Mechanism of Deoxyribonucleic Acid Degradation by an Acid Deoxyribonuclease from the Snail *Helix aspersa* Müll.†

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ABSTRACT: The acid deoxyribonuclease extracted from the hepatopancreas of the snail (*Helix aspersa* Müll.) is specific for deoxypolymers. Glycosylated (T4 DNA) and nonglycosylated DNA, as well as poly[d(A-T)]·poly[d(A-T)], are hydrolyzed, whereas poly[d(G-C)] is not. The enzyme is an endonuclease since it opens covalently closed PM2 circular molecules of DNA. The initial velocity of the enzyme is 30 times higher when acting on native DNA than on denatured DNA. The denatured DNA is not an inhibitor of the enzyme. Circular molecules of PM2 untwisted by the sequential use of pancreatic DNase and *Escherichia coli* polynucleotide ligase are hydrolyzed at the same rate as twisted natural ones. The enzyme hydrolyzes DNA according to both diplotomic (single hit) and haplotomic (double hit) mechanisms. It yields products bearing the monoesterified phosphate attached to position 3′. The oligonucleotides produced after an exhaustive digestion (average chain length 6.4) contains barely detectable mono- and dinucleotides and very few trinucleotides. Their average length ranges between 4 and 10.

The hepatopancreas of the common snail (*Helix aspersa* Müll.) contains an acid DNase. In the previous paper (Laval and Paoletti, 1972) we have described its purification, established its main physical and chemical properties, and compared it with another acid DNase: the beef spleen endonuclease. Availability of this enzyme in a pure form, free of contaminating enzymatic activities such as exonuclease, phosphatase, and unspecific phosphodiesterase, enables us to study its specificity, according to the origin and the secondary and tertiary structures of the DNA used as substrate, and its initial kinetics of action (haplotomic *vs.* diplotomic mode of hydrolysis). The nature of oligonucleotides produced after digestion of DNA, *i.e.*, their average length and the localization of the phosphate terminal were also investigated.

Materials and Methods[1]

Urea is a Merck product and is recrystallized from ethanol prior to use. DEAE-cellulose (Selectacel, type 20, capacity 1 mequiv/g) is purchased from Schleicher and Schuell, Keene, N. Y.). Sucrose is an Analar product, and Tris is from Sigma, St. Louis, Mo. Solutions and buffers are made with glass-distilled water and are sterilized prior to their utilization. Dialysis tubings are boiled twice in a 10% sodium carbonate–0.01 M EDTA solution, washed with water until a neutral pH is reached, and sterilized. Dialysis tubing is handled with sterile surgical gloves.

Enzymes. Helix endonuclease (fraction VIII) and beef spleen acid DNase have been described in the preceeding paper (Laval and Paoletti, 1972). Spleen phosphodiesterase, pancreatic DNase (3-times recrystallized), and snake venom phosphodiesterase are purchased from Worthington Biochemical Corp., Freehold, N. J. The preparation of polynucleotide ligase from *Escherichia coli* is already described (Paoletti *et al.*, 1971).

Nucleic Acids. Calf thymus DNA (CTS) is prepared as described by Signer and Schwander (1949). The molar extinction coefficient, measured at 260 nm as moles of nucleotide per liter, ϵ (P), is 6900. Sedimentation coefficient is 24 S.

The preparation of ^{3}H-labeled *E. coli* DNA has been described already (Laval *et al.*, 1970) (Laval and Paoletti, 1972). ^{14}C-labeled *E. coli* is prepared by the same method as used to prepare ^{3}H-labeled *E. coli*. Its specific activity is 3.2×10^{6} cpm/μmole. When using denatured DNA, ^{14}C-labeled DNA is denatured just before the experiment by heating for 10 min in a boiling water bath (15 μg/ml in 0.005 M NaCl then chilling in an ice bath. Hyperchromicity at 260 nm is 25%. By reference to the C_0t curve established by Britten and Kohne (1968) for *E. coli* DNA, one may assume that after 90 min of incubation there is less than 1% renatured DNA in our conditions.

DNA from [^{3}H]thymidine-labeled T7 phage is extracted according to Richardson *et al.* (1964), except for the first step of purification of phages which is performed by phase partition (Philipson *et al.*, 1960). The specific activity of T7 DNA is 2.4×10^{6} cpm/μmole; 74% of this DNA sediments in an alkaline sucrose gradient as an homogeneous component during zone sedimentation.

