Endonuclease II of *Escherichia coli*. Degradation of Double- and Single-Stranded Deoxyribonucleic Acid†

Sheik-Mumtaz Hadi, Dollie Kirtikar, and David A. Goldthwait*,‡

ABSTRACT: A procedure for the 1600-fold purification of endonuclease II of *Escherichia coli* is described. The assay is based upon the ability of the enzyme to degrade alkylated DNA. The enzyme can also make a limited number of single-strand breaks in phage T7 and T4 native DNA, but approximately 1000-fold more enzyme is required for this hydrolysis than for the hydrolysis of alkylated or depurinated reduced DNA. Single-strand cleavage of native T7 DNA at 45° requires considerably less enzyme than at 37° which suggests that adeninethymine rich areas may be the site of cleavage. Also at 45°, there is an increase in the number of single-strand breaks. The enzyme degrades nonalkylated d(A-T) but does not degrade nonalkylated or alkylated dG:dC. The enzyme makes a limited number of breaks in single-stranded DNA of mol wt 22×10^6 but no breaks if the same DNA has a mol wt of 5×10^6. The most obvious interpretation of the limited endonucleolytic attack on native and denatured DNA is that the substrate for the enzyme is an area in the DNA molecule probably rich in adenine and thymine base pairs, where there is a change in the secondary structure from double strandedness to single strandedness. Native DNA is hydrolyzed by the enzyme to give 5′-phosphomonoesters as determined by the polynucleotide kinase reaction. Indirect evidence is presented that partially depurinated DNA, either reduced or converted to the oxime derivative, is hydrolyzed by endonuclease II to yield a 5′-phosphomonoester of the altered nucleotide residue.

Endonuclease II was originally isolated and purified on the basis of its ability to degrade DNA alkylated with methyl methanesulfonate (Friedberg and Goldthwait, 1969). Subsequently, its mechanism of action on alkylated DNA was studied and it was noted that the enzyme also made a limited number of single-strand breaks in native T4 and T7 DNA (Friedberg *et al.*, 1969). It was concluded that these two activities were due to the same enzyme. In addition to its activity on alkylated DNA, the enzyme has since been found to degrade partially depurinated DNA (Hadi and Goldthwait, 1971). The present article deals with further purification of the enzyme and its activity on native DNA. The possible nature of sites on native and single-stranded DNAs has been explored.

Materials and Methods

Enzymes. Polynucleotide kinase was prepared from T4 infected *Escherichia coli B* cells (Richardson, 1965). Alkaline phosphatase was purchased from the Worthington Co. and chromatographed on DEAE-cellulose (Garen and Levinthal, 1960) and was free of endonucleolytic activity on double- or single-stranded DNA.

DNA polymerase, fraction VII (Richardson *et al.*, 1964), was obtained from General Biochemicals Inc. DNase I and micrococcal DNase were from Sigma Chemical Co.

DNA Preparations. The T7 phage was grown in *E. coli B* and purified (Summers and Szybalski, 1968). [^{3}H]Thymine-labeled T7 phage was grown similarly and purified by centrifugation. The specific activity was generally 1600 cpm/nmol of nucleotide. The T7 phage DNA used for these experiments gave *s* values consistently lower than those reported (Studier, 1965). The *s* value determined in the analytical centrifuge of the double-stranded DNA was 27.9 ± 1.11 (an average of eight determinations made at a concentration of 25 μg/ml) and the single-stranded DNA was 31.4 ± 0.85 (an average of six determinations). T7 phage was also obtained from Dr. Studier and the *s* value of the double-stranded DNA observed in this laboratory was 31.2 while the value for the single-stranded DNA was 34.4. Reported values were 32.0 and 37.2 (Studier, 1965).

The preparation of T4 DNA, unlabeled and [^{3}H]thymine labeled, has been described (Melgar and Goldthwait, 1968). The specific activity of the labeled preparation was generally 1200 cpm/nmol of nucleotide. The procedure for isolating [^{3}H]thymine-labeled *Bacillus subtilis* DNA of Young and Spizizen (1961) was employed. Salmon sperm DNA was obtained from Sigma Chemical Co. Concentrations were determined by optical density.

The endonuclease assay with alkylated DNA entrapped in a polyacrylamide gel and the exonuclease assay using *B. subtilis* DNA have been described (Friedberg and Goldthwait, 1969).

Unlabeled d(A-T) and dG:dC polymers were obtained from Miles Laboratories Inc. [^{3}H]Thymine-labeled poly[d-(A-T)] was prepared (Schachman *et al.*, 1960). The reaction mixture contained, in a total volume of 1 ml, 60 μmol of potassium phosphate buffer, pH 7.4, 1 μmol of dATP, 1 μmol of [^{3}H]thymine-labeled dTTP, 6 μmol of $MgCl_2$, 10 μmol of β-mercaptoethanol, 60 nmol of poly[d(A-T)] primer, and 15 units of DNA polymerase. The incubation was for 2 hr at 37° after which the reaction was terminated by adding NaCl to a final concentration of 0.2 M and heating at 70° for 5 min. The reaction mixture was then dialyzed against 1 l. of 0.2 M NaCl and 0.02 M potassium phosphate buffer, pH 7.5, at 4° and finally against 0.02 M potassium phosphate buffer, pH 7.5, for 6 hr. The specific activity of the product obtained was 400 cpm/nmol. ^{32}P-labeled poly(dG:dC) was prepared (Radding *et al.*, 1962). The reaction mixture contained in a total volume of 1 ml, 60 μmol of potassium phosphate buffer, pH 7.5, 0.8

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TABLE I: Purification of Endonuclease II.

Fraction	Procedure	Total Units[a] (μmol of DNA Released/hr)	Recovery of Units (%)	Sp Act. (μmol of DNA Released/mg of Protein per hr)	Purification
I	Crude	950	100	0.056	1
II	Streptomycin sulfate	940	99	0.07	1.3
III	Ammonium sulfate (45–80% fraction)	500	52.5	0.144	2.6
IV	Phosphocellulose	448	42.3	2.37	42
V	Sephadex G-100	300	31.6	9.6	172
VI	DNA-cellulose	246	26	94.2	1682

[a] One unit of activity is defined as the amount of enzyme capable of liberating 1 μmol of DNA nucleotide/hr from a standard amount of DNA gel alkylated with methyl methanesulfonate (Friedberg and Goldthwait, 1969).

