

Partial purification and characterization of four endodeoxyribonuclease activities from *Escherichia coli* K-12

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ABSTRACT

Four hitherto undescribed endodeoxyribonucleases, temporarily designated A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, and B, have been isolated from *E. coli* K-12. Each requires Mg<sup>++</sup> and is not stimulated by ATP or S-adenosylmethionine. A<sub>3</sub> is strongly inhibited by Fe<sup>+++</sup> and weakly inhibited by ATP, S-adenosylmethionine, and DPN, whereas B is inhibited by caffeine. Each can be purified free of exonuclease or DNA-3'-phosphatase. A<sub>1</sub> (molecular weight approximately 72,000) cleaves single-stranded, circular fd DNA to form 3'-hydroxyl termini and introduces nicks and breaks in the closed, double-stranded replicative form DNA of fd (fd RFI). A<sub>2</sub> (molecular weight approximately 46,000) cleaves fd DNA and introduces nicks and breaks in RFI, forming 3'-hydroxyl- and 5'-phosphoryl termini. A<sub>3</sub> (molecular weight approximately 38,000) cleaves fd DNA to form 3'-hydroxyl termini and introduces only nicks in fd RFI. Irradiation of the RFI with ultraviolet light markedly increases the rate of hydrolysis by A<sub>3</sub>. B appears to form 3'-phosphoryl termini with fd DNA, but its characterization is highly preliminary due to its instability.

In the course of purifying the *recBC*<sup>†</sup> deoxyribonuclease of *Escherichia coli* K-12<sup>1</sup> it was noted that endodeoxyribonuclease activity eluted from DEAE-cellulose prior to the *recBC* enzyme. Unlike the *recBC* enzyme this activity was not stimulated by ATP. It was not abolished by tRNA as is *E. coli* endonuclease I<sup>2</sup> and was present in mutants lacking endonuclease I. In addition, it was distinguishable from *E. coli* endonuclease II<sup>3</sup> by absolutely requiring Mg<sup>++</sup>, and it differed from the restriction enzymes of *E. coli* which require ATP and require, or are stimulated by S-adenosylmethionine<sup>4,5</sup>. This communi-

<sup>†</sup>Abbreviations used are: *recBC* DNase, the deoxyribonuclease controlled by the *recB* and *recC* genes of *E. coli* (this enzyme has also been designated exonuclease V); S-AM, S-adenosylmethionine; fd RF, the single-stranded, circular replicative form of fd DNA; RFI, covalently closed circles; RFII, circles with at least one strand of the helix opened; RFIII, linear forms produced by opening both strands of the helix of RFI or RFII at the same point; nick, the opening of one phosphodiester bond of fd RF; break, the opening of the two strands of fd RF at the same place on the molecule so as to convert RFI or RFII to RFIII; UV-RF, RF treated with ultraviolet light; DTT, dithiothreitol (Cleland's reagent); [γ-<sup>32</sup>P]ATP, ATP labeled with <sup>32</sup>P in the γ-phosphoryl group; BSA, bovine serum albumin; BAP, bacterial alkaline phosphatase.

cation describes the resolution of this side fraction into four subfractions and presents preliminary observations of the properties of these activities.

### MATERIALS AND METHODS

*Enzymes* -- *E. coli* exonuclease I was the hydroxylapatite fraction of Lehman and Nussbaum<sup>6</sup>. Snake venom phosphodiesterase, pancreatic DNase, micrococcal nuclease, and bacterial alkaline phosphatase were purchased from the Worthington Biochemical Corporation. The alkaline phosphatase used in determining the base composition of 5'-termini was further purified on DEAE-cellulose<sup>7</sup>. One unit of phosphatase releases 1  $\mu$ mole of Pi from 5'-AMP in one hour at 37°. Polynucleotide kinase was the hydroxylapatite fraction assayed according to Richardson<sup>8</sup> and Jacquemin-Sablon and Richardson<sup>9</sup>. *E. coli* exonuclease III was prepared and assayed by the exonuclease assay as described by Richardson and Kornberg<sup>10</sup>.

*DNA* -- <sup>3</sup>H-labeled fd phage DNA and fd replicative form DNA (labeled and unlabeled) from fd sB-1<sup>o</sup> were prepared as previously described<sup>11</sup>. DNA bearing <sup>32</sup>P-labeled 3'-phosphoryl termini was prepared by partial digestion of <sup>32</sup>P-labeled *E. coli* DNA with micrococcal DNase as described by Richardson and Kornberg<sup>10</sup>. UV - fd RF was prepared by irradiating for seven minutes 0.1 ml of 0.44 mM [<sup>3</sup>H]RF in a thin film (area 6.5 mm<sup>2</sup>) on a watchglass which was at 0°, 35 cm from a Westinghouse G15 T8 Sterilamp. The incident energy was 46 ergs·mm<sup>-2</sup>·sec<sup>-1</sup>. This level of irradiation forms about 100 thymine dimers per DNA molecule, monitored as described previously<sup>12</sup>. DNA and RNA concentrations are expressed as the molarity of nucleotide residues.

*Other Materials* -- ATP was purchased from Sigma Corporation; all other labeled nucleotides were purchased from P. L. Biochemicals, Incorporated. [ $\gamma$ -<sup>32</sup>P]ATP was prepared by the method of Gynn and Chapell<sup>13</sup>. Enzyme-grade ammonium sulfate and density gradient grade sucrose were purchased from Mann Research Laboratories. DEAE-cellulose, type 40, was from the Brown Company; Phosphocellulose, type 11, was purchased from Whatman. Streptomycin sulphate and aldolase were purchased from Calbiochem.