Poly[d(A-T)]·poly[d(A-T)] and poly[d(G·C)] are purchased from Miles Research laboratories. DNA from bacteriophage PM2 is prepared as described by Paoletti *et al.* (1971). Its

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[1] Abbreviations used are: solution A, standard solution made up of ethidium bromide (1 μg/ml) dissolved in 0.01 M Tris-HCl (pH 7.8)–0.02 M NaCl–0.005 M EDTA; IF, fluorescence intensity; ΔIF, variation of IF expressed as units on the scale of the PMQ II Zeiss spectrometer equipped with ZF M 4 fluorimeter. We define a single-strand break (ss break) as the hydrolysis of a phosphodiester linkage on one strand only. When two ss breaks on each individual strand either face each other, or are sufficiently close they generate a double-strand break (ds break), *i.e.*, a rupture of the duplex. The maximal distance between the breaks on opposite strands which allows the duplex to be stable depends on the environmental conditions, temperature, and ionic strength (Freifelder and Trumbo, 1969). When an enzyme delivers only ss breaks, its mechanism of action is defined as haplotomic, when it delivers ds break, this mechanism is defined as diplotomic (Bernardi, 1968).

superhelix density is 0.047 as determined by Revet *et al.* (1971) and Paoletti (unpublished data); untwisted PM2 DNA is prepared by the simultaneous action of pancreatic DNase and *E. coli* ligase as described by Paoletti *et al.* (1971).

Assays

Assays of Helix DNase. Enzyme activity is assayed in most cases as described in the preceeding paper (Laval and Paoletti, 1972). The assay is also performed according to Kunitz (1949). Hyperchromic shift is followed in a jacketed silica cell using a Zeiss PMQII spectrophotometer.

Viscosity Measurements. For deciding between an haplotomic and a diplotomic mode of action of the enzyme, we follow the changes of viscosity of an enzyme DNA mixture using a viscosimeter Lecomte du Nouy "Couette" type, at shear gradient 15 sec^{-1}, temperature 25°. Kinetic data obtained by following the intrinsic specific viscosity during enzymatic degradation of the DNA are treated according to Schumaker *et al.* (1956). The apparent number of strands, n, is obtained from the slope of $\log (1 - R)/R$ *vs.* $\log t$. R is equal to M_t/M_0 or η_t/η_0, where M_t, M_0, η_t, and η_0 are, respectively, average molecular weight and the specific viscosity at digestion time t and zero time.

Fluorescence Measurements. The rate at which the first break is induced on each DNA molecule is determined by establishing the kinetics of the opening of closed circular PM2 DNA. For this purpose, we choose the fluorometric method described by Paoletti *et al.* (1971) based on the differential affinity of ethidium bromide for open and closed DNA circular molecules.

Assay of Exonucleases. The liberation of uranyl perchloric soluble material is used as a measurement of enzyme activity. To 400 μg of native or *Helix* nuclease treated DNA in 500 μl of the proper buffer is added 1 ml of a cold aqueous solution of 2.5% $HClO_4$ and 0.25% uranyl acetate. After 5 min at 0°, the precipitate is centrifuged 10 min at 12,000*g*. Absorbance of the supernatant is read at 260 nm against an appropriate blank.

Zone Sedimentation of DNA on Sucrose Density Gradients. Sedimentation is carried out in linear concentration gradients of sucrose (5–20%) according to Richardson (1966). Neutral conditions are 0.01 M Tris-HCl–1 M NaCl–0.001 M EDTA (pH 8.0). Alkaline conditions are 0.11 M NaOH–0.89 M NaCl–0.001 M EDTA (pH 12.0). Gradients are prepared by use of a gradient maker device and a polystatic pump (Büchler, Fort Lee, N. Y.).

The sample of T7 [^{3}H]DNA (0.64 μg in 0.1 ml) is layered on 4.2 ml of sucrose. In neutral conditions, DNA is centrifuged for 165 min at 35,000 rpm at 4° in the rotor SW 39 in a Spinco Model L2-65 centrifuge. Fractions (2 drops) are collected on Whatman fiber paper GF/C, dried, and counted. Recovery of radioactive material was better than 90%. In alkaline conditions, DNA is centrifuged 120 min at 52,000 rpm in a SW 65 rotor.

Both under neutral and alkaline conditions the fraction of DNA sedimenting in the undegraded form, as well as in the degraded one, is established by measuring the relative weight of the area cut off under the corresponding peak.

Assuming that the breaks are randomly distributed among all the molecules, the Poisson's law applies: $p = e^{-m}$ where p is the fraction of DNA still sedimenting in an undegraded form and m the average number of ds break per DNA molecule (in neutral conditions) or the average number of ss break per strand (in alkaline conditions).

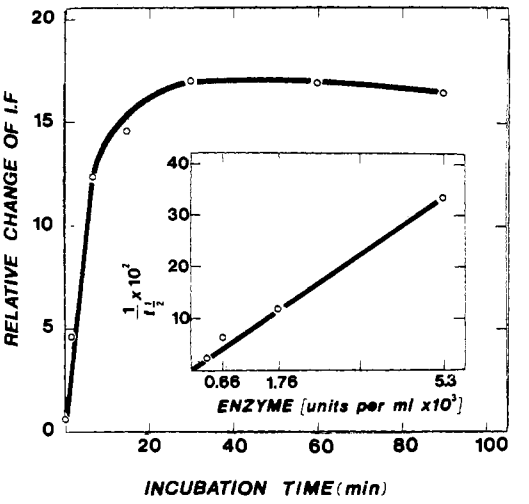

FIGURE 1: Fluorometric determination of the hydrolytic rate of *Helix* endonuclease. *Helix* DNase (0.0033 unit) in 5 μl of 0.1% ovalbumin is added to PM2 DNA in 420 μl of 0.1 M Na acetate buffer (pH 5.6) (DNA concentration: 9.91 μg/ml). After different times at 37°, 40 μl of the mixture is widthdrawn and added to 800 μl of solution A. For fluorometric determinations, see Materials and Methods. Insert: proportionality between the hydrolytic rate and the enzyme concentration. An experiment similar to that described above is run PM2 DNA concentration is 1.60 μg/ml. For definition of $t_{1/2}$, see text. Increasing amounts of *Helix* DNase are added.

