*DEOXYRIBONUCLEASE IV: A NEW EXONUCLEASE FROM MAMMALIAN TISSUES**

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Communicated by Theodore Shedlovsky, December 12, 1968

Abstract.—An exonuclease which specifically degrades double-stranded DNA has been isolated from rabbit tissues. The enzyme has an approximate molecular weight of 42,000, requires a divalent metal ion as cofactor, and attacks DNA at the 5′-terminal ends, thereby liberating 5′-mononucleotides. It degrades several synthetic polydeoxynucleotides of single repeating base sequences more rapidly than DNA from natural sources. The specificity of this mammalian enzyme resembles that of several microbial enzymes (phage λ exonuclease and DNA polymerase) which appear to be required for repair and recombination of DNA.

As part of a general survey of mammalian DNA-modifying enzymes, we have investigated the major DNase activities in extracts from myeloid and lymphoid tissues. Two types of DNases from animal tissues have previously been characterized: DNase I, which is usually isolated from pancreas;[1] and DNase II, which is localized in lysosomes.[2] An exonuclease which attacks RNA and single-stranded DNA[3] is also present in lysosomes, and endonucleases similar to DNase I but with different substrate specificities occur in mitochondria[4] and in brain extracts.[5] At alkaline pH values, the lysosomal enzymes show very little activity, and the activity of DNase I is inhibited by specific proteins[6] in crude cell extracts from lymphoid organs. In such extracts, the major DNase activities are two Mg^{++}-dependent exonucleases localized in cell nuclei. We have called these enzymes DNase III and IV in accord with the numbering system introduced by Laskowski.[7]

DNase III from normal rabbit tissues[8] attacks single-stranded DNA from the 3′-terminal end and appears to be widely distributed. An enzyme with very similar properties has previously been observed in mouse and rat tumor tissue[9, 10] as well as in HeLa cells [11] and baby hamster kidney cells.[12] DNase IV, the subject of the present communication, attacks double-stranded polydeoxyribonucleotides from the 5′-terminal end, thereby liberating 5′-mononucleotides. This enzyme has the unusual property of degrading polynucleotides of repeating base sequence more rapidly than native DNA.

Experimental.—Frozen rabbit organs from adult animals were obtained from Pel-Freez Biologicals, Inc. Terminal deoxynucleotidyltransferase was purified from calf thymus.[13] Polynucleotide kinase was purified from *E. coli* B infected with an amber mutant of phage T4 (T4 H39x).[14] Phage λ-induced exonuclease from *E. coli* was a gift from Dr. C. M. Radding.

Pancreatic DNase, *E. coli* alkaline phosphatase, venom phosphodiesterase, micrococcal nuclease, spleen phosphodiesterase, catalase, and lysozyme were obtained from Worthington. Crystalline bovine serum albumin was purchased from Armour Pharmaceutical Co. γ-^{32}P-ATP (2 mc/μmole) was prepared enzymatically.[15] 3′-dAMP was obtained by degradation of poly dA with micrococcal nuclease and spleen exonuclease.

Sources of other compounds were as follows:‡ dATP, dATP-^{3}H, dTTP, and dTTP-^{3}H, from Schwarz Bioresearch, Inc.; 5′-deoxymononucleotides, from Calbiochem; ATP, from Sigma; phosphocellulose grade P1, from Whatman; hydroxyapatite, from Clarkson Chemical Co.; Sephadex G-200, from Pharmacia; Brij 58, from Ruger Chemical Co.; $^{32}P_i$, from New England Nuclear Co.; and collodion bags (porosity less than 5 mμ), from Schleicher and Schuell. Determinations of protein and radioactivity were as previously described.[16]

Polynucleotides: Poly dA, poly dT, poly dA-^{3}H (5.2 μc/μmole), and poly dT-^{3}H (4.3 μc/μmole) were synthesized with terminal deoxynucleotidyltransferase to an average chain length of 300 residues.[17] Poly dA-^{3}H, labeled with ^{14}C at the 3′-OH end, was prepared by incubating dATP-^{3}H with the enzyme for 24 hr followed by a 40-min pulse of dATP-^{14}C (5–10 nucleotide residues added). Thymus DNA with several single-strand breaks,[16] poly dA-^{3}H, and poly dAT-^{3}H were labeled with ^{32}P at their 5′-terminal ends by polynucleotide kinase and γ-^{32}P-ATP.[18]