μmol of [α-^{32}P]dGTP, 0.8 μmol of dCTP, 6 μmol of $MgCl_2$, 10 μmol of β-mercaptoethanol, 60 μmol of poly(dG:dC) primer, and 30 units of DNA polymerase. The incubation was for 5 hr at 37°. The reaction was terminated and the mixture dialyzed as described for poly[d(A-T)]. The specific activity of the product was 2500 cpm/nmol.

Isotopes, Etc. [γ-^{32}P]ATP and [α-^{32}P]GTP were obtained from International Chemical and Nuclear Corporation and [^{3}H]thymine-labeled dTTP was from Schwarz BioResearch. Phosphocellulose was the product of H. Reeve Angel & Co. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Inc.

Results

Enzyme Purification (Fractions I–III). The procedure has been altered to allow purification of larger quantities of material to a considerably higher specific activity (Table I). The modifications have been made only after fractions I, II, and III. Frozen cells of *E. coli* K-12 (200 g) were broken with glass beads and centrifuged at low speed and then at high speed (fraction I). Streptomycin sulfate was added to a final concentration of 0.8%, the mixture was centrifuged, and the supernatant fraction was saved (fraction II). The enzyme was then precipitated by ammonium sulfate in a fraction between 45 and 80% (fraction III). The details of these steps have been published (Friedberg and Goldthwait, 1969).

Phosphocellulose Chromatography (Fraction IV). A phosphocellulose column (20 × 6.5 cm) was equilibrated with 0.04 M potassium phosphate buffer, pH 6.5, containing 10^{-4} M dithiothreitol (buffer B). Protein (5 g) of fraction III (in 140 ml of buffer B) was dialyzed against buffer B for 16 hr and applied to the column (Figure 1). The column was first eluted with 0.05 M NaCl in buffer B until the optical density at 280 mμ dropped to 0.1 or less. The enzyme was then eluted with 0.3 M NaCl in buffer B. Fractions of 25 ml were collected and assayed for activity on alkylated and native DNA. Fractions obtained with 0.3 M NaCl which were active only on alkylated DNA were combined (endonuclease I was eluted with 0.05 M NaCl). The pooled fractions of endonuclease II were precipitated with 80% ammonium sulfate, and the pellet was taken up in 10 ml of buffer B. The solution was dialyzed against the same buffer for 16 hr and centrifuged. The supernatant fraction (fraction IV) was applied to a Sephadex column.

Sephadex G-100 Fractionation (Fraction V). A Sephadex G-100 column (3 × 85 cm) was washed with buffer B plus 10% glycerol (buffer C). Five milliliters of fraction IV (95 mg of protein or one-half of the total protein) was placed on the column which was eluted with buffer C. Fractions of 2.5 ml

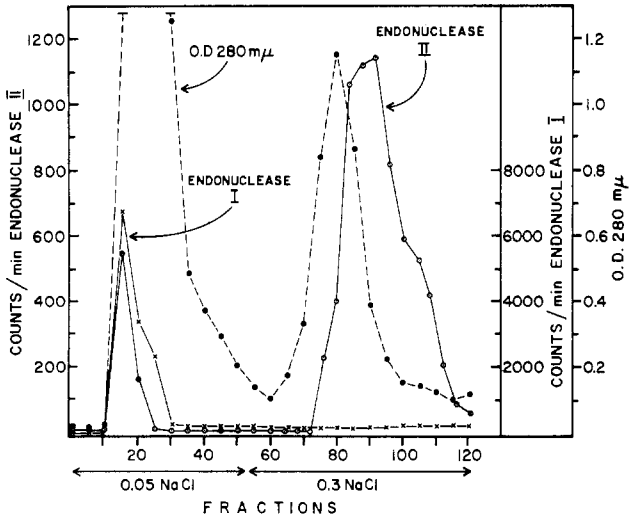

FIGURE 1: Chromatography of endonuclease II on a phosphocellulose column. The release of alkylated DNA in the endonuclease II assay in fractions 11–24 is due to endonuclease I. The endonuclease I assay is based on the release of nonalkylated DNA from the gel.

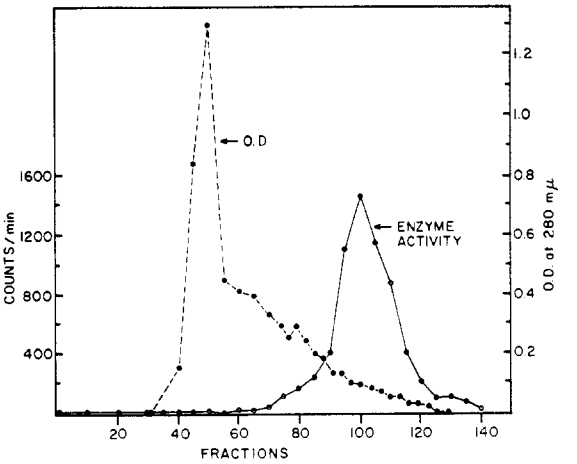

FIGURE 2: Gel filtration of endonuclease II on a Sephadex G-100 column.

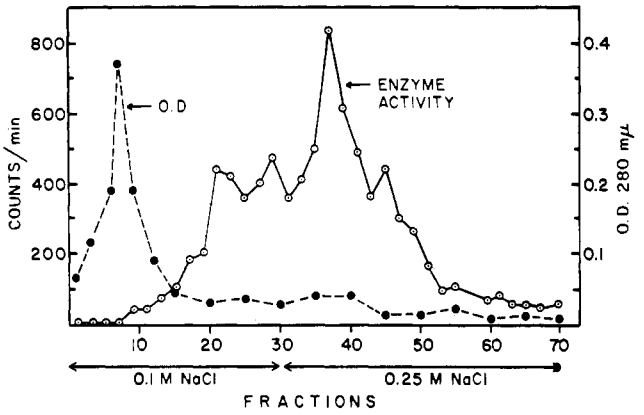

FIGURE 3: Elution of endonuclease II from a DNA-cellulose column.

