 nucleotides.

Reduced Depurinated DNA. Reductions were performed with sodium borohydride with or without ^{3}H label. For non-radioactive reduction the tube, containing 250 μl alkylated-depurinated [^{3}H]DNA or [^{32}P]DNA in NaCl/Cit, 0.01 M $MgCl_2$, and 250 μl 0.2 M sodium borate, was cooled at 0 °C; the pH of the mixture was about 9.5. After addition of 50 μl $NaBH_4$ (3.75 mg) in 0.05 M NaOH, the tube was kept at 0 °C for 30 min. Addition of 50 μl of 1 M H_3PO_4 produced a vigorous bubbling and the pH fell to about 7.5. The solution was then dialyzed against NaCl/Cit, 0.01 M $MgCl_2$, at 4 °C. For radioactive reduction, acid-depurinated or alkylated-depurinated DNA, unlabelled or labelled with ^{32}P, was used. Sodium boro-[^{3}H]hydride (200 Ci/mol; New England Nuclear) was dissolved in cold 0.05 M NaOH to a final concentration of 10 μg/μl. 2 ml DNA (200 μg) solution was dialyzed against 0.01 M sodium borate buffer, pH 9.8. Portions of 50, 25 and 25 μl of the labelled hydride solution were successively added and the mixture was left at room temperature for 1 h after each addition; 200 μl non-labelled sodium borohydride (15 mg) also in 0.05 M NaOH was finally added. After 30 min the excess hydride was destroyed with 0.2 ml 1 M sodium acetate buffer, pH 5.0. When the hydrogen release was completed (60 min), the solution was dialyzed at 4 °C for a week against six changes of 2 l NaCl/Cit, 0.01 M $MgCl_2$, adjusted to pH 6.0. A control DNA that had not been depurinated was used to check that most of the ^{3}H was localized at the apurinic sites (Table 1).

Table 1. *Radioactivities of DNA samples reduced with sodium boro-[^{3}H]hydride*

Sample	^{3}H
	dis. min^{-1} μg^{-1}
Untreated	470
Alkylated-depurinated	16500
Acid-depurinated	2320

Labelled Sonicated DNA. E. coli [^{3}H]DNA (50 μg/ml in 0.05 M Tris · HCl, 0.8 mM $MgCl_2$, pH 8.0) was sonicated with eight 30-s pulses of a Branson W-185C sonicator at the lowest output (20 W) using the microtip.

Labelled 3'-OH/5'-Phosphate Nicked DNA. The reaction mixture contained 200 μg *E. coli* [^{3}H]DNA and 0.5–5 units pancreatic deoxyribonuclease (Sigma Chemicals Co.) in 1.5 ml 0.1 M NaCl, 0.01 M sodium citrate, 0.017 M Tris · HCl, 0.17 mM $MgCl_2$, pH 7.5. After an incubation of 15 min at 37 °C, the solution was heated for 10 min at 77 °C, then cooled on ice. This treatment inactivated the enzyme without denaturing the DNA.

Labelled 3'-Phosphate/5'-OH Nicked DNA. The reaction mixture contained 30 μg *E. coli* [^{32}P]DNA and 1 or 1.6 units of micrococcal nuclease (Worthington) in 1 ml 0.05 M glycine, 0.01 M $CaCl_2$, pH 9.2. After a 15-min incubation at 37 °C, the solution was dialyzed at 4 °C against NaCl/Cit, 0.01 M $MgCl_2$, to eliminate the calcium.

Doubly Labelled 3'-OH/5'-Phosphate Nicked DNA. The reaction mixture contained: 250 µl 3'-OH/5'-phosphate nicked [^{32}P]DNA (45 µg); 500 µl 0.4 M glycine, 0.04 M $MgCl_2$, 6 mM 2-mercaptoethanol, pH 9.2; 500 µl of an aqueous solution of dATP, dGTP, dCTP (100 nmol each) and 4.5 µCi [^{3}H]dTTP (18.9 Ci/mmol; New England Nuclear); 750 µl purified DNA polymerase I (3.75 units). After a 30-min incubation at 37 °C, the mixture was heated for 15 min at 45 °C; the DNA was purified by phenol extraction [14] and the aqueous solution was extensively dialyzed against NaCl/Cit, 0.01 M $MgCl_2$.

Labelled Alkylated DNA from T7 Bacteriophage. T7 phages were labelled with [^{3}H]thymidine as described by Verly *et al.* [15]; the DNA, isolated by the phenol method of Mandell and Hershey [14], had a specific radioactivity of 240000 dis. min^{-1} μg^{-1}. The T7 [^{3}H]DNA in 0.15 M $NaNO_3$, 0.2 M sodium phosphate, pH 7, was mixed with an equal volume of 0.02 M methylmethanesulfonate in the same buffer, and incubated for 2 h at 37 °C. After cooling to 4 °C, the reaction mixture was dialyzed against NaCl/Cit, 0.01 M $MgCl_2$. The alkylated T7 [^{3}H]DNA contained approximately 165 methyl groups/strand.

The Enzymes

Endonuclease VI. Endonuclease VI was purified according to Verly and Rassart [3]; two preparations were used: prep. IV, after the phosphocellulose step, was in 0.04 M sodium phosphate, 0.15 M NaCl, pH 6.5; prep. V, after Sephadex G-75, was in 0.05 M Tris · HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8.0, with an equal volume of glycerol. Both were kept at −20 °C. For the experiments, these preparations were diluted with a suitable buffer.

The assay measures the release of acid-soluble radioactivity from alkylated-depurinated [^{3}H]DNA [2]. To 20 µl of NaCl/Cit, 0.01 M $MgCl_2$, containing 1 µg alkylated-depurinated [^{3}H]DNA, were added 10 µl enzyme solution in 0.05 M Tris · HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 8.0; the mixture was incubated for 10 min at 37 °C. After cooling on ice, 100 µl NaCl/Cit containing 200 µg calf thymus DNA (Sigma Chemicals Co.) and perchloric acid to a final concentration of 5% were successively added; after centrifugation, the acid-soluble radioactivity was measured. Results were corrected for controls without enzyme, which were always below 1%. 1 unit enzyme activity releases 10% of the total radioactivity in acid-soluble form.

Exonuclease III. The phosphatase assay measures the release of radioactive inorganic phosphate from 3'-phosphate/5'-OH nicked [^{32}P]DNA [16]. The reaction mixture contained the enzyme and 0.6 µg of the substrate in 0.2 ml 0.1 M potassium phosphate, 15 mM 2-mercaptoethanol, pH 7.0; after an incubation of 30 min, the acid-soluble radioactivity which was not adsorbed on Norit was measured. The exonuclease assay measures the conversion of sonicated DNA into acid-soluble nucleotides [16]. The unit of exonuclease III is defined as the activity causing the release of 1 nmol acid-soluble nucleotides in 30 min.

