Deoxyribonucleic Acid Nucleases

I. THE USE OF A NEW METHOD TO OBSERVE THE KINETICS OF DEOXYRIBONUCLEIC ACID DEGRADATION BY DEOXYRIBONUCLEASE I, DEOXYRIBONUCLEASE II, AND ESCHERICHIA COLI ENDONUCLEASE I*

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SUMMARY

A new method for the assay of DNA endonuclease activity has been devised. It is based on the release of DNA fragments from DNA entrapped in a polyacrylamide gel. The average molecular weight of the fragments released is approximately 400,000. This method has been used to examine the kinetics of DNA degradation. Hydrolysis by DNase I as a function of time and also as a function of enzyme concentration with Mg²⁺ as the divalent cation showed an initial lag. This was not observed with DNase II or Escherichia coli endonuclease I. The difference in kinetics is considered to represent the difference between a "double hit" and a "single hit" mechanism.

This paper describes a new assay for DNA endonuclease activity. The method depends upon the liberation of large fragments of DNA of average molecular weight approximately 400,000 from a polyacrylamide gel in which high molecular weight isotopically labeled DNA is entrapped. This assay has been used to examine the kinetics of DNA hydrolysis as a function of time and of enzyme concentration. Previous workers have provided evidence that DNase I hydrolyzes both strands of native DNA by a "double hit" mechanism (1-5). This mechanism requires more than one encounter between enzyme and DNA for a break in both strands of the DNA. Both DNase II (3, 4) and Escherichia coli endonuclease I (5, 6) hydrolyze native DNA by a predominantly "single hit" mechanism—the cleavage of both DNA strands during one encounter between enzyme and DNA. The pattern of some of the kinetic data reported here with DNase I differs from that obtained with DNase II and endonuclease I. The different patterns are considered to be a reflection of the different mechanisms.

MATERIALS AND METHODS

Bacteria and Bacteriophage— Stocks of unlabeled T4 phage were prepared with E. coli SW 1485 in a modified Fraser-Jerrel medium (7) with glucose as a carbon source. For the synthesis of 32P T4 phage, E. coli B3 (kindly provided by Dr. A. W. Kosinski) was grown in Tris-casamino acids-glucose medium (8) to 4 x 10⁹ cells per ml. Then 1.5 to 2.0 μC of 32P-sodium phosphate per ml were added and growth was continued to 7 x 10⁹ cells per ml. T4 phage was then added at a multiplicity of 10.

Phage DNA was also labeled with 3H-thymine. E. coli B3 was grown overnight to stationary phase in 50 ml of nutrient broth. Cells were centrifuged, washed once with the Tris-casamino acids medium (8), resuspended in 5 ml of the same medium, and inoculated into 1 liter of this medium with glucose and 20 mg of unlabeled thymine, 90 μg of 3H-thymine (containing 2.5 μC), and 10⁻³ M potassium phosphate. Growth was continued at 37° to a cell concentration of 5 x 10⁹ per ml. Then 5 mg of fluorodeoxyuridine and 20 μg of uridine, plus T4 phage at a multiplicity of infection of 10, were added. After lysis, the phage were harvested by centrifugation (9).

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Extraction of Phage DNA—DNA was obtained by phenol extraction of phage according to the method of Mandell and Hershey (10). The DNA was dialyzed extensively against 0.05 M Tris-HCl, pH 7.2. Labeled ³²P-DNA preparations were made immediately after phage purification. DNA with a specific activity of 1000 to 2000 cpm per μmole of DNA nucleotide was used. Phage DNA labeled with tritium was more stable in the gel and zero time control values were lower than those obtained with ³²P-labeled DNA. ³²P-DNA was used in the assay for only 2 weeks after its biosynthesis.