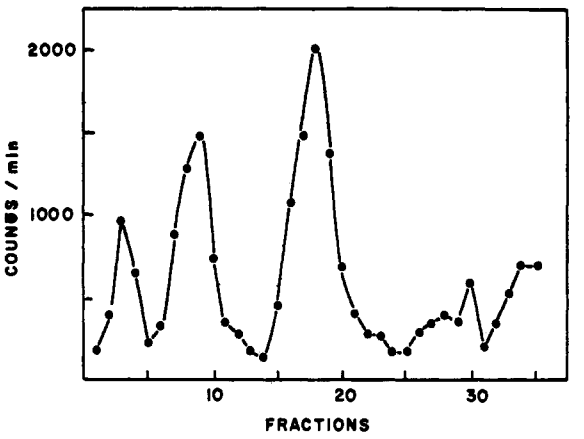

FIGURE 4: Polyacrylamide gel pattern of endonuclease II of DNA-cellulose fraction.

were collected. The endonuclease II activity and the protein profile are shown in Figure 2. The active fractions were pooled and stored at −20°.

DNA-Cellulose Chromatography (Fraction VI). Salmon sperm was used to prepare single-stranded DNA-cellulose (Alberts, 1968). The DNA-cellulose was suspended in 0.05 M Tris-HCl (pH 8.0)–0.1 mM dithiothreitol plus 10% glycerol (buffer D), and a column of 2 cm × 5 cm was prepared. To eliminate any release of DNA from the column during elution of the enzyme, the column was first washed with 0.25 M NaCl in buffer D until no ultraviolet (uv) absorbing material was eluted, and then rewashed with buffer D. Fraction V (100 ml) containing 7.5 mg of protein (one-half of the preparations) was applied. The column was then washed with a small amount of buffer D and the elution was started with 0.1 M NaCl in buffer D (Figure 3). The molarity of NaCl was changed to 0.25 at fraction 30, and 5-ml fractions were collected. The active fractions between numbers 20 and 50 were pooled and concentrated by dialysis under negative pressure against 0.05 M Tris-HCl, pH 8.0, containing 0.1 mM β-mercaptoethanol plus 20% glycerol. The A_{280}/A_{260} ratio of the concentrated fraction was 1.7.

After electrophoresis of the DNA-cellulose fraction in polyacrylamide gel at pH 8.3, three major peaks of activity were observed (Figure 4). The pattern was the same when the fractions were tested on alkylated DNA by the gel assay either with or without Mg^{2+}. Since there was no activity of the DNA-cellulose fraction on native DNA when tested by the gel assay, it was concluded that all three peaks were endonuclease II. The contaminating exonuclease was observed in two peaks which were different from the endonuclease peaks. Three major bands were stained with Coomassie Blue, but they did not correspond exactly with the enzyme activity. These observations indicate that the enzyme is still only partially purified.

Isoelectrofocusing (Fractions VIIa, b, and c). The LKB electrofocusing system 8100-10 was used. The electrofocusing column dimensions were 54 cm × 5.4 cm with a volume of 110 ml. The ampholytes in a pH range of 3–10 were used as a 1% solution in a sucrose density gradient containing β-mercaptoethanol. The sample, 4.2 ml of fraction VI with 2.9 mg of protein, was layered in the center of the gradient. The electrofocusing was done at a constant current of 2 mA for 60 hr at 3°. Fractions of 3.0 ml were collected from the bottom at a rate of 1 fraction/min (Figure 5). Aliquots of 0.02 ml from even-numbered fractions were assayed immediately for endonuclease II and exonuclease activity. The pH determinations were done at 15°. The ampholytes interfered with accurate protein determination. Fraction VIIa which formed a band at pH 4.3 was contaminated with exonuclease while fraction VIIb at pH 6.1 was not. Fraction VIIb was only partially stable when frozen in 10% glycerol. A third fraction (VIIc) which focused over a broad pH range between 7.8 and 9.0 was also observed. Similar results were obtained on runs from different preparations. These data suggest that the three peaks observed on the polyacrylamide gels were similar to those observed by isoelectrofocusing and were probably due to different net charges rather than to variable degrees of aggregation. A value of 3.6 S has been estimated for the enzyme (Friedberg *et al.*, 1969).

Exonuclease Contaminant. The endonucleolytic activity was contaminated with an exonuclease up through fraction VI. This exonuclease in fraction VI was active on *B. subtilis* double-stranded DNA in Tris-HCl buffer, pH 8.0, 10^{-3} M β-mercaptoethanol, and 5×10^{-3} M $MgCl_2$ but not on T4 DNA. In the absence of $MgCl_2$, the activity decreased to less than one-tenth of the control value. [^{3}H]TMP was identified by thin layer chromatography as the only degradation product of a short incubation of fraction VI with [^{3}H]TMP-labeled *B. subtilis* DNA. In attempts other than isoelectrofocusing to separate endo- and exonucleolytic activities, a small percentage of the endonuclease, free of exonuclease, was occasionally eluted in a pH gradient from phosphocellulose columns before the main fraction containing both activities. No separation of activities was obtained with DEAE-, carboxymethyl-, or single-stranded DNA-cellulose columns or with column electrophoresis. (The separation, originally observed on a preparative polyacrylamide gel column (Friedberg and

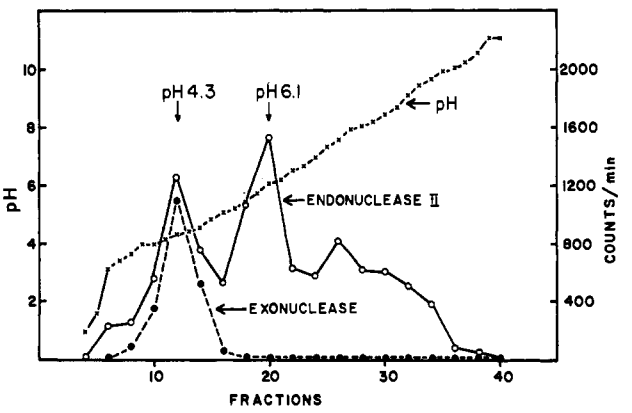

FIGURE 5: Isoelectrofocusing of endonuclease II.

