Studies on T3-induced Ribonucleic Acid Polymerase

III. PURIFICATION AND CHARACTERIZATION OF THE T3-INDUCED RIBONUCLEIC ACID POLYMERASE FROM BACTERIOPHAGE T3-INFECTED ESCHERICHIA COLI CELLS*

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SUMMARY

A procedure for the isolation and purification of bacteriophage T3-induced RNA polymerase from T3-infected Escherichia coli cells is presented. The procedure described leads to the isolation of polymerase preparation that displays a single protein band by polyacrylamide gel electrophoresis and is free of detectable ribonuclease and deoxyribonuclease activities. Other enzyme activities absent from such preparations include inorganic pyrophosphatase, nucleoside di-phosphokinase, DNA polymerase, Escherichia coli RNA polymerase, nucleoside triphosphatase, polynucleotide phosphorylase, polyriboadenylate polymerase, and polyphosphate kinase.

T3-induced RNA polymerase has been characterized with regard to various biochemical parameters. The enzyme is a single polypeptide chain of molecular weight 105,000 ± 5,000. The enzyme activity is completely dependent on the presence of Mg²⁺, all four nucleoside triphosphates, and T3 DNA. T7 DNA shows only 2 to 5% of activity as that obtained with T3 DNA, while other native DNA preparations examined are inactive. The T3 RNA polymerase is highly sensitive to salt concentrations above 0.03 M or to reagents which react with sulfhydryl groups such as p-hydroxymercuribenzoate and N-ethylmaleimide.

The enzyme catalyzes a T3 DNA-dependent ³²Ppexchange into nucleoside triphosphates. However, no pyrophosphorylisis of free RNA can be demonstrated. The ³²Ppexchange reaction can occur with GTP alone. The exchange reaction with the other three nucleoside triphosphates, present either alone or in combination, does not occur except when GTP is included in the reaction.

The effect of Escherichia coli RNA chain termination factor, ρ, on the T3 RNA polymerase reaction has been studied. It has been found that ρ factor has no effect on the rate, yield, or size of RNA formed in the T3 RNA polymerase-catalyzed reactions.

Since the original discovery by Chamberlin et al. (3) of the synthesis of a new DNA-dependent RNA polymerase in bacteriophage T7-infected Escherichia coli cells, reports from this laboratory (1, 2) and that of Bautz et al. (4) have described the isolation of a new DNA-dependent RNA polymerase in Escherichia coli infected with bacteriophage T3. The phage-induced polymerase was shown to be physically and biochemically distinct from Escherichia coli RNA polymerase. The phage polymerase uses only T3 DNA as template and is inactive with native T2, T4, E. coli, calf-thymus DNA, or with poly[d(A-T)]. T7 DNA was shown to be approximately 5% as active as T3 DNA. In contrast Escherichia coli RNA polymerase is nonspecific in its DNA requirements. T3 RNA polymerase can, however, transcribe poly[dG·dC] to make poly(G) exclusively, which indicates that the polymerase may initiate chains only with GTP. It has subsequently been shown (2) that RNA chains made by T3 RNA polymerase with native T3 DNA template are initiated exclusively with GTP and that completed RNA chains are released free of template DNA. Polymerase is also released in this process and, acting catalytically, reinitiates new RNA chains.

The purpose of this communication is to report the purification and characterization of the T3-induced RNA polymerase and various properties of the polymerase reaction. Such enzyme preparations have already been used to study various aspects of the transcription process catalyzed by T3 RNA polymerase (2, 5). The accompanying paper (6) describes the reaction of the purified enzyme with denatured DNA templates.

EXPERIMENTAL PROCEDURE

Materials

Preparation of T3-Bacteriophage Lysate and T3 Phage-infected cells—The growth medium (Medium A) contained, per liter, 10 g of glucose, 10 g of Casamino acids, 0.1 g of yeast extract, 0.3 g of MgSO₄, 5 g of NaCl, 2 g of NH₄Cl, 0.03 g of CaCl₂, 7 g of KH₂PO₄, and 3 g of K₂HPO₄. For the preparation of crude T3 (wild type) lysates, E. coli B (strain Sy 106) was grown aerobically at 37°C. When the optical absorbance of growing bacteria reached 0.5 to 0.6 at 600 nm, the culture was infected with phage T3 at a multiplicity of 0.2. After lysis occurred (30 to 40 min), 1 ml of CHCl₃ and 24 g per liter of NaCl were added, and the lysate was centrifuged for 10 min at 5000 × g to remove debris (crude bacteriophage lysate).

For the preparation of T3 phage-infected cells, E. coli Sy 106 were grown at 30°C aerobically in Medium A. When the optical absorbance of bacteria reached 0.8 at 600 nm, the culture was
infected with phage T3 at a multiplicity of 8. Sixteen minutes after infection, the culture was rapidly cooled at 5°, and the cells were harvested in a Sharples continuous flow centrifuge which was cooled to 5°. The infected cells were stored at −40° until use. Under the above condition of infection, cell lysis was complete after 28 min.

**Isolation of DNA**—T3 phage was further purified from the crude bacteriophage lysate by precipitation with polyethylene glycol followed by extraction of the precipitated material with 1 M KCl solution as described by Yamamoto et al. (7). Subsequently, bacteriophage was purified by isopycnic banding in CsCl.

T3 and other phage DNAs were isolated from phage preparations purified through the CsCl step by phenol extraction as described by Sadowski and Hurwitz (8). T7 [3H]DNA was isolated as described by Richardson (9).