DNA Polyacrylamide Gel Preparation—Isotopically labeled DNA was incorporated in a polyacrylamide gel (11). An acrylamide stock solution contained a final concentration of

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‡ Recipient of National Institutes of Health Research Career Award Fellowship 5-K06-GM21444.
The gel was washed by suspension and centrifugation six times with 10 volumes of 0.05 M Tris-HCl, pH 7.2, and 0.15 M KCl. Two milliliters of the acrylamide stock solution were mixed with 1 ml of a DNA solution containing 0.25 mg per ml of DNA in 0.05 M Tris-HCl, pH 7.2, plus 0.15 M KCl. To this was added 0.25 ml of a riboflavin solution which contained 40 mg per liter. The solution was introduced into a vertical glass tube 15 cm in length by 1 cm in diameter which was stopped at the bottom and this was then aerated through a Pasteur pipette with nitrogen for 10 min. Five-tenths of a milliliter of a milliliter of distilled water, previously aerated with nitrogen, was layered on top of the solution. In the presence of overhead fluorescent lighting, polymerization was complete in 60 min. (The polymeracylamide did not polymerize easily with high concentrations of DNA and the gel which did polymerize was more easily fragmented.) After polymerization of the gel, the tube was unstopped and the gel was forced out with a stainless steel rod slightly less than 1 cm in diameter. It was then transferred to a 10-ml syringe and fragmented by passage through a 50-mesh screen. The gel was washed by suspension and centrifugation six times with 10 volumes of 0.05 M Tris-HCl buffer, pH 7.2, containing 0.15 M KCl and 2 × 10−4 M EDTA, six times with 0.05 M Tris-HCl, pH 7.2, containing 2 × 10−3 M EDTA, and finally six times with 0.05 M Tris-HCl, pH 7.2. For assays of DNase I, the gel was suspended in 0.05 M Tris-HCl buffer, pH 7.2, and the volume was adjusted to contain 25 to 35 mg of DNA per ml of gel suspension. For other DNase assays, the gel was washed with EDTA as above and then washed with and finally resuspended in the buffer to be used in the reaction. The average gel preparation contained approximately 186 μmole of DNA nucleotide per ml of gel volume after it was packed by centrifugation at 2000 × g for 5 min.

**Assay of DNase I**—Nuclease assays were performed as follows. The stock suspension of DNA gel was stirred to produce a uniform suspension with a magnetic stirrer. Aliquots, 1.0 ml, were removed with a 1.0-ml pipette, the tip of which had been allowed to fast pipetting. Aliquots taken under these conditions contained an amount of isotopically labeled DNA which varied usually ±5%. Divalent metal ions required for DNase I activity were added to the reaction mixtures and the reactions were started by the addition of the enzyme. The final volume of the incubation mixture was 1.5 ml and all incubations were at 37°C. The reaction was stopped by the addition of 0.5 ml of 0.1 M EDTA. The tubes were centrifuged at 2,000 rpm for 5 min and 1.0 ml of the supernatant fraction was removed for counting. 32P counting was done with a Tracerlab low background gas flow counter and 3H counting in a scintillation counter. A small piece of Millipore membrane in the scintillation fluid prevented a high percentage of the quenching. Reactions were run in duplicate. When a low percentage of the total counts in the gel was liberated, the accuracy of the method was ±10% or better. When larger percentages were liberated, the accuracy decreased. On storage, the 32P gel released counts slowly and for this reason occasional washes were advisable. When the DNA gel was incubated without enzyme, but with buffer and either Mg2+ or Mn2+, approximately 200 counts were liberated from a gel containing 20,000 cpm; with DNA labeled with tritium, the amount released was in the range of 50 to 100 out of 10,000 to 20,000. Control tubes lacking enzyme were run in each experiment.

**Assay of DNase II and E. coli Endonuclease I**—For assays of DNase II, the gel was washed with and finally resuspended in 0.05 M sodium acetate, pH 4.7, containing 0.01 M sodium citrate. To stop reactions catalyzed by DNase II, 0.5 ml of 0.3 M Tris-HCl buffer, pH 9.6, which contained 0.9 μM MgSO4, was added to each reaction mixture. For assays of E. coli endonuclease I, the DNA gel was washed and suspended in 0.05 M Tris-HCl, pH 7.2, with additions as indicated. Reactions were stopped by the addition of 0.5 ml of 0.1 M EDTA.

