Proceedings of the National Academy of Sciences
Vol. 67, No. 1, pp. 434–441, September 1970

An Endonuclease Activity from *Escherichia coli* Absent from Certain *rec*⁻ Strains*

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Communicated by Paul Berg, June 5, 1970

Abstract. A new endonuclease activity from *Escherichia coli* which cleaves circular, single-stranded DNA in the presence of added nucleoside triphosphate has been purified. The activity has not been detected in extracts from certain *rec*⁻ strains and is therefore implicated in genetic recombination.

Introduction. During studies of the specificity of the restriction endonuclease from *Escherichia coli* B, we observed a contaminating nuclease activity which reduced the infectivity of the single-stranded circular DNA of coliphage fd. Further experiments have shown that this inactivation was due to endonucleolytic cleavage of the phage DNA. This endonuclease requires or is greatly stimulated by added nucleoside triphosphate and has other properties in common with an exonuclease activity recently reported by Buttin and Wright,[1,2] Oishi,[3] and Barbour and Clark.[4] In particular, the endonuclease activity is lacking in extracts from mutants of the *recB* and *recC* genes of *E. coli* K12. This report describes the purification and some characteristics of this endodeoxyribonuclease.

Materials and Methods. Materials: Phage fd was obtained from Hoffman-Berling. *E. coli* Hfr C6-2 (*thy*⁻) was provided by David Pratt. All other *E. coli* strains were obtained from Dr. John Clark and have been described.[4]

ATP was purchased from Calbiochem; other unlabeled nucleotides were purchased from P. L. Biochemicals. Thymidine, ^{14}C- and ^{3}H-labeled, was from Schwarz BioResearch. ADP-C-P, the methylene-bridged analog of ATP, was from Miles Laboratories; streptomycin sulfate and enzyme-grade ammonium sulfate were from Mann Research Laboratories. DEAE-cellulose (type 40) and phosphocellulose (P11) were purchased from the Brown Co. and Whatman, respectively. Biogel P300 and agarose 0.5 M (10%) were from Bio-Rad Laboratories. Pancreatic DNase was purchased from Worthington; exonuclease I was either the DEAE-cellulose fraction described by Lehman,[5] or was purified as a by-product of the endonuclease purification (glycerol gradient).

Replicative form I (RF)-I DNA from phage fd was prepared as described by Linn and Arber.[6] T7 DNA was the gift of Dr. David Hinkle. Labeled fd phage DNA was prepared by growing *E. coli* Hfr C6-2 to a density of 10^8 cells/ml in glucose-casamino acids medium,[7] then adding either [^{14}C]thymidine or [^{3}H]thymidine to a final concentration of 0.4 μCi/ml (9.6 μg/ml), or 10 μCi/ml (4 μg/ml), respectively. The cells were then infected with fd phage at a multiplicity of infection of 3–5, and grown for 8 hr at 37°C. After removal of the cells, the phage were purified by two cycles of differential centrifugation. The DNA was obtained by incubating the phage with 0.5% sodium dodecyl sulfate for 10 min at 25°C, extracting twice with buffer-saturated phenol, and dialyzing extensively.

Endonuclease assay: The assay measured the amount of fd DNA nucleotide that

is made sensitive to exonuclease I. The reaction mixture (0.15 ml) contained 50 mM glycylglycine–NaOH, pH 7.0, 10 mM $MgCl_2$, 0.33 mM ATP, 3.8 nmol [^{3}H]-labeled fd DNA (3.2×10^3 cpm/nmol), 1 unit[8] of exonuclease I, and endonuclease. The mixture was incubated for 30 min at 37°C, then chilled on ice. Bovine serum albumin (0.5 mg in 0.05 ml) and 0.25 ml of 7% trichloroacetic acid were added and the reaction mixture was kept at 0°C for 5 min. After centrifugation at 25,000 × *g* for 5 min, the supernatant fluid was removed and its radioactivity determined in scintillation fluid (9.1 g PPO, 0.61 g POPOP, 1250 ml Triton X-100, 2140 ml toluene). Blank values without endonuclease gave less than 0.8% of the input radioactivity soluble in acid. One unit of endonuclease converts 1 nmol of fd DNA-nucleotide to a form sensitive to exonuclease in 30 min at 37°C. The assay was linear in the range 0.06–0.75 unit. Infectivity assays[6] confirmed that the DNA that remained resistant to exonuclease I retained its infectivity and therefore was still circular. Protein was determined by the method of Lowry *et al.*[9]

Purification of the endonuclease: Unless otherwise indicated, all operations were performed at 0–4°C, and centrifugation was for 10 min at 16,000 × *g*.