DNA Polymerase I. DNA polymerase I from *E. coli* corresponding to fraction IV of Richardson *et al.* [17], was purchased from P.L. Biochemicals; it was contaminated by several nucleases and particularly by endonuclease VI. 3 mg of this preparation, dissolved in 1 ml 0.05 M Tris · HCl, 0.01 M 2-mercaptoethanol, pH 7.5, was applied to a 1.6 × 60 cm column of Sephadex G-70 (Pharmacia) equilibrated with the same buffer; the elution was at a rate of 8 ml/h. The enzyme was assayed by the method of Richardson *et al.* [17]. The most active fractions were pooled; the purified enzyme had no detectable activity on untreated or depurinated DNA.

Using calf thymus DNA activated with pancreatic deoxyribonuclease, the enzyme unit is defined as the activity causing the incorporation of 10 nmol nucleoside triphosphates into acid-insoluble polynucleotides in 30 min at 37 °C [18].

Other Enzymes. E. coli alkaline phosphatase, snake venom phosphodiesterase and spleen phosphodiesterase were bought from Worthington Biochemical Corporation. Polynucleotide 5'-hydroxyl-kinase was a gift from Dr P. Bannon of our laboratory; it was purified from T4-phage-infected *E. coli* B following the method of Richardson [19].

Other Techniques

Chromatographic Separation of Oligonucleotides. The method of Junowicz and Spencer [20] was used. A DEAE-Sephadex (Pharmacia) column of 1 × 53 cm was equilibrated in 5 mM Tris · HCl, 7 M urea, pH 7.6 buffer (Tris/urea), at 65 °C. The samples of DNA solution, 10-fold diluted with Tris-urea, were applied onto the column and the elution was carried out, at a rate of 30 ml/h, with 400 ml of a 0−0.4 M LiCl linear gradient in Tris/urea. The ^{3}H and/or ^{32}P radioactivities were measured in the 2.5-ml fractions. Calibration has shown that mononucleotides were eluted at 0.19 M, and dinucleotides at 0.33 M LiCl. After the gradient, the LiCl molarity was abruptly raised to 1 M to elute the large DNA pieces.

Determination of the Number of Breaks in T7 DNA Strands. The denatured T7 [^{3}H]DNA was centrifuged on neutral sucrose gradients and the average number of breaks per strand was determined from the sedimentation profile by computer analysis following the technique of Gillespie *et al.* [21] adapted by Crine and Verly [22].

Radioactivity Measurements. The sample to be analyzed was diluted with water to a volume of 1 ml and prepared for counting by addition of 10 ml of a

scintillation mixture made of 1 part Triton X-100 and 2 parts toluene containing 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP); radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. For tritium, an internal [^{3}H]-hexadecane standard was used. For ^{32}P, the yield was near 100%, but a standard was used to correct for decay and calculate 0 time values. For both radionuclides, the results are given in disintegrations/min.

The radioactivity of precipitates collected on fiberglass filters was measured in 10 ml toluene containing 0.4% (w/v) PPO and 0.01% (w/v) dimethyl-POPOP. The results are presented in counts/min.

NaOH Treatment. To 1 vol. of DNA solution was added 1 vol. of 0.4 M NaOH; after 15 min at 37 °C, the mixture was neutralized with 1 vol. of 0.4 M HCl. This treatment was found adequate to hydrolyze a phosphoester bond near each apurinic site [2].

Determination of Acid-Soluble Radioactivity. To x ml radioactivity DNA solution (50–500 µl containing between 0.4 and 10 µg DNA) were added 0.1 ml calf thymus DNA (200 µg) in NaCl/Cit and $2(x + 0.1)$ ml 7.5% perchloric acid. After shaking, the mixture was left on ice for 5 min, centrifuged for 15 min at $12000 \times g$, and the ^{32}P and/or ^{3}H radioactivities of the supernatant were measured.

Formamide Denaturation. The DNA solution (1 ml) was dialyzed at 4 °C against 250 ml of 95% formamide [formamide freshly redistilled under reduced pressure, 0.015 M EDTA (95/5; v/v), adjusted to pH 7] with two changes. The temperature was then raised at 37 °C for 2 h. The formamide was finally eliminated by dialysis against a suitable buffer.

EXPERIMENTS AND RESULTS

The preparations of endonuclease VI used in these experiments were either prep. IV or prep. V of Verly and Rassart [3] (see Materials and Methods).

Restriction of Action of Prep. IV on Alkylated T7 DNA at Alkali-Labile Sites

Alkylated [^{3}H]DNA from bacteriophage T7 (8 µg in 1 ml NaCl/Cit, 0,01 M $MgCl_2$), containing approximately 165 methyl groups/strand, was incubated at 37 °C with prep. IV. A control, without enzyme, was run in parallel. After 0, 1, 2 and 3 h, aliquots were taken from assay and control, incubated for 15 min at 37 °C in 0.2 M NaOH, then neutralized; centrifugation on neural sucrose gradients enabled us to determine the average number of breaks per strand, Four successive experiments were carried out; individual results, means and standard errors are presented in Table 2. Before alkylation, the DNA, after treatment with NaOH, contained an average of 0.3 break/strand. The table shows that, after alkylation, the NaOH-treated DNA contained an average of 6.5 breaks/strand; this number increased during incubation at 37 °C to reach 18 breaks/strand after 3 h. But, on the other hand, Table 2 shows clearly that, at any time during incubation, the number of breaks recorded was the same whether the enzyme was present or not.

Action of Prep. IV on Reduced Alkylated-Depurinated DNA

One of two tubes containing 250 µl alkylated-depurinated [^{3}H]DNA in NaCl/Cit, 0.01 M $MgCl_2$ (15 µg/ml) was reduced with $NaBH_4$ (see Materials and Methods), while the other was treated identically without hydride. Both solutions were subsequently dialyzed against NaCl/Cit, 0.01 M $MgCl_2$ at 4 °C. Aliquots (60 µl) of the alkylated-depurinated [^{3}H]-DNA reduced or not were mixed with 30 µl of either prep. IV (7 units), or 0.4 M NaOH, or NaCl/Cit, 0.01 M $MgCl_2$ (control). After 15 min at 37 °C the mixtures were cooled in ice and the acid-soluble radioactivities were measured; the results obtained for the enzyme and NaOH were corrected for the control.