Poly dAT-^{3}H (25 μc/μmole) and poly dG:dC-^{3}H (3.4 μc/μmole) were obtained from Biopolymers, Inc. The poly dAT-^{3}H was further purified by gel filtration on Sephadex G-200 and had an average chain length of 1,100 residues. Poly rA-^{3}H (16.7 μc/μmole) and poly rU were obtained from Miles Laboratories, Inc. Calf thymus DNA was obtained from Worthington, and ^{32}P-labeled DNA was isolated from *E. coli*.[16] Aliquots from both samples were reduced to an approximate length of 600 nucleotides per strand by sonic irradiation in a Raytheon well-type sonicator. DNA-^{3}H from human embryonic kidney cells and DNA-^{14}C from adenovirus type 2 were gifts from Dr. W. Doerfler. DNA-^{32}P from phage T7 was a gift from Dr. W. Colli.

Assay for DNase IV activity: The assay measures the conversion of poly dAT-^{3}H to acid-soluble products. The standard assay mixture (0.1 ml) contained 0.4 mμmole dAT-^{3}H in 0.05 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes)-KOH, pH 8.5, 0.002 *M* $MgCl_2$, 0.002 *M* mercaptoethanol, 0.05% bovine serum albumin, and a limiting concentration of enzyme. After incubation at 37° for 15 min, the samples were chilled to 0°, an equal amount of cold 7% perchloric acid was added, and, after centrifugation, the radioactivity of the supernatants was determined. One unit of DNase IV converts 1 mμmole poly dAT to an acid-soluble form under the conditions described. The assay was linear over the range of 0.003–0.1 unit with purified DNase IV. In crude enzyme preparations (fractions I and II; see below), the assay was linear only in the range 0.003–0.015 enzyme units.

Size determinations: Analytical gel filtration and alkaline sucrose gradient centrifugations were performed as previously described.[16] Zone centrifugation of DNase IV was performed on fraction II according to Martin and Ames[19] with the use of catalase, bovine serum albumin, and lysozyme as standards. The 5–20% sucrose gradients contained 0.3 *M* KCl, 0.05 *M* Tris-HCl, 0.002 *M* mercaptoethanol, and 0.001 *M* EDTA, pH 7.1.

Analysis of reaction products: The radioactive acid-soluble products released from polynucleotides by DNase IV were mixed with a pancreatic DNase digest of nonradioactive DNA as carrier and chromatographed on DEAE cellulose in 0.02 *M* sodium acetate, pH 4.8, with a linear gradient of 0–0.4 *M* NaCl. To separate 5′-dAMP and 3′-dAMP, samples mixed with authentic nonradioactive 5′-dAMP and 3′-dAMP were chromatographed on Whatman No. 3-MM paper for 20 hr in a mixture of 80% saturated $(NH_4)_2SO_4$, 18% 1 *M* sodium acetate, and 2% isopropanol.

Subcellular localization: Subcellular fractions were obtained by differential centrifugation.[16] The mitochondrial fraction was further purified by sucrose density gradient centrifugation.

Purification of DNase IV from rabbit bone marrow: A summary of the purification procedure is given in Table 1. All operations were performed at 4°, all solutions were 10^{-3} *M* in mercaptoethanol, and centrifugations were for 15 min at 12,000 × *g*. Leucine was added to some solvents to inhibit aminopeptidase activity.[20]

(a) *Crude extract:* 50 gm of frozen rabbit bone marrow were extracted with 5 vol of 0.1 *M* NaCl, 0.05 *M* Tris-HCl, 0.02 *M* leucine, and 0.001 *M* EDTA, pH 7.4; debris and fat were removed as previously described[16] (fraction I).

(b) *Ammonium sulfate fractionation:* Fraction I was brought to 52% saturation in $(NH_4)_2SO_4$ and centrifuged 30 min later. The supernatant was brought to 70% saturation for 30 min. The precipitate was recovered and redissolved in 15–20 ml 0.2 *M* NaCl, 0.01 *M* sodium phosphate, 0.02 *M* leucine, and 5×10^{-4} *M* EDTA, pH 7.2, and dialyzed against the same solvent (fraction II).

(c) *Phosphocellulose chromatography:* Fraction II was dialyzed against the column buffer (0.01 *M* sodium phosphate and 5×10^{-4} *M* EDTA, pH 7.2) and, after 3 hr, was chromatographed on phosphocellulose (2.5 × 20 cm). The column was washed with 30 ml of the same buffer, then by 100 ml 0.04 *M* NaCl, 0.02 *M* sodium phosphate, and 5×10^{-4} *M* EDTA, pH 7.2. The enzyme was eluted with a linear gradient between 150 ml of the latter solvent and 150 ml of 0.5 *M* NaCl, 0.02 *M* sodium phosphate, and 5×10^{-4} *M* EDTA, pH 7.2. The enzyme appeared immediately before the major peak of eluted protein (fraction III).