**Other Materials**—Unlabeled and labeled ribonucleoside triphosphates and [3P]Pi were obtained from Schwarz BioResearch or from New England Nuclear Corp. [γ-32P]Nucleoside triphosphates and bacteriophage T2 [3H]RNA were obtained as described previously (10, 11). DEAE-cellulose (DE-52) and phosphocellulose (P-11) were from Whatman. Bovine serum albumin (twice reconstituted, obtained from Pentex Inc.) was dialyzed against 1 mM EDTA for 48 hours followed by dialysis against water for 24 hours according to Singer (12). E. coli RNA polymerase was obtained as described previously (13). Protein factor p was isolated and purified from E. coli MRE-600 as described by Roberts (14). Incubation of 5 μg of p factor with either 10 nmoles of f2 bacteriophage [3H]RNA or 10 nmoles of T7 [3H]DNA for 30 min at 37° did not alter the sedimentation profile of either polymer, indicating the absence of detectable DNA or RNA endonucleases in p factor preparations.

**Methods**

**Assay of T3 RNA Polymerase**—The conditions of the assay, unless otherwise specified, were as follows. Reaction mixtures (0.5 ml) contained 50 mM Tris-HCl buffer, pH 7.8, 20 mM MgCl₂, 4 mM dithiothreitol, 20 μg of dialyzed bovine serum albumin, 20 nmoles of T3 DNA, 20 nmoles each of ATP, UTP, CTP, and GTP, 100 nmoles of [3P]Pi, (1 to 2 × 10⁶ cpm per μmole), and 2 to 10 units of T3 RNA polymerase. Incubation was for 30 min at 37°, after which reactions were terminated by adding 0.5 ml of 0.2 M EDTA, 0.2 ml of 0.1 M PPi, pH 6.0, and 0.2 ml of a 10% suspension of Norit. The reaction mixtures were gently mixed in ice for 5 min, followed by addition of 2 ml of 0.01 M PPi (adjusted to pH 6.0 with KH₂PO₄), and were centrifuged for 5 min at 10,000 g. The Norit pellet was suspended in 2 ml of 0.01 M PPi, pH 6.0, filtered through a glass fiber filter (Whatman GF/C soaked with 0.01 M PPi, pH 6.0) and washed four times with 3-ml aliquots of 0.01 M PPi, pH 6.0. The filters were placed in glass scintillation vials, dried under gentle heat, and counted in toluene 2,5-diphenyloxazole (POPOP)-1,4-bis[2-(5-phenyl-oxazolyl)]benzene (PPOPOP) in a liquid scintillation counter.

**Preparation of [3P]RNA Substrates for Pyrophosphorolysis**—Native T3 DNA or single-stranded φX-174 DNA-directed T3 RNA polymerase products labeled with [3P]Pi were prepared as follows. Two separate RNA polymerase reaction mixtures were prepared; one contained 20 nmoles of native T3 DNA, while the other contained 20 nmoles of φX-174 DNA instead of T3 DNA. [α-32P]GTP (1 × 10⁶ cpm per μmole) was the labeled triphosphate used. All other additions were the same as described under "Assay of T3 RNA Polymerase." Both reaction mixtures were incubated with 10 units of T3 RNA polymerase at 37° for 1 hour and terminated by the addition of sodium dodecyl sulfate (0.5% final concentration) and EDTA (0.04 M final concentration). Each reaction mixture was layered onto a Sephadex G-100 column (1.4 × 25 cm), previously equilibrated and washed with a solution containing 0.1 M Tris-HCl buffer, pH 7.8, 0.02 M KCl, and 10⁻⁴ M EDTA. The column was then washed with the above buffer solution, and 0.5 ml fractions were collected. The labeled RNA product emerged from the column as a single symmetrical peak. Under these conditions over 95% of the [3P]Pi present in the RNA product was precipitated with 5% CCl₄-COOH. With native T3 DNA, T3 RNA polymerase produces RNA chains free of template DNA (2), while with single-stranded DNA templates (e.g. φX-174 DNA), the RNA produced is present as DNA-RNA hybrids (6).

**Other Methods**—Protein was determined by the turbidometric method of Bücher (16).

**RESULTS**

**Purification of T3 RNA Polymerase**

Unless otherwise indicated, all operations were carried out at 0–4° and all buffer solutions contained 5 × 10⁻⁴ M EDTA and 10⁻⁴ M 2-mercaptoethanol. The purification procedure is described below, while the results of a typical preparation from 30 g of T3-infected E. coli cells are summarized in Table 1.

**Preparation of Crude Extracts**—Thirty grams of partially thawed T3-infected E. coli cells were ground with 60 g of Alcoa alumina powder A-301 in a precooled mortar until a fine paste was obtained. The mixture was then extracted with 90 ml of extraction buffer which contained 25 mM Tris-HCl buffer, pH 7.8, 10 mM MgCl₂, and 30 mM NiCl₂ (Buffer A). The suspension was centrifuged for 15 min at 23,000 × g in a Sorvall centrifuge. The supernatant fluid was decanted, and the pellet was extracted with 30 ml of Buffer A. The combined supernatants from the two extractions were recentrifuged in the Sorvall as described above. The supernatant fluid (S-20) was then centrifuged for 3 hours in the Spinco ultracentrifuge at 150,000 × g.
in a No. 50 Ti rotor. The supernatant fluid obtained after high speed centrifugation contained about 8 to 10% of T3 RNA polymerase activity and was discarded. To each pellet were added 5 ml of Buffer A containing 1 mM NH₄Cl. The pellets were gently homogenized in a glass homogenizer and the suspension was incubated at 4°C for 1 hour. The suspension was then centrifuged for 15 min at 30,000 × g, and the supernatant fluid was recentrifuged for 3 hours at 150,000 × g. The supernatant fluid was adjusted to 30% saturation by the addition of solid ammonium sulfate (165 g per liter). After 20 min of gentle stirring, the precipitate was removed by centrifugation at 30,000 × g for 15 min, and the supernatant fluid was adjusted to 50% saturation with solid ammonium sulfate (130 g per liter). After 30 min of gentle stirring, the precipitate containing T3 RNA polymerase was collected by centrifugation at 80,000 × g for 20 min in a Spincor rotor No. 30, and dissolved in 5 ml of 50 mM Tris-HCl, pH 7.8, containing 10% glycerol. The solution was dialyzed for 6 hours against 1 liter of 50 mM Tris-HCl, pH 7.8, containing 10% glycerol (ammonium sulfate I fraction).