Table 2. *Action of endonuclease VI on alkylated T7 DNA*
Alkylated T7 DNA (8 µg), containing approximately 165 methyl groups/strand, was incubated at 37 °C with 1 ml NaCl/Cit, 0.01 M $MgCl_2$ (control) or with 200 units prep. IV in 1 ml of the same buffer. After the indicated time, aliquots were denatured with NaOH and the average number of breaks per strand was determined. The table gives the results of four successive experiments and the means with their standard errors (from P. Crine)

Expt.	Average number of breaks per strand							
	0 h		1 h		2 h		3 h	
	enzyme	control	enzyme	control	enzyme	control	enzyme	control
1	6.81	5.49	12.32	9.56	12.58	12.57	17.90	19.20
2	6.40	8.10	10.80		12.60	15.80	17.90	17.80
3	5.80	6.30	9.90	11.20	13.10	12.10	16.30	19.50
4	6.40	7.30	10.80	13.60	15.90	14.80	19.00	16.50
Mean	6.35 ± 0.65	6.79 ± 0.56	10.95 ± 0.69	11.45 ± 0.83	13.54 ± 0.79	13.81 ± 0.88	17.77 ± 0.56	18.25 ± 0.69

Table 3. *Action of NaOH and endonuclease VI on reduced alkylated-depurinated DNA*
Aliquots (60 µl) of alkylated-depurinated [^{3}H]DNA or of alkylated-depurinated [^{3}H]DNA reduced with $NaBH_4$ (0.4 µg DNA) in NaCl/Cit, 0.01 M $MgCl_2$, were incubated for 15 min at 37 °C with 30 µl prep. IV (7 units), or 30 µl 0.4 M NaOH, or 30 µl of buffer (control). The acid-soluble radioactivity was measured and corrected for the corresponding control; the results are expressed as fractions of the substrate radioactivity

Substrate	Proportion of radioactivity in acid-soluble fraction	
	NaOH	prep. IV
Alkylated-depurinated DNA	0.404	0.212
Reduced alkylated-depurinated DNA	0.011	0.254

Table 3 shows that reduction of alkylated-depurinated DNA slightly increased the endonuclease VI activity; on the other hand, it suppressed the action of NaOH.

EDTA and Divalent Cations

Prep. IV (50 units/ml) and the alkylated-depurinated [^{3}H]DNA solution (15 µg/ml) were dialyzed against NaCl/Cit. Aliquots of 20 µl from the two solutions were mixed in several test-tubes which received, in addition, 4 µl 100 mM $MgCl_2$ or 4 µl EDTA solution of concentration ranging from 0 to 100 mM. After a 30-min incubation at 37 °C, the acid-soluble radioactivity was measured and the results were corrected for controls without enzyme. Table 4 shows that endonuclease VI was active whether Mg^{2+} was present or not in the incubation medium. On the other hand, EDTA strongly inhibited the enzyme activity at concentration greater than 1 mM.

To investigate further the role of Mg^{2+}, prep. V (45 units/ml) and the alkylated-depurinated [^{3}H]DNA (15 µg/ml) were dialyzed against 0.1 M Tris · HCl, 10 mM EDTA, pH 8.0, in order to remove the divalent cations, then against the same buffer without EDTA; when aliquots from the two solutions were mixed and incubated at 37 °C, no enzyme activity could be found. The activity was, however, restored on addition of Mg^{2+}, 20 mM being the optimum concentration. Among the other divalent cations tested, Ca^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} were unable to replace Mg^{2+}, although Ca^{2+} had a slight activity (Table 4).

pH Requirement

Solutions of 0.05 M Tris · HCl buffers, containing 10 mM $MgCl_2$, were adjusted to different pH ranging from 6.5 to 9.5. Aliquots of the alkylated-depurinated [^{3}H]DNA were dialyzed against each of these buffers, whereas aliquots of prep. IV were diluted 100-fold with the same buffers containing 0.1 % bovine serum albumin. Portions of 50 µl of enzyme and substrate solutions of the same pH were mixed. All the mixtures were incubated for 15 min at 37 °C and the acid-soluble radioactivity measured; the results were corrected for controls without enzyme. The optimum pH for the endonuclease VI activity was 8.5.

Table 4. *Effect of EDTA and divalent cations on endonuclease VI activity*
For the first experiment, substrate and prep. IV were extensively dialyzed against NaCl/Cit. For the second experiment, substrate and prep. V were extensively dialyzed against 0.1 M Tris · HCl, pH 8.0, containing 10 mM EDTA, then against the same buffer without EDTA. Each assay was made of 20 µl alkylated-depurinated [^{3}H]-DNA (0.3 µg) solution and 20 µl dialyzed enzyme (about 1 unit); 4 µl EDTA or the chosen cation solution was added to reach the indicated concentration (mM). After 30 min at 37 °C, the acid-soluble radioactivity was measured and corrected for a suitable control without enzyme; the corrected values are expressed as fractions of the substrate total radioactivity

Experiment	Addition	Concn	Proportion of radioactivity in acid-soluble fraction
		mM	
1	EDTA	0.0	0.155
		0.1	0.140
		0.5	0.140
		1.0	0.033
		5.0	0.003
		10.0	0.006
	Mg^{2+}	10.0	0.160
2	Mg^{2+}	0.0	0.000
		2.0	0.010
		5.0	0.036
		10.0	0.092
		20.0	0.139
		30.0	0.116
		40.0	0.103
		50.0	0.094
		70.0	0.031
		100.0	0.014
	Ca^{2+}	0.0	0.000
		2.0	0.001
		5.0	0.008
		10.0	0.009
		20.0	0.006
		30.0	0.008
		40.0	0.006
		50.0	0.002
	Mn^{2+}	10.0	0.000
	Zn^{2+}	10.0	0.000
	Cu^{2+}	10.0	0.000

Exonuclease and Phosphatase Activities of Prep. V

Some acid-soluble radioactivity was liberated by prep. V from normal [^{3}H]DNA, but the amount was considerably increased after sonication; analysis of the