**Materials**—DNase I (bovine pancreas DN-C) and DNase II (bovine spleen, DN-II) were purchased from Sigma. E. coli endonuclease I, Fraction IV (12), was kindly supplied by Dr. I. R. Lehman. Crystalline bovine serum albumin (Calbiochem), used for dilution of DNase I, did not have DNase activity when tested by the gel assay, by viscosity measurements, or by examination of single stranded DNA in an alkaline sucrose gradient (13). 32P as inorganic phosphate and thymine methyl 3H were obtained from New England Nuclear.

### Results

#### Size of DNA Retained in Gel—A series of experiments was done to determine the size of the DNA which could be retained by the gel after polymerization in the presence of the DNA. The results are shown in Table I. When the gel was polymerized in the presence of E. coli DNA with an approximate molecular weight of 550,000, only 3% was retained in the gel. However, 79% of the native T4 DNA was retained. Thus the polymerization of polyacrylamide in a solution containing DNA allows the trapping of DNA molecules only if the DNA has a relatively large molecular weight.

#### Size of DNA Released from Gel by DNase I—Experiments were then done to determine the size of the T4 DNA released from the gel by DNase I. Because the fragments liberated from the gel into the supernatant fraction were also degraded by the enzyme, three different approaches to the determination of the size of material released from the gel were made.

The first involved varying times of incubation followed by the determination by a sucrose density gradient technique (14) of the sedimentation coefficient of the peak of radioactivity of

### Table I

<table>
<thead>
<tr>
<th>DNA Type</th>
<th>Apparent S value</th>
<th>DNA in gel</th>
<th>Approximate molecular weighta</th>
<th>% retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli, sonicated treated</td>
<td>7.8b</td>
<td>3.2</td>
<td>5.5 × 10^4</td>
<td>74</td>
</tr>
<tr>
<td>E. coli</td>
<td>9.5b</td>
<td>17.0</td>
<td>1.0 × 10^5</td>
<td>48</td>
</tr>
<tr>
<td>T4</td>
<td>57.3b</td>
<td>79.0</td>
<td>1.0 × 10^5</td>
<td>99</td>
</tr>
</tbody>
</table>

a Approximate molecular weight was calculated with the formula of Crothers and Zimm (15).
b The apparent S value was determined by examination in the analytical ultracentrifuge at a concentration of 0.05 mg per ml and was uncorrected for concentration.

c The apparent S value was determined by sucrose density gradient sedimentation (14) with a reference sample of calf thymus DNA, the S20,w of which was 20.1 as determined in the analytical ultracentrifuge.
the DNA present in the supernatant fraction. The results are shown in Table II. At the earliest times, molecules were present with an average apparent S value of 7.3 and an approximate molecular weight of $3.9 \times 10^4$. After 10 min when 11.7% of the DNA had been released, the average molecular weight had started to decrease. Similar results were obtained with either Mg$^{2+}$ or Mn$^{2+}$ as the activating ion with DNase I. An indication of size distribution of the released DNA is given in Fig. 1 for the samples shown in Table I, Experiment 1.

The second approach was to vary the reaction volume in order to decrease the final concentration of the DNA released into the supernatant fraction while the enzyme concentration was kept constant. This, in effect, decreased the substrate concentration in the supernatant fraction. In Table III, the data show that the rate of release of DNA into three different reaction volumes was constant. However, the average sedimentation value of the released fragments differed and in the case of the largest volume was 7.3 S. This shows that dilution does affect the size of the DNA fragments isolated. It is probable that shorter incubation times would have produced slightly larger fragments.

A third approach was the addition of unlabeled DNA in solution to dilute the liberated isotopic DNA and thereby decrease the rate of degradation of the labeled DNA. Data from such an experiment, shown in Table III, indicate that the maximum average sedimentation value obtained was 8.4. It is apparent from these three approaches that an approximate average molecular weight of the fragments released from the gel was from 4 to $7 \times 10^4$. The lower figure is preferable since it is an underestimation rather than an overestimation of the average size of the fragments released.

Thus, for retention of DNA in polyacrylamide gel, its molecular weight must be relatively high. Native T4 DNA has a molecular weight greater than $100 \times 10^6$ (16). The requirement for high molecular weight DNA is apparent both from experiments in which DNA with an average sedimentation coefficient of 7.8 was not retained in the gel after polymerization and washing, and from experiments showing that the DNA released enzymatically from the gel had an S value of 7.3 to 8.4.

