SEPARATION OF DEOXYRIBO-OLIGONUCLEOTIDASES INDUCED BY INFECTION WITH BACTERIOPHAGE T2*

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Communicated by J. M. Buchanan, June 11, 1965

In 1962, Aposhian and Kornberg\(^1\) described a procedure for partial purification of the DNA polymerase induced by infection of Escherichia coli with bacteriophage T2. They observed that their purest preparation of phage-induced DNA polymerase exhibited deoxyribonuclease activity when assayed with heat-denatured DNA as substrate. Recently, Lucas and Kornberg\(^2,3\) purified the DNA polymerase induced by the mutant bacteriophage T4Am82. Their most highly purified preparation also evinced an exonuclease activity. This nuclease attacks oligonucleotides prepared by the partial degradation of DNA much more rapidly than it attacks heat-denatured DNA. This activity, therefore, has a substrate specificity resembling that of the T2-induced exonuclease described by Oleson and Koerner.\(^4\)

These results raise the question of the relationship between the oligonucleotidase and DNA polymerase activities induced by infection with bacteriophage T2.

In this paper we will present evidence that the T2-induced exonuclease previously studied in this laboratory is readily separable from T2-induced DNA polymerase. We will also show that, closely associated with the T2-induced DNA polymerase, there is another distinct nuclease with high activity on partially degraded DNA.

Materials and Methods.—Assays: T2-induced DNase was assayed with use of partially degraded salmon sperm DNA for substrate.\(^4\)

T2-induced DNA polymerase was assayed by the procedure of Aposhian and Kornberg\(^1\) with the modification that partially degraded salmon sperm DNA was used as primer.\(^3\) The radioactive substrate was C\(^{14}\)-labeled dATP (1500 cpmp/mumole; obtained from Schwarz BioResearch, Inc.). The DNA polymerase activity is expressed in the units defined by Richardson et al.\(^5\)

E. coli DNA polymerase was assayed using "activated" thymus DNA for primer as described by Richardson et al.\(^4\)

Analytical procedures: Protein was measured by the biuret method of Mokrasch and McGilvery.\(^6\) The protein concentration in the effluent solutions from chromatographic columns was determined by measuring the absorbance at 280 mp.\(^6\) Nucleic acid contamination of the protein was estimated by the method of Warburg and Christian as described by Layne.\(^7\)

Preparation of the cell-free extract: Cell-free extracts of E. coli infected with bacteriophage T2 were prepared as previously described,\(^4\) with the exception that the cells were disrupted by sonic disintegration (Branson Sonifier, model S-110) after suspension in a solution 0.01 M with respect to Tris-acetate buffer, pH 7.5; 0.002 M with respect to EDTA; and 0.01 M with respect to magnesium acetate. Extracts were stored at \(-15\)°.

Fractionation of the extract: The extract (50 ml) was thawed, and a soluble fraction and precipitate were prepared by use of protamine sulfate as previously described.\(^4\) The protamine supernatant solution was further fractionated with ammonium sulfate and prepared for DEAE-cellulose chromatography as previously described.\(^4\) The protamine precipitate was resuspended by sonic vibration in half the crude extract volume of a solution which was 0.01 M with respect to Tris-acetate buffer, pH 7.5; 0.002 M with respect to EDTA; and 0.002 M with respect to 2-mercaptoethanol. This suspension was centrifuged (10,000 \(\times\) g for 10 min); the supernatant solution was collected, and the precipitate was washed with the above buffer (0.1 vol of the crude extract). These supernatant solutions derived from the protamine precipitate were then combined. Pancreatic ribonuclease was added to the combined supernatants to achieve a final concentration of