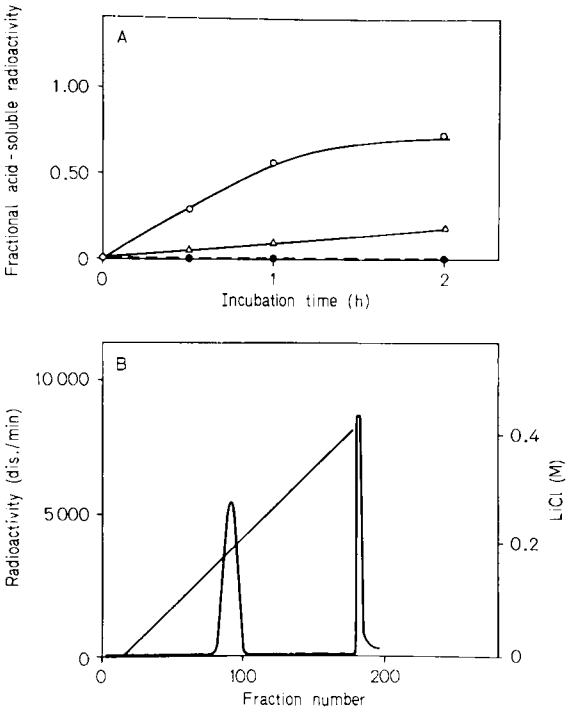

Fig. 1. *Exonucleolytic activity of prep. V on sonicated DNA.* (A) Several tubes containing 20 µl solution of untreated (△) or sonicated (○) [^{3}H]DNA (1 µg) and 10 µl prep. V (1 unit) were incubated at 37 °C for various times before measuring the acid-soluble radioactivity. The results are expressed as fractions of the total radioactivity. Controls without enzyme were treated with NaOH before determining the acid-soluble radioactivity; the values were at all times negligible for normal as well as for sonicated [^{3}H]DNA (●). (B) The reaction mixture (0.1 ml) containing prep. V (1.5 units) and sonicated [^{3}H]DNA (2.5 µg), after a 2-h incubation at 37 °C, was 10-fold diluted with Tris/urea and submitted to chromatography on DEAE-Sephadex in the presence of 7 M urea (see Materials and Methods). The column was eluted with a linear 0–0.4 M LiCl gradient, then with 1 M LiCl. Radioactivity (dis./min) is given for each collected fraction

incubation medium on DEAE-Sephadex indicated the presence of mononucleotides (Fig. 1) Incubation of 3′-OH/5′-phosphate nicked [^{3}H]DNA with prep. V also released labelled mononucleotides (Table 5).

Aliquots (200 µl) of alkylated-depurinated [^{32}P]-DNA (6 µg), reduced with $NaBH_4$ or not, were incubated at 37 °C for 60 min with 200 µl prep. V (18 units). Another 200-µl aliquot of the non-reduced alkylated-depurinated [^{32}P]DNA was incubated at 37 °C for 15 min with 200 µl 0.4 M NaOH. All mixtures were subsequently analyzed on DEAE-Sephadex. Table 5 shows that prep. V released mononucleotides from alkylated-depurinated DNA and that this exonucleolytic activity, which followed the endonucleolytic cleavage near apurinic sites, was not affected by reduction of the apurinic sites with $NaBH_4$. On the other hand, no significant amount of mononucleotides was set free from non-reduced alkylated-depurinated DNA by NaOH treatment.

[^{32}P]DNA, activated with pancreatic deoxyribonuclease, was incubated with DNA polymerase I and ^{3}H-labelled deoxynucleoside triphosphates (see Materials and Methods); this treatment left a 3′-OH/5′-phosphate nicked double-stranded DNA and the strand pieces between the nicks were labelled with ^{3}H at the 3′-end and with ^{32}P at the 5′-end. To several tubes were added 40 µl NaCl/Cit, 0.01 M $MgCl_2$, containing 0.6 µg of this 3′-OH/5′-phosphate nicked doubly-labelled DNA, and 20 µl prep. V (1.8 units); the incubation was stopped at various times to determine the ^{3}H and ^{32}P acid-soluble radioactivities. Fig. 2 shows that ^{3}H was released in the acid-soluble fraction much faster than ^{32}P which suggests that, starting from the nicks, the exonuclease degraded the DNA strands in the 3′→5′ direction.

Table 5. *Exonucleolytic action of prep. V on 3′-OH/5′-phosphate nicked DNA, alkylated-depurinated DNA, and reduced alkylated-depurinated DNA*

Acid-soluble radioactivity and radioactivity in mononucleotides are expressed as percentages of the substrate radioactivity. (A) The solution (60 µl) of 3′-OH/5′-phosphate nicked [^{32}P]DNA (11 µg) was incubated at 37 °C for 60 min with 60 µl prep. V (5.5 units). A control was carried out where prep. V was replaced by buffer. At the end of the incubation, 20-µl aliquots were used to measure the acid-soluble radioactivity, and the remaining solution was chromatographed on DEAE-Sephadex (see Materials and Methods). (B, C) The solution (200 µl) of alkylated-depurinated [^{32}P]DNA (6 µg), reduced with $NaBH_4$ or not, was incubated at 37 °C for 60 min with 200 µl prep. V (18 units). A 200-µl aliquot of the non-reduced alkylated-depurinated [^{32}P]DNA was also incubated at 37 °C for 15 min with 200 µl 0.4 M NaOH. All mixtures were subsequently analyzed on DEAE-Sephadex

[^{32}P]DNA	Prep. V	NaOH	Radioactivity	
			acid-soluble	mononucleotides
			% of substrate	
A. 3′-OH/5′-P nicked DNA	−	−	43	0
	+	−	43	9
B. Alkylated-depurinated	−	+	42	0
	+	−	50	17
C. Reduced alkylated-depurinated	+	−	49	15

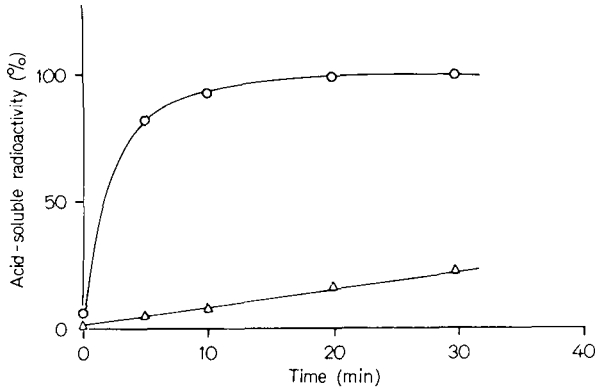

Fig. 2. *Direction of the exonuclease activity of prep. V.* Doubly labelled 3′-OH/5′-phosphate nicked DNA, whose strand pieces between nicks were labelled with ^{3}H at the 3′-end and with ^{32}P at the 5′-end, was incubated with prep. V and the appearance of ^{3}H (○) and ^{32}P (△) in the acid-soluble fraction (% total radioactivity of each radionuclide) was measured at different times