*DNase Assays* -- The endonuclease assay for single-stranded DNA measures the transformation of circular fd phage DNA to a form susceptible to exonuclease I. Unless otherwise noted, this assay was used in the studies described below. The standard assay (0.15 ml) contained 20 mM MgCl<sub>2</sub>, 0.67 mM DTT, 1 mM 2-mercaptoethanol, 67 mM buffer, 3.75 nmoles of <sup>3</sup>H-labeled fd DNA (2-6 x 10<sup>3</sup> cpm per nmole), and 2 units<sup>6</sup> of exonuclease I. Buffers used were Tris-maleate, pH 6.3; glycylglycine-NaOH, pH 7.0; Tris-HCl, pH 8.2; lysine-NaOH, pH 9.0; glycine-NaOH, pH 9.5; glycine-NaOH, pH 10.5. After 30 minutes

at 37° the DNA rendered soluble in acid was quantitated as previously described<sup>11</sup>. One unit of endonuclease converts 1 nmole of fd DNA nucleotide to a form susceptible to exonuclease I in 30 minutes. The standard assay system was modified by the addition of 0.25 units of BAP or 2.0 units of exonuclease III when assaying endonuclease which formed 3'-phosphoryl termini.

The endonuclease assay for double-stranded DNA measures by velocity sedimentation, the conversion of <sup>3</sup>H-labeled fd RFI to RFII, RFIII, or small fragments. The reaction mixture was the same as for the assay with single-stranded DNA, except that 3.5 nmoles of <sup>3</sup>H-labeled RFI were used in a reaction volume of 0.17 ml and exonuclease I was omitted. After 60 minutes at 37° the reaction was stopped by adding 0.17 ml of 0.2 M EDTA and the mixture was divided into two equal volumes which were "layered onto" alkaline and neutral sucrose gradients for velocity sedimentation. The quantity of RFI converted to RFII ("nicking") or to double-stranded linear form ("breaking") was calculated from the sedimentation data. This assay is considered to be only semi-quantitative because of variability in the recovery of DNA (50-100%).

*DNA Phosphatase Assay* -- The DNA phosphatase assay measures the release of inorganic phosphate from 3'-phosphoryl-terminated DNA<sup>10</sup>.

*Infectivity Assay of fd DNA and fd RFI* -- Assays of the infectivity of DNA preparations were carried out by the method of infectious centers, in which competent spheroplasts are first exposed to the potentially infective DNA and then are plated with virus-susceptible (indicator) bacteria<sup>5</sup>.

*Polynucleotide Kinase Treatment* -- Reaction conditions for polynucleotide kinase were: 50 mM Tris-HCl, pH 8.2; 20 mM MgCl<sub>2</sub>; 2 mM KPO<sub>4</sub>, pH 6.7 (to inhibit residual alkaline phosphatase, if present) and the indicated amount of [ $\gamma$ -<sup>32</sup>P]ATP and polynucleotide kinase.

*Sucrose Gradient Sedimentation* -- All DNA sedimentation was done at 4° in polyallomer tubes with the Spinco SW 50.1 rotor at 50,000 rpm. Previously formed 5% to 20% sucrose gradients (5.2 ml) contained 0.25 M NaCl, 20 mM Tris-HCl, pH 8.2, and 5 mM EDTA and were centrifuged for 300 minutes (neutral sedimentation), or contained 0.25 M NaOH and 5 mM EDTA and were centrifuged for 110 minutes (alkaline sedimentation). Fractions were collected from a hole in the bottom of the tube, neutralized if alkaline, and the radioactivity determined by liquid scintillation counting.

Enzyme sedimentation for purification or molecular weight determination was at 4° in polyallomer tubes with the SW 65L rotor at 65,000 rpm for 6 hours. Previously formed 5% to 20% sucrose gradients contained 10 mM Tris-HCl, pH 7.5, 1 ml 2-mercaptoethanol and 0.1 ml DTT. Fractions were collected from a hole

in the bottom of the tube and endonuclease activity was determined by the standard assay.

*Other Methods* -- For liquid scintillation counting, samples were brought to 0.9 ml and mixed with 10 ml scintillator (9.1 g of 2,5-diphenyloxazole (PPO), 0.61 g of 1-4-bis [2-(5-phenyloxazolyl)] benzene (POPOP), 1,250 ml of Triton X-100, 2,190 ml of toluene). Non-aqueous samples on chromatography paper were placed in 5 ml of non-aqueous scintillator (12 g PPO, 0.3 g POPOP, 3 liters toluene).

Liquid samples and Norit suspensions to be counted in the gas-flow Geiger counter were dispensed onto planchettes, neutralized with NaOH if acidic, mixed with a drop of 5% sodium dodecylsulfate and dried under a heat lamp. Samples on chromatography paper were dried and placed on planchettes for counting. Protein concentration was estimated by the method of Lowry *et al.*<sup>15</sup> using BSA as a standard.

### RESULTS

#### *Purification of enzymes*

All operations were carried out at 0-4°, and, unless otherwise indicated, all buffers contained 0.1 mM DTT.

*Preparation of Extract* -- 12 liters of *E. coli* JC 4583 (an endonuclease I - deficient K-12 strain<sup>16</sup>) were grown and collected by centrifugation as previously described<sup>11</sup>, then resuspended in 50 ml of 50 mM glycylglycine-NaOH, pH 7.0,  $5 \times 10^{-4}$  M DTT (Buffer A). Following cell disruption by sonic irradiation (5 episodes of 45 seconds each at 110 watts) the cell debris was removed by centrifugation at 16,000 x g for 10 minutes and the absorbance of the extract at 260 nm was adjusted to 200 with Buffer A (Fraction I).

*Streptomycin Precipitation* -- To Fraction I was slowly added with stirring 0.087 volume of freshly prepared 5% streptomycin sulfate solution. After a total of 30 minutes stirring, the precipitate was removed by centrifugation and discarded (Fraction II).

*Ammonium Sulfate Precipitation* -- Solid ammonium sulfate (37.6 g/100 ml) was added slowly with constant stirring to Fraction II. After 30 minutes the precipitate was collected by centrifugation at 27,000 x g for 15 minutes and resuspended in sufficient Buffer A to give an absorbance at 260 nm of 350 (Fraction III).