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38 µg/ml, and the solution was incubated for 20 min at 30°. The solution was then made 0.01 M with respect to magnesium chloride. Crystalline pancreatic deoxyribonuclease was added to achieve a final concentration of 1 µg/ml, and incubation was continued at 30° for 30 min. The resulting precipitate was removed by centrifugation (15,000 X g for 15 min) and discarded. The supernatant solution was made 0.1 M with respect to Tris-acetate buffer, pH 7.5, and ammonium sulfate was added to attain 55% saturation. After centrifugation (15,000 X g for 15 min), the precipitate was collected and dissolved in sufficient buffer solution (0.025 M with respect to Tris-acetate buffer, pH 7.5; 0.002 M with respect to EDTA; and 0.002 M with respect to 2-mercaptoethanol) to give a final protein concentration of 2.4%. The ammonium sulfate fractions derived from the protamine supernatant and protamine precipitate were desalted by gel filtration with Sephadex G-25. Both fractions were then subjected to chromatography with DEAE-cellulose at pH 7.5 as previously described, with the following exceptions: the columns were 1.1 cm diameter X 15 cm long; the limits of the linear gradient were 0.02 and 0.35 M NaCl; and the total gradient volume was 200 ml. Fractions of 3 ml were collected, and these were assayed for DNase, T2-induced DNA polymerase, and E. coli DNA polymerase. The most active fractions of the T2-induced exonuclease (Fig. 1A, peak Z) and of the T2-induced DNA polymerase (Fig. 1B, peak Y) were subjected to rechromatography (Fig. 2).

In preparation for rechromatography, an aliquot of the T2-induced exonuclease fraction was diluted fourfold with a solution 0.02 M with respect to potassium phosphate buffer, pH 7.5; 0.005 M with respect to EDTA; and 0.01 M with respect to 2-mercaptoethanol. Prior to rechromatography, a portion of the T2-induced DNA polymerase fraction was dialyzed against 6 vol of the potassium phosphate buffer-EDTA-mercaptoethanol solution. Aliquots of T2-induced DNA polymerase and of T2-induced exonuclease were also combined and dialyzed for rechromatography. Rechromatography of the three preparations was carried out on columns of DEAE-cellulose 0.33 cm diameter X 5.5 cm long. The protein was eluted with a linear gradient of 0.02 M-0.35 M NaCl. Both the starting solution and the limiting solution also contained the potassium phosphate buffer-EDTA-mercaptoethanol solution described above. The total gradient volume was 14.5 ml, and fractions of 0.5 ml were collected.

Results and Discussion.—Both the fractionation with protamine and the chromatography with DEAE-cellulose (Table 1; Fig. 1) separate the T2-induced exonuclease described by Oleson and Koerner from the T2-induced DNA polymerase. The bulk of the T2-induced exonuclease is found in the protamine supernatant; the bulk of the T2-induced DNA polymerase is recovered from the protamine precipitate. The T2-induced exonuclease is eluted from DEAE-cellulose with 0.2 M NaCl; the T2-induced DNA polymerase is eluted with 0.07 M NaCl.

In extracts from infected E. coli, the T2-induced exonuclease accounts for approximately 70 per cent of the total DNase active on partially degraded DNA. Although this nuclease is accompanied by a trace of T2-induced DNA polymerase after protamine fractionation, it is free of detectable DNA polymerase activity following chromatography with DEAE-cellulose. In the chromatogram, the trace of T2-induced DNA polymerase can be identified as a small but distinct peak (Fig. 1A). The ratio of DNase activity to DNA polymerase activity in the chromatographic fractions containing T2-induced exonuclease is less than 3 X 10^-3.

The major portion of the activity characteristic of T2-induced DNA polymerase is found in the soluble fraction derived from the protamine precipitate. There is also an appreciable level of DNase activity in this fraction. Much of this DNase activity is separable from the activity of the T2-induced DNA polymerase by chromatography with DEAE-cellulose; however, a portion of the DNase activity appears on the chromatogram as a peak which parallels the peak of DNA polymerase activity (Fig. 1B).
FIG. 1.—Chromatography of protein fractions from T2-infected E. coli. (A) Protein from protamine supernatant solution; (B) protein from protamine precipitate. ———, DNase activity assayed with partially degraded DNA; cross-hatching, T2-induced DNA polymerase activity; ---, A_{290} of effluent; ---, concentration of sodium chloride in effluent solution. X, E. coli Exonuclease IV; Y, T2-induced DNA polymerase; Z, T2-induced exonuclease. E. coli DNA polymerase is precipitated with protamine and appears in fractions 20–30 on the chromatogram. Experimental details are described in the text.

On rechromatography, both the T2-induced exonuclease and the nuclease accompanying the T2-induced DNA polymerase are eluted at their characteristic concentrations of NaCl (Fig. 2A and B). Furthermore, when the two fractions are combined and rechromatographed, the two nucleases are separated and appear at their characteristic regions on the chromatogram (Fig. 2C).