### Table II

**Size of DNA after release from gel by DNase I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (min)</th>
<th>DNA released (mg)</th>
<th>Apparent S value</th>
<th>Approximate molecular weight</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2.5</td>
<td>7.3</td>
<td>$3.9 \times 10^4$</td>
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<td></td>
<td>5</td>
<td>6.2</td>
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<td>$3.9 \times 10^4$</td>
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<td></td>
<td>10</td>
<td>11.7</td>
<td>7.0</td>
<td>$3.3 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>8.4</td>
<td>6.2</td>
<td>$2.1 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>23.0</td>
<td>4.9</td>
<td>$0.7 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>38.0</td>
<td>3.4</td>
<td>$&lt;1 \times 10^4$</td>
</tr>
</tbody>
</table>

**TABLE III**

**Effect of varying reaction volume and amount of unlabeled DNA on rate of reaction and size of DNA released**

In Experiment 2, 35.8 mmoles of $^{32}$P-DNA were present in the gel; unlabeled T4 DNA was added to two of the tubes. The reaction was for 10 min at 37°. The reaction was stopped by adding EDTA at a final concentration of 50 mM. Aliquots of the supernatant fraction were counted and other aliquots were sedimented in 5 to 20% sucrose density gradients at 25,000 rpm, 4°, for 17 hours. Thirty-two 10-drop fractions were collected from the bottom of the centrifuge tubes and aliquots of each fraction were taken for the determination of $^{32}$P-DNA and of the optical density at 260 mp of a marker of E. coli transfer RNA. Sedimentation coefficients were calculated according to Martin and Ames (14).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reaction volume (ml)</th>
<th>Unlabeled DNA added (mmoles)</th>
<th>Rate of release (mmoles/min)</th>
<th>Apparent S value</th>
<th>Approximate molecular weight (X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.46</td>
<td>5.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0</td>
<td>0.32</td>
<td>6.0</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0</td>
<td>0.45</td>
<td>7.3</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>0</td>
<td>0.42</td>
<td>6.5</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>24</td>
<td>0.39</td>
<td>7.6</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>72</td>
<td>0.95</td>
<td>8.4</td>
<td>6.9</td>
</tr>
</tbody>
</table>
Fig. 2. The effect of varying amounts of DNA gel on the amount of DNA fragments released by DNase I and DNase II. Reaction mixtures for DNase I contained 50 mM Tris-HCl (pH 7.2), 150 mM KCl, 6.6 mM MgCl₂, 0.04 μg of DNase I, and different amounts of ³²P-DNA as DNA gel in a final volume of 6.0 ml. The reaction was stopped as indicated under "Materials and Methods." For DNase II reaction mixtures contained 50 mM sodium acetate buffer (pH 4.7), 10 mM sodium citrate, 0.5 mM MgSO₄, 0.8 μg of DNase II, and different amounts of ³²P-DNA as DNA gel in a final volume of 6.0 ml. DNase II reactions were stopped by the addition of 0.5 ml of a solution which contained 0.3 M Tris-HCl, pH 9.6, and 0.2 M MgSO₄. All incubations were at 37° for 30 min.

Effect of Varying Amounts of DNA Gel—A DNA gel concentration curve is shown in Fig. 2 for both DNase I and DNase II. It is evident that, when increasing amounts of DNA gel are added, the rate of release of DNA increases in a linear fashion. This is not evident that the enzyme is unsaturated with DNA, since addition of gel represents addition of DNA which is contained in a specific volume of gel. The amount of DNA per ml of gel packed by centrifugation at 1000 × g for 5 min was approximately 186 mpmoles. The addition of varying amounts of gel is thought to represent an addition of varying amounts of reaction volume which the enzyme must penetrate to act upon the substrate.