*Potassium Phosphate Fractionation* -- To Fraction III was added 0.14 volumes of 4 M K<sub>2</sub>HPO<sub>4</sub>. After stirring for 10 minutes the precipitate was removed by centrifugation at 27,000 x g for 10 minutes and to the supernatant fraction was added an additional 0.41 volumes of 4 M K<sub>2</sub>HPO<sub>4</sub> (based on the initial

volume of Fraction III). After stirring for 30 minutes the precipitate was collected by centrifugation at 27,000 x g for 20 minutes and resuspended in 15 ml of Buffer A (Fraction IV).

*DEAE-Cellulose Chromatography* -- A column (4 x 20 cm) of DEAE-cellulose was equilibrated with 2 liters of Buffer A. Fraction IV was extensively dialyzed versus Buffer A and applied to the column. The column was washed with 200 ml of Buffer A then the enzyme was eluted with a linear gradient (1,500 ml total volume) from 0 to 0.4 M NaCl in Buffer A (Fig. 1). The active fractions were pooled into Pool A (0.13 - 0.18 M NaCl) and Pool B (0.20 - 0.28 M NaCl), then solid ammonium sulfate was added to each (42.6 g/100 ml) with stirring for 30 minutes. The precipitates were collected by centrifugation at 27,000 x g for 15 minutes and each was taken up in a minimum volume of 10 mM  $KPO_4$ , pH 6.7 (Buffer B) (Fraction VA and Fraction VB).

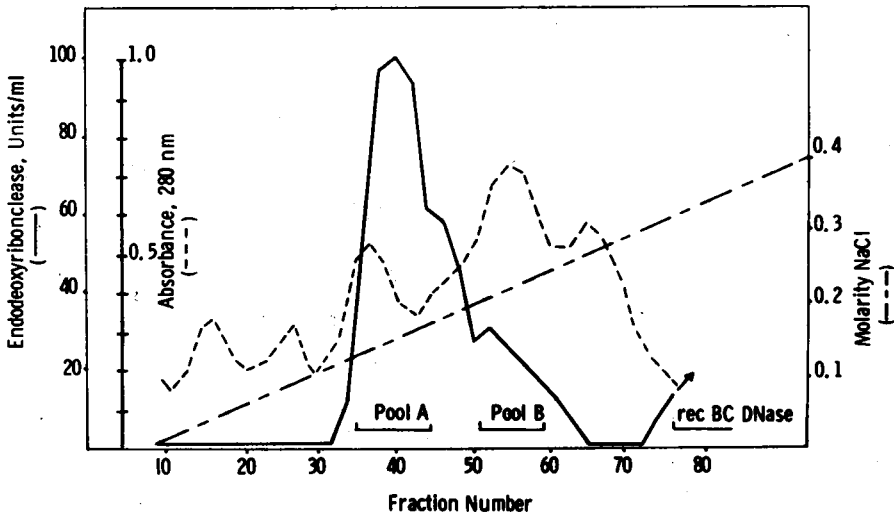


Fig. 1. DEAE-cellulose chromatography. Elution was as described in the text. The *recBC* DNase eluted after 0.3 M NaCl.

*Phosphocellulose Chromatography* -- Fractions VA and VB were dialyzed extensively versus Buffer B. Two phosphocellulose columns, Column A (2 x 16 cm) and Column B (1.3 x 12 cm), were equilibrated with Buffer B, then the dialyzed fractions were loaded onto the respective columns. Column A was eluted successively with 50 ml of Buffer B, 100 ml of 0.1 M  $KPO_4$ , pH 6.7, and a linear gradient (total volume 500 ml) from 0.10 M  $KPO_4$ , pH 6.7 - 0.50 M  $KPO_4$ , pH 6.7. Three separate peaks of nuclease activity were identified and pooled as Fraction VI A<sub>1</sub>, Fraction VI A<sub>2</sub>, and Fraction VI A<sub>3</sub>, eluting at 0.13 - 0.17 M  $KPO_4$ , 0.25 - 0.29 M  $KPO_4$ , and 0.33 - 0.40 M  $KPO_4$ , respectively (Fig.

2). Column B was eluted successively with 20 ml of Buffer B, 50 ml 0.1 M  $KPO_4$ , pH 6.7, and a linear gradient (total volume 150 ml) from 0.10 M  $KPO_4$ , pH 6.7 to 0.50 M  $KPO_4$ , pH 6.7. One peak of endonuclease activity (Fraction VIB) was identified, eluting at 0.30 - 0.35 M  $KPO_4$  (Fig. 2). It should be noted that prior to being assayed for endonuclease activity, all fractions from the phosphocellulose columns were dialyzed *versus* 0.01 M Tris-HCl, pH 8.2 because the  $KPO_4$  in the fractions interfered markedly with the assays, presumably by precipitation of  $Mg^{++}$  at alkaline pH.

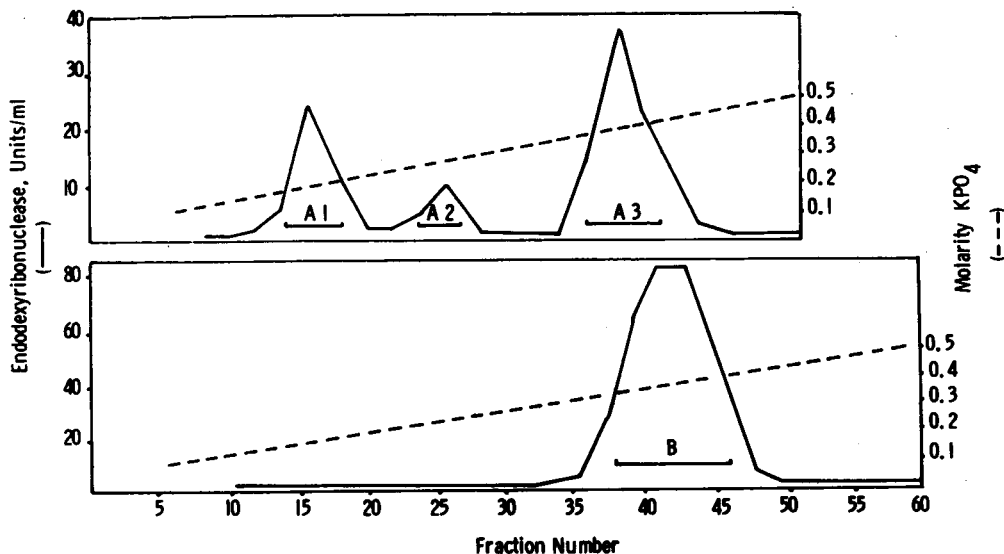


Fig. 2. Phosphocellulose chromatography. The columns were eluted and the individual fractions were dialyzed, then assayed as described in the text. Fractions from Column A were 9.5 ml, from Column B, 2.5 ml.