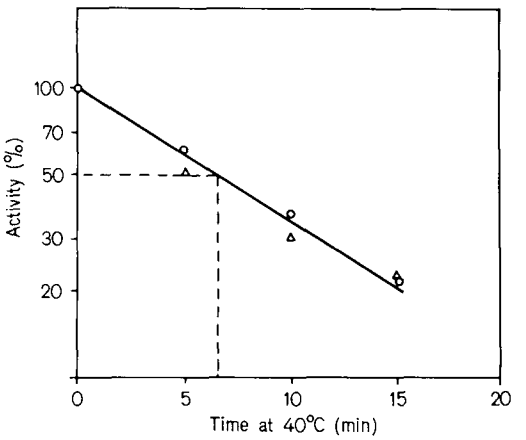

Fig. 3. *Thermosensitivities of the endonuclease VI and exonuclease III of prep. V.* Aliquots of prep. V were heated at 40 °C for 0–15 min. The residual endonuclease VI activity was tested on alkylated-depurinated [^{3}H]DNA (○), and the residual exonuclease III activity on sonicated [^{3}H]DNA (△). The results, as percentages of initial activities, are plotted on a semi-logarithmic scale

To look for a 3′-phosphatase activity, 20-µl aliquots of 3′-phosphate/5′-OH nicked [^{32}P]DNA (0.6 µg) were mixed with up to 50 µl (= 4.5 units) of prep. V, and the volume was completed to 200 µl with 0.01 M potassium phosphate, 1.5 mM 2-mercaptoethanol, pH 7.0. After 30 min at 37 °C, the mixture was cooled on ice and the acid-soluble radioactivity, which was not adsorbed on Norit, was measured. Radioactive inorganic phosphate was released by prep. V proportionally to the number of nicks present in the substrate (results not shown).

The thermosensitivities of the endonuclease VI and exonuclease activities of prep. V were compared. Aliquots (150 µl) of prep. V (4.5 units) were heated at 40 °C for 0–15 min in polypropylene test-tubes, then quickly cooled in ice. The remaining endonucleolytic activity was tested on alkylated-depurinated [^{3}H]DNA as usual, while the remaining exonucleolytic activity was tested on sonicated [^{3}H]DNA. The results, expressed as percentages of the initial activities, were plotted on a semi-logarithmic scale; Fig. 3 shows that the half-lives of the exonuclease and of endonuclease VI were not significantly different and were about 7 min.

The Phosphoester Bond Hydrolyzed by Endonuclease VI: Nature of the Ends Resulting from the Nicking

Aliquots (20 µl) of prep. V (1 unit) were mixed with 20 µl NaCl/Cit, 0.01 M $MgCl_2$, containing 1 µg alkylated-depurinated [^{32}P]DNA, and incubated at 37 °C. At times ranging from 0–30 min, the total acid-soluble radioactivity and the acid-soluble radioactivity not adsorbed on Norit were measured. Although the endonuclease had the expected activity, the amount of radioactive inorganic phosphate released was negligible (2.4 pmol). This result suggests that the endonuclease VI did not leave a 3′-phosphate, which would have been hydrolyzed by the 3′-phosphatase activity of prep. V (producing 150 pmol radioactive inorganic phosphate), but rather a 3′-OH.

Alkylated-depurinated DNA (600 µg) in 500 µl NaCl/Cit, 0.01 M $MgCl_2$, was mixed with 500 µl prep. IV (25 units) in the same buffer, or 500 µl buffer, or 500 µl 0.4 M NaOH; all three mixtures were incubated at 37 °C for 60 min, then cooled in ice. They were subsequently denatured with formamide and dialyzed at 4 °C successively against NaCl/Cit containing 6% formaldehyde and against NaCl/Cit alone. Aliquots (20 µl) from each reaction mixture were mixed with 165 µl 0.1 M Tris · HCl, pH 8.0, containing 1 unit of *E. coli* alkaline phosphatase or with 165 µl of the same buffer without the enzyme. All tubes were incubated at 37 °C for 30 min, then cooled in ice and supplemented with 100 µl 0.6 M $MgCl_2$ and 5 µl 1.02 M 2-mercaptoethanol; to two of the tubes (with and without phosphatase treatment), were added 10 µl 0.05 M sodium phosphate, 0.25 M KCl, pH 7.5, whereas, to two other tubes (with and without phosphatase treatment), were added 10 µl of the same buffer containing 6.5 units of polynucleotide 5′-hydroxyl-kinase. All tubes finally received 20 µl 0.25 mM [γ-^{32}P]-ATP (10^{12} dis. min^{-1} $mmol^{-1}$) and were incubated at 37 °C for 60 min. After addition of 100 µl NaCl/Cit containing 10 µg calf thymus DNA, 300 µl 0.1 M sodium pyrophosphate, and 2.5 ml 0.4 M trichloroacetic acid containing 0.02 M sodium pyrophosphate, the mixtures were filtered through glass-fiber discs (Whatman GF/C); the discs were washed with 0.4 M trichloroacetic acid containing 0.02 M sodium pyrophosphate, dried, and counted for radioactivity. The

Table 6. *Nature of the 5′-ends in alkylated-depurinated DNA treated by NaOH or endonuclease VI*
Alkylated-depurinated DNA was incubated at 37 °C for 60 min with prep. IV, or with NaOH, or with neither of them (control). The DNA was denatured with formamide and half of the samples were treated with alkaline phosphatase. The DNA was then incubated with polynucleotide kinase in the presence of [γ-^{32}P]ATP; an incubation without the latter enzyme was run in parallel. The DNA was precipitated with trichloroacetic acid, collected on fiber-glass filters and the incorporated radioactivity determined. Using the [γ-^{32}P]-ATP specific radioactivity, the number of γ-P atoms incorporated/10^6 daltons DNA was calculated; the results were corrected for the corresponding samples without polynucleotide kinase (blanks) and also for the control values (from Y. Paquette)

Treatment	Alkaline phosphatase	γ-P atoms from ATP/10^6 daltons DNA	
		corrected blank	corrected control
Control	–	1.0	0.0
	+	3.7	0.0
Endonuclease VI	–	2.1	1.1
	+	13.5	9.8
NaOH	–	1.2	0.1
	+	12.9	9.2

results given by the specimens treated with the polynucleotide 5′-hydroxyl-kinase were corrected for the values obtained without the enzyme; knowing the [γ-^{32}P]ATP specific radioactivity, the number of γ-P atoms from ATP incorporated/10^6 daltons of DNA was calculated. Table 6 shows that enzyme and NaOH gave approximately the same number of 5′-ends that could be labelled after alkaline phosphatase treatment; on the other hand, without alkaline phosphatase treatment, the number of 5′-ends that could be labelled was negligible in both cases. It can be concluded that the single-strand breaks, introduced in alkylated-depurinated DNA by NaOH or endonuclease VI, are limited by 5′-phosphate.