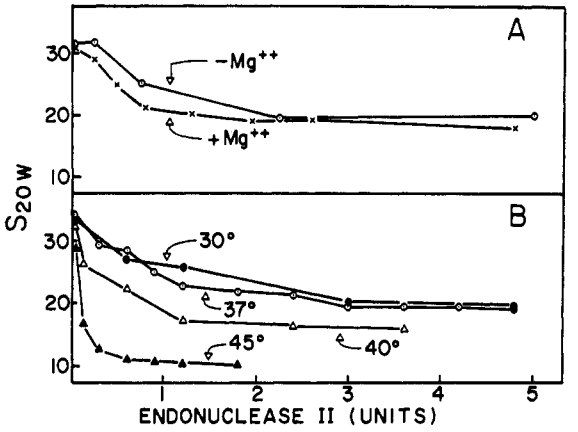

FIGURE 6: Enzymatic degradation of native T7 DNA with and without magnesium and at varying temperatures. (A) The incubation mixtures of 1.0 ml contained 75 nmol of T7 DNA, 5×10^{-3} M $MgCl_2$ or 10^{-4} M 8-hydroxyquinoline, 10^{-4} M β-mercaptoethanol, 0.05 M Tris-HCl, pH 8, and enzyme (fraction VI). The incubation was at 37° for 1 hr. Control samples contained buffer instead of enzyme. The reaction was stopped by heating at 60° for 10 min and an aliquot of the reaction mixture was diluted with NaCl and NaOH to give a final concentration of NaCl of 0.9 M and NaOH of 0.1 M. EDTA was added to a final concentration of 10^{-2} M. The solution was placed in a 12-mm single sector cell with a charcoal-filled Epon centerpiece. Centrifugation in a Spinco Model E analytical ultracentrifuge was at 20° and at 32,000 rpm. Scanner tracings at 260 mμ were obtained at 4-min intervals. (B) The incubation mixtures of 0.6 ml contained 39 nmol of T7 DNA, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M β-mercaptoethanol, 0.05 M Tris-HCl, pH 8.0, 0.18 ml of glycerol, and enzyme (fraction VI) as indicated. Incubation was for 1 hr and the reaction was stopped by the addition of 10 μmol of EDTA, 7.5 μmol of Tris-HCl, pH 8.0, 0.9 mmol of NaCl, and 0.1 mmol of NaOH to give a final volume of 1.0 ml. Samples were examined in the analytical ultracentrifuge as in part A and the *s* values were multiplied by a factor of 1.1, determined experimentally by comparison of the sedimentation value obtained without and with glycerol at 20°.

Goldthwait, 1969) was invariably accompanied by a loss of 80–90% of the endonuclease activity.)

Amount of Enzyme Required to Degrade Native and Depurinated Reduced DNA. A considerably larger amount of enzyme was required for the degradation of native T4 and T7 than for alkylated or depurinated DNA. A comparison of the units of enzyme *vs.* the *s* values of the native and the depurinated reduced substrates, measured with radioactive T4 DNA by the sucrose gradient method, is shown in Table II. Approximately 1000-fold more enzyme was required to make single-strand breaks in native DNA. This was not a matter of a difference in the number of sites in the two different substrates, because the limit degradation was approximately the same. Several properties of the enzyme which degraded native DNA were similar to those of the enzyme which degraded alkylated DNA (Friedberg *et al.*, 1969) and depurinated reduced DNA (Hadi and Goldthwait, 1971) and this indicated that the degradation of native DNA was due to endonuclease II itself and not due to a contaminant. One reason for the difference in the amount of enzyme required (Table II) could be that the K_m for sites present in native DNA is different than the K_m for the sites in alkylated DNA. Another reason could be that sites in native DNA may be transient because they are created by "breathing" of the DNA (Printz and von Hippel, 1965). Experiments described in the next section suggest that the latter explanation accounts for some if not all of the difference.

Degradation of Native T7 DNA. Previous experiments indicated that endonuclease II made a limited number of single-strand breaks in native T4 DNA and also made some single-strand breaks in native T7 DNA although these experiments were not adequate to establish the limit of degradation for T7 DNA (Friedberg *et al.*, 1969). Figure 6A shows the *s* values of single-stranded DNA after incubation of native T7 DNA with endonuclease II, with and without Mg^{2+}. These data indicate that the enzyme recognizes only a limited number of sites in the double-stranded DNA. The presence of these sites is not dependent upon divalent metal, and when 0.15 M NaCl was present in the incubation mixture, the degree of degradation was not altered. Six determinations of the *s* value of the single-stranded DNA of the T7 phage used for these experiments gave an average value of 31.4 ± 0.85 and the molecular weight calculated (Studier, 1965) was 8.5×10^6. The limit degradation product with an *s* value of 19 had a mol wt of 2.5×10^6. The enzyme, therefore, made an average of 2.4 breaks/single strand. When the degradation of labeled T7 DNA was examined by the sucrose gradient technique (Figure 7) the untreated single-stranded DNA migrated at approximately 29 S relative to rat liver ribosomal RNA of 28 S and the degradation product migrated at approximately 20 S. The pattern indicates that single-strand breaks were made in both strands. It is possible that three breaks are made in one strand and two

TABLE II: A Comparison of the Amount of Endonuclease II Required to Degrade Native and Depurinated Reduced DNA.[a]

Enzyme Units	*s* Value of Substrate Determined in 0.1 N NaOH: Native DNA	*s* Value of Substrate Determined in 0.1 N NaOH: Depurinated Reduced DNA
None	43.0	41.5
9.6×10^{-5}		34.4
9.6×10^{-4}		27.0
9.6×10^{-3}	43.0	25.8
4.8×10^{-2}	43.0	25.8
9.6×10^{-2}	40.0	
1.9×10^{-1}		22.8
2.4×10^{-1}	37.0	
3.8×10^{-1}		24.0
4.8×10^{-1}	31.4	
6.7×10^{-1}	28.5	

[a] Depurinated reduced DNA prepared by heating 166 nmol of tritiated T4 DNA in a volume of 2.0 ml of 0.1 M sodium citrate, pH 3.5, containing 10^{-3} M EDTA at 40° for 6 min followed by rapid cooling. The pH was adjusted to 6.5 with NaOH and potassium phosphate buffer, pH 6.5, was added to a final concentration of 0.5 M in a final volume of 3.0 ml. A concentrated solution of $NaBH_4$ was added three times at 15-min intervals to give a final concentration of 0.25 M. The solution was kept at room temperature for 1 hr and finally dialyzed against 500 ml of 0.05 M Tris-HCl, pH 8, at 4° for 16 hr. The reaction mixtures containing 0.05 M Tris-HCl, pH 8, 6.8 nmol of native or depurinated reduced DNA, 5×10^{-3} M $MgCl_2$, 10^{-4} M β-mercaptoethanol, and enzyme (fraction VI) in a total volume of 0.2 ml. Incubation was at 37° for 1 hr and the reaction was stopped as described in Figure 7. Centrifugation conditions in the alkaline sucrose gradient were also the same.