*DEAE-Cellulose Concentration* -- Fractions VI A<sub>1</sub>, VI A<sub>2</sub>, VI A<sub>3</sub>, and VI B were concentrated by adsorption onto DEAE-cellulose followed by one-step elution. Columns of 12 ml, 6 ml, 1 ml, and 1 ml, respectively, were equilibrated with 0.02 M  $KPO_4$ , pH 6.7 (Buffer C). Subfractions of step VI were applied, and the columns were eluted with two column volumes of Buffer C, then with two column volumes of 0.4 M KCl in Buffer C. Fractions of 2 ml, 1.5 ml, 0.5 ml, and 0.5 ml were collected from the respective columns, and the most active fraction from each was saved (Fractions VII A<sub>1</sub>, VII A<sub>2</sub>, VII A<sub>3</sub>, VII A<sub>4</sub>).

*Sucrose Gradient Sedimentation* -- Fractions VII A<sub>1</sub> and VII A<sub>2</sub> were further purified by layering 0.180 ml onto a 5.2 ml gradient of sucrose (5 - 20%) and sedimenting as described in "Methods". The most active fractions which were free of exonuclease and phosphatase activities (as determined by

the release of acid-soluble nucleotide, or  $P_i$ , respectively, under standard reaction conditions) were identified and stored at 0° (Fractions VIII A<sub>1</sub> and VIII A<sub>2</sub>).

TABLE I. *Purification of DNases*

Fraction		Volume <i>ml</i>	Protein <i>mg/ml</i>	Specific Activity <sup>a</sup> <i>units/mg protein</i>
I.	Crude Extract	75	45.2	29
II.	Streptomycin	222	14.5	40
III.	Ammonium Sulfate	85	29	40
IV.	Potassium Phosphate	26.5	56	52
V.	DEAE Fractions			
	Pool A	17	3.1	328
	Pool B	24	1.6	208
VI.	Phosphocellulose Pools			
	A <sub>1</sub>	77	0.17	82
	A <sub>2</sub>	55	0.04	400
	A <sub>3</sub>	108	0.01	2,700
	B	22	0.04	1,125
VII.	DEAE Concentrates			
	A <sub>1</sub>	2	2.28	86
	A <sub>2</sub>	1.5	0.77	76
	A <sub>3</sub>	0.5	0.735	2,067
	B	0.5	0.460	474
VIII.	Sucrose Gradients			
	A <sub>1</sub>	0.26 <sup>b</sup>	0.096	104 <sup>c</sup>
	A <sub>2</sub>	0.26 <sup>b</sup>	0.216	115 <sup>c</sup>

<sup>a</sup>All assays were done in glycine-NaOH, pH 9.5.

<sup>b</sup>Only portions (0.180 ml) of Fractions VII A<sub>1</sub> and VII A<sub>2</sub> were taken for purification on sucrose gradients.

<sup>c</sup>These were not the most active fractions from the gradient, but were selected to be free from contaminating exonuclease and phosphatase activities.

The purification is summarized in Table I. Unless otherwise indicated, the studies below utilized Fractions VII A<sub>1</sub>, VII A<sub>2</sub>, VII A<sub>3</sub>, or VII B.

#### *Storage and Stability of the Preparation*

The purification was routinely carried out through step VII (DEAE-cellulose concentration) and each fraction was stored either over liquid nitrogen or at 0°. Fractions VII A<sub>1</sub>, VII A<sub>2</sub>, and VII A<sub>3</sub> were stable for at

least eight weeks during storage over liquid nitrogen, but each lost 25 - 50% of its activity upon each cycle of freezing and thawing. At 0°, Fractions VII A<sub>1</sub> and VII A<sub>2</sub> proved to be stable for eight weeks, whereas Fraction VII B decayed with a half-life of approximately 24 hours. While at 0° Fraction VII A<sub>3</sub> underwent a progressive loss of activity when assayed at pH 9.5 (the optimal pH of the fresh preparation) with a concomitant rise in activity at pH 7.0 (see below). This phenomenon of "aging" required 8 to 10 weeks.

The sucrose gradient fraction, VIII A<sub>1</sub>, had stability properties similar to Fraction VII A<sub>1</sub>, but Fraction VIII A<sub>2</sub> proved to be unstable under any conditions of storage with a half-life of approximately four hours. Consequently, it was necessary to characterize it, then use it immediately after preparation.

### *Properties of the Purified Enzymes*

In order to characterize the four endonuclease activities, and in particular to decide whether they represented four individual unrelated enzyme species, various physical and catalytic properties of each were examined. It should be noted, however, that the results with endonuclease B are preliminary since its instability precluded both extensive characterization and complete separation from residual endonuclease A<sub>3</sub>.

*Influence of pH on Rate of Reaction* -- The optimal pH for endonucleases A<sub>1</sub> and A<sub>2</sub> were 7.5 and 7.0 respectively. However, in both cases approximately 30% of the activity at the optimal pH was manifest at pH 9.5. The activity of endonuclease B had an optimum pH 7.0, but was detectable at 9.5.