Ammonium Sulfate II—Ammonium sulfate I fraction (10 ml) was diluted with an equal volume of buffer containing 10 mM Tris-HCl, pH 7.8, and 0.5 M KCl. Fifty grams of DEAE-cellulose, wet weight, previously equilibrated with 20 mM Tris-HCl buffer, pH 7.8, containing 0.25 M KCl, were added. The suspension was stirred for 20 min and was subsequently applied to a column of DEAE-cellulose (1.4 × 30 cm) equilibrated with 20 mM Tris-HCl, pH 7.8, and 0.25 M KCl. The column was then washed with the same buffer solution containing 5% glycerol. Under these conditions most proteins were unretarded, while nucleic acids were retained. The elution of protein from the column was followed by measuring the absorbance of the effluent at 280 nm. Fractions containing the bulk of the unretarded polymerase activity which showed at least a 3-fold increase in specific activity relative to the ammonium sulfate II fraction were pooled (Sephadex G-200 eluate, Fraction IV).

Phosphocellulose Chromatography—Fraction IV was immediately applied to a phosphocellulose column (2 × 8 cm), previously equilibrated with 50 mM Tris-HCl, pH 7.8. The column was then washed with 10 ml of the above buffer, followed by 30 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 5% glycerol. A linear gradient of 60 ml total volume from 0.1 M to 0.4 M potassium phosphate buffer (pH 7.5) containing 10% glycerol was applied. The active fractions were pooled and were dialyzed for 6 hours against 500 ml of 0.1 M Tris-HCl buffer (pH 7.8) and 50% glycerol. This dialysis procedure resulted in a concentration of the pooled fraction. The dialyzed fraction (phosphocellulose eluate, Fraction V) was stored at −20°C.

Properties of Purified Enzyme

Stability—T3 RNA polymerase was routinely stored at the Fraction V stage at −20°C. Under these conditions, less than 15% of loss of polymerase activity occurred over a 2-month period. We have also added dialyzed bovine serum albumin to Fraction V to a concentration of 1 mg per ml of albumin. Under these conditions the enzyme preparation was found to be stable for over 6 months at −20°C or at −90°C. Repeated freezing and thawing or storage at 0°C caused marked loss of enzyme activity; over 60 to 70% of enzyme activity was lost in less than 2 weeks.

Purity—When 20 μg of the Fraction V enzyme were analyzed by native polyacrylamide gel electrophoresis according to the method of Maizel (17) at pH 8.0, a single band migrating toward the anode was detected. However, we have not tried to show that the enzymatic activity coincides with this protein band. (No protein bands were observed migrating towards the cathode at pH 4.5.) Purified polymerase (Fraction V) was also analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1). The single band observed provided further evidence of homogeneity and also indicates that T3 RNA polymerase is composed of a single type of polypeptide chain.

Nuclease Activities in Purified Polymerase Preparation (Fraction V)

Endonuclease Activity towards RNA and DNA—Incubation of 10 nmoles of either f2 bacteriophage [3H]RNA or T7 [3H]DNA with 40 units of T3 RNA polymerase for 1 hour did not alter the sedimentation profile of either polymer, indicating the absence of RNA and DNA endonucleases in the T3 RNA polymerase preparation (Fig. 2).

Exonuclease Activity—When 32P-labeled f2 bacteriophage RNA (20 nmoles, specific radioactivity, 1 × 10^6 cpm per n mole) or T3 RNA polymerase product (20 nmoles, specific activity, 2.5 × 10^6 cpm per n mole) or H-labeled native or heat-denatured T7 DNA was incubated with 100 units of T3 RNA polymerase for 16 hours at 37°C, less than 0.1% of radioactivity was converted to the 5% CCl₃COOH-soluble form. The experimental conditions used were similar to those described in a typical T3 RNA
polymerase reaction mixture except that all four nucleoside triphosphates were omitted, and 32P-labeled RNA or 3H-labeled T7 DNA replaced T3 DNA.

**Assay for Other Enzyme Activities in Purified T3 RNA Polymerase**—Measurement of the following enzyme activities was carried out under conditions described for the routine T3 RNA polymerase assay (see “Methods”) except that DNA and ribonucleoside triphosphates were omitted and incubation was for 2 hours at 37° with 40 units of T3 RNA polymerase (Fraction V). These enzyme activities were assayed as described previously (13).

**Inorganic Pyrophosphatase**—T3 RNA polymerase incubated with 200 nmoles of 32P-labeled inorganic pyrophosphate (10⁸ cpm per μmole) did not catalyze detectable formation of P₁ (<2 pmoles).

**Nucleoside Diphosphokinase**—T3 RNA polymerase incubated with 25 nmoles of [γ-32P]UTP (specific activity, 5 x 10⁶ cpm per μmole) and unlabeled ADP (200 nmoles) did not yield detectable [γ-32P]ADP (<1 pmoles).