Size Determination of Oligonucleotides Produced by an Extensive Hydrolysis of DNA. The oligonucleotides are separated on a DEAE-cellulose–urea column according to Tomlinson and Tener (1963). The ultraviolet transmission of the effluent is continuously monitored at 254 nm using a LKB recorder (Uppsala, Sweden). The optical density of the fractions is measured in a Zeiss PMQ II spectrophotometer. The experimental chromatographic pattern is analyzed by the Dupont de Nemours Curve Resolver 310 which computes the proportion of each isoplith. The length of each separated isoplith is established from (i) the molarity of the elution from the column and (ii) the ratio of phosphatase-sensitive phosphorus to total phosphorus. Phosphorus determination is carried according to Martin and Doty (1949) as modified by Carrara and Bernardi (1968).

Results

Hydrolysis of DNA of Different Sources. Calf thymus DNA, T4 DNA which bears glycosylated hydroxymethylcytosine nucleotides, and poly[d(A-T)]·poly[d(A-T)] are all found to be equally good substrates (Kunitz assay) for the enzyme. Poly(dG)·poly(dC) does not show any hyperchromic effect. After sedimentation through an alkaline gradient (pH 12.5), the patterns of endonuclease-treated and nontreated poly(dG)·poly(dC) are similar. Therefore, no single-strand breaks are revealed. This result rules out a limited attack of this polymer by *Helix* nuclease. Its significance will be discussed in the third paper of this series (Laval *et al.*, 1972)[2] which is relative to the bases specificity of this enzyme which is unable to hydrolyze a dpCpX phosphodiester sequence.

Helix DNase is able to perform endonucleolytic breaks as shown by two different experiments. (i) This enzyme is able open up covalently closed PM2 DNA circular molecules (Figure 1). This opening, which expresses the initial breaking events, can be followed by ethidium bromide fluorimetry

[2] In preparation.

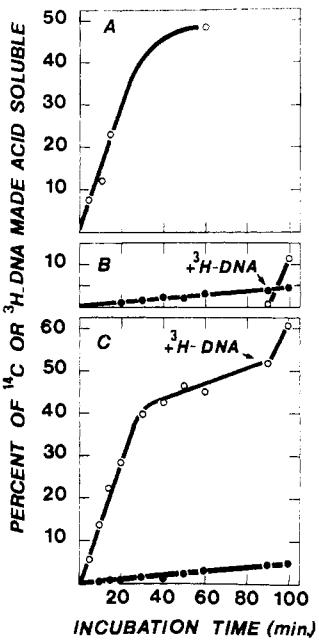

FIGURE 2: Activity of *Helix* DNase, according to secondary structure of DNA. Three series of reaction mixtures are set up, containing either ^{3}H labeled native or ^{14}C-labeled heat denatured or both ^{3}H-labeled native and ^{14}C-labeled *E. coli* denatured DNA, as described in Materials and Methods, except that incubation temperature is 49°. The mixtures were preincubated at 49° for 3 min prior to addition of 0.095 unit of enzyme per assay. (A) ^{3}H-labeled native DNA. (B) ^{14}C-labeled heat-denatured DNA; + [^{3}H]DNA indicates the addition of 15.8 nmoles of native [^{3}H]DNA in a volume of 10 μl. (C) Mixture of ^{3}H-labeled native and ^{14}C-labeled denatured DNA. + [^{3}H]DNA: *vide supra* B. (○——○) Per cent of total [^{3}H]DNA rendered acid soluble. (●——●) Per cent of total [^{14}C]DNA rendered acid soluble.

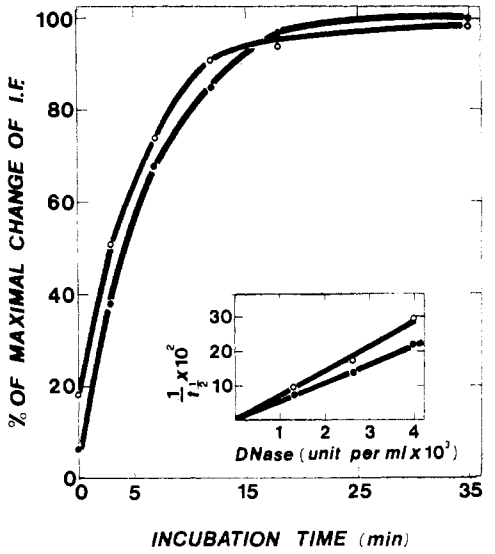

FIGURE 3: Comparative rate of opening of twisted and detwisted covalently closed circular PM2 DNA by *Helix* endonuclease. *Helix* DNase (0.004 unit) in 5 μl of 0.1% ovalbumin is added to PM2 DNA in 980 μl of 0.1 M Na acetate buffer (pH 5.6). DNA concentration for twisted closed DNA: 4.15 μg/ml, for detwisted closed DNA 5.20 μg/ml. After different times at 37°, 150 μl of each mixture is withdrawn and added to 800 μl of solution A. For fluorometric determinations, see Materials and Methods. Twisted DNA (●——●) IF before addition of enzyme, 65.4, ΔIF, 16.2; detwisted DNA (○——○) IF before addition of enzyme, 64.2, ΔIF; 33.8. IF of solution A alone is 17.0. Insert: rate of opening *vs.* enzyme concentration. Same experimental conditions as before.