The effect of pH on A<sub>3</sub> was complicated: upon preparation the enzyme had a sharp pH optimum near 9.5 with little or no activity at pH 7.0. On storage in liquid nitrogen this pattern remained unchanged but on storage at 0° (in glycylglycine-NaOH, pH 7.0) the enzyme lost activity at pH 9.5 (to less than one-fourth the initial activity) and assumed an amount of activity at pH 7.0 roughly equal to that initially present at 9.5.

*Effect of Polyvalent Cations* -- All four endonuclease activities required Mg<sup>++</sup>, the optimal concentration being 0.01 - 0.02 M. They were also strongly inhibited by EDTA. A unique property of endonuclease A<sub>3</sub> was a strong inhibition by Fe<sup>+++</sup>: 0.1 mM FeCl<sub>3</sub> reduced the activity by 25%, and 7.5 mM FeCl<sub>3</sub> inhibited both the "fresh" (pH 9.5) and the "aged" (pH 7.0) forms of the enzyme by greater than 90%. Neither A<sub>1</sub> nor A<sub>2</sub> was affected by Fe<sup>+++</sup>; a small depression of B was observed which was probably due to contamination by endonuclease A<sub>3</sub>. While Fe<sup>++</sup> and Co<sup>++</sup> also slightly inhibited A<sub>3</sub>, the presence of the trivalent ions during incubation could not be ruled out. Neither 0.1 mM Ca<sup>++</sup> nor Zn<sup>++</sup> affected any of the activities.



*Effect of tRNA* -- When each endonuclease was assayed in the presence of 26  $\mu\text{M}$  tRNA (that amount capable of completely inhibiting endonuclease I<sup>2</sup>) an inhibition of 20 - 40% was seen. This inhibition was consistently seen but distinctly less than that expected for endonuclease I. (We have observed that tRNA inhibits endonuclease I at least 99% when assayed under our standard conditions.)

*Effect of Other Possible Cofactors* -- ATP, a cofactor for the *recBC* and restriction DNases of *E. coli*<sup>1,4,5</sup>, S-AM, a cofactor for the restriction DNases of *E. coli*<sup>4,5</sup>, and DPN, the cofactor for DNA ligase of *E. coli*<sup>17</sup> were without effect on the activities of A<sub>1</sub>, A<sub>2</sub>, or B. On the other hand, 0.13 mM ATP, 20  $\mu\text{M}$  S-AM, and 1  $\mu\text{M}$  DPN each consistently inhibited A<sub>3</sub> by approximately 50%, whether it was in the "fresh" or "aged" forms. These concentrations are those optimal for the other enzymes noted above. Neither 0.13 mM cyclic AMP nor 0.1 mM vitamin B<sub>6</sub> had any effect on these endonucleases. Caffeine was without effect on A<sub>1</sub>, A<sub>2</sub>, or A<sub>3</sub>. However, at pH 7.0, 0.67 mM caffeine inhibited B by 30 - 70%; at pH 9.6 there was no effect. A more systematic study of this inhibition was precluded by contamination of B by A<sub>3</sub>, and by our inability to control whether this A<sub>3</sub> was in the fresh (pH 9.5) form or the aged (pH 7.0) form.

*Effect of Bacterial Alkaline Phosphatase (BAP) and DNA Phosphatase (E. coli Exonuclease III)* -- The standard assay for these endonucleases measures the conversion of single-stranded, circular fd DNA to a form susceptible to exonuclease I. Since exonuclease I degrades molecules only if they have a 3'-hydroxyl terminus<sup>6</sup>, this assay detects breaks in which 3'-phosphomonoester termini are produced only if these termini are removed by a phosphomonoesterase. To test whether 3'-phosphoryl termini were present, bacterial alkaline phosphatase or DNA phosphatase were added to reaction mixtures. With neither A<sub>1</sub> nor A<sub>2</sub> was there any effect, even with Fractions VIII A<sub>1</sub> or VIII A<sub>2</sub> with which we could detect no removal of <sup>32</sup>P<sub>i</sub> from [<sup>32</sup>P]DNA containing 3'-phosphomonoester groups. In the case of A<sub>3</sub>, digestion with fractions free of phosphatase were unaffected by exonuclease III, and inhibited by 30 - 50% in the presence of BAP. In the case of B, BAP or exonuclease III stimulated the assay two- to five-fold. This stimulation was found even when DNA was treated with the endonuclease, then heated to inactivate endonuclease before being exposed to the exonuclease I and phosphomonoesterase. These results imply that A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> form 3'-hydroxyl groups when cleaving fd DNA, but B forms at least some 3'-phosphoryl groups. Whether B forms only 3'-phosphoryl groups is unknown because of the presence of some A<sub>3</sub> in Fraction VII B.

*Size of Endonucleases* -- The endonucleases sedimented through sucrose gradients with an  $S_{20,W}$  of 4.7, 3.5, and 3.1 for  $A_1$ ,  $A_2$ ,  $A_3$ , respectively, relative to aldolase ( $S_{20,W} = 8.0$ ) added as a marker. Using the method of Martin and Ames<sup>18</sup> these values were used to estimate molecular weights of 72,000, 46,000, and 38,000. Recently, more purified  $A_3$  has been observed to sediment with an apparent molecular weight of approximately 20,000. The activity of B could not be recovered from gradients.

*Digestion Products Obtained with Endonucleases*

*Absence of Exonuclease and DNA Phosphomonesterase Activity* -- As noted above,  $A_1$ ,  $A_2$ , and  $A_3$  could be purified free from DNA-3'-phosphatase activity. In addition,  $A_1$  and  $A_2$  could be separated from any detectable exonuclease activity on a sucrose gradient, whereas  $A_3$  and B were free of exonuclease after phosphocellulose chromatography. The absence of exonuclease was verified by (1) the fact that no material was made acid-soluble from single-stranded DNA in the absence of added exonuclease I, and (2) the lack of non-sedimentable material during digestion of the double-stranded RF, even after all the RF had been cleaved (Fig. 3). With  $A_2$  the RF could be degraded to nonsedimentable material, but until the bulk of material became so degraded, no DNA was observed at the top of the gradient.