**Nucleoside Triphosphatase**—T3 RNA polymerase incubated with 25 nmoles of one of the four γ-32P-labeled ribonucleoside triphosphates (specific activity, 2 x 10⁸ cpm per μmole) did not yield detectable Norit-nonadsorbable 32P (<1 pmoles).

**DNA Polymerase**—The assay utilized dAT copolymer as primer and was carried out as described by Richardson et al. (19) with [α32P]TTP (2 x 10⁶ cpm per μmole) as the labeled deoxyribonucleoside triphosphate. There was no detectable incorporation of radioactivity into an acid-insoluble form (<5 pmoles).

**Polyphosphate Kinase**—Incubation of T3 RNA polymerase with 25 nmoles of [γ-32P]ATP (specific activity, 10⁸ cpm per μmole) did not convert 32P into an acid-insoluble form (<0.5 pmoles), indicating the absence of polyphosphate kinase (20).

**Polyribonucleotide Phosphorylase**—T3 RNA polymerase incubated with 100 nmoles of poly(I) + poly(C) and 100 nmoles of [3H]ATP (specific activity, 4 x 10⁶ cpm per μmole) did not give rise to any significant acid-insoluble radioactivity (<0.2 pmoles).

**Myokinase**—T3 RNA polymerase incubated with 20 nmoles of [γ-32P]ATP and 50 nmoles of 5'-AMP did not give rise to detectable 32P into an acid-insoluble form (<0.1 pmoles).

**Polyribonucleotide Phosphorylase**—T3 RNA polymerase incubated with 50 nmoles of ADP and 10 nmoles of 32P (specific activity, 2 x 10⁶ cpm per μmole) did not yield detectable Norit-adsorbable 32P (<2 pmoles).

**Molecular Weight**—The sedimentation constant of T3 RNA polymerase was determined by polacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The mobilities of T3 RNA polymerase relative to several marker proteins were measured. As shown in Fig. 4, the molecular weight obtained in this way was 105,000 ± 5,000.

The subunit molecular weight of T3 RNA polymerase was determined by polacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The mobilities of T3 RNA polymerase relative to several marker proteins were measured. As shown in Fig. 4, the molecular weight obtained in this way was 105,000 ± 5,000. Since the molecular weight of the polymerase was the same under native as well as denaturing conditions, T3 RNA polymerase probably consists of a single polypeptide chain.

Fig. 1 (left). Polyacrylamide gel electrophoresis of T3 RNA polymerase. T3 RNA polymerase (Fraction V; 5 μg) was subjected to polyacrylamide gel electrophoresis (6.5%) in the presence of sodium dodecyl sulfate at pH 7.0 according to the methods described by Maizel (17). The gels were stained with Coomassie blue and destained electrophoretically. The authors are indebted to Dr. P. Gupta of this institution for considerable help in the gel electrophoresis experiments.

Fig. 2 (right). Sedimentation profile of f2 bacteriophage RNA and T7 DNA after treatment with T3 RNA polymerase. Reaction mixtures (0.3 ml) contained 0.1 M Tris-HCl buffer, pH 7.8, 20 mM MgCl₂, 4 mM dithiothreitol. In Experiment A, 10 nmoles of f2 [3H]RNA (15,000 cpm per nmole) and in Experiment B, 14 nmoles of T7 [3H]DNA (31,000 cpm per nmole) were added. The T7 DNA was heated at 70° for 4 min and fast cooled before use to insure disaggregation. Reaction mixtures were incubated at 30° with 40 units of T3 RNA polymerase for 30 min. In the experiment presented in A, the reaction was terminated by the addition of EDTA to 0.04 M and sodium dodecyl sulfate to 0.5%. The reaction mixture was stored at 0° for 15 min and the precipitate formed was removed by centrifugation. The supernatant containing RNA was subjected to formaldehyde treatment to disrupt secondary structure by the following procedure. Sodium phosphate buffer, pH 7.7 (final concentration 0.1 M), formaldehyde to 3% (v/v) were added and the mixture was heated at 63° for 15 min followed by cooling to 20° according to Boedtker (18). Aliquots were layered onto 5 ml of 5 to 20% sucrose gradients. Gradients contained 0.1 M sodium phosphate buffer, pH 7.7, 1.1 M formaldehyde, and 0.2% sodium dodecyl sulfate. Centrifugation was for 4 hours at 45,000 rpm in the SW 50.1 rotor at 20°. The acid-insoluble radioactivity was measured in fractions (0.15 ml) collected from the bottom of the tube. In the experiment presented in B, the reaction was terminated by the addition of 0.06 ml of a solution containing 1.15 M NaOH, 0.3 M EDTA, and 1.85 M NaCl. The entire mixture was layered onto a 5 to 20% alkaline sucrose gradient (containing 0.7 M NaCl, 0.3 M NaOH, and 10⁻⁴ M EDTA) and centrifuged in an SW 50.1 rotor for 185 min at 49,000 rpm at 5°. Fractions were collected directly into Bray's solution from a hole pierced in the bottom of the tube and counted in a Packard Tri-Carb liquid scintillation counter.
Catalase  Alkaline Phosphatase  Aldolase  Ovalbumin