(d) *Hydroxyapatite chromatography:* After dialysis against 0.05 *M* potassium phosphate, pH 7.5, fraction III was applied to a column of hydroxyapatite (1 × 6 cm) in the same buffer. After being washed with 50 ml, the enzyme was eluted with a linear gradient between 50 ml 0.05 *M* potassium phosphate, pH 7.5, and 50 ml 0.5 *M* potassium phosphate, pH 7.5. The most active fractions (12 ml) were pooled, concentrated 15-fold by vacuum dialysis in a collodion bag, and dialyzed against 0.2 *M* KCl, 0.02 *M* Tris-HCl, 0.02 *M* leucine, and 5×10^{-4} *M* EDTA, pH 7.5 (fraction IV).

All fractions of the enzyme lost about 50% of their activity in two days at 4°. To preserve activity, 0.02 ml 10% Brij 58 was added per 1 ml of fraction IV, and the enzyme was stored at −20°. After 1 month, 40% of the activity remained. The purified enzyme was free from DNA polymerase and terminal deoxynucleotidyltransferase activity, as well as from interfering nucleases.

TABLE 1. *Purification of DNase IV from rabbit bone marrow.*

Fraction	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)
I. Crude extract	240	2640	0.22	581
II. Ammonium sulfate	30	420	1.1	462
III. Phosphocellulose	40	19	5 6	106*
IV. Hydroxyapatite	1	3	21	63

* Only one third of the total enzyme activity adsorbed to the column. The adsorbed and unadsorbed activities had identical substrate specificity and kinetics of heat inactivation, indicating that they were due to the same enzyme.

Results.—Properties of the purified enzyme: The enzyme showed optimal activity at pH 8.5 and required a divalent cation. At an optimal concentration of 0.002 *M*, Mn^{++} was twice as effective as Mg^{++}. No activity was observed with Ca^{++}, Fe^{++}, or Zn^{++}. Exposure to 3×10^{-4} *M* p-hydroxymercuribenzoate completely inactivated the enzyme. Fifty per cent inhibition was obtained when the standard assay mixture contained either 0.08 *M* NaCl or 0.033 *M* potassium phosphate. At 52°, heat inactivation of the enzyme activity followed first-order kinetics with a half life of one minute. After five minutes, less than 1 per cent of the activity remained, indicating that the more heat-stable DNases I, II, and III were not present.

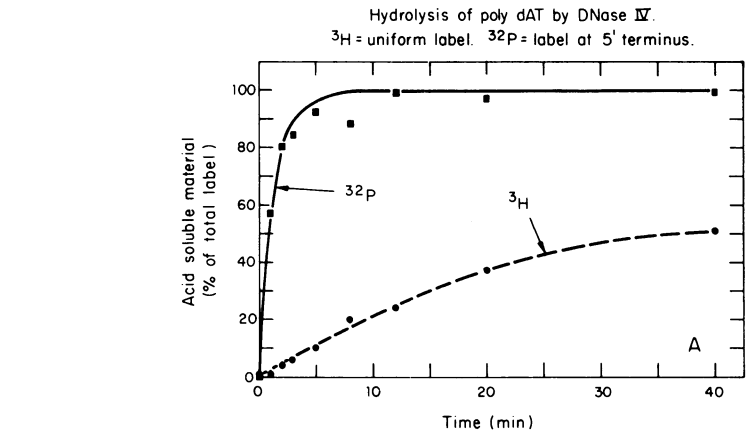
Molecular weight and frictional properties: The sedimentation coefficient of DNase IV activity was 3.4 ± 0.3*S* and the Stokes radius 29.5 ± 1.0 *A*. Assuming a partial specific volume of 0.725, a molecular weight of 42,000 ± 4,000 and a frictional ratio of 1.3 are obtained.[21] The molecular weight of the activity

obtained directly by gel filtration was $M = 43{,}000 \pm 6{,}000$, which is in good agreement with the previous value.

Products of reaction and mode of degradation: After 35 per cent hydrolysis of dA-^{3}H:dT by DNase IV, the major radioactive acid-soluble product was 5'-dAMP. The amount of liberated dinucleotide was less than 10 per cent and of oligonucleotides less than 3 per cent of that of mononucleotide. The acid-soluble products from DNA with 5'-^{32}P terminal labels were also identified as the four major 5'-deoxymononucleotides by paper chromatography.