The nuclease activity in the DNA polymerase fraction accounts for approximately 10 per cent of the total phage-induced activity which cleaves partially degraded DNA.

The two T2-induced oligonucleotidases can be distinguished from all DNases found in uninfected E. coli strain B. Both enzymes preferentially attack partially degraded DNA, whereas endonuclease I and exonuclease III have relatively low activity on this substrate. Both enzymes can be distinguished from exonuclease II by the low total activity of the latter enzyme in crude extract and by chromatographic separation (Table 2). Both enzymes can also be separated from exonuclease IV by chromatography (Table 2). However, the T2-induced exonuclease is eluted at a sodium chloride concentration similar to that required for elution of E.
2. Protamine precipitation

Fractionation from exonuclease V by its graphic

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1. Crude extract
2. Protamine precipitation
   (a) Supernatant solution
   (b) Resuspended precipitate
3. Fractionation of protamine supernatant
   (a) Ammonium sulfate
   (b) DEAE-cellulose
      (1) Exonuclease IV
      (2) T2 DNA polymerase-nuclease
      (3) T2 exonuclease
1. Fractionation of protamine precipitate
   (a) Ammonium sulfate
   (b) DEAE-cellulose
      (1) Exonuclease IV
      (2) T2 DNA polymerase-nuclease
      (3) T2 exonuclease

### TABLE 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Units per mg protein</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Units per mg protein</th>
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<td>1. Crude extract</td>
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<td>610</td>
<td></td>
<td></td>
<td>2,850</td>
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<tr>
<td>2. Protamine precipitation</td>
<td></td>
<td></td>
<td></td>
<td>53</td>
<td>100,000</td>
<td>595</td>
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<tr>
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<td></td>
<td>106</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>3. Fractionation of protamine supernatant</td>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>21,500</td>
<td>286</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>1,050</td>
</tr>
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</table>

* The reason for inhibition of DNA polymerase activity in this fraction has not been determined by experiment. Possibly it is correlated with the high relative activity of the T2-induced exonuclease.† Specific activity was calculated for the more active fractions comprising at least 75% of the total activity in the peak.

coli exonuclease I. It is distinguishable from exonuclease I by its relatively low activity on heat-denatured DNA, and it is distinguishable from exonuclease V by its much higher level of total activity in crude extracts (Table 2) and by its greater lability to heat.

The ratio of activity of DNase to DNA polymerase in the most active chromatographic fractions of T2-induced DNA polymerase is equal to 4.5. Earlier, Aposhian and Kornberg assayed T2-induced DNA polymerase for DNase, and they observed a nuclease to polymerase ratio equal to 0.01 when they used heat-denatured DNA as a substrate. (This ratio is calculated by conversion of their units of DNA polymerase activity to those used in this paper.) We attribute the relatively high activity observed in the present study to the fact that this DNase cleaves oligonucleotides much more rapidly than it cleaves heat-denatured DNA.

It is of interest that our most purified DNA polymerase preparation is capable of cleaving oligonucleotides more rapidly, when assayed under optimal conditions for hydrolysis, than it is capable of effecting DNA synthesis, when assayed under

### TABLE 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration of NaCl for elution* (M)</th>
<th>Estimated DNase activity in 50 ml of crude extract (DNase units)</th>
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<tr>
<td>T2 DNA polymerase-nuclease</td>
<td>0.04-0.09</td>
<td>12,800</td>
</tr>
<tr>
<td>E. coli DNA polymerase-nuclease II.</td>
<td>0.08-0.18</td>
<td>310</td>
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<tr>
<td>T2 exonuclease</td>
<td>0.18-0.23</td>
<td>104,000</td>
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<tr>
<td>Exonuclease I</td>
<td>0.21-0.28</td>
<td>7,000</td>
</tr>
<tr>
<td>Exonuclease V</td>
<td>0.18-0.30</td>
<td>20,500</td>
</tr>
<tr>
<td>Exonuclease V</td>
<td>0.18-0.30</td>
<td>7,000</td>
</tr>
</tbody>
</table>

* Chromatography was carried out on DEAE-cellulose at pH 7.5 as described in the text.
optimal conditions for polymerization. Since the same preparation of partially degraded DNA can be used as primer for assay of DNA polymerase and as substrate for assay of DNase, this comparison can be made on the basis of similar availability of free 3'-hydroxyl end groups in both assays. The answer to the question of whether the T2-induced DNA polymerase activity and its accompanying oligonucleotidase activity are enzymatic properties of a single protein or of two different proteins awaits the isolation of a homogeneous preparation of phage-induced DNA polymerase.