6641

5 10 15 20

Bottom Fraction No.  Top

Fro. 3. Glycerol gradient sedimentation of T3 RNA polymerase. T3 RNA polymerase (Fraction V, 100 units) containing 1 mg per ml of bovine serum albumin was dialyzed for 4 hours against a buffer containing 50 mM Tris-HCl (pH 7.8), 0.1 mM NaCl, 1 mM EDTA, and 5% glycerol. The dialyzed enzyme, mixed with 1 mg of crystalline rabbit muscle aldolase and 45 µg of beef heart catalase in a total volume of 5.2 ml, was layered onto a 10 to 30% linear glycerol gradient (5.2 ml) containing 0.05 M Tris-HCl buffer, pH 7.5, 0.1 mM NaCl, 1 mM dithiothreitol, and 0.1 mM EDTA. Two marker gradients were run in parallel. One contained catalase (45 µg), crystalline muscle aldolase (1 mg) and bacterial alkaline phosphatase (0.5 mg); the other contained DNA polymerase I (2.5 units as defined by Richardson et al. (19)) and muscle aldolase (1 mg). The gradients were centrifuged for 20 hours at 4°C at 50,000 rpm in the SW 50.1 rotor. Fractions of 0.2 ml were collected from each tube and each fraction was assayed for each of the enzyme activities included in the gradient. T3 RNA polymerase was assayed as described under "Methods" while DNA polymerase I was assayed by the method of Richardson et al. (19). Aldolase was assayed by the method of Jagannathan et al. (22); catalase was assayed according to Chance and Maehly (23). Eighty percent of T3 RNA polymerase activity applied to the gradient was recovered. O---O, DNA polymerase I activity; ●—●, T3 polymerase activity.

Characterization of T3 RNA Polymerase Reaction

General Requirements of Reaction—Maximal T3 RNA polymerase activity depends on the presence of all four ribonucleoside triphosphates, Mg++, T3 DNA, and a sulfhydryl reagent (dithiothreitol). Omission of any one of the four ribonucleoside triphosphates, Mg++, or T3 DNA led to no detectable RNA synthesis (<0.2 nmole). As reported previously (1, 2, 4), the T3 RNA polymerase reaction is specific for T3 DNA among a variety of native DNA preparations tested so far (e.g. DNA from calf thymus, E. coli, bacteriophages T2 and T4). When assayed with T7 DNA with 3 to 30 units of T3 RNA polymerase, the rate of RNA synthesis is only 2 to 5% of that obtained with T3 DNA.

pH Optimum and Ion Requirements—The maximal rate of RNA synthesis catalyzed by T3 RNA polymerase occurred at pH 7.8 (Fig. 5). The polymerization reaction was absolutely dependent on the presence of Mg++. At 4, 10, and 30 mM MgCl₂, the reaction rate was 36, 66, and 64%, respectively, of that obtained at 20 mM. MnCl₂ (1 to 5 mM) was inactive when used in place of MgCl₂.

The T3 RNA polymerase reaction was markedly inhibited by monovalent salts above 0.03 M salt concentration (Fig. 6). The reaction was completely inhibited by N-ethylmaleimide or p-hydroxymercuribenzoate (Table II).

Ribonucleoside Triphosphate Requirement—The polymerase reaction exhibited typical Michaelis-Menton kinetics with increasing concentration of UTP in the presence of 0.5 mM of each of the other three ribonucleoside triphosphates (Fig. 7). The apparent Kₘ for UTP was calculated to be 0.75 × 10⁻⁴ M. Similar results were obtained for ATP (apparent Kₘ = 1 × 10⁻⁴ M) and for CTP (apparent Kₘ = 0.8 × 10⁻⁴ M) (data not shown). The effect of GTP concentration on polymerase activity is shown in Fig. 8. It is evident that there is a lag in nucleotide incorporation at low concentration of GTP. Extrapolation of the linear portion of the double reciprocal plot, as indicated in Fig. 8B, to the abscissa, the apparent Kₘ for GTP was calculated to be 1.2 × 10⁻⁴ M. If the nonlinear region...
FIG. 6. Effect of salt concentration on T3 RNA polymerase activity. Varying amounts of salt were added to the standard assay mixture at the indicated concentration. The other conditions of the assay were as described under "Methods." O---O, KCl; △---△, NH₄Cl; ●---●, NaCl; ■---■, (NH₄)₂SO₄.

TABLE II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate (4 × 10⁻⁷ M)</td>
<td>96</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate (8 × 10⁻⁷ M)</td>
<td>28</td>
</tr>
<tr>
<td>p-Hydroxymercuribenzoate (2 × 10⁻⁶ M)</td>
<td>4</td>
</tr>
<tr>
<td>N-Ethylmaleimide (2 × 10⁻⁶ M)</td>
<td>65</td>
</tr>
<tr>
<td>N-Ethylmaleimide (10⁻⁵ M)</td>
<td>8</td>
</tr>
</tbody>
</table>

of the data presented in Fig. 8B, obtained at low concentration of GTP, was plotted as 1/V versus 1/S, a linear curve was obtained (Fig. 8C). It is known from previous studies (25) that the first two nucleotides at the 5' end of RNA chains synthesized with T3 RNA polymerase are both guanine. Thus, the sequence at the 5' end of RNA chains is 5'pppGpGp... If the rate-limiting step in RNA synthesis is the formation of the first phosphodiester bond (initiation reaction) through a bimolecular reaction between 2 GTP molecules, the rate of RNA synthesis will be second order with respect to GTP concentration. Thus a plot of 1/V versus 1/S will give a linear curve at low concentrations of GTP. With other nucleoside triphosphates which are only involved in sequential elongation of nucleotides to the 3' end of the nascent RNA chain, the rate of RNA synthesis will be expected to be a first order reaction with respect to substrate concentration. Thus for these three triphosphates 1/V versus 1/S should give a straight line. This is what was obtained for UTP, CTP, and ATP (data for UTP are shown in Fig. 7B).