TABLE III: Degradation of Synthetic Polymers by Endonuclease II.[a]

Polymer	Enzyme Units	$S_{20,w}$
d(A-T)	None	9.45
	4.8×10^{-2}	9.45
	9.6×10^{-2}	5.1
dG:dC	None	4.72
	1.9×10^{-1}	4.65
	3.9×10^{-1}	4.75
	9.7×10^{-1}	4.40

[a] The incubation mixtures (0.3 ml) contained 120 nmol of poly[d(A-T)] or 105 nmol of poly(dG:dC), 5×10^{-3} M $MgCl_2$, 10^{-4} M β-mercaptoethanol, 0.05 M Tris-HCl, pH 8, and enzyme (fraction VI). Incubation was at 37° for 30 min; at the end of the reaction 0.1 ml of 0.1 M EDTA was added, then NaCl and NaOH were added to final concentrations of 0.9 and 0.1 M, respectively. Analysis in the Model E analytical ultracentrifuge was as noted in Figure 6. For the d(A-T) polymer the speed was 48,000 rpm while for the dG:dC polymer it was 60,000 rpm. Tracings were made every 8 min.

in the other. Although the enzyme (fraction VI) was contaminated with a small amount of an exonuclease active on sonicated DNA, there is no evidence in Figure 7 of exonuclease activity on T7 DNA.

Figure 6B shows the effect of the temperature of the incubation on the amount of enzyme required and on the extent of degradation. At 30 and 37° the amount of enzyme necessary as well as the extent of degradation are similar. At 45°, the amount of enzyme required for half-maximal degradation was markedly reduced and the DNA molecule was cleaved at more sites. All incubation experiments shown in Figure 6B were done in 30% glycerol to protect the enzyme. Incubation of the enzyme at 45° for 30 min resulted in 74% inactivation while at 37° the inactivation was 25%. Thus, the amount of active enzyme present during incubation at 45° is even less then the amount indicated. The results are compatible with the enzymatic degradation of specific A-T rich sites in native DNA which are converted at elevated temperatures to partially single-stranded areas such as those seen in denaturation maps (Inman, 1966). The results are not due to depurination by heat, as T7 DNA, preheated under exactly the same conditions to 45°, did not undergo more single-strand cleavage by the enzyme at 37° than unheated T7 DNA.

Degradation of Synthetic Polymers by Endonuclease II. Evidence is provided in Figure 8 and Table III that d(A-T) but not dG:dC is degraded by endonuclease II. The degradation of d(A-T) requires approximately the same amount of enzyme as is needed for the cleavage of native DNA at 37°, and use of the enzyme fraction obtained by isoelectrofocusing ensured that the degradation of d(A-T) was not due to an exonuclease contaminant. No significant degradation of dG:dC was observed. When dG:dC was alkylated with half-sulfur mustard, and treated with similar amounts of enzyme, no degradation was observed.

Degradation of Single-Stranded DNA by Endonuclease II. T4 DNA, denatured with alkali, was degraded to a molecule of approximately 25 S by endonuclease II. This is shown in Figure 9 (top). When double-stranded DNA was incubated under identical conditions with excess enzyme (0.58 unit) and the products examined in an alkaline sucrose gradient (Figure 9, bottom) the extent of hydrolysis was found to be only slightly less than that noted with the single-stranded material. The latter degradation was due to single-strand breaks (Friedberg and Goldthwait, 1969).

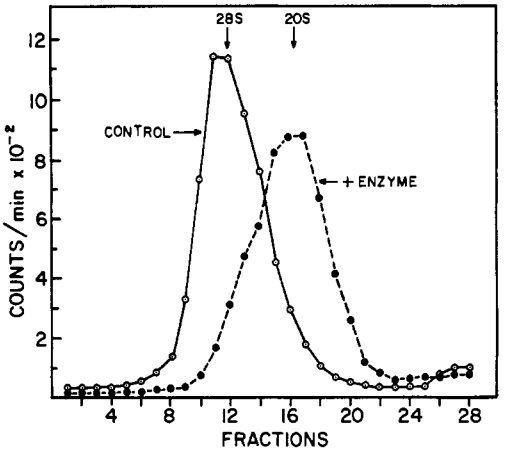

FIGURE 7: Degradation of T7 DNA by endonuclease II. The reaction mixture contained in a total volume of 0.5 ml 18 nmol of tritiated T7 DNA 5×10^{-3} M $MgCl_2$ or 10^{-4} M 8-hydroxyquinoline, 10^{-4} M β-mercaptoethanol, 0.05 M Tris-HCl, pH 8, and 2.55 units of enzyme (fraction VI). The tubes were incubated for 1 hr at 37° and the reaction was terminated by the addition of 0.02 ml of 10% sodium dodecyl sulfate and 0.05 ml of 0.2 M EDTA. An aliquot of the reaction mixture was layered on a 5–20% alkaline sucrose density gradient in 0.1 M NaOH–0.9 N NaCl–10^{-3} M EDTA. The marker was rat liver ribosomal RNA run in a neutral sucrose gradient. The centrifugation was for 3 hr at 35,000 rpm at 20° in a Spinco SW-56 rotor.