These results indicate that the enzyme attacks its substrate by releasing mononucleotides from one or both ends of the molecule. Further support for an exonucleolytic mode of action was obtained from a study of partially degraded dAT-^{3}H by zone centrifugation in alkaline sucrose. After 5 per cent of the polymer had been digested to acid-soluble products, the remaining material showed only a very small reduction in the sedimentation coefficient (less than 10%) and little change in the distribution of sedimentation coefficients when compared to the intact polynucleotide. Similarly, after 0.5 per cent hydrolysis of uniformly labeled DNA-^{32}P to acid-soluble material, no significant amount of endonucleolytic degradation could be detected.[22]

Site of attack: In order to determine whether the enzyme attacks its substrate



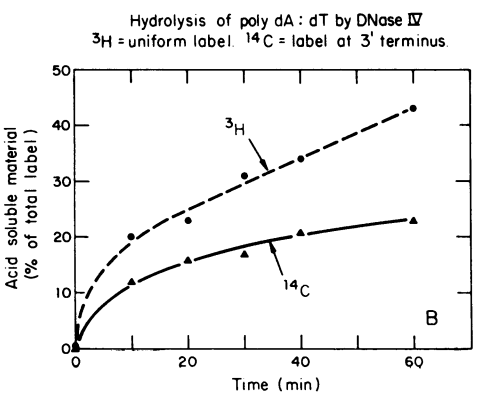


FIG. 1.—Kinetics of hydrolysis of terminally labeled polydeoxyribonucleotides by DNase IV. 0.12 unit of enzyme was added per 0.1 ml aliquot of the standard assay mixture, followed by incubation at 37° for various lengths of time.

(*A*) Degradation of uniformly labeled poly dAT-^{3}H with a 5'-terminal ^{32}P label. Of the ^{32}P released, more than 99% was released as nucleotides (material adsorbing to charcoal).

(*B*) Degradation of poly dA:dT. The dA strand was uniformly labeled with ^{3}H and had a 3'-terminal ^{14}C label.

preferentially from the 3'- or 5'-terminal end, the kinetics of hydrolysis of doubly labeled polymers were followed (Fig. 1). The 5'-terminal label was released very rapidly in the form of mononucleotides, and there was no preferential release of label from the 3'-terminal end. Thus DNase IV attacks double-stranded polydeoxyribonucleotides in an exonucleolytic fashion from the 5'-terminal end, thereby liberating 5'-mononucleotides. The data further suggest that the enzyme attacks different polynucleotide chains randomly rather than degrading one chain at a time in a processive way. A similar mode of action has been described for λ exonuclease[23] and for the exonucleolytic activity of *E. coli* DNA polymerase.[24]

Substrate specificity: DNase IV does not attack single-stranded polydeoxynucleotides such as poly dA-^{3}H or poly dT-^{3}H at a detectable rate (i.e., <2% of the rate observed with poly dAT-^{3}H). A more sensitive test was provided by using poly dA-^{3}H labeled with ^{32}P at its 5'-terminus; less than 2 per cent of the terminal label was released by 0.1 unit of DNase IV under standard assay conditions. Double- or single-stranded RNA polymers were not attacked by the enzyme: the rates of release of acid-soluble material from poly rA-^{3}H:rU and poly rA-^{3}H were less than 2 and 5 per cent, respectively, of that observed with poly dAT. The double-stranded homopolymers poly dA-^{3}H:dT and poly dA:dT-^{3}H were attacked at the same rate as poly dAT-^{3}H. Prior removal of the terminal 5'-phosphate residue by alkaline phosphatase did not affect the rate of degradation of poly dAT-^{3}H. Poly dG:dC-^{3}H was attacked at one third of the rate of poly dAT. Lambda exonuclease also was found to attack poly dAT three times faster than poly dG:dC.

With native *E. coli* DNA-^{32}P, DNase IV showed a surprisingly slow rate of hydrolysis compared to dAT, while λ exonuclease attacked both these substrates at comparable rates (Fig. 2). Similar results were obtained with phage T7 DNA, kidney DNA, adenovirus DNA, and sonicated *E. coli* DNA. That the slow degradation of native DNA was indeed due to DNase IV and not to a contaminating enzyme was indicated by the heat lability and metal ion requirements of the activity. Moreover, labeled 5'-terminal nucleotides were selectively