Pyrophosphate Exchange Reaction—T3 RNA polymerase catalyzed the incorporation of 32PP into ribonucleoside triphosphates. The pyrophosphate exchange reaction was dependent on the presence of ribonucleoside triphosphates, Mg²⁺, and T3 DNA (Table III). The exchange was abolished by DNase and unaffected by RNase. No detectable 32PP incorporation occurred in the absence of T3 DNA or its replacement with either T2 DNA or calf thymus DNA. With T7 DNA, however, a slower rate of exchange reaction relative to T3 DNA occurred. Thus the specificity for template DNA for the pyrophosphate exchange reaction is similar to that for the polymerization reaction catalyzed by T3 RNA polymerase. The ribonucleoside triphosphate requirements for pyrophosphate exchange reactions are shown in Table IV. It is evident that GTP is absolutely required for the 32PP exchange reaction. This result is in keeping with the observations reported previously from this laboratory that in the T3 RNA polymerase reaction, RNA chains are initiated exclusively with GTP (2). It should be noted (Table IV) that GTP alone can support the 32PP exchange reaction. The rate of incorporation of 32PP into ribonucleoside triphosphates did not substantially increase if in addition to GTP, any one of the other three ribonucleoside triphosphates either alone or in combination was added to the polymerase reaction.

The product of the exchange reaction was identified as nucleoside triphosphates by Dowex 1 (Cl⁻) chromatography. With GTP as the sole nucleotide in the reaction mixture, all incorporated 32P cochromatographed with GTP (Table V, Experiment A). In the presence of all four ribonucleoside triphosphates, the incorporated 32PP was found in regions corresponding to all four nucleoside triphosphates (Table V, Experiment B).
FIG. 8. Effect of GTP concentration on T3 RNA polymerase activity. Reaction mixtures (0.25 ml) containing 6 units of T3 RNA polymerase were prepared as described under "Methods" except that the concentration of GTP was varied as indicated in the figure while the concentrations of the three other triphosphates were kept at 0.5 mM. Incubation was for 15 min at 37°. The reaction was terminated and the acid-insoluble radioactivity was determined as described under "Methods." A, direct plot of rate of [3H]UMP incorporated in nanomoles per 15 min (V) versus micromolar GTP concentration (S). B, double reciprocal plot of 1/V versus 1/S. C, plot of 1/V versus 1/S².

Pyrophosphorolysis of RNA—It has previously been shown that in the T3 RNA polymerase reaction, RNA chains formed with native T3 DNA were released free of template DNA as free RNA (2), while with single-stranded DNA, e.g. with φX-174 DNA, RNA chains were isolated as DNA-RNA hybrids (6). Incubation of 32P-labeled T3 RNA polymerase products synthesized with either native T3 DNA or φX-174 DNA, with 500 mM PPi, and 40 units of T3 RNA polymerase for 1 hour did not lead to any detectable acid-soluble radioactivity. The amount of RNA used in these experiments was 10 nmole of nucleotides (containing 1 x 10⁶ cpm) and was isolated by Sephadex gel filtration of 32P-labeled T3 polymerase products as described under "Methods." Hence DNA template was also present in the isolated RNA product used in the pyrophosphorolysis assays. The other conditions of the assay were the same as those described under "Methods." The DNA present was also in the isolated RNA products used in the pyrophosphorolysis assays. Under "Methods," DNA was also shown to be present in the isolated RNA product used in the pyrophosphorolysis assays. The DNA and RNA were isolated by C. plot of 1/V versus 1/S².

Effect of E. coli RNA Chain Termination Factor 1 on T3 RNA Polymerase Reaction

Two mechanisms of termination of in vitro RNA synthesis have been distinguished in the transcription of DNA catalyzed by E. coli RNA polymerase. At high ionic strength (>0.15 M KCl), termination of in vitro RNA synthesis occurs with the reaction: A

**TABLE III**

**Requirements for exchange reaction between 32PPi and nucleoside triphosphates**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Norit adsorbable #P (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complete system</td>
<td>0.42</td>
</tr>
<tr>
<td>2. Complete system, omit enzyme</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>3. Complete system, omit Mg⁺⁺</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>4. Complete system, omit T3 DNA</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>5. Complete system, omit T3 DNA, add T2 DNA or calf thymus DNA</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>6. Complete system, omit T3 DNA, add T7 DNA</td>
<td>0.13</td>
</tr>
<tr>
<td>7. Complete system + DNase (10 μg)</td>
<td>0.02</td>
</tr>
<tr>
<td>8. Complete system + RNase (10 μg)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

**TABLE IV**

**Influence of nucleoside triphosphates on the 32PPi exchange reaction**

Reaction mixtures (0.2 ml) were as described under "Assay for Pyrophosphate Exchange" except that addition of ribonucleoside triphosphates was as indicated in the table. Incubation was at 37° for 30 min with 12 units of T3 polymerase. The conversion of 32PPi into Norit-adsorbable form was measured as described under "Methods."

<table>
<thead>
<tr>
<th>Additions</th>
<th>Norit-adsorbable #P (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP + UTP + GTP + CTP</td>
<td>1.4</td>
</tr>
<tr>
<td>ATP + UTP + CTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ATP + GTP + CTP</td>
<td>1.3</td>
</tr>
<tr>
<td>UTP + GTP + ATP</td>
<td>1.4</td>
</tr>
<tr>
<td>GTP + UTP + CTP</td>
<td>1.5</td>
</tr>
<tr>
<td>ATP + GTP</td>
<td>1.4</td>
</tr>
<tr>
<td>GTP + CTP</td>
<td>1.3</td>
</tr>
<tr>
<td>GTP + UTP</td>
<td>1.5</td>
</tr>
<tr>
<td>GTP alone</td>
<td>1.2</td>
</tr>
<tr>
<td>ATP or UTP or CTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(ATP + CTP) or (ATP + UTP) or (CTP + UTP)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Identification of products of \(^{32}P\)P, exchange reaction