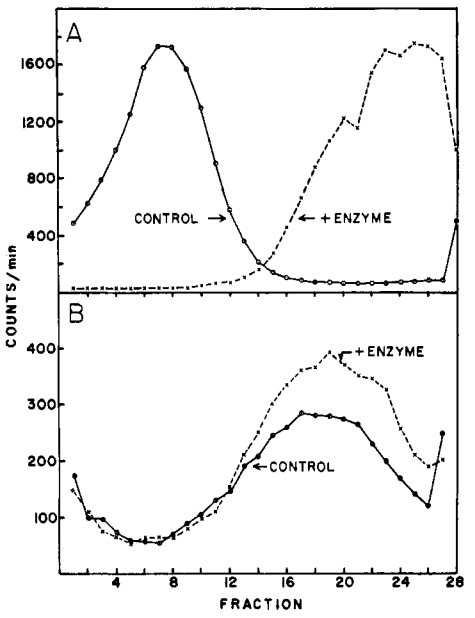

FIGURE 8: Degradation of poly[d(A–T)] but not of poly[d(G:C)] by endonuclease II. The reaction mixture contained in a total volume of 0.3 ml, 4.5 nmol of [^{3}H]thymine-labeled poly[d(A-T)] or 5.5 nmol of [^{32}P]GMP-labeled poly[d(G:C)], 5×10^{-3} M $MgCl_2$, 10^{-4} M β-mercaptoethanol, 0.05 M Tris-HCl, pH 8, and enzyme (fraction VI). For poly[d(A-T)], 0.18 unit of exonuclease-free endonuclease II obtained at pH 6.1 by electrofocusing (fraction VIIb) was used and for poly[d(G:C)], 0.30 unit of the DNA-cellulose fraction VI was used. The incubation was for 30 min at 37° and the reaction was terminated by the addition of 0.01 ml of 10% sodium dodecyl sulfate and 0.02 ml of 0.2 M EDTA. Aliquots of the rection mixtures were layered on 5–20% alkaline sucrose gradients. The centrifugation of poly[d(A-T)] was at 38,000 rpm for 16 hr and of poly[d(G:C)] at 50,000 rpm for 44 hr at 20° in a Spinco SW-56 rotor.

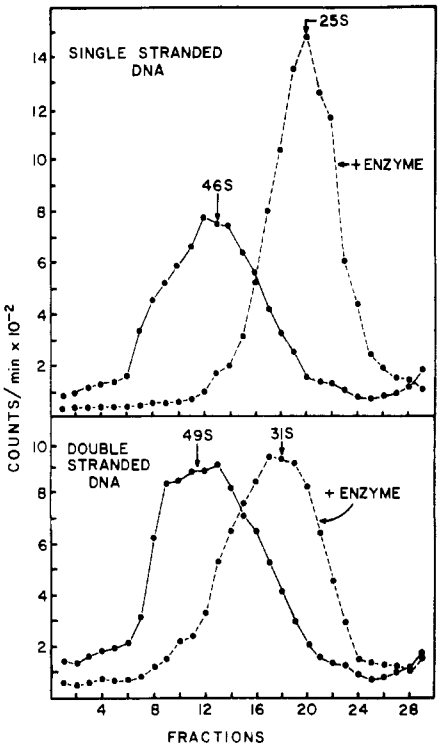

FIGURE 9: Degradation of double-stranded and alkali-denatured single-stranded T4 DNA by endonuclease II. Tritiated T4 DNA (30 nmol with a specific activity of 1500 cpm/nmol) in 0.5 ml of 0.05 M Tris-HCl, pH 8, was denatured by addition of 0.03 ml of 2 M NaOH. The solution was kept at room temperature for 5 min and then 0.08 ml of 1.1 N HCl–0.2 M Tris was added to bring the pH to 8. The denatured DNA was immediately used in the incubation mixtures. For native DNA, 0.03 ml of 2 M NaCl was added along with 0.08 ml of 0.2 M Tris-HCl, pH 8.0. The reaction mixture of 0.2 ml contained 0.05 M Tris-HCl, pH 8, 5 nmol of native or denatured DNA, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M β-mercaptoethanol, and 0.57 unit of the enzyme (fraction VI). After incubation at 37° for 1 hr, 0.01 ml of 10% sodium dodecyl sulfate and 0.02 ml of 0.2 M EDTA were added to the reaction mixture. The total reaction mixture was layered on a 5–20% alkaline sucrose gradient and centrifuged in a Spinco SW-56 rotor at 28,000 rpm for 3 hr at 20°.

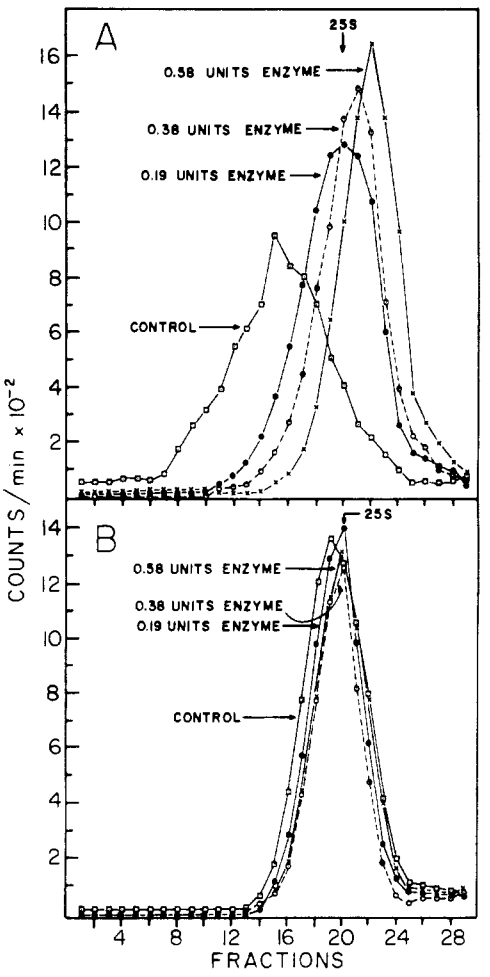

FIGURE 10: Enzymatic degradation of alkali-denatured but not of heat-denatured DNA. (A) Alkali-denatured DNA was prepared as described in Figure 7. Before incubation with enzyme, it was dialyzed against glass-distilled water for 48 hr at 4°. The incubation mixture contained 0.05 M Tris-HCl, pH 8, 7 nmol of DNA, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M β-mercaptoethanol, and the enzyme (fraction VI) as indicated in a total volume of 0.2 ml. After incubation for 1 hr at 37° the reaction was stopped as described in Figure 7. The centrifugation conditions in the alkaline sucrose gradient were also the same as in Figure 7. (B) Heat-Denatured DNA. To the denatured dialyzed DNA, prepared as noted above in A, Tris-HCl, pH 8, was added to a final concentration of 0.05 M; the solution was heated at 100° for 5 min and cooled rapidly. The reaction mixtures were the same as described in A. Centrifugation in alkaline sucrose gradients was done at 28,000 rpm for 3 hr at 20° in a Spinco SW-56 rotor.