Six liters of *E. coli* K12 (JC 4583 *rec*$^+$) were grown in L broth[10] at 37°C with forced aeration to a density of 6–8 × 10^8 cells/ml. The cells were harvested by centrifugation for 5 min at 4000 × *g* and resuspended in 40 ml of buffer A (0.05 M glycylglycine–NaOH, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol). The cell suspension was disrupted with three 45-sec pulses from a Branson Sonifier (100 W output) and the debris was removed by centrifugation and discarded.

The absorbance of the extract at 260 nm was adjusted to 200 with buffer A (final volume, 123 ml) and 14.7 ml of freshly prepared 5% streptomycin sulfate was added with stirring. After 35 min at 0°C the precipitate was removed by centrifugation. To the supernatant fluid (123 ml) was added 39.2 g of ammonium sulfate. After 45 min at 0°C the precipitate was removed by centrifugation and dissolved in 15 ml of buffer A (final volume, 22 ml). This fraction was dialyzed overnight against two 1-liter portions of 0.05 M glycylglycine–NaOH, pH 7, 5 mM 2-mercaptoethanol, 3 mM EDTA.

The dialyzed material was then incubated at 25°C until 75–80% of the ultraviolet-absorbing material at 260 nm was rendered soluble in 7% perchloric acid (45–60 min). After removal of insoluble material by centrifugation, the autolysate was passed onto a 10% agarose column (4.5 × 85 cm) previously equilibrated with buffer B (0.05 M glycylglycine–NaOH, pH 7, 1 mM EDTA, 0.1 mM DTT). The enzyme was eluted with buffer B just after the void volume.

Agarose fractions with a specific activity greater than 25 were pooled, mixed with 0.1 volume of glycerol (final volume, 180 ml), and applied to a 2.5 × 18 cm DEAE-cellulose column equilibrated with buffer C (0.05 M glycylglycine–NaOH, pH 7, 1 mM EDTA, 0.1 mM DTT, 10% glycerol). The column was washed with 150 ml of buffer C, then eluted with a linear gradient of 0.05–0.55 M NaCl in buffer C (total volume, 800 ml). The enzyme was eluted in approximately 0.38 M NaCl. Fractions with a specific activity greater than 100 were pooled (volume, 92 ml) and concentrated by precipitation with 37.5 g of ammonium sulfate as described above. The pH was maintained at 7.0 by the addition of 0.19 ml of 1 N NaOH. The precipitate was dissolved in 10 ml of buffer D (10 mM KPO_4, pH 6.7, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol) and the material was dialyzed against 500 ml of buffer D. The dialyzed material was diluted to 3 mg protein/ml and applied to a phosphocellulose column (1.5 × 11 cm) equilibrated with buffer D. The column was washed with buffer D and the enzyme appeared in the void volume of the column. The protein from this fraction (40 ml) was precipitated with 15.6 g of ammonium sulfate as above, then dissolved in 1.5 ml of buffer D.

The concentrated phosphocellulose fraction was purified further by layering 0.25 ml upon 5 ml of a 20–40% glycerol gradient (containing 10 mM KPO_4, pH 6.7, 0.1 mM EDTA, 0.1 mM dithiothreitol) and sedimenting for 12 hr at 50,000 rpm in a Spinco SW 50.1 rotor at 4°C. The endonuclease activity sedimented about halfway down the tube.

Alternatively, additional purification was achieved by reverse-flow chromatography on a Biogel P300 column (2.5 × 100 cm) equilibrated with buffer D. Phosphocellulose

fraction (1 ml) was put onto the column and the column was washed with buffer D. The endonuclease activity was eluted about 100 ml past the void column. Fractions free of exonuclease I were pooled and concentrated by putting them onto a 1 ml DEAE-cellulose column equilibrated with buffer D, washing with 3 ml of buffer D, then eluting with 0.5 M NaCl in buffer D. The concentrated fraction was finally dialyzed against buffer D.

Results. Detection and purification of the endonuclease: In order to detect specifically the endonuclease activity, we used as an assay the ability of the enzyme to render fd phage DNA susceptible to exonuclease I from *E. coli*. Exonuclease I is unable to hydrolyze intact circular fd DNA, but causes rapid release of deoxyribonucleoside-5'-phosphates once the integrity of the DNA is interrupted by endonuclease.[8,11] The release of acid-soluble mononucleotide was then used to monitor the degree of endonucleolytic cleavage.