Two reaction mixtures were prepared; one contained 20 nmoles of GTP as the only ribonucleoside triphosphate, and the other contained 20 nmoles of each of the four ribonucleoside triphosphates. The other conditions of the two reaction mixtures were as described under "Methods." The specific activity of \(^{32}P\)P was \(10^7\) cpm per nmole and 12 units of enzyme were used. After incubation at 37°C for 30 min, the reaction was terminated and the nucleotides were adsorbed to Norit as described under "Methods." The Norit was washed three times by suspending it in 2 ml of 0.01 M HCl, pH 6.0, and the supernatant fluid was removed each time by low speed centrifugation. The nucleotides were then eluted from Norit with 1 \(\times\) NH\(_3\) in 50% ethanol solution. The eluate was evaporated to dryness and suspended in a small volume of H\(_2\)O. After adding ATP, UTP, CTP, and GTP (2 \(\mu\)mole of each) to both reaction mixtures as carrier, each solution was applied to a column of Dowex 1 (Cl\(^{-}\)) (1 \(\times\) 10 cm, 100 to 200 mesh, 2% cross-linked) which had been extensively washed with water. Each column was sequentially developed with 100 ml of each of the following reagents in the following order: (a) 10 mM HCl; (b) 10 mM HCl + 40 mM LiCl; (c) 10 mM HCl + 80 mM LiCl; (d) 10 mM HCl + 0.12 mM LiCl; (e) 10 mM HCl + 0.15 mM LiCl. Fractions of 10 ml were collected. The elution profiles of the nucleotides were determined by measuring the absorbance of the eluates at 260 and 280 nm. The above separation procedure completely resolved CTP, ATP, UTP, and GTP which were eluted sequentially in this order from the column. The fractions were analyzed for \(^{32}P\). The total \(^{32}P\) radioactivity in each of the nucleoside triphosphate region was calculated. The recovery of completely resolved CTP, ATP, GTP, and UTP which were eluted by the addition of \(^{32}P\)P to both reaction mixtures was greater than 90%.

<table>
<thead>
<tr>
<th>Region of chromatogram</th>
<th>Experiment A: with GTP alone</th>
<th>Experiment B: with all four triphosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP</td>
<td>&lt;100</td>
<td>23,200</td>
</tr>
<tr>
<td>ATP</td>
<td>&lt;100</td>
<td>36,800</td>
</tr>
<tr>
<td>GTP</td>
<td>120,400</td>
<td>32,600</td>
</tr>
<tr>
<td>UTP</td>
<td>&lt;100</td>
<td>28,800</td>
</tr>
</tbody>
</table>

lease of RNA chains from DNA. Polymerase is also released in this process and, acting catalytically, reinitiates new RNA chains (26-28). At low ionic strength (<0.1 M KCl), however, the presence of a protein factor, \(p\), is necessary to bring about termination and release of RNA chains (14). Polymerase is not released and reinitiation of RNA chains does not occur (14, 28, 29). The over-all effect of \(p\) factor on transcription of DNA catalyzed by \(E. coli\) RNA polymerase can be summarized as follows. (a) Addition of \(p\) factor to an \(E. coli\) RNA polymerase reaction depresses RNA synthesis without affecting the initial rate of RNA synthesis. (b) RNA chains formed in the presence of \(p\) factor are smaller and more homogeneous in size than are those formed in its absence. It was of interest to investigate whether \(p\) factor affected in vitro RNA synthesis catalyzed by \(T3\) RNA polymerase.

As shown in Table VI, \(p\) factor had no effect on either the rate or extent of RNA synthesis catalyzed by \(T3\) RNA polymerase. The concentration of \(p\) factor used in this experiment was sufficient to cause over 50% depression of the rate of nucleotide incorporation catalyzed by \(E. coli\) RNA polymerase. Variation of the concentration of \(p\) factor in a \(T3\) RNA polymerase reaction over a 20-fold range (1 to 20 \(\mu\)g) also had no effect on RNA synthesis catalyzed by \(T3\) RNA polymerase (data not shown). In addition, the presence of a large excess of \(T3\) RNA polymerase had no effect on the \(p\)-mediated inhibition of (\(T4\) DNA-directed) RNA synthesis by \(E. coli\) RNA polymerase (Table VII). In these experiments, \(p\) factor was first incubated with \(T3\) RNA polymerase in the absence of nucleoside triphosphates, and the reaction was initiated by addition of \(E. coli\) RNA polymerase and nucleoside triphosphates. Since \(T3\) RNA polymerase is specific for \(T3\) DNA and does not copy \(T4\) DNA, nucleotide incorporation under these conditions is solely a measure of the \(E. coli\) RNA polymerase activity. As shown in Table VII, the extent of inhibition of the \(E. coli\) RNA polymerase activity by \(p\) factor was identical whether or not \(p\) factor was preincubated with \(T3\) RNA polymerase.

The effect of \(p\) factor on the size of RNA produced by either \(E. coli\) or \(T3\) RNA polymerase was studied (Fig. 9). In agree-
ment with results obtained with T4, T7, and λ DNA templates (14, 28). ρ factor decreased the average size of RNA transcribed from T3 DNA by E. coli RNA polymerase from a molecular weight $2.2 \times 10^6$ to approximately $6 \times 10^4$ (Fig. 9A). In contrast, ρ factor had no effect on the average size of RNA formed from T3 DNA template transcribed by T3 RNA polymerase (Fig. 9B). Similar results were obtained with T7-induced RNA polymerase by Goldberg and Hurwitz (30).