Localization of the Phosphoester Bond Hydrolyzed by Endonuclease VI

Exonuclease Activity of Prep. V. An aliquot (140 µl) of the solution of alkylated-depurinated DNA (7 µg) reduced with sodium boro[^{3}H]hydride was incubated for 1 h at 37 °C with 140 µl prep. V (15 units); the mixture was then chromatographed on DEAE-Sephadex: the radioactivity was released only with large DNA fragments when the LiCl concentration was raised ro 1 M (results not shown), suggesting that the reduced apurinic site was not excised by the exonuclease activity of prep. V. Confirmatory evidence was given in the following experiment. Aliquots (20 µl) of the solution

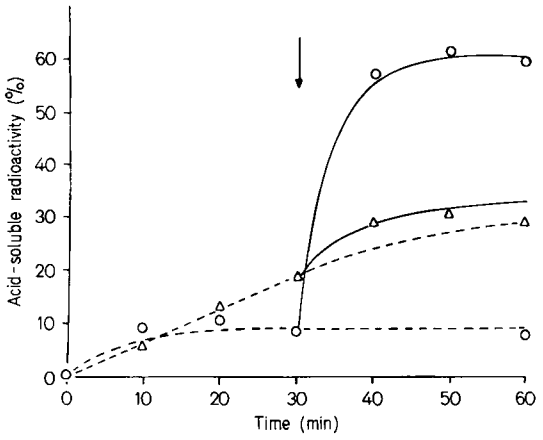

Fig. 4. *Excision, by DNA polymerase I, of apurinic sites reduced with sodium borohydride from DNA previously treated with endonuclease VI.* Acid-depurinated [^{32}P]DNA, reduced with sodium [^{3}H]borohydride, was incubated at 37 °C with prep. V; the release of the two radionuclides in the acid-soluble fraction (% total radioactivity of the nuclide) was followed as a function of time in some tubes (- - -). After 30 min DNA polymerase I and the four deoxyribonucleoside triphosphates were added (arrow) to other tubes, and the acid-soluble radioactivity was also followed (——). (○) ^{3}H; (△) ^{32}P

of acid-depurinated[1] [^{32}P]DNA (1.4 µg) reduced with sodium [^{3}H]borohydride and 20 µl prep. V (1.8 units) was incubated at 37 °C in several tubes, and the ^{3}H and ^{32}P radioactivities in the acid-soluble fraction were measured at different times. Fig. 4 shows the appearance of a small amount of acid-soluble ^{3}H, resulting from the endonuclease VI activity, which was quickly at a plateau; there was a similar release of acid-soluble ^{32}P which, however, continued to rise owing to the exonuclease activity of prep. V. In some tubes, after 30 min, were added 50 µl 0.4 M glycine, 0.04 M $MgCl_2$, 6 mM 2-mercaptoethanol, pH 9.2 buffer, 50 µl water containing 10 nmol of each of the four deoxynucleoside triphosphates, and 50 µl of the purified DNA polymerase I (1 unit). Fig. 4 shows that the ^{32}P release was not much increased on addition of DNA polymerase I, whereas it was this enzyme which set free most of ^{3}H. The conclusion is that the apurinic site was excised by DNA polymerase I. Analysis of the acid-soluble fraction on DEAE-Sephadex indicated that most of the ^{32}P was in mononucleotides, whereas the reduced apurinic site labelled with ^{3}H was released in dinucleotides and trinucleotides.

Snake Venom and Spleen Phosphodiesterases: Three tubes containing 100 µl prep. V (9 units) and 100 µl of a solution of alkylated-depurinated [^{32}P]DNA (10 µg) reduced with sodium boro[^{3}H]hydride, were incubated for 30 min at 37 °C, then heated at 100 °C for 15 min and cooled in ice. The first tube was kept as a control. To the second tube was added 100 µl NaCl/Cit, 0.01 M

[1] DNA polymerase I was inactive on alkylated-depurinated DNA pretreated with endonuclease VI.

Table 7. *Action of snake venom and spleen phosphodiesterases on alkylated-depurinated [^{32}P]DNA reduced with sodium boro[^{3}H]hydride and treated with endonuclease VI*
Alkylated-depurinated [^{32}P]DNA reduced with sodium boro[^{3}H]-hydride was incubated with prep. V and subsequently denatured by heating at 100 °C. The doubly labelled DNA was exposed to snake venom phosphodiesterase or, after a treatment with alkaline phosphatase, to spleen phosphodiesterase. The acid-soluble ^{3}H and ^{32}P were then determined, and also the ^{3}H and ^{32}P radioactivity appearing in the mononucleotide peak of a DEAE-Sephadex chromatography. The results are expressed as percentages of the corresponding nuclide radioactivity in the substrate DNA; correction is made for a control, which was not exposed to either of these two phosphodiesterases

Phosphodiesterase	Amount	Radioactivity			
		acid-soluble		mononucleotides[a]	
		^{32}P	^{3}H	^{32}P	^{3}H
	units/μg DNA	%			
None		23	26	7	1
Snake venom	0.04	45	48	14	1
	corrected	22	22	7	0
Spleen	0.2	39	46	22	25
	corrected	16	20	15	24

[a] Or molecules carrying the same electric charge as mononucleotides at pH of the DEAE-Sephadex chromatography.

$MgCl_2$, containing 0.4 unit of snake venom phosphodiesterase, before incubation at 37 °C for 30 min. To the third tube was added 100 μl 0.3 M glycine, pH 8.8, containing 2 units of *E. coli* alkaline phosphatase; after 30 min at 37 °C, the medium was completed with 200 μl 0.3 M succinate, 0.01 M $MgCl_2$, 0.03 M 2-mercaptoethanol, pH 6, and 100 μl water containing 2 units of spleen phosphodiesterase, and the incubation was carried out at 37 °C for another 30 min. Aliquots were taken from each tube to measure the acid-soluble radioactivity, while the rest of the solution was chromatographed on DEAE-Sephadex. Table 7 shows that snake venom phosphodiesterase, which hydrolyzes DNA strands from 3′-OH in the 3′→5′ direction, released ^{32}P into mononucleotides but very little ^{3}H; on the other hand, the spleen phosphodiesterase, which degrades DNA strands from 5′-OH in the 5′→3′ direction, released ^{3}H in molecules behaving chromatographically as mononucleotides. Both results agree with the hypothesis that the strand pieces, obtained by denaturation of the reduced alkylated-depurinated DNA treated with prep. V, carried the reduced apurinic sites at their 5′-end. Spleen phosphodiesterase probably removed a ^{3}H-reduced deoxyribose 3′-phosphate. The rate of this reaction seemed, however, lower than the subsequent removal of nucleoside 3′-phosphates, since the ^{32}P release was rather high as compared to that of ^{3}H (Table 7).