The endonuclease was purified (Table 1) as described in *Materials and Methods*.

TABLE 1. *Purification of the endonuclease.*

Fraction	Protein (mg)	Units of activity	Units/mg
Extract	1970	...	...
Streptomycin	1850	...	...
Ammonium sulfate	688	...	...
Autolysis	690	...	...
Agarose	576	19,000	31
DEAE-cellulose	53	8,300	155
Phosphocellulose	36	7,000	196
Glycerol gradient*	1.8	5,800	3200
Biogel P300*	0.40	1,200	3000

Fractionation and assays are described in *Materials and Methods*.
* These values have been corrected for the fact that only a fraction of the phosphocellulose fraction was used for these steps.

The activity could not be reproducibly assayed before the agarose gel step, perhaps because of the presence of inhibitory material in the crude preparations. After agarose gel filtration, however, a distinct endonuclease activity was observable which was markedly stimulated by ATP. The enzyme was further purified on DEAE-cellulose, phosphocellulose, and finally by filtration through Biogel P300 or by sedimentation through a glycerol gradient. Either the Biogel or the glycerol gradient resulted in a fraction which was about 100 times as pure as the agarose eluate; however, the two fractions differed somewhat in their properties (see below). The agarose fraction was stable for several months at 0°C, whereas more purified fractions were stabilized by storage either over liquid nitrogen or at −20°C in 30% glycerol.

Properties of the enzyme: The endonuclease has a broad pH optimum between 6.5 and 8.2 with a maximum at pH 7. The enzyme is most active in glycylglycine buffers, and is markedly inhibited by phosphate. Magnesium ion is required for activity; maximum activity was found at 10 mM.

Purified enzyme also required ATP for maximal activity. The enzyme fractions from the agarose, DEAE-cellulose, phosphocellulose, and glycerol gradient steps of the purification were stimulated 5- to 8-fold by the presence of 0.3 mM ATP. About half of this stimulation was observed in 0.1 mM ATP, whereas higher concentrations (0.5–2.0 mM) gave about 70% of the stimulation. In

contrast, enzyme obtained from Biogel P300 had an absolute requirement for ATP (Table 2). The different response to ATP may not necessarily be due to the removal of a contaminant activity that does not require ATP, since no such activity has been detected elsewhere in the column eluate. Furthermore, the effect of ATP upon the exonuclease activity of the preparation (see below) was similarly affected by the Biogel step.

TABLE 2. *Cofactor requirements of the Biogel P300 fraction.*

Compound present	nmoles fd phage DNA sensitive to exonuclease I	Maximal activity (%)
None	<0.01	<1
ATP		
0.066 mM	0.24	39
0.13 mM	0.37	60
0.33 mM	0.62	100
0.66 mM	0.46	79
dATP	0.40	64
dTTP	0.17	27
GTP	0.18	29
4 XTP's (0.13 mM each)	0.28	45
4 dXTP's (0.13 mM each)	0.55	88
ADP-C-P	<0.01	<1
ATP + SAM (0.013 mM)	0.30	49
ATP + caffeine (0.5 mM)	0.61	98
ATP + tRNA (0.06 mM)	0.62	100

The reaction mixtures (0.15 ml) contained 50 mM glycylglycine–NaOH, pH 7.0, 10 mM $MgCl_2$, 3.8 nmoles [^{3}H]-fd DNA (3.2×10^3 cpm/nmole), 1 unit of exonuclease I, and 0.63 unit of endonuclease. Compounds were tested at a concentration of 0.33 mM unless otherwise noted. After 30 min at 37°C the acid-soluble radioactivity was determined as in *Materials and Methods*.

The effect of some other compounds upon the endonuclease activity of the Biogel fraction are shown in Table 2. GTP, dATP, and dTTP were somewhat less effective as stimulators than ATP, as were mixtures of the four ribo- or four deoxyribonucleoside triphosphates. The ATP analog, β,γ-methylene ATP (ADP-C-P), had no stimulatory effect, however. Neither caffeine, and inhibitor of dark repair of ultraviolet-irradiated DNA in *E. coli*,[12] nor tRNA, an inhibitor of endonuclease I from *E. coli*,[13] affected this activity.

Although no extensive analysis was performed to study the size of the enzyme, the activity had a sedimentation coefficient of 11–12 S during the glycerol gradient purification step (determined using catalase as a marker). This value would indicate a molecular weight in the range of 300,000 daltons.