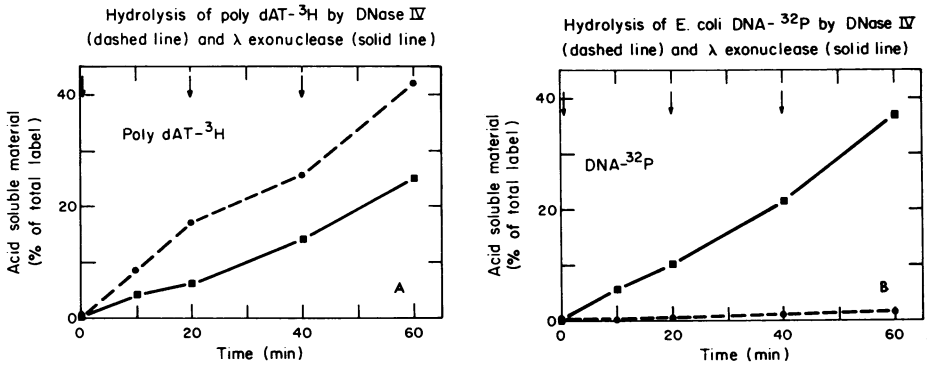


FIG. 2.—Kinetics of hydrolysis of dAT-^{3}H and *E. coli* DNA-^{32}P by DNase IV and λ exonuclease. Each tube (0.1 ml) contained 0.4 mμmole polynucleotide. At 0, 20, and 40 min, 0.05 unit DNase IV or 0.01 unit[23] λ exonuclease were added to each 0.1-ml incubation mixture (*arrows*). ●———● = DNase IV; ■———■ = λ exonuclease.

released at similar rates from both DNA and poly dAT. The latter observation implies that DNase IV releases the first nucleotides in DNA more rapidly than subsequent ones. The apparent low general activity with DNA was not due to inactivation of the enzyme by the DNA, as addition of a twofold excess of sonicated DNA in the standard assay interfered but little (20% inhibition) with dAT degradation.

Subcellular and cellular distribution: After differential centrifugation, 70 per cent of the total DNase IV activity was found in the cytoplasm, while 30 per cent was retained in nuclei that had been washed twice. No activity was detected in extracts from a purified mitochondrial fraction.

DNase IV was present in several rabbit tissues, with highest specific activities occurring in bone marrow, lymph node, and lung extracts. The enzyme from lung was purified and was indistinguishable from the bone marrow enzyme.

Discussion.—DNase IV is the first mammalian exonuclease to be described which is specific for double-stranded DNA. Enzymes with this property have been postulated to play important roles in both repair and recombination of DNA. We have been particularly interested in enzymes of this type in lymphoid and myeloid tissues.[25, 16]

In its substrate requirements and mode of attack, DNase IV resembles two well-characterized microbial nucleases: *E. coli* DNA polymerase and λ exonuclease. These three enzymes can degrade double-stranded polydeoxyribonucleotides by liberating 5′-mononucleotides from the 5′-terminal ends. Of the two microbial enzymes, the λ exonuclease forms part of the general recombination system of the bacteriophage,[26] while *E. coli* DNA polymerase might be involved in excision and repair of DNA among other synthetic functions.

DNase IV degrades synthetic polynucleotides at a much faster rate than native DNA, a property which it apparently has in common with *E. coli* DNA polymerase.[27] Two general models may be advanced to account for this unusual substrate preference. Either a "stop signal" for the enzyme occurs within the sequence of native DNA in the form of a specific di- or trinucleotide sequence or a methylated base, or else the enzyme is sensitive to the local conformation of the double-stranded polymer at the site of attack. In synthetic polynucleotides, extensive chain slippage is known to occur[28] which would allow a particular local conformation to be constantly re-formed. A number of specific examples of such sites of attack may be visualized. The enzyme might prefer a 5′-end matched with a 3′-end at the opposite strand, i.e., a protruding single-stranded 3′-terminus at the complementary strand could inhibit the enzyme. Alternatively, the enzyme may work most efficiently at a nick in a double helix. Experiments are in progress to elucidate this point.

* Supported by grant GB 6546 from the National Science Foundation and by grant AM 04256 from NIH.

† Fellow of the Helen Hay Whitney Foundation.

‡ *Abbreviations used:* Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; TTP, thymidine triphosphate; poly dA, a homopolymer of deoxyadenylate; poly dT, a homopolymer of thymidylate; poly dA-^{3}H, poly dA labeled with ^{3}H; other radioactive compounds are designated in an analogous way; poly dA:dT, a double-stranded polymer composed of poly dA and poly dT; poly dG:dC, a double-stranded polymer composed of polydeoxyguanylate and polydeoxycytidylate; poly dAT, an alternating double-stranded copolymer of deoxyadenylate and thymidylate; poly rA, polyriboadenylic acid; poly rU, polyuridylic acid.

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