which is based on the measurement of $t_{1/2}$, time at which the increase of the fluorescence intensity due to the opening of the DNA circles is half the maximum increase when all the circles have been opened. There is an average of 0.69 opening event per PM2 circular molecule at $t_{1/2} \cdot t_{1/2}$ is reciproccal to the hydrolytic rate (Paoletti *et al.*, 1971).

In our experimental conditions [DNA concentration, 9.9 μg/ml; temperature, 37°; solvent, 0.1 M acetate (pH 5.6)] the opening rate, as followed by fluorometry, is proportional to the enzyme concentration, in the range used for this work (Figure 1, insert).

Each unit of enzyme activity (1 nmole of DNA made acid soluble per minute at 37°) delivers 415 hits per PM2 DNA molecule per minute or 69 hits per 1×10^6 molecular weight of DNA and per minute.

(ii) Few breaks only reduce the molecular weight of T7 DNA as judged by zone centrifugation in neutral as well as in alkaline conditions (see below). This observation implies that the breakage is an endonucleolytic one. Moreover, during the action of the enzyme there is no increase of radioactivity detected at the meniscus (see below, Figure 4). Therefore, during the initial stages of degradation there is no detectable liberation of small oligonucleotides; hence the enzyme preparation is not contaminated by an exonuclease, and the endonuclease molecule is devoid of detectable exonucleolytic activity.

Effect of the Secondary Structure of DNA on Enzymatic Rate. Native and heat-denatured *E. coli* labeled DNA are compared as substrates for *Helix* endonuclease either alone or as a mixture. At 49°, the initial velocity of the attack is 30 times more rapid for the native form than for denatured DNA (Figure 1). The digestion of native DNA proceeds rapidly until about 45% of the substrate is rendered acid soluble and then slows down suddenly. An explanation for the suddenness of this slowing down when only a part of the DNA has been hydrolyzed could be provided by a specificity for an organized secondary structure. Another explanation would assume that denatured DNA and/or the melted products of the enzyme action are inhibitors of the enzyme. This latter explanation is ruled out by experiments shown on Figure 2. The rate of attack of DNA does not depend on the addition in the reaction medium of denatured DNA, or of reaction products of low molecular weight (Figure 2C). However, although these products do not inhibit DNA hydrolysis, they might still competitively inhibit the reaction at its end.

The results obtained at 37° support the hypothesis according to which the enzyme is specific for a duplex structure: the slowing of the rate of enzyme action occurs when more than 70% of the substrate is rendered acid soluble, instead of 45% at 49° (Figure 2A). Such a result could be expected since the double-stranded structure of comparable size is more stable at 37° than at 49° in our conditions. In each of these experiments, it has been verified that no partial denaturation of the enzyme occurs during the reaction: no loss of activity is observed when native DNA is added after 90 min of enzyme action at 49° on either native DNA (Figure 2C) or on denatured DNA (Figure 2B, C).

Effect of Supercoiling of Circular DNA on Enzyme Activity. It has been demonstrated (Dean and Lebowitz, 1971; unpublished data from our laboratory; Paoletti and Le Pecq, 1971) that supercoiling to which a positive free energy is associated causes alterations in the double-helical structure of native closed circular DNA. Since from the above

TABLE I: Average Number of Double-Strand Breaks and Single-Strand Breaks per Molecule of T7 DNA after a Limited Hydrolysis by *Helix* DNase.

	Sedimentation in Neutral Sucrose[a]		Sedimentation in Alkaline Sucrose[a]		
Hydrolysis Time (min)	32.0S Native DNA/ Total DNA[b]	Average Number of ds Breaks per T7 DNA Molecule[c]	37.2S DNA/ Total DNA[b]	Average Number of ss Breaks per T7 DNA Strand[c]	Ratio ds Breaks/ ss Breaks per T7 DNA Molecule
0	1	0	0.74	0.3	
10	0.64	0.4	0.28	1.3	0.15
20	0.15	1.9	0.08	2.5	0.38

[a] Data taken from T7 DNA zone sedimentation in Figure 4. [b] Total DNA is the DNA sedimenting at the position of native T7 marker (32S) or denatured T7 marker (37.2S) (Studier, 1965) plus the DNA sedimenting in the degraded form. [c] For the calculation method, see Materials and Methods as well as text.