Effect of Varying Amounts of DNA per Unit Volume of Gel—To determine whether the DNA is present at a saturating level within the gel, an experiment was done in which varying amounts of DNA were incorporated into a standard amount of gel (70, 140 and 280 mpmoles of DNA per ml of packed gel). The ratio of release by DNase I with 1.3 mM MgCl₂ and 100 mM KCl was for the 140-mpmole concentration approximately 2 times the rate with the 70-mpmole concentration and with 280 mpmoles per ml it was approximately 2.6 times the 70 mpmole per ml concentration. With DNase II similar results were obtained. For these two enzymes, saturating levels of DNA are not present in the gel. When larger amounts of DNA were added to the polymerization mixture, the process of polymerization was

FIG. 3. The effect of time on the liberation of total and acid-soluble counts from the double stranded DNA gel by DNase I. The reaction mixture contained 32 mpmoles of ³²P-DNA as DNA gel, 50 mM Tris-HCl (pH 7.2), 150 mM KCl, 6.6 mM MgCl₂, and 0.01 μg of DNase I in a volume of 1.5 ml.

FIG. 4. The effect of time on the liberation of single and double stranded DNA from the gel by DNase I. The reaction mixture was similar to that described in the legend for Fig. 2 with the exception of the addition of equal volumes of ³²P single stranded DNA gel and ³H-double stranded DNA gel.
partially inhibited. In most of the experiments reported in subsequent papers, gel with 180 to 200 μmoles of DNA per ml of packed gel was used.

Kinetics with DNase I—The rate of release of isotopic DNA

from the gel catalyzed by DNase I in the presence of $6.6 \times 10^{-3} \text{ M MgCl}_2$ and $0.15 \text{ M KCl}$ is indicated in Fig. 3. The acid-soluble counts were 10 to 15% of the total counts released during the early phase of the reaction. Since the gel contained 32 μmoles of DNA, one would expect an increased percentage of acid-soluble counts in the later phase of the reaction when as much as 20 μmoles of DNA had been liberated. It is clear that the major amount of ³²P released in the early part of the reaction is not acid-soluble. This would be predicted by the size distribution of DNA (Fig. 1). The lag in the first 10 min which is present in Fig. 3 has been noted in four experiments with DNA gel when Mg²⁺ was the divalent metal added. It was also noted in two experiments when acid-soluble oligo- and mononucleotides were measured. This lag in the degradation of DNA with DNase I plus Mg²⁺ is considered to be related to the mechanism of action of the enzyme.

A number of other possible explanations for the lag period were investigated. The first was the time required for the enzyme to diffuse into the gel. Three observations rule out this explanation. First, the lag phase was absent when single stranded DNA was released from the gel by DNase I. Fig. 4 shows the release of both single stranded and double stranded DNA in the same reaction mixture as a function of time. In this experiment, each incubation tube contained gel with ³H-thymine-labeled double stranded DNA and gel with ³²P-labeled single stranded DNA. Thus one DNA served as a control for the other. Here the lag is apparent only with the double stranded DNA. The second observation that rules out enzyme diffusion
The effect of varying the enzyme concentration on the rate of liberation of DNA from the gel is shown in Fig. 5. When the incubation is in the presence of Mg²⁺ ion, a lag occurs at very low concentrations of enzyme as emphasized by the inset while at higher enzyme concentrations the rate of release is proportional to the enzyme concentration. The lag is not related to enzyme inactivation in the presence of buffer and metal as previous incubation of a low concentration of enzyme for as long as 60 min did not result in loss of activity. The lag was also observed when acid-soluble degradation products were measured instead of the total counts released from the gel. When single stranded DNA in the gel was used as a substrate with DNase I plus Mg²⁺, the enzyme concentration curve had no lag.

No inhibition of the rate of release of DNA from the gel was noted when increasing amounts of gel without DNA were added to a standard reaction mixture. Amounts of gel lacking DNA up to 2.5 times the DNA gel were added. Thus the gel itself does not adsorb the enzyme.

**Kinetics with DNase II**—The release of ³²P-DNA from the gel by DNase II as a function of time is shown in Fig. 6. In this case the release of acid-soluble material is even less than with DNase I. Note that there is no lag period in the release of DNA with this enzyme. DNase II has been shown by others to degrade DNA with kinetics which are interpreted as a single hit mechanism. A further discussion of this is presented (17). Also, after an initial rapid phase of enzyme action, there is a slow phase. This has been observed with another assay method (18). Time curves with varying amounts of enzyme were diphasic with the apparent break in the curve occurring between 10 and 20 min after an amount of DNA released which varied directly with the amount of enzyme added. When 0.02 μg of DNase II was used (inset, Fig. 6) again a diphasic curve was noted with the rapid phase occurring during the first 10 min.