Effect of certain *rec*$^-$ mutations upon the enzyme activity: In order to gain insight into the biological role of this enzyme, we examined extracts from mutants of bacteria defective in functions that might utilize an endodeoxyribonuclease. Since the wild-type activity could not be assayed reproducibly in early fractions, all extracts were taken through the agarose gel filtration step. As shown in Table 3, the ATP-stimulated endonuclease activity was missing in extracts from bacteria carrying the *recB21* and/or *recC22* mutations. Furthermore, if the agarose fractions were put through DEAE-cellulose, comparable column fractions from the *recB* or *recC* extracts lacked the ATP-stimulated

TABLE 3. *ATP-stimulated endonuclease activity in extracts from rec⁻ cells.*

Fraction	Source of extract	Units of activity per mg protein −ATP	+ATP
Agarose	JC 4583 (*rec*⁺)	4.5	32.3
	JC 4588 (*recA56*)	3.5	9
	JC 6720 (*recB21 recC22*)	4.0	3.6
	JC 6721 (*recC22*)	6.8	5.5
	JC 6722 (*recB21*)	6.8	5.0
DEAE	JC 4583 (*rec*⁺)	31	140
	JC 4588 (*recA56*)	24	104
	JC 6720 (*recB21 recC22*)	5.6	2
	JC 6721 (*recC22*)	2.2	<0.02
	JC 6722 (*recB21*)	0.8	<0.02

Extracts were prepared and assayed for endonuclease activity as described in *Materials and Methods*, except that ATP was omitted from the reaction mixture where indicated.

activity, and there was no evidence for such an activity elsewhere in the column eluates from the mutant extracts. Extracts from *E. coli recA56*, however, contained the activity (Table 3), as did extracts from *E. coli* B. The inhibitory effect of ATP upon the residual activity in extracts from *recB* and/or *recC* mutants, and the reduced activity of the corresponding DEAE-cellulose fractions in the absence of ATP (Table 3) were consistently observed.

One explanation for the lack of activity in extracts from the *recB* and *recC* strains would be the presence of an inhibitor. Indeed, DEAE-cellulose fractions from these strains strongly inhibited the ATP-dependent endonuclease purified from the *rec*⁺ or *recA* strains (Table 4). Heating of the mutant fractions inactivated the inhibitory effect. There was no evidence that such an inhibitor exists in the equivalent *rec*⁺ fractions, nor did an inhibitor appear upon further purification of the endonuclease. However, the presence of a small amount of inhibitor in the *recA* extracts has not been ruled out. The implications of this inhibitory effect are under study and will be published elsewhere.

TABLE 4. *Effect of extracts from mutant strains upon the endonuclease activity.*

Fractions present	nmoles DNA susceptible to exonuclease I
rec⁺	0.29
recA56	0.66
recB21	<0.01
recC22	<0.01
rec⁺ + *recB21*	<0.01
rec⁺ + *recC22*	0.02
recA56 + *recB21*	0.02
rec⁺ + *recB21* (heated)	0.25
rec⁺ + *recC22* (heated)	0.28

Assays were performed as described in *Materials and Methods*. Where indicated, 2 μg of the Biogel P300 fraction from the *rec*⁺ strain, or 15 μg of the DEAE-cellulose fractions obtained from *recA*, *recB*, or *recC* strains were added. Heated fractions were treated for 5 min at 90°C and cleared by centrifugation before use. Control experiments verified that exonuclease I was entirely unaffected by the *recB* or *recC* fractions. Similar inhibitory effects were found with the comparable agarose fractions from *recB* or *recC* strains.

Effect of the enzyme upon single-stranded DNA: Purified enzyme from *rec*⁺ cells converted single-stranded circular DNA to a form that was sensitive to exonuclease I. Acid-soluble fragments were also produced in the absence of exonuclease I, even early in the reaction when a large proportion of the circles remained intact. This degradation required ATP and was also found if the fd DNA had been previously nicked with pancreatic DNase (Table 5). When the effect of the enzyme upon fd circles was studied by sedimentation of the treated DNA in alkaline sucrose gradients (Fig. 1),