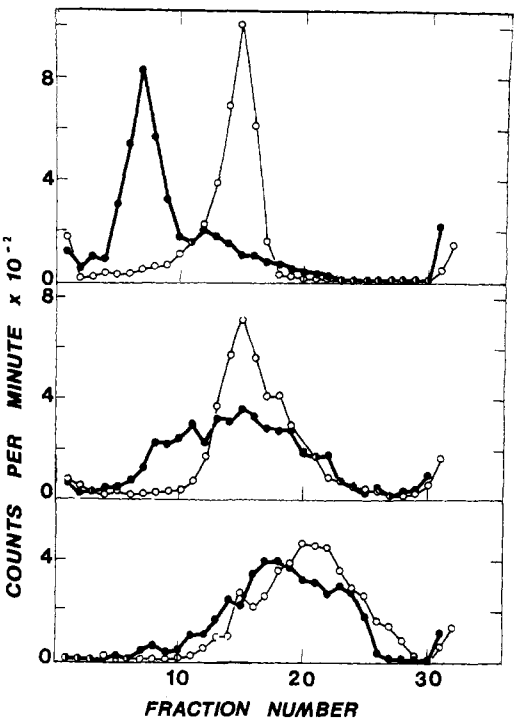

FIGURE 4: Zone sedimentation of T7 [^{3}H]DNA degraded by *Helix* endonuclease. Incubation mixture contains 6 μg/ml of boiled pancreatic RNase, 5.32 μg of T7 [^{3}H]DNA in 0.05 M acetate buffer (pH 5.6), and 0.0017 unit of *Helix* DNase; final volume 800 μl. After 0, 10, and 20 min at 37°, 250 μl is withdrawn, poured over 10 μl of 1 M Tris to stop the enzymatic reaction, and kept at 0°, 0.1 ml is layered either on an alkaline or on a neutral sucrose gradient, radioactivity is determined on fractions after centrifugation as described in Materials and Methods. For the sake of simplicity, neutral and alkaline sucrose gradients are superimposed. (●——●) Alkaline gradient, (○——○) neutral gradient.

data *Helix* endonuclease recognizes the secondary structure of DNA, it was interesting to determine whether the enzyme reacts in the same way toward twisted natural PM2 circular DNA and detwisted DNA (allomorphic structures), prepared by the removal of the supercoils after opening up and closing the circles through the joint action of pancreatic DNase and *E. coli* polynucleotide ligase. An answer to this question is provided by the data of Figure 3. *Helix* endonuclease induces a number of breaks per minute and per molecule of PM2 closed circular DNA, which does not depend on the twisting of the DNA, in the limits of sensivity of the fluorometric method used here.

Predominant Diplotomic Mode of Action of Helix Nuclease. If an endonuclease produces only ss breaks on DNA it will not modify its average molecular weight during the initial phase of its action whereas it will decrease the average molecular weight of its strands, measured after an alkaline denaturation.

In contrast, if an endonuclease produces only ds breaks on DNA, the average molecular weight of the duplex DNA will decrease as soon as the enzymatic attack is initiated; its value will parallel that of the separated strand. In the first case (ss breaks), the ratio between ds breaks and ss breaks per molecule will be initially 1 per 200 to reduce the molecular weight to half as shown by Thomas (1956) and Freifelder and Trumbo (1970) in environmental conditions similar to those used here. In the second case (ds breaks) it should be 1 per 2, *i.e.*, 0.5.

The number of ss breaks and ds breaks is evaluated by zone sedimentation as described in Material and Methods. Table I gives the ratio of ds breaks to ss breaks per molecule during the initial phase of attack of T7 DNA by *Helix* nuclease. For a very small average number of ss breaks per strand (about 1), the ratio of ds breaks to ss breaks per molecule is already high (0.15).

When the average number of ss breaks per strand is doubled, the ratio of ds breaks to ss breaks per molecule (0.38) is already nearly equal to the theoretical ratio for diplotomic enzyme (0.5). Consequently, *Helix* endonuclease acts as a diplotomic enzyme delivering breaks facing each other or very close on opposite strand. However, at least during the initial stage of DNA attack, some ss breaks are produced, therefore, both haplotomic and diplotomic mechanisms are at work, the latter being widely predominant. For instance, for an average delivery of 5 ss breaks per molecule, about 80% of these breaks are located in such a position as to form ds breaks. Such a phenomenon has also been reported for several other nucleases (Bernardi, 1968).

The kinetics of DNA degradation by *Helix* DNase followed by viscosity reflects these findings.

When the kinetic data related to the changes of viscosity of DNA under the *Helix* nuclease action are treated according to Schumaker *et al.* (1956) allowing the determination of the apparent number of strand *n*, *n* is found to be 1.3 for *Helix* DNase.

Control experiments establish that in identical conditions hog spleen DNase (predominantly diplotomic) has an *n* value of 0.9 and pancreatic DNase (haplotomic) of 1.5, similar to the finding of Bernardi (1968).