Summary.—A cell-free extract was prepared from Escherichia coli infected with bacteriophage T2 and was subjected to a fractionation procedure involving precipitation with protamine and chromatography with DEAE-cellulose. The fractions were assayed for deoxyribonucleases which would attack a substrate of mixed oligonucleotides derived from partially degraded DNA. The fractions were also assayed for the DNA polymerase activity of normal cells and for the DNA polymerase activity induced by phage T2. The T2-induced exonucl ease previously isolated from this system, the T2-induced DNA polymerase, and the E. coli DNA polymerase were readily obtained as separate fractions. The T2-induced exonuclease accounted for 90 per cent of the T2-induced deoxyribo-oligonucleotidase activity in the extract. Another deoxyribonuclease which degrades oligonucleotides more rapidly than heat-denatured DNA was found to be closely associated with the T2-induced DNA polymerase activity. The two phage-induced nucleases can be distinguished from the known nucleases of uninfected E. coli.

Fig. 2.—Rechromatography of T2-induced exonuclease and T2-induced DNA polymerase fractions obtained from DEAE-cellulose chromatography. ---, DNase activity assayed with partially degraded DNA. Fractions which showed high activity for T2-induced exonuclease (Fig. 1A, peak Z) and for T2-induced DNA polymerase (Fig. 1B, peak Y) were saved from the first chromatographic procedure. Rechromatography was conducted separately with aliquots of the T2-induced exonuclease fraction and the T2-induced DNA polymerase fraction. Aliquots of the two fractions were also mixed, and the mixture was subjected to rechromatography. The experimental details are described in the text. (A) T2-induced exonuclease (3500 DNase units); (B) T2-induced DNA polymerase-nuclease (1200 DNase units); (C) mixed fractions (1000 DNase units of exonuclease and 1000 DNase units of DNA polymerase-nuclease).
STUDIES ON THE PHYSIOLOGY OF RAT LIVER POLYRIBOSOMES: QUANTITATION AND INTRACELLULAR DISTRIBUTION OF RIBOSOMES*

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Communicated by Paul Doty, May 27, 1965

The central role of polyribosomes in cytoplasmic protein synthesis has been demonstrated in a wide variety of organisms. In the rat liver system, polysomes, when isolated, are aggregates of 83S ribosomes joined together, at least in part, by material sensitive to ribonuclease and resistant to trypsin and deoxyribonuclease, presumably messenger RNA. Consistent with presently favored hypotheses of the mechanism of polysome function, involving reattachment to messenger RNA of monosomes from a relatively large cytoplasmic pool,1, 2 investigators have observed a high proportion of "free" (70–85S) ribosomes in preparations derived from many sources. As techniques for polyosome isolation have improved, however, the proportion of 70–85S ribosomes has diminished.3, 4

Early in the course of our investigation of rat liver polysome physiology, it became apparent that 260-mu absorption of sucrose gradient fractions is not an accurate indication of the quantity of polysomal RNA. Indeed, most of the 260-mu optical density in the 70–85S region is due to the metalloprotein, ferritin.5 On correcting for ferritin, the proportion of deoxycholate-liberated monosomes is reduced to less than 5 per cent of total cytoplasmic ribosomal RNA. Evidence will be presented that no additional free and/or deoxycholate-liberated cytoplasmic monosomes are being degraded, compartmentalized, or otherwise selected against during isolation procedures and sucrose gradient analysis. Those free ribosomes that do exist in liver extracts are primarily confined to the nucleus.

Materials and Methods.—6-C14-Orotic acid hydrate, 5 mc/mimole, was obtained from New England Nuclear Corporation and dissolved in dilute NaOH before intraperitoneal injection. Sodium deoxycholate and sodium dodecyl sulfate (from Mann Research Laboratories, and Merck,

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* This investigation was supported by a research grant from the U.S. Public Health Service (AI-04479).

11 Jorgensen, S. E., unpublished experiments.