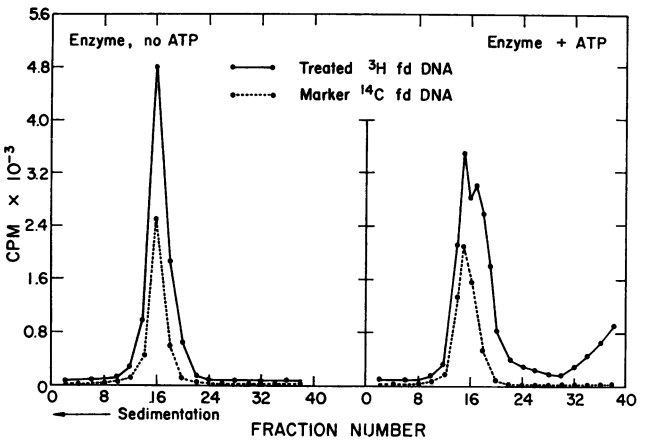

FIG. 1. Sedimentation of fd DNA treated with enzyme. The reaction mixture (0.05 ml) contained 50 mM glycylglycine–NaOH, pH 7.0, 4.5 mM $MgCl_2$, 6.3 nmol [^{3}H]-labeled fd DNA (3.2×10^3 cpm/nmol), 3.5 units of Biogel P300 fraction endonuclease, and 0.33 mM ATP where indicated. After 1 hr at 37°C the reaction mixture was chilled, made 20 mM in EDTA, and diluted to 0.15 ml with distilled water. Before sedimentation, NaOH was added to 0.25 N, and [^{14}C]-labeled fd DNA was added as marker. The samples were layered onto a 5 ml sucrose gradient (5–20%) containing 0.25 N NaOH and 1 mM EDTA. Sedimentation was at 50,000 rpm in a Spinco SW 50.1 rotor for 315 min at 4°C. Fractions (8 drops) were collected from a hole in the bottom of the tube and counted as described in *Endonuclease Assay*, except that 0.6 ml of 0.1 N HCl was added to the fractions before the scintillation fluid.

Under these conditions the intact circles sedimented at a rate of 19 S, whereas the linear molecules sedimented at about 17 S, a value that corresponds to the trailing shoulder on the curve obtained for the treated DNA.

it appeared that the enzyme acted in two steps: cleavage of the circles to linear molecules then degradation of linear molecules to nonsedimentable material. The latter degradation, once initiated, must be comparatively rapid, since no appreciable amount of material of intermediate length was present. No ATP-stimulated breakdown to small fragments has been observed with fractions from *recB* or *recC* cells.

Effect of the enzyme upon double-stranded DNA: As shown in Table 5, the purified enzyme preparation degraded native T7 DNA to products that were soluble in acid. This degradation was also dependent upon the presence of ATP (Table 5), but had a pH optimum of 9.5. When the products were analyzed

TABLE 5. *Effect of the enzyme on several DNA species.*

		nmol DNA made acid-soluble with exonuclease I	
	ATP	Absent	Present
Intact fd DNA	−	0.003	0.003
	+	0.217	0.624
Nicked fd DNA	−	0.006	3.77
	+	0.340	3.78
T7 DNA	−	0.002	
	+	0.091	

Reaction mixtures contained 50 mM glycylglycine–NaOH, pH 7.0, 10 mM $MgCl_2$, and 0.64 unit of enzyme. ATP (0.33 mM), 1 unit of exonuclease I, 3.8 nmol of [^{3}H]-fd DNA (3.2×10^3 cpm/nmol), or 1.1 nmol of native T7 phage DNA (3.0×10^4 cpm/nmol) were added as indicated. After 30 min at 37°C acid-soluble fragments were quantitated as described in *Materials and Methods*. DNA from phage fd was nicked with pancreatic DNase until 2% of the molecules remained intact as measured by infectivity in a spheroplast assay.[6]

by sedimentation in alkaline or neutral sucrose gradients (Fig. 2), there was no evidence for substantial endonucleolytic fragmentation of the DNA. Thus, under conditions where 30–40% of the T7 DNA was converted to acid-soluble fragments in the presence of enzyme and ATP, the sedimentation pattern of the remaining molecules remained unchanged from that obtained in controls where either enzyme or ATP were omitted. These results suggest an exclusively exonucleolytic action upon native DNA. We have also observed that if the double-stranded, unnicked, RF-I DNA from phage fd was exposed to an amount of enzyme that would make an equivalent amount of the single-stranded circular phage DNA completely susceptible to exonuclease I, about 85% of the double-stranded molecules remained completely intact as measured by band sedimentation in alkali and infectivity. In this way the activity upon native DNA was similar to that reported by others[1–4] for an ATP-stimulated exonuclease.