Metaperiodate Treatment. Aliquots (20 μl) of prep. V (1.2 units) in NaCl/Cit, 0.01 M $MgCl_2$, were incubated for 30 min at 37 °C with 20 μl of the same buffer containing alkylated-depurinated DNA (1 μg) reduced with sodium boro[^{3}H]hydride. To one-half of the tubes, was added 20 μl 0.05 M sodium metaperiodate, 1 M sodium acetate, pH 5.0; after 0 – 60 min at 37 °C, the reaction was stopped by addition of 10 μl 20% glycerol. The other tubes were treated identically except that the sodium metaperiodate was omitted. The metaperiodate treatment did not, at any time, increase the acid-soluble radioactivity (results not shown).

DISCUSSION

The Exonuclease Activity of Prep. V

We previously published [2] that purified endonuclease VI did not produce acid-soluble material when incubated with double-stranded DNA. However, when DNA of low molecular weight or containing many single-strand breaks is used, an increase of the acid-soluble fraction is detected. The effect is greatly enhanced when the DNA is sonicated or activated with pancreatic deoxyribonuclease. Analysis of the acid-soluble fraction shows that it is constituted of mononucleotides, which indicates that the preparation contains an exonuclease (Fig. 1, Table 5).

Yajko and Weiss [12] and Weiss [13] have presented genetic and biochemical data from which they concluded that exonuclease III and endonuclease II were two activities of the same protein. Because their assay on DNA alkylated with methylmethanesulfonate did not allow to distinguish between endonuclease II (active on alkylated sites) and endonuclease VI (active on apurinic sites), we wanted to know whether it was not rather endonuclease VI and exonuclease III which were the same protein. Because pure endonuclease VI is very unstable, we used the enzyme at the previous step of purification (prep. V); at this stage, the preparation still contains several proteins, but it is free of any activity on alkylated sites (see later).

Exonuclease III has a 3′→5′ exonuclease activity on double-stranded DNA and a 3′-phosphatase activity [16]. Both activities were found in prep. V (Fig. 2 shows an exonuclease activity working in the 3′→5′ direction), which thus contained exonuclease III. On heating prep. V at 40 °C, the endonuclease VI and exonuclease III activities disappeared simultaneously with a half-life of 7 min (Fig. 3). We have checked that the ratio of exonuclease III and endonuclease VI activities remained approximately the same through several steps of the enzyme purification (prep. III, IV and V, according to Verly and Rassart [3]). Our observations are, however, not sufficient to decide that exonuclease III and endonuclease VI are the same pro-

tein, but, taken together with the genetic data of Yajko and Weiss [12] and Ljungquist *et al.* [6], they strongly support this hypothesis. Recently, however, Kirtikar *et al.* [23] have published that endonuclease II, apurinic acid endonuclease and exonuclease III were three different enzymes, but Ljungquist and Lindahl [24] suggest that the endonuclease for apurinic sites followed by these authors was endonuclease IV rather than endonuclease VI.

We observed that, after the endonuclease VI cleavage near apurinic sites, exonuclease III released mononucleotides from alkylated-depurinated DNA and that the reaction rate was unchanged by reduction of the apurinic sites (Table 5).

The Specificity of Endonuclease VI

The exonuclease III activity may interfere with the demonstration of the specificity for apurinic sites of endonuclease VI by the acid-soluble technique. We already mentioned that DNA of low molecular weight or containing many single-strand breaks, on incubation with prep. V, yielded a detectable acid-soluble fraction. A similar difficulty can be met with alkylated DNA. The demonstration that endonuclease VI has no action on alkylated sites rests on the observation that the acid-soluble fraction is the same whether the alkylated DNA is incubated with the enzyme and then treated with NaOH, or incubated for the same time without enzyme and also treated with NaOH [2,3]. However, when one does not utilize a freshly prepared alkylated DNA and many apurinic sites are already present, the acid-soluble radioactivity is greater when there is an incubation with the enzyme. Such a result might erroneously lead to the conclusion of an action on alkylated sites.

To avoid these difficulties, we have used T7 phage DNA and the centrifugation on sucrose gradient after NaOH denaturation. Strands of T7 DNA contain about 40000 nucleotides and, if the number of internal breaks (primary or secondary to the action of endonuclease VI on apurinic sites) is small (it does not exceed 20/strand in our experiments), removal of a few nucleotides from the strand pieces does not alter their sedimentation profile; by contrast with the acid-solubility test, this technique is remarkably insensitive to the action of exonuclease III.

Paquette *et al.* [2] had shown that, after NaOH denaturation, the sedimentation profile of T7 DNA was not altered by an incubation with purified endonuclease VI. The same result is obtained when the enzyme is incubated with denaturated T7 DNA. The method would have detected 1 break/100000000 daltons of DNA. Our data thus indicate that endonuclease VI has no action on normal strands whether in double-stranded or in single-stranded DNA.

We show in Table 2 that the sedimentation profile of denaturated alkylated T7 DNA, containing 165 methyl groups/strand, was not altered when the NaOH treatment was preceded by an incubation with purified endonuclease VI; this means that the enzyme acts only on alkali-labile sites. If NaOH is known to introduce breaks near apurinic sites [25,26], it is without effect or rather stabilizes the glycosidic bond of alkylated bases [27]. The conclusion is that endonuclease VI has no action on alkylated sites, either of the endonuclease or of the *N*-glycosidase type; it is thus different from endonuclease II of Friedberg and Goldthwait [7], which was a mixture of enzymes, and from the better defined endonuclease II of Kirtikar and Goldthwait [10].

Some General Properties of Endonuclease VI

Dialysis against EDTA inactivates the enzyme (Table 4); it thus seems that divalent cations are needed for its activity. The inactivated enzyme can be restored to full activity with Mg^{2+} ions; Mn^{2+}, Zn^{2+} and Cu^{2+} are inactive, whereas Ca^{2+} has only a slight activity. Mg^{2+} might thus be the physiological activator of endonuclease VI. Although citrate is a mild chelator for Mg^{2+}, extensive dialysis against NaCl/Cit does not decrease the enzyme activity: the activator cation thus appears firmly bound to the enzyme.