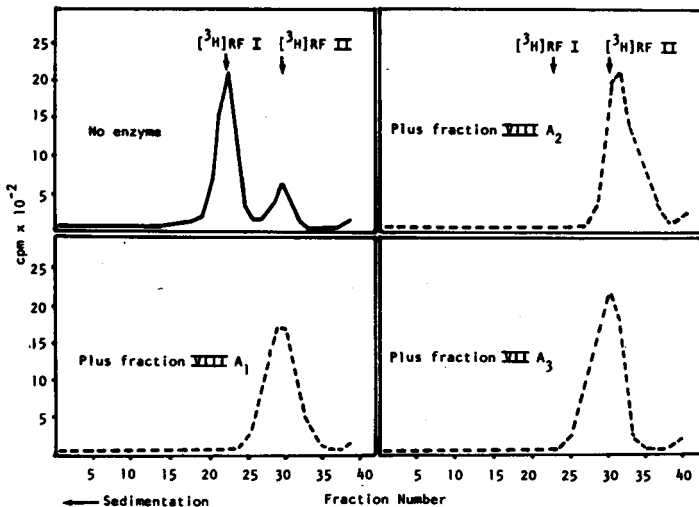


Fig. 3. Extensive degradation of fdRF. Reaction mixtures contained 3.5 nmoles of [<sup>3</sup>H]RF (7 x 10<sup>3</sup> cpm per nmole) and 2.0 units of VIII A<sub>1</sub>, 0.4 unit of VIII A<sub>2</sub>, or 4.0 units of VII A<sub>3</sub>. After incubation at pH 9.5 they were sedimented through alkaline sucrose gradients.

*Mode of Attack on Duplex Circular Substrates* -- To detect whether the enzymes digested duplex DNA by "nicking" single strands or "breaking" double strands, the products of digestion of fd RF by limiting amounts of enzymes (i.e., levels which left significant residual RFI) were analyzed by sedimentation in neutral and alkaline sucrose gradients (Fig. 4). Fractions VIII A<sub>1</sub> and VIII A<sub>2</sub> were able to catalyze both nicks and breaks in RFI as evidenced by the presence of material of less than unit length in both the alkaline and neutral gradients. However, Fraction VII A<sub>3</sub> appeared to catalyze only nicks. In addition to the above findings it was observed that when the digestion products from the experiment of Figure 3 (in which no RFI remained) were analyzed for residual infectivity, A<sub>1</sub> and A<sub>2</sub> were seen to have essentially eliminated the infectivity, whereas A<sub>3</sub> had little effect (Table II). This finding reinforces the evidence that endonucleases A<sub>1</sub> and A<sub>2</sub> catalyze double-stranded breaks in RF (which result in the loss of infectivity) whereas A<sub>3</sub> catalyzes only nicks (with no loss of infectivity).

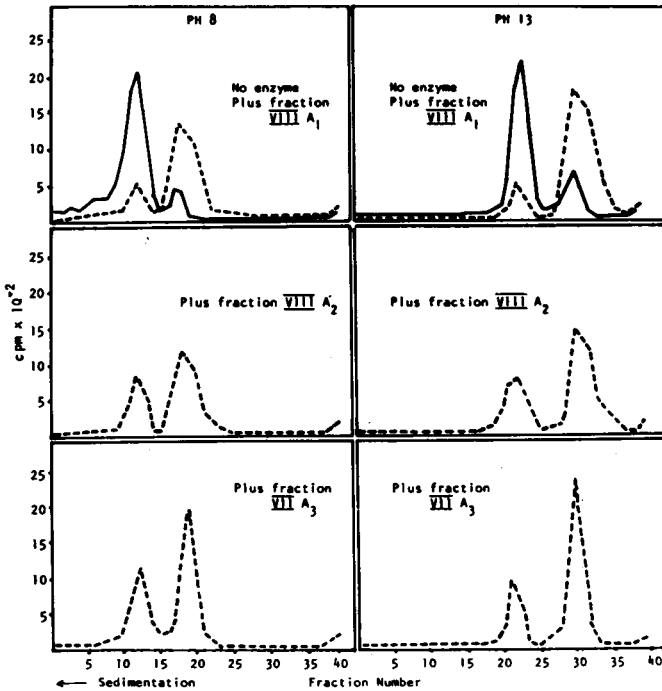


Fig. 4. Partial digestion of fd RF. Reaction mixtures contained 3.5 nmoles of [<sup>3</sup>H]RF (7 x 10<sup>3</sup> cpm per nmole) and 1.0 unit of VIII A<sub>1</sub>, 0.2 unit of VIII A<sub>2</sub> or 2.0 units of VII A<sub>3</sub>. After incubation they were sedimented through neutral or alkaline sucrose gradients. The large peak in the control gradients is RFI; the smaller peak is RFII in the neutral gradient (RFIII runs 10% more slowly than RFII) and unit length, single-stranded circular or linear molecules in the alkaline gradient.

TABLE II. Infectivity of RF Digests<sup>a</sup>

Enzyme used in Digestion	Volume of Diluted Digest Plated μl	Plaques Per Plate <sup>b</sup>
None	25	20, 18
	100	55, 70
VIII A <sub>1</sub>	25	0, 0
	100	0, 0
VIII A <sub>2</sub>	25	0, 1
	100	0, 0
VII A <sub>3</sub>	25	21, 13
	100	104, 61

<sup>a</sup>Digestion products from the experiment described in Fig. 3 were diluted 1:600 then assayed for infectious centers.

<sup>b</sup>Pairs represent duplicate plates.