Although the enzyme degraded alkali-denatured single-stranded DNA, it did not degrade heat-denatured single-stranded DNA which was considerably smaller (Figures 10A and B). With increasing amounts of enzyme, the sedimentation coefficient of the alkali-denatured DNA decreased to 25 and then very slowly below this, while there was no evidence of significant enzymatic degradation of the heat-denatured DNA with similar amounts of enzyme. Under the conditions used, heat denaturation alone degraded the DNA to material with a value of 25 S.

The experiments with heat-denatured DNA, which were repeated three times, prove that there is no nonspecific single-strand endonuclease present in this preparation of endonuclease II. They do suggest that there are a limited number of sites present in the higher molecular weight single-stranded DNA which are eliminated when the DNA is partially degraded by heating at 100° for 5 min. Either the enzyme recognizes these sites on the single strands of alkali-denatured DNA, and these sites are near the points of the cleavage which occur during denaturation at 100°, or there are a limited number of sites on the single-stranded DNA, such as alternating d(A-T) regions, which can form intramolecular double-stranded regions in the single strands, some portions of which are susceptible to enzymatic hydrolysis. The degradation of the DNA at 100° would separate these regions physically so that in dilute solution the formation of double-stranded regions would be less likely. At present it is impossible to distinguish between these alternatives.

The enzyme degradation products of the alkali-denatured DNA have an s value of about 25 and an approximate mol wt of 5×10^6 (Studier, 1965). This suggests that there are perhaps as many as 11 of these sites in each strand of T4 DNA which are recognized by the enzyme. With partially degraded double-stranded T4 DNA which had single strands of mol wt $\sim 22 \times 10^6$, the enzyme made 3–4 breaks per single strand or 8–11 per single strand of undegraded T4 DNA (Friedberg and Goldthwait, 1969). Therefore, the limited extent of degradation of single-stranded T4 DNA is approximately the same as the limited degradation of double-stranded T4 DNA. This suggests that perhaps the same sites are involved in the en-

TABLE IV: Incorporation of ^{32}P into Various DNA Preparations Incubated with Polynucleotide Kinase and [γ-^{32}P]ATP.[a]

Expt	DNA Preparation	DNA Concn (nmol)	Prior Treatment of DNA (pmol)			
			None	Phosphatase	Endonuclease II	Endonuclease II + Phosphatase
1	Native	27.0	0.26	0.63	0.50	2.45
2	Native	27.0	0.14	0.20	0.21	1.18
3	Depurinated 50°, 20 min, reduced	14.1	0.45	1.04	0.57	0.92
4	Depurinated 70°, 6 min, reduced	18.0	0.97	1.40	0.90	1.20
5	Depurinated 50°, 20 min, NH_2OH	27.0		4.00		4.64
6	Micrococcal DNase (A)	30	5.00			
	Depurinated Reduced (B)	20	2.07			
	A + B	50	10.40			
	DNase I	6.2	1.18	8.65		

[a] T4 native, depurinated reduced, or depurinated hydroxylamine treated DNA (50–110 nmol) was incubated in reaction mixtures (0.5 ml) containing 0.05 M Tris-HCl, pH 8.0, 5×10^{-3} M $MgCl_2$, and 10^{-4} M β-mercaptoethanol. Endonuclease II (1–5 units of fraction VI) was present in one reaction mixture while a second served as a control. After incubation at 37° for 1 hr the incubation mixtures were each divided into two halves (0.25 ml each). After addition of 0.1 ml of water, 2 M NaOH was added to give a final concentration of 0.08 M. The solution was kept at room temperature for 5 min before adding sufficient 1 N HCl to bring the pH to 8. The solution was made 0.05 M with further addition of Tris-HCl, pH 8, and 5.5 units of alkaline phosphatase was added. The mixture (0.5 ml) was incubated at 37° for 1 hr; additional enzyme (5.5 units) was added after 30 min. At the end of the incubation period, the reaction mixtures were again divided into two halves (0.25 ml each). To one, polynucleotide kinase was added with additions noted below. The enzyme was omitted from the other. The reaction mixtures, with a total volume of 0.6 ml, contained 0.05 M Tris-HCl buffer, pH 8, 0.01 M $MgCl_2$, 0.005 M β-mercaptoethanol, 0.025 M potassium phosphate buffer, pH 7.5, 2–5 nmol of [γ-^{32}P]ATP (sp act. 1.8–8.5 $\times$ 10^8 cpm/μmol), and 8 units of polynucleotide kinase. Incubation was for 1 hr at 37°; additional enzyme (8 units) was added at the end of 30 min. The reaction was stopped by the addition of 1 mg of bovine serum albumin, 0.4 ml of 0.1 M sodium pyrophosphate, and 1 ml of 7% $HClO_4$. The tubes were kept at 0° for 30 min before centrifugation and during several washings of the precipitate with cold 1% $HClO_4$. Finally the precipitate was dissolved in 1 ml of 1 M NH_4OH and counted with Aquasol (New England Nuclear) scintillation fluid. All reactions were run in duplicate and the picomoles of ^{32}P incorporated into the total DNA are reported.

zymatic degradation of both the double- and single-stranded DNA.

Cleavage of Native DNA to Give 5′-Phosphate Groups. Studies with polynucleotide kinase were done to establish the products formed by the hydrolysis of the phosphodiester bond. Incubation of native T4 DNA with or without endonuclease II and with or without alkaline phosphatase was done prior to incubation with the kinase. The results are shown in Table IV, experiments 1 and 2, where maximum labeling of the DNA by the kinase was observed with endonuclease II only after treatment with alkaline phosphatase. These data indicate that hydrolysis by endonuclease II produces 3′-hydroxyls and 5′-phosphates. The number of phosphate groups added to the DNA after treatment with endonuclease II and alkaline phosphatase is equivalent to 1 per 15,000–17,000 nucleotides or approximately 10–12 per intact single strand. This agrees reasonably well with the number of single-strand breaks made by the enzyme in a T4 DNA molecule as noted above.