The endonuclease VI optimum pH is 8.5. The enzyme is very thermolabile; in the conditions that we used, its half-life was 7 min at 40 °C (Fig. 3).

Reduction of alkylated-depurinated DNA makes it a better substrate for endonuclease VI (Table 3) whereas it suppresses the hydrolytic action of NaOH. The aldehyde function on C-1′ is thus not required for the enzyme action and the mechanism of alkaline hydrolysis of depurinated DNA cannot serve as a model to understand how endonuclease VI works. Indeed, at alkaline pH the nick formation is mostly the result of β-elimination and the hydrolyzed phosphoester bond is on the 3′ side of the apurinic site, whereas the bond hydrolyzed by the enzyme seems to be on the 5′ side (see later).

The Phosphoester Bond Hydrolyzed by Endonuclease VI

The observation that depurinated DNA, after a treatment with endonuclease VI, becomes a substrate for DNA polymerase I of *E. coli* [4] indicates the formation of a 3′-OH. This 3′-OH might, however, result from the 3′-phosphatase activity of the exonuclease III present in our preparation. But we have shown that the team endonuclease VI/exonuclease III does not release radioactive inorganic phosphate from alkylated-depurinated [^{32}P]DNA, so that we can conclude that the hydrolysis of a phosphoester bond near

the apurinic site, by endonuclease VI, leaves a 3′-OH. On the other hand, polynucleotide 5′-hydroxylkinase can catalyze the transfer of the γ-phosphate from ATP on alkylated-depurinated DNA treated with endonuclease VI only after an incubation with *E. coli* alkaline phosphatase (Table 6). This result indicates the presence of a 5′-phosphate in the nick produced by endonuclease VI.

Three different sets of experiments seem to point to the conclusion that the phosphoester bond hydrolyzed by endonuclease VI is on the 5′ side of the apurinic site. Exonuclease III, which works in the 3′→5′ direction from the nick produced by endonuclease VI, leaves the apurinic site in the DNA molecule. After denaturation of depurinated DNA treated with endonuclease VI, analysis with snake venom and spleen phosphodiesterases suggests that the apurinic site remains at the 5′ end of the strand pieces (Table 7). We do not know, however, if an apurinic site at the 3′ end might have blocked the action of snake venom phosphodiesterase and exonuclease III. If the hydrolyzed phosphoester bond had been the immediate neighbour on the 3′ side of the apurinic site, oxidative cleavage of the resulting diol with metaperiodate would have released the aldehyde function (labelled by reduction with sodium boro[^{3}H]hydride) of the apurinic site in a small acid-soluble fragment; the result of this experiment was negative.

The phosphoester bond hydrolyzed by the human endonucleases is also on the 5′ side of the apurinic site leaving a 5′-phosphate; but this 5′ end, after a phosphatase treatment, cannot be phosphorylated by polynucleotide 5′-hydroxyl-kinase suggesting that the 5′-OH belongs to the base-free deoxyribose [28]. Our results are quite different; it is thus possible that the phosphoester bond hydrolyzed by *E. coli* endonuclease VI is not in the immediate vicinity of the apurinic site.

Molecular Mechanism of the Repair of Depurinated DNA in vitro

We had previously shown that depurinated DNA was repaired *in vitro* when incubated with endonuclease VI, DNA polymerase I and the four deoxynucleoside triphosphates, polynucleotide ligase and its coenzyme [4]. The observation that DNA polymerase I removes, in an oligonucleotide, the apurinic sites from depurinated DNA treated with endonuclease VI (Fig. 4) allows us to propose a scheme for the repair in a *E. coli* system [29] (Fig. 5).

Endonuclease VI hydrolyzes a phosphoester bond on the 5′ side of the apurinic site, leaving a 3′-OH and a 5′-phosphate. Exonuclease III degrades the incised strand, from the nick, in the 3′→5′ direction, leaving the apurinic site in DNA. DNA polymerase I starts polymerizing from the 3′-OH left by exonuclease III,

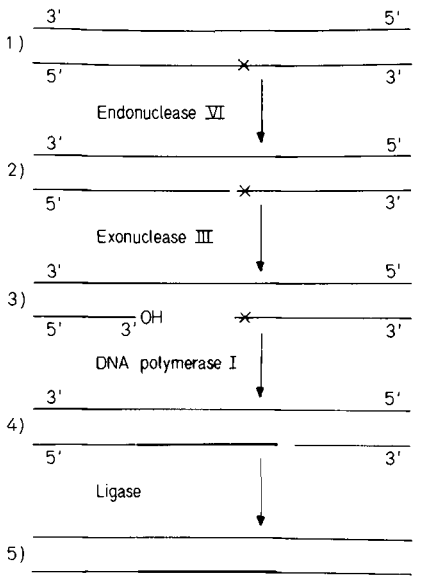

Fig. 5. *Molecular mechanism of depurinated DNA repair.* The cross indicates an apurinic site (1). Endonuclease VI hydrolyzes a phosphoester bond on the 5′ side of the apurinic site leaving 3′-OH and 5′-phosphate ends (2). Exonuclease III removes a few nucleotides in the 3′→5′ direction leaving the apurinic site in the DNA molecule (3). DNA polymerase I starts to synthesize DNA from the 3′-OH end of the gap and excises the apurinic site in a di- or trinucleotide before catalyzing a nick translation in the 3′ direction (4). Ligase can close this nick at any time (5)

fills the gap introduced by this activity, excises the apurinic site in a di or trinucleotide, then catalyzes the translation of a nick in the 5′→3′ direction. This nick, limited by a 3′-OH and a 5′-phosphate is finally closed by polynucleotide ligase.

Exonuclease III might, in this repair, work as an antiligase: removing a few nucleotides prevents the closing, by polynucleotide ligase, of the nick just opened by endonuclease VI. This function of exonuclease III is, however, not an absolute requirement for the repair of depurinated DNA: absence of endonuclease VI/exonuclease III does not increase the sensitivity of *E. coli* to methylmethanesulfonate as much as the absence of DNA polymerase I; endonuclease VI is then probably replaced by endonuclease IV, which is devoid of exonuclease activity [6]. We, however, plan to investigate whether, in the presence of polynucleotide ligase, the DNA polymerase I excision of the apurinic site is not more efficient when the incision step is catalyzed by endonuclease VI rather than by endonuclease IV.

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