The relative activities of A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> ("aged") on RFI *versus* single-stranded fd DNA can be estimated from the experiment of Figure 4, in which 1 unit of A<sub>1</sub> and 0.2 unit of A<sub>2</sub> each nicked or broke roughly 2.2 nmoles RF, whereas 2.0 units of A<sub>3</sub> catalyzed the alteration of approximately 1.6 nmoles of RF. Noting that the time of incubation of the enzymes with the RF was twice that for the determination of the standard (fd DNA) units, it appears that in terms of the number of nicks or breaks introduced per 30 minutes, A<sub>1</sub> is equally active, A<sub>2</sub> roughly five times more active, and A<sub>3</sub> approximately one-half as active on double-stranded as on single-stranded DNA. (This calculation takes into account the fact that the RF has double the number of nucleotides as the phage DNA.) Although a precise analysis of the limit of the endonuclease reactions was not attempted, it has been noted that A<sub>1</sub> and A<sub>2</sub> can endonucleolytically reduce the DNA to small, non-sedimentable oligonucleotides, whereas A<sub>3</sub> leaves rather large fragments.

When the RF was irradiated with ultraviolet light and then exposed to endonucleases A<sub>1</sub>, A<sub>2</sub>, or B, no increased rate of hydrolysis was observed. On the other hand, this DNA was distinctly more sensitive to A<sub>3</sub> (Fig. 5). Although this difference was consistently observed, it should be emphasized that A<sub>3</sub> was not specific for the irradiated DNA and we made no attempt to

correlate the increased sensitivity to any particular photoproduct. In fact, the irradiated DNA was shown to contain roughly 100 thymine dimers, and the enzyme was not observed to degrade the DNA so extensively as one break per dimer.

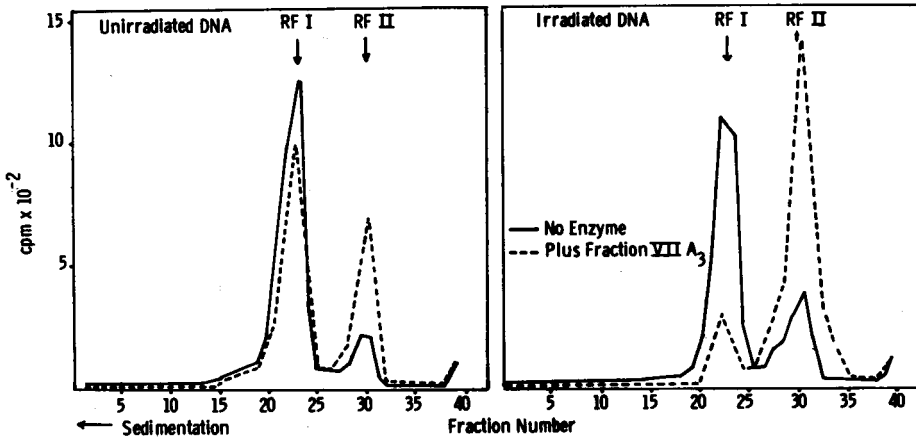


Fig. 5. Comparison of ultraviolet-irradiated and unirradiated RF as substrate for endonuclease  $A_3$ . Reaction mixtures contained 1 unit of enzyme and 3.5 nmoles of  $[^3H]RF$  ( $7 \times 10^3$  cpm per nmole), irradiated where indicated. After incubation they were sedimented through alkaline sucrose gradients.

*Analysis of fd DNA Digestion Products by Phosphorylation with Polynucleotide Kinase* -- Digestion products of fd DNA and fd replicative form DNA were tested for 5'-termini which would accept  $^{32}PO_4$  from  $[\gamma-^{32}P]ATP$  in the presence of polynucleotide kinase as described by Weiss, Live, and Richardson<sup>7</sup> with and without prior treatment of the digest with phosphomonoesterase. Reproducible results based on significant incorporation were obtained only in the case of  $A_2$ , mainly due to the more extensive digestion of DNA with this enzyme. Following phosphorylation, the  $A_2$  digests were hydrolyzed to mononucleoside-5'-phosphates, separated by paper chromatography, and the amount of label incorporated into each nucleotide was measured (Table III). A significant amount of  $^{32}P$  incorporation was found to occur only after treatment of the digest with phosphomonoesterase. Since DNA with existing 5'-phosphomonoester groups is not phosphorylated by polynucleotide kinase it is evident that endonuclease  $A_2$  produces 5'-phosphoryl termini. If the frequency of labeling of the four nucleotides is examined (Table III) and compared to base composition of the DNAs, it is seen that, while not specific, the enzyme has some bias for breaks next to guanine and against breaks adjacent to thymine.

TABLE III. Phosphorylation of Digestion Products of Endonuclease A<sub>2</sub> by Polynucleotide Kinase

DNA Substrate	Nucleotide	pmole <sup>32</sup> P Incorporated		Fraction Total <sup>a</sup>	Overall DNA Base Composition
		-BAP	+BAP	%	%
fd DNA	dAMP	0.13	3.11	21	24
	dGMP	0.19	5.47	37	20
	dTMP	0.18	3.05	21	34
	dCMP	0.22	3.00	21	22
	Total	0.72	14.63		
fd RF	dAMP	.05	0.75	25	29
	dGMP	.08	1.00	34	21
	dTMP	.05	0.48	17	29
	dCMP	.07	0.67	23	21
	Total	.25	2.90		

<sup>a</sup>From samples treated with BAP.