The number of 5′-phosphates in the DNA not treated with endonuclease II in experiment 1 is 1 per 73,000 nucleotides which would indicate 1–2 breaks per single strand of the original T4 DNA. However, in experiment 2, the predicted increase with phosphatase alone was not observed. The blanks in these experiments were approximately 20–25% of the values observed with no prior treatment and the data are not accurate enough to predict the molecular weights of the DNA single strands. The DNA used for experiment 7 was from another preparation.

A series of experiments was also done with depurinated reduced DNA and with depurinated hydroxylamine treated DNA in the hope that more sites could be observed per DNA molecule than with native DNA. The results of a typical experiment are shown in Table IV, experiments 3, 4, and 5, which indicate that there is no significant difference in the reaction with labeled ATP and polynucleotide kinase after incubation of depurinated reduced DNA with endonuclease II with or without alkaline phosphatase. The most reasonable interpretation of the findings in experiments 3–5 is that the phosphodiester bond on the 5′ side of the depurinated reduced residue has been cleaved by endonuclease II, and, after treatment with alkaline phosphatase, the remaining 5′-terminal deoxyribitol (experiments 3 and 4) or oxime (experiment 5) is not a substrate for the polynucleotide kinase. A similar inactivity of the kinase on the hydroxyl group adjacent to a thymine dimer has been observed (Kushner *et al.*, 1971).

To rule out inhibition of the polynucleotide kinase by its binding to the depurinated reduced DNA, experiment 6 was done. This shows that the presence of depurinated reduced DNA (B) did not inhibit the action of polynucleotide kinase on DNA with 5′-hydroxyl groups prepared with the micrococcal nuclease (A and A plus B). Experiment 7 is a control which shows that degradation with DNase I requires phosphatase in order to observe maximum incorporation. Further experiments with the kinase indicated that partially depurinated DNA underwent nonenzymatic hydrolysis of phosphodiester bonds, and this created 5′-phosphate monoesters

as predicted by the β-elimination reaction (Brown and Todd, 1955).

Discussion

Endonuclease II has been purified by assaying for hydrolysis of alkylated DNA. The presence of three distinct fractions of endonuclease II separated by isoelectrofocusing or by gel electrophoresis suggests a variable number of amide groups or variable degradation such as that seen with the DNA polymerase (Cavalieri and Carroll, 1968). Although the peak of enzyme activity observed in a sucrose gradient is reasonably symmetrical, the nature of the differences between the three fractions of enzyme is not clear. Evidence that endonuclease II and not a contaminant was responsible for the single-strand breaks made in native DNA has been provided (Friedberg and Goldthwait, 1969). This was based on the absence of a metal requirement for activity as well as a stimulation with Mg^{+} noted with both substrates plus comparable inhibition of hydrolysis of both substrates with specific levels of EDTA and of *p*-chloromercurisulfonate. The fact that the activity on native DNA has persisted in the present purification even through fraction VIIb makes it more certain that the activities on alkylated and native DNA are due to the same enzyme.

However, the amounts of enzyme required for cleavage of native DNA in all preparations have been several orders of magnitude greater than for alkylated DNA. The demonstration that at 45° the amount of enzyme needed for hydrolysis of native DNA could be reduced by approximately tenfold (conditions which resulted in considerable inactivation of enzyme) strengthened the hypothesis that in native DNA there are a limited number of sites where single stranding occurs, sites presumably rich in A·T base pairs. The requirement for less enzyme at the elevated temperature would be explained by the increased "breathing" or single stranding per unit of time of an A + T rich area (Printz and von Hippel, 1965). The observation that the size of the limit degradation was smaller at 45° than at 37° is consistent with the hypothesis of new areas of significant "breathing" for enzymatic cleavage at the higher temperature. The degradation of non-alkylated d(A-T) by the most purified fraction is further support for the concept of A + T rich areas in double-stranded native DNA which are subject to enzymatic cleavage.

The limited degradation of the high molecular weight (22×10^6) single-stranded DNA by the enzyme to a size of approximately 5×10^6, but the absence of significant degradation of the same DNA when its molecular weight was reduced to approximately 5×10^6 by heating at 100° suggests that the sites of cleavage were eliminated when the size of the substrate was reduced by heating. This would not be predicted if a specific base sequence in a single strand was the substrate unless the heating produced nonenzymatic cleavage of these specific sites. This seem unlikely. An alternative is that the enzyme recognizes areas of DNA where there is a change from double strandedness to single strandedness. Intramolecular double-stranded areas might be formed, for example, by several d(A-T) stretches in a single strand. These might form easily in the high molecular weight material, but in the low molecular weight material formation would be unlikely because the double-stranded regions would be intermolecular. To date we have no evidence for or against this proposal. Finally, the secondary structure of DNA, very rich in A·T base pairs, has been noted by X-ray scattering experiments to be different from the usual B conformation (Bram, 1971). It is possible that this is the substrate in native DNA for the enzyme.

References

Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D., and Ferris, F. L. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 209.

Bram, S. (1971), *Nature (London), New Biol. 232*, 174.

Brown, D., and Todd, A. (1955), *in* The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic Press, p 409.

Cavalieri, L. F., and Carroll, E. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 951.

Friedberg, E. C., and Goldthwait, D. A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 934.

Friedberg, E. C., Hadi, S. M., and Goldthwait, D. A. (1969), *J. Biol. Chem. 244*, 5879.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 35*, 470.

Hadi, S. M., and Goldthwait, D. A. (1971), *Biochemistry 10*, 4986.

Inman, R. B. (1966), *J. Mol. Biol. 18*, 464.

Kushner, S. R., Kaplan, J. C., Ono, H., and Grossman, L. (1971), *Biochemistry 10*, 3325.

Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243*, 4401.

Printz, M. P., and von Hippel, P. H. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 363.

Radding, C. M., Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2069.

Richardson, C. C. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 158.

Richardson, C. C., Schildkraut, D. L., Aposhian, H. Y., and Kornberg, A. (1964), *J. Biol. Chem. 239*, 222.

Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A. (1960), *J. Biol. Chem. 235*, 3242.

Studier, I. W. (1965), *J. Mol. Biol. 11*, 373.

Summers, W. C., and Szybalski, W. (1968), *Virology 34*, 9.

Young, F. E., and Spizizen, J. (1961), *J. Bacteriol. 81*, 823.