Localization of the Phosphomonoester and Hydroxy End Groups on the Oligonucleotides Provided by Helix Nuclease. (a) In order to ascertain if the *Helix* nuclease yields oligonucleotides bearing 3′-P or 5′-P end groups, advantage has been taken of the specificity of the spleen and venom exonucleases. Spleen exonuclease initiates attack at free 5′-OH

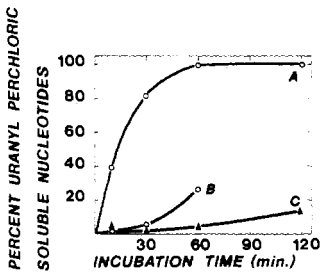

FIGURE 5: Action of spleen and venom exonucleases on DNA nicked by *Helix* nuclease. Thymus DNA (CTS) (1.375 mg) in 0.05 M sodium acetate buffer (pH 5.6) is treated with 14 units of *Helix* endonuclease in 1.5 ml for 25 min, then boiled for 10 min to destroy the enzyme activity (15.6% of DNA is rendered perchloric acid soluble while there is no uranyl perchloric acid soluble material as described in Materials and Methods). This partially hydrolyzed DNA (HP-DNA), is then used for the following experiments. Curve A: 400 μg of HP-DNA, 8 μmoles of acetic acid, and 2 units of spleen phosphodiesterase (SPH). Final volume 500 μl; pH 5.0. Curve B: HP-DNA is replaced by native DNA, other conditions are the same for curve A. Curve C: 400 μg of HP-DNA, 2 μmoles of $MgCl_2$, 24 μmoles of Tris base, and 20 μg of venom phosphodiesterase (VPH). Final volume, 500 μl; pH 9.0. Aliquots (50 μl) are withdrawn at various times and mixed with 150 μl of carrier DNA (1 mg/ml) and 200 μl of uranyl. Perchloric acid soluble material is determined as described in Materials and Methods. All experiments are run at 37°.

and liberates 3′-P mononucleotides. It does not attack polynucleotides with a 5′-phosphomonoester end group. The snake venom initiates hydrolysis at the 3′-OH end, yielding 5′-P mononucleotides. It does not attack polynucleotides with a 5′-phosphomonoester end group.

Figure 5 shows that a limited digest of calf thymus DNA by *Helix* nuclease is a good substrate for spleen exonuclease (curve A), while it is not for venom exonuclease (curve C). The slow and delayed appearance of mononucleotides upon action of the venom enzyme probably reflects its contamination by an endonuclease as described by Laskowski (1967). The same explanation [*i.e.*, presence of traces of the acid endonuclease, described by Bernardi and Griffé (1964) in the exonuclease preparation] holds for the delayed effect of spleen exonuclease on native DNA.

These experiments demonstrate that the oligonucleotides produced by the *Helix* nuclease bear 3′-phosphomonoester end groups.

(b) The same conclusion is reached by an experiment with polynucleotide kinase which has been shown to have an absolute requirement for 5′-OH ends (Richardson, 1965). Upon incubation of DNA with *Helix* DNase, oligonucleotides appear which can be phosphorylated by polynucleotide kinase [see paper III of this series (Laval *et al.*, 1972)[2]]. Advantage has been taken of this property to identify the 5′-OH-terminal nucleotide in *Helix* DNase digests.

Determination of the Length of the Oligonucleotides Liberated after an Exhaustive Degradation of DNA by Helix Endonuclease. The average length of the oligonucleotides of an exhaustive digest is 6.4 as determined by the ratio of phosphatase-sensitive phosphorus to total phosphorus.

When these oligonucleotides are fractionated according to their length on a DEAE-cellulose–urea column (Figure 6) the relative proportion of each isoplith can be estimated (Table II). No oligonucleotide of chain length higher than 12 is present. No mononucleotides and very few, if any, dinucleotides and trinucleotides are recovered, even if the column is purposely overloaded. These data confirm that the enzyme preparation is devoid of an exonucleolytic activity either embodied in the endonucleolytic protein or present as a contaminant.

TABLE II: Chromatography of *Helix* Digest on DEAE-Cellulose–Urea Column.[a]

Proportion of Each Isoplith (Per Cent of Total Absorbance)						
<IV	IV	V	VI	VII	VIII	>VIII
1.0	5.5	16.5	23.7	19.2	13.4	20.7

[a] Incubation mixture of the digest contains 500 OD_{260} of DNA (CTS) and 1.670 units of *Helix* DNase in 0.05 M Na acetate buffer (pH 5.6), and 2 drops of toluene; final volume: 15 ml. Incubation: 37° for 19 hr. Isopliths are fractionated on a DEAE-cellulose–urea column (see Figure 6). IV to VIII refer to tetra- to octanucleotides.

Discussion

Helix nuclease is an endonuclease since it is able to open covalently closed circular DNA molecules. It is free of detectable amounts of contaminating true exonuclease *sensu stricto* since it does not yield mono- or dinucleotides. Moreover, it yields only one band in polyacrylamide gel electrophoresis.