LEGEND TO TABLE III. Incubations (0.075 ml) with 5.6 units Fraction VIII A<sub>2</sub> were carried out for two hours at pH 7.5 with 9.6 nmoles of <sup>3</sup>H-labeled fd phage DNA (1.4 x 10<sup>3</sup> cpm per nmole), then the mixtures were made 0.05 M in Tris-HCl, pH 8.2 and heated at 90° to terminate the endonuclease reaction. The mixtures were chilled to 0° and 2.3 units of alkaline phosphatase were added to each where indicated. After incubation at 65° for 30 minutes they were chilled and each mixture was made 2 mM in KPO<sub>4</sub>, pH 6.7 (to inhibit residual alkaline phosphatase) and 1.2 nmoles of [γ-<sup>32</sup>P]ATP (4.2 x 10<sup>3</sup> cpm per pmole) was added along with 6 units of polynucleotide kinase to a final volume of 0.100 ml. After 30 minutes at 37° reactions were terminated with 0.1 ml 0.1 M ATP, 0.1 ml 2 mg/ml salmon sperm DNA, and 25 μl 7N perchloric acid. After 5 minutes at 0°, the precipitates were collected by centrifugation at 16,000 x g for 5 minutes, suspended in 0.1 ml 0.1 N NaOH, and reprecipitated with 0.02 ml 7 N perchloric acid and 0.5 ml distilled water. Precipitates were collected and resuspended once again in the same manner and the mixtures were made 0.15 M in Tris-HCl, pH 8.2 and 1 mM in ATP, extracted with buffer-saturated phenol, then dialyzed extensively to remove residual phenol and ATP. The samples were concentrated under a stream of nitrogen to 0.15 ml and made 0.05 M in Tris-HCl, pH 8.2, and 5 mM in MgCl<sub>2</sub>, mixed with 5 μl pancreatic DNase (2 mg/ml), and incubated for 90 minutes at 37°. Six μl 1 M glycine-NaOH, pH 10.4 and 3 μl snake venom phosphodiesterase (2 mg per ml, 3 x 10<sup>4</sup> units per ml) were then added. (One unit converts 1 nmole oligonucleotides to mononucleotides in 30 minutes at 37° at pH 9.2.) After six hours at 37° the mixtures were concentrated to 0.2 ml and spotted onto Whatman No. 1 paper with 0.1 μmole each deoxyribonucleoside-5'-phosphate and chromatographed (descending) in saturated ammonium sulfate-1 N sodium acetate-isopropyl alcohol

(80:18:2) for 17 hours. Spots were identified under ultraviolet light and each was cut out and counted in a gas flow counter. The remaining paper was cut into sections and counted in the same way. No significant counts remained at the origin and approximately 8% of the counts recovered were randomly distributed between identifiable spots. Recovery of the [ $^3\text{H}$ ]nucleotide ranged from 30 to 35%. Blank values for reactions without  $A_2$  (0.18 - 0.34 pmole per nucleotide and 0.03 - 0.05 pmole per nucleotide with and without BAP treatment, respectively) have been subtracted from the values shown. The experiment with fd RF was the same except that the incubation mixture contained 2.5 nmoles of unlabeled fd RF and 2.8 units  $A_2$ . Blank values for reactions without enzyme (0.03 - 0.05 pmole per nucleotide and 0.02 - 0.03 pmole per nucleotide with and without BAP treatment, respectively) have been subtracted from the values shown.

TABLE IV. *Characteristics of Endonucleases*

Fraction	VIII A <sub>1</sub>	VIII A <sub>2</sub>	VII A <sub>3</sub>	VII B
pH Optimum	7.5 (broad)	7.0 (broad)	9.5 (fresh) 7.0 (aged)	7.0
Fe <sup>+++</sup> Inhibition	none	none	> 90%	little or none
Inhibition by ATP, S-AM, DPN	none	none	50%	none
Inhibition by Caffeine	none	none	none	30-70%
Stimulation of Exonuclease I Assay by BAP or Exonuclease III	none	none	none	2- to 5-fold
Approximate Molecular Weight	72,000	46,000	38,000	---
Action on RF	nicks, breaks	nicks, breaks	nicks	---
Product Size with RF	small	small	large	---
Preference for UV-Irradiated RF	none	none	yes	---

#### DISCUSSION

A question of primary importance is whether the four activities reported here represent four independent enzymes, or whether at least some of them are related, but resolve during purification due to aggregation, proteolysis, chromatography artifacts, etc. Table IV summarizes some of the catalytic properties of the endonucleases, and from these observations it seems likely that  $A_3$  and B are independent enzymes. On the other hand,  $A_1$  and  $A_2$  might be

related, since neither has any property which clearly distinguishes it from the other--i.e.,  $A_2$  might be a smaller form of  $A_1$  which is more stable under reaction conditions, but less stable after sucrose gradient sedimentation.

Another important question is the biological function of these enzymes. We have examined some mutants in DNA replication, repair, recombination, and restriction and have not as yet detected any alteration of the properties of these endonuclease activities. The preference of  $A_3$  for ultraviolet-irradiated RF and the inhibition of B by caffeine (a reported inhibitor of dark repair<sup>19</sup>) might suggest, however, that these enzymes are involved in DNA repair, although no definitive evidence for such a role has been obtained.

The stimulation by BAP or exonuclease III of digestion by exonuclease I of fd DNA circles nicked by endonuclease B is evidence that this enzyme makes 3'-phosphoryl groups. Such an endonuclease would be unique for *E. coli*, since all other DNases reported to date from this organism form 3'-hydroxyl groups. Unfortunately, the instability of this enzyme has precluded its further characterization and purification from contaminating enzymes.

The basis of conversion of  $A_3$  to a form with a pH optimum of 7 and a strong activity on RF DNA is unknown. The two forms cosediment in sucrose gradients and are inhibited by the same reagents. For these reasons, the conversion is not likely due to aggregation of subunits, a major change in conformation of the enzyme, or the decay of the activity of one enzyme with the concomitant appearance of the activity of an unrelated enzyme. Finally  $A_3$  is possibly the same enzyme reported by Champ and Gold<sup>20</sup>. Using a markedly different method of purification, they isolated an endonuclease from *E. coli* K-12-1100 which was active on both single-stranded and double-stranded DNA with a pH optimum of 9.5 and an absolute requirement for  $Mg^{++}$ . It was reported to catalyze only a limited number of nicks in RF (one per molecule under their conditions). Their preparation retained a small amount of exonuclease activity and was not further characterized.  $A_3$  has been noted to be a small basic protein and as such is similar to endonuclease I. Indeed, in the presence of tRNA, endonuclease I has been shown to put a limited number of nicks into duplex, circular DNA<sup>21</sup>. However, the latter activity was absent from the endonuclease I mutant, *E. coli* 1100, whereas this mutation has no effect upon the enzymes described here. In addition,  $A_3$  is not inactivated by endonuclease I antiserum (under conditions where endonuclease I is inactivated) and  $A_3$  does not copurify with endonuclease I.



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