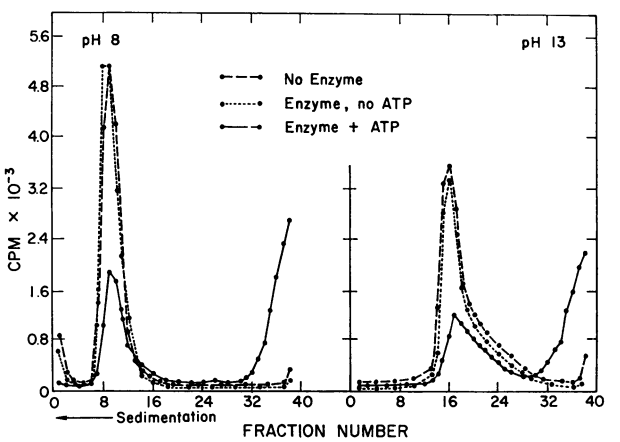

FIG. 2. Sedimentation of T7 DNA treated with enzyme. The reaction mixtures (0.05 ml) contained 50 mM glycylglycine–NaOH, pH 7.0, 4.5 mM $MgCl_2$, and 2.5 nmol of [^{3}H]-labeled T7 DNA (3×10^4 cpm/nmole). Where indicated, 4 units of endonuclease or 0.33 mM ATP were added. After 30 min at 37°C the reaction mixture was chilled, made 20 mM in EDTA, and diluted to 0.15 ml with distilled water. Each mixture was analyzed by layering 75 μl onto neutral and alkaline sucrose gradients (5–20%) and sedimenting at 50,000 rpm for 2.5 hr in a Spinco SW 50.1 rotor at 4°C. Neutral gradients contained 0.25 M NaCl, 0.02 M Tris, pH 8.2, 1 mM EDTA, and alkaline gradients contained 0.25 N NaOH, 1 mM EDTA. Fractions were collected and radioactivity was determined as described in Fig. 1.

Discussion. The enzyme preparation reported here is able to cleave fd-DNA circles, thus making them susceptible to exonuclease I. We do not know at this time, however, whether all or only a specific portion of the fd DNA circle is susceptible to this cleavage. The enzyme preparation also degrades open rings to acid-soluble products. This latter degradation does not appear to occur by random endonucleolytic scission of the population of substrate molecules; it acts either in an exonucleolytic fashion or preferentially upon molecules whose degradation has already commenced. When native DNA is used as substrate, on the other hand, the enzyme appears capable only of exonucleolytic degradation. Intact closed circles are relatively inert to the enzyme, and linear duplexes show no significant amount of endonucleolytically cleaved intermediates.

The role of ATP remains obscure. The degree of stimulation is dependent upon which fraction from the purification is utilized. Only one fraction absolutely requires ATP. It is quite possible that these differences represent alteration of the enzyme in some way, rather than removal of a contaminant.

The absence of enzyme activity in extracts from *recB* and *recC* mutants implicates this nuclease in recombination *in vivo*. However, the presence in fractions from the mutants of a heat-sensitive inhibitor of the ATP-dependent endonuclease activity makes it premature to conclude exactly the relationship between the products of the *recB* and *recC* genes and the nuclease activity. The exonuclease activity upon native DNA observed by Oishi[3] and by Barbour and Clark[4] was reported to be functional in the presence of crude extracts from *recB* and *recC* strains. Indeed, we find that under their assay conditions (pH 9.2), aliquots from the *recB* and *recC* fractions which almost completely inhibit the endonuclease activity (Table 4) only slightly inhibit (25%) the degradation of native DNA. Thus, the inhibitor is apparently selective in its action. The lack of temperature-sensitive mutants for these genes limits the experiments that may be done to clarify this point.

Finally, the complex characteristics of the enzyme preparation reported here are remarkably similar to those observed by Anai *et al.*[14] for an ATP-requiring nuclease from *Micrococcus lysodeikticus* (*M. luteus*). Unfortunately, no mutant studies for the *M. lysodeikticus* enzyme are available.

We are grateful to Mrs. Helen Mobach for her excellent technical assistance, and to Drs. A. J. Clark, Stephen Barbour, and Gerard Buttin for generously communicating their results prior to publication.

Abbreviations: ADP-C-P, β,γ-methylene adenosine triphosphate; tRNA, transfer RNA; SAM, *S*-adenosylmethionine; XTP, ribonucleoside triphosphate; dXTP, deoxyribonucleoside triphosphate; fd RF-I DNA, the double-stranded, unnicked, replicative form of phage fd DNA.

* This investigation was supported by biomedical sciences support grant FR-7006 from the General Research Support Branch, National Institutes of Health, and by grant 5 RO1 CA11092-02 from the National Cancer Institute.

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