The *Helix* enzyme can only accommodate oligonucleotides at least eight monomers long, since the heptanucleotides and smaller oligonucleotides would yield either mono-, di-, or trinucleotides which are not found after isosteach separation. An upper limit of the length of the oligonucleotides completely sensitive to the enzyme is represented by decanucleotides. These are barely detectable. Thus, ability of the oligonucleotides to be attacked by the *Helix* enzyme is highly length dependent, in the range 8–10 nucleotides. Such a dependency is also found for other depolymerizing enzymes, such as lysozyme which degrades pentamers and hexamers 500 and 3750 times faster than tetramers (Jollès, 1964).

One cannot rule out the possibility that such a narrow range of average chain length could merely be the consequence of an accumulation of sequences of bases resistant to hydrolysis, because the enzyme is highly specific for specific base sequences as demonstrated in the accompanying paper (Laval *et al.*, 1972).[2] However, the published results (Lehman *et al.*, 1962) on the mode of action of endonuclease I from *E. coli* does not favor this hypothesis. This nuclease has a molecular weight close to that of the *Helix* enzyme and gives also mainly heptanucleotides after exhaustive digestion of DNA in spite of absence of known base sequences specificity.

Another explanation for the relatively precise and short average length of the oligonucleotides produced by *Helix* enzyme lies on the assumption that their size depends on the size of the enzymatic protein. The mean radius of a protein molecule having a molecular weight of 30,000, such as *Helix* DNase, should be in the range of 1.5–4 nm depending on its shape. Accordingly, it should accommodate a zone 5–12 base pairs long on the duplex DNA, which is in good agreement with the actual average length of oligonucleotides resulting from an exhaustive action of the *Helix* DNase (6.4 nucleotides). Such an hypothesis implies that breaks or some truly endonucleolytic breaks are accompanied by the adjacent removal of one or several oligonucleotide(s). Such a mechanism is an exo–endonucleolytic one, provided that one uses the

definition of an exonuclease previously given by Paoletti *et al.* (1971). "An enzyme which catalyzes a stepwise attack beginning either at the termini of a DNA molecule or at single-strand interruptions within the molecule, sequentially liberating mono- or *oligonucleotides*."

The association of an exonucleolytic mechanism of action with an endonucleolytic has already been demonstrated for the *Micrococcal* nuclease (Williams *et al.*, 1961) and for *E. coli* endonuclease I (Paoletti *et al.*, 1971). It must be noted that the latter enzyme yields products whose average size is similar to that of the products of the *Helix* enzyme.

One can argue that no oligonucleotide whose average length ranges around 6.4 is detected during the initial stage of attack of DNA by *Helix* DNase. However, it can be shown that the amount of such oligonucleotides, which could have been eventually liberated by the exo–endonucleolytic mechanism, is below the threshold of their detection in the sedimentation experiments which lies around 2% of the total material.

During the time elapsed in our longest experiments (20 min) (Figure 4C) each DNA molecule has received on the average around 100 endonucleolytic breaks as established from the fluorometric data. If each endonucleolytic break were accompanied by the removal of a short piece of oligonucleotides whose average length is 8, $100 \times 8 = 800$ nucleotides would have been removed during this period. This corresponds to only 800/90,000, *i.e.*, less than 1% of total DNA which is a nondetectable amount of radioactivity. Experiments devised for directly establishing the exo–endonucleolytic mode of action of *Helix* endonuclease are in progress.

From the pioneering effort of Laskowsky (1961, 1967) to define rules for the classification of nucleases, the only criteria which remains unambiguous is the cleavage of the internucleotide bond on the 3′-phosphate *vs.* the 5′-phosphate side. *Helix* enzyme is a 3′-phosphomonoester former, a property which seems so far to be a characteristic of acid DNase: spleen, thymus ones (Bernardi, 1968), *Bothrops atrox* venom (Georgatsos and Laskowski, 1962). To our knowledge, the only exception to this rule could be an endonuclease extracted from *Octopus vulgaris*, active at pH 6.0, which yields 3′-phosphate ends (Georgatsos and Antonoglou, 1966). A nuclease from *Micrococcus pyogenes* yields also 3′-phosphate oligonucleotides, in spite of being active at alkaline pH. But this enzyme is nonspecific for the sugar (Cunningham *et al.*, 1956).

The rate of attack on closed DNA circles is not affected by twisting. Therefore, the affinity of the enzyme for the circles is not modified by the existence of an excluded volume. Moreover, the modification of the secondary structure of the supercoils (Dean and Leibowitz, 1971) is not recognized by *Helix* endonuclease. An identical independence of enzyme action toward the supercoiling has also been found for pancreatic DNase and beef spleen acid DNase (our unpublished results). These observations should be brought together with those of Mitsui *et al.* (1970), obtained on left-hand polynucleotides, showing that twelve different enzymes of nucleic acid metabolism, among which are pancreatic DNase, exonuclease I and II of *E. coli*, micrococcal nuclease, spleen phosphodiesterase, and *M. luteus* exonuclease, do not recognize left-handed polynucleotides from the natural right-handed ones.