When DNase II was previously incubated with varying amounts of unlabeled DNA in buffer and MgSO₄ for 10 min and then the reaction was started by the addition of ³²P-DNA gel, an inhibition of the rate of release of DNA from the gel was observed. The effect of varying the enzyme concentration on the rate of liberation of DNA is shown in Fig. 5. When the incubation is in the presence of Mg²⁺ ion, a lag occurs at very low concentrations of enzyme as emphasized by the inset while at higher enzyme concentrations the rate of release is proportional to the enzyme concentration. The lag is not related to enzyme inactivation in the presence of buffer and metal as previous incubation of a low concentration of enzyme for as long as 60 min did not result in loss of activity. The lag was also observed when acid-soluble degradation products were measured instead of the total counts released from the gel. When single stranded DNA in the gel was used as a substrate with DNase I plus Mg²⁺, the enzyme concentration curve had no lag.

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Incubation period. However, no change was observed. Also, these results were obtained with an assay time of 30 min.

The binding of DNase II to fragments of DNA released from the gel present inhibited the release of DNA from the gel. With DNase I, the addition of unlabeled DNA when $M_{\text{g}}^{2+}$ was added. This is shown in Fig. 7. This suggests that some similar curvilinear aspect of the diphasic curve noted in Fig. 6, the effect of varying the enzyme concentration on DNA release was tested with an S-min enzyme dilutions were made in a solution composed of 0.25 ammonium sulfate, 0.05 mM KCl, 6.6 mM Tris-HCl buffer (pH 7.2), and 1 mg per ml of crystalline bovine serum albumin. Incubation was for 30 min at 37 °C.

Noted which was proportional to the amount of unlabeled DNA added. This is shown in Fig. 7. This suggests that some complex may be formed between unlabeled DNA and DNase II which decreases the effective enzyme concentration. This does not appear to be related to the formation of oligonucleotides since previous incubation of DNase II with a constant amount of unlabeled DNA for periods of time varying from 0 to 60 min did not alter the degree of inhibition by the added DNA. With DNase I, the addition of unlabeled DNA when $M_{\text{n}}^{2+}$ was present inhibited the release of DNA from the gel. With DNase I plus $M_{\text{n}}^{2+}$ added unlabeled DNA stimulated the release of DNA from the gel. This effect has not been investigated. The binding of DNase II to fragments of DNA released from the gel may be the explanation of the diphasic curve noted in Fig. 6. The effect of varying the enzyme concentration on the rate of release of DNA by DNase II is shown in Fig. 8. The data suggest a curvilinear rather than a linear relationship. It is clear that no lag in the curve exists as was noted with DNase I. These results were obtained with an assay time of 30 min. Because of the possibility that the curve could be due to some aspect of the diphasic curve noted in Fig. 6, the effect of varying enzyme concentration on DNA release was tested with an 8-min incubation period. However, no change was observed. Also, similar curvilinear results were obtained when the release of acid-soluble material was measured.

**Kinetics with Endonuclease I of E. coli**—The liberation of $^{32}$P-DNA from the gel catalyzed by the E. coli endonuclease I as a function of time is shown in Fig. 9. This endonuclease appears to have no lag period, nor does it show any diphasic curve such as was noted with DNase II (Fig. 6). With this assay, single stranded DNA was released from the gel at approximately 1.2 times the rate of double stranded DNA. An enzyme concentration curve is shown in Fig. 10. This curve has no lag and is similar to that obtained with DNase II (Fig. 8).

**Discussion**

The method described in this paper allows a rapid and easy distinction between endo- and exonuclease activity because DNA fragments of an average molecular weight of approximately $4 \times 10^6$ are released and it is possible by measuring total and acid-soluble isotope released to show that well characterized endonucleases release primarily acid-insoluble material. All of the counts liberated by a pure exonuclease should be acid-soluble. A purified snake venom phosphodiesterase preparation (19), normally considered to be an exonuclease, was tested with this assay and showed liberation of counts only 2% of which were acid-soluble. This showed that the enzyme preparation had considerable endonuclease activity.