**DISCUSSION**

The present communication extends previous studies on the T3-induced RNA polymerase (1, 2). In this paper, we have reported studies on the detailed purification procedure and physical and chemical characterization of T3 RNA polymerase as well as studies on requirements of the polymerase reaction.

The final enzyme preparation examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis displayed a single protein band. The enzyme preparation has been examined for nuclease contamination and, within the limitations of the assays employed, appears to be free of RNase and DNase activities. Many other known enzyme activities involving nucleoside triphosphates were also absent.

The properties of T3 RNA polymerase resemble RNA polymerase isolated from E. coli as well as other sources. All have absolute requirements for a DNA template, a divalent cation, and all four nucleoside triphosphates. All catalyze PPi exchange reactions with nucleoside triphosphates. Where studied, evidence has been obtained that RNA products contain a nucleoside triphosphate at the initiating 5' terminus (31–34). However, T3 RNA polymerase shows very little RNA synthesis activity; with 3 to 5,000 units of enzyme, T3 RNA polymerase is at best 5% as active on T7 DNA as on T3 DNA. This template specificity distinguishes T3 RNA polymerase from T7 RNA polymerase described by Chamberlin et al. (3). In contrast, in the DNA-dependent PPi exchange reaction with nucleoside triphosphates catalyzed by T3 RNA polymerase, T7 DNA was nearly 30% as active as T3 DNA (Table III).

A comparison of the biochemical properties of T7 and T3 RNA polymerases (1–4) shows that the two polymerases behave similarly. Both T3 and T7 RNA polymerases are single polypeptide chains of molecular weight approximately 165,000 ± 5,000. Both enzymes have similar requirements for RNA synthesis. Both enzymes are insensitive to rifampicin but sensitive to salt concentrations above 0.03 M. Both enzymes, in addition to transcribing the homologous DNA, work efficiently with the homopolymer pair poly(dG·dC) to make poly(G) exclusively. This result suggests that promoter sites recognized by these two polymerases may be GC-rich regions of the DNA.

However, the T3 RNA polymerase appears to have a more restricted initiation site, since this enzyme will only use native T3 DNA and is virtually inactive with T7 DNA. The T7 RNA polymerase, however, can utilize native DNA from either T3 or T7 as template (4).

T3 RNA polymerase catalyzes a T3 DNA-dependent PPi exchange reaction with nucleoside triphosphates. The requirements for the PPi exchange reaction were found to be the same as that observed for RNA synthesis except that the PPi exchange reaction can occur with GTP alone. In the absence of GTP, the exchange of PPi with any one of the other three ribonucleoside triphosphates ATP, UTP, and CTP, present either alone or in combination, does not occur. This result is in keeping with observations reported previously from this laboratory that RNA chain initiation by T3 RNA polymerase proceeds exclusively by the incorporation of an intact GTP to form the 5' terminus of the RNA (2). The second nucleotide incorporated by forming a phosphodiester bond with the initiating GTP has also been identified to be guanine. Thus the sequence at the 5'-triphosphate end of RNA chains is pppGpGp . . . (25).
observation that the PP1 exchange reaction can occur with GTP alone is most readily explained as a consequence of a polymerization step to form pppGpG followed by pyrophosphorylation of the newly formed phosphodiester bond.

Results presented in this paper also demonstrate that transcription of T3 DNA by T3 RNA polymerase is unaffected by the E. coli RNA chain termination factor, p. It has been shown with E. coli RNA polymerase that the presence of p factor is necessary to bring about termination and release of RNA chains (14). Addition of p factor to an E. coli RNA polymerase reaction mixture causes inhibition of RNA synthesis without affecting the initial rate of RNA synthesis. p appears to interact with E. coli RNA polymerase at specific DNA sites leading to the formation of RNA chains of well defined length (30). The addition of p factor to T3 RNA polymerase reaction, on the other hand, is without effect on either the rate, yield, or size of RNA produced. Similar results have been obtained by Dunn et al. (35).

The inability of p factor to affect transcription by T3 RNA polymerase may be due to the absence of signals for p-mediated termination in the regions of the T3 genome which are transcribed by T3 RNA polymerase. Alternatively, and perhaps more likely, p may be specific for E. coli RNA polymerase and be unable to interact with T3 polymerase under any condition.

Termination of in vitro RNA synthesis has been shown to occur in the T3 RNA polymerase reaction with the release and reinitiation of RNA chains from the T3 DNA template (2). If RNA produced in vitro by T3 RNA polymerase is found to terminate improperly when compared to in vivo RNA formed in T3-infected cells, we must consider the possibility that there exists a phage-coded termination factor specific for T3 RNA polymerase.

Finally, we have calculated that with native T3 DNA template and under the condition of the assay employed for T3 RNA polymerase in this paper, the rate of RNA synthesis per mole of T3 RNA polymerase is approximately 10 times faster than that obtained per mole of E. coli RNA polymerase. The above calculation indicates a higher degree of efficiency of catalytic function for T3 RNA polymerase than for E. coli RNA polymerase.

The biological role of T3 RNA polymerase is clear. RNA products made in vitro by purified T3 RNA polymerase have been shown to contain all of the sequences present in "late" mRNA isolated from T3-infected cells (5, 35). Thus the enzyme is responsible for transcription of late mRNA during the life cycle of bacteriophage T3.

REFERENCES

Studies on T3-induced Ribonucleic Acid Polymerase: III. PURIFICATION AND CHARACTERIZATION OF THE T3-INDUCED RIBONUCLEIC ACID POLYMERASE FROM BACTERIOPHAGE T3-INFECTED ESCHERICHIA COLI CELLS

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