These data indicate that most of known nucleases recognize only an extreme change of the secondary structure (native *vs.* denatured). They contrast with an observation reported by Hayashi and Hayashi (1971). These authors compared the allomorphic forms of the replicative form (RF DNA) of ϕX-174, for their template activity, *i.e.*, the rate of *in vitro* synthesis by *E. coli* DNA-dependent RNA polymerase. Twisted RFI DNA which does not have a break on either strand, has a rate of transcription which is about three times higher than that of untwisted RFII DNA, derived from RFI by introducing a single-strand break on either strand by pancreatic DNase.

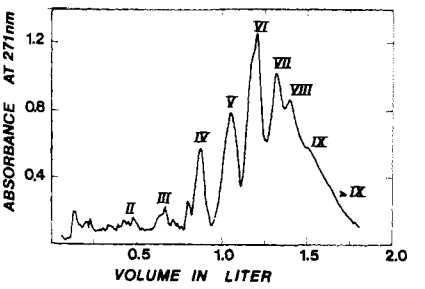

FIGURE 6: DEAE-cellulose chromatogram in 7 M urea of the exhaustive digest of calf thymus DNA by *Helix* DNase. A DEAE-cellulose column (2.5 × 27 cm) is loaded with 475 OD_{271} units. A linear gradient is set between 0 and 0.3 M NaCl in 0.1 M sodium acetate (pH 4.7) at 25°. Roman numerals give the lengths of oligonucleotides determined as described in Methods. Digestion conditions are given in Table II. Recovery of absorbing material is 99% of the absorbance of the input.

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Rate of Depurination of Native Deoxyribonucleic Acid†

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ABSTRACT: The rate of depurination of double-stranded *Bacillus subtilis* DNA, radioactively labeled in the purine residues, has been followed as a function of temperature, pH, and ionic strength. In a Mg^{2+}-containing buffer of physiological ionic strength, the rate constant for depurination of DNA is 4×10^{-9} sec^{-1} at 70° and pH 7.4. The activation energy of the reaction is 31 ± 2 kcal/mole. These data strongly indicate that depurination of DNA occurs at a physiologically significant rate under *in vivo* conditions and consequently that the lesions introduced in this fashion must be repaired.

When neutral DNA solutions are exposed to high temperatures, two kinds of structural changes occur. Within a narrow temperature interval around the helix–coil transition temperature, T_m, the macromolecular structure of the DNA is denatured, and the two strands separate when all hydrogen bonds have been broken (Doty *et al.*, 1960). A different, slower inactivation process also takes place, which is due to heat-induced degradation of the primary structure and may be detected as an irreversible loss of biological activity of transforming DNA (Lerman and Tolmach, 1959; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; van Sluis and Stuy, 1962; Strack *et al.*, 1964; Alberts and Doty, 1968). The latter reaction occurs both above and below the T_m, and is faster at low pH (Roger and Hotchkiss, 1961). This irreversible inactivation is probably to a large extent due to depurination of the DNA (Tamm *et al.*, 1952; Greer and Zamenhof, 1962), and/or to the formation of chain breaks (Tamm *et al.*, 1953) and cross-links (Freese and Cashel, 1964) occurring as a consequence of depurination. Other types of heat-induced DNA degradation could also be relevant, *e.g.*, deamination of cytosine residues (Shapiro and Klein, 1966), destruction of deoxyribose residues, and hydrolytic cleavage of pyrimidine–glycosyl bonds (Shapiro and Danzig, 1972).

As these various heat-induced processes are associated with finite activation energies, they should also proceed at physiological temperatures, though at greatly reduced rates. Local denaturation of the DNA double helix, followed by rapid renaturation, has been observed at temperatures well below the T_m, and this structural motility may be of physiological relevance in the interaction of various proteins with DNA (Printz and von Hippel, 1965). Degradation of the covalent DNA structure may also occur at a physiologically significant rate at moderate temperatures.

Greer and Zamenhof (1962) demonstrated that purine bases were released in detectable quantities from DNA at neutral pH and temperatures near the T_m, and that the activation energy for depurination of denatured DNA was approximately 28 kcal/mole in 0.005 M phosphate buffer (pH 6.8). These results indicate that depurination of DNA could take place at a relevant rate also under physiological conditions. However, the liberation of purines in a DNA solution stored for 2 months at 37° was below the limit of chemical detectability. The possibility therefore remained that the rate of depurination of double-stranded DNA would be extremely slow, or show a strong temperature dependence, so that the reaction would be of negligible importance in living cells. For this reason, we have reinvestigated the rate of depurination of double-stranded DNA as a function of temperature and pH, employing bacterial DNA isotopically labeled in the purine residues. The results show that depurination of DNA in solution takes place at a significant rate even under conditions that presumably resemble those *in vivo*.

Materials and Methods

DNA Preparations. A purine-requiring mutant of *Bacillus subtilis* (strain 168 *purB* 6) was obtained from Dr. A. Adams. The bacteria were grown in the competence medium C^+ of O'Sullivan and Sueoka (1967), supplemented with 5×10^{-5} M [8-^{14}C]adenine (20 mCi/mmole). The bacteria were harvested in the late logarithmic growth phase, washed once with 0.15 M NaCl–0.1 M EDTA (pH 7.0) containing 10^{-4} M adenine, suspended in the same solvent, and treated with 1 mg/ml of

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