The method can be used with double stranded DNA, double stranded alkylated DNA, and single stranded DNA and will therefore be useful in the purification of as yet uncharacterized endonucleases. One limitation is that both strands of a double stranded DNA molecule must be cleaved in order to observe liberation of DNA from the gel. Cleavage of single strands in neighboring areas appears to be effective as observed with DNase I. The method is extremely sensitive and levels of DNase I as low as 0.0005 μg per ml can be measured (17).

The enzymatic hydrolysis of double stranded DNA can occur by two different mechanisms. The first has been termed a single hit mechanism in which one encounter between enzyme and DNA results in hydrolysis of both strands. The second has been called double hit mechanism in which each encounter of enzyme with DNA results in cleavage of only one of the two strands. DNase I or pancreatic DNase in the presence of $M_{\text{n}}^{2+}$ hydrolyzes DNA by a mechanism which is predominantly double hit. This has been shown by assays involving viscosity (1), light scattering (2, 3), and ultracentrifugation (4, 5). DNase II, and acid DNase obtained from various animal sources, hydrolyzes DNA by a predominantly single hit mechanism. This has been observed by techniques of light scattering (3) and ultracentrifugation (4). Endonuclease I of E. coli degrades DNA by a single hit mechanism similar to DNase II. This has been established by light scattering and viscometry (6) and ultracentrifugation (5) techniques. Although the single hit mechanism of hydrolysis of DNA by DNase II does not preclude some single strand hydrolysis (3), the action is predominantly a simultaneous scission of both strands. One would predict that any method which measures endonuclease activity and which depends upon cleavage of both strands of native DNA should show differences in kinetics between the single and double hit mechanism. In fact, the basis for describing the double hit mechanism of DNase I was due to kinetics which showed a lag in hydrolysis of double stranded DNA (1, 2).

It is clear that, with the gel assay used for these experiments, a lag in the release of DNA fragments as a function of time occurs with DNase I plus $M_{\text{n}}^{2+}$ (Fig. 3), but not with DNase II (Fig. 6) or with E. coli endonuclease I (Fig. 9). Several experiments
were done to rule out possible artifacts as reasons for the lag with DNase I. The most convincing evidence that the lag represents a double hit mechanism is that it does not occur with single stranded DNA under the same conditions, and that it can be eliminated with Mn$^{2+}$ and other metals which, by other criteria, shift the mechanism of hydrolysis to single hit kinetics. The latter experiments are presented in a subsequent paper (17).

The enzyme concentration curve with DNase I (Fig. 5) shows a lag which is not seen with DNase II (Fig. 8) or E. coli endonuclease I (Fig. 10), or with DNase I plus Mg$^{2+}$ when the substrate is single stranded DNA. It is also of interest that the nonlinear enzyme concentration curve noted with DNase II and endonuclease I has been observed with DNase I and Mn$^{2+}$ as is discussed (17).

Unlabeled DNA when added to the incubation mixtures with DNase II produces significant inhibition (Fig. 7). With DNase I plus Mn$^{2+}$ inhibition by unlabeled DNA was also observed (Table III) but, with Mg$^{2+}$, the rate of release was stimulated. The reason for this stimulation is not known, but might be related to the effect of nucleotides in the presence of Mg$^{2+}$ on the activity of DNase I. Experiments showing this point are presented (17). A possible explanation for the diphasic time curve with DNase II is that the enzyme binds to DNA fragments released from the gel and this process decreases the enzyme concentration available to act on DNA in the gel. A similar diphasic curve has been observed with DNase II by measurement of the increase of phosphomonoester or of ultraviolet absorption (18). It is possible that DNase II does not dissociate readily from the DNA after hydrolysis. Further investigation is required for a satisfactory explanation.

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**REFERENCES**

Deoxyribonucleic Acid Nucleases: I. THE USE OF A NEW METHOD TO OBSERVE THE KINETICS OF DEOXYRIBONUCLEIC ACID DEGRADATION BY DEOXYRIBONUCLEASE I, DEOXYRIBONUCLEASE II, AND ESCHERICHIA COLI ENDONUCLEASE I
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