PURIFICATION AND CHARACTERIZATION OF DNA-DEPENDENT RNA POLYMERASE FROM PSEUDOMONAS PUTIDA A.3.12

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JAMES CARL JOHNSON
1967

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF DNA-DEPENDENT RNA POLYMERASE FROM *PSEUDOMONAS PUTIDA* A.3.12

by James Carl Johnson

The purification of DNA-dependent RNA polymerase has been described. The enzyme has been purified to a specific activity of 5,590 mμ moles of CTP converted to a TCA insoluble form per hour per mg of protein. The increase in specific activity was 160 fold over that of the Initial Extract and the recovery of activity was 20%. The enzyme could be stored at -196°C for several months without detectable losses of activity.

The synthesis of RNA by RNA polymerase from *P. putida* was absolutely DNA dependent. The reaction exhibited a broad pH optimum between 8 and 9. RNA synthesis did not occur below pH 7. No RNA synthesis was observed in the absence of divalent cations. Mg^{++} and/or Mn^{++} stimulate the synthesis of RNA and maximum stimulation was observed when both cations were present. When assayed at either high substrate concentrations or high DNA concentrations, an inhibition of RNA synthesis was observed. When assayed at temperatures below 30°C, the synthesis of RNA was observed to have a lag phase which was diminished by preincubating the enzyme with DNA.

James Carl Johnson

A new and unique assay for the pyrophosphate formed by the catalytic action of RNA polymerase during RNA synthesis was developed. The pyrophosphate linked formation of NADPH was observed in a coupled reaction which involved the enzymes uridinediphosphoglucose pyrophosphorylase, phosphoglucomutase and glucose-1-phosphate dehydrogenase. It was established that the formation of pyrophosphate as observed by this assay was catalyzed by DNA-dependent RNA polymerase.

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By

James Carl Johnson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1967

DEDICATION

To Phyl

ACKNOWLEDGMENTS

The author wishes to express his words of gratitude and recognition of Dr. John A. Boezi whose guidance and counsel have been invaluable during the course of this study.

I wish to thank Dr. R. G. Hansen and Sam Bass for the gift of the UDPG-pyrophosphorylase and the advice on its use.

Words of thanks are also given to Dr. Lucy F. Lee, Dr. R. L. Armstrong, Ken Payne and Gary Gerard for helpful discussions and to Mrs. M. DeBacker for technical assistance.

I also wish to recognize the Department of Biochemistry of Michigan State University and the National Institutes of Health from which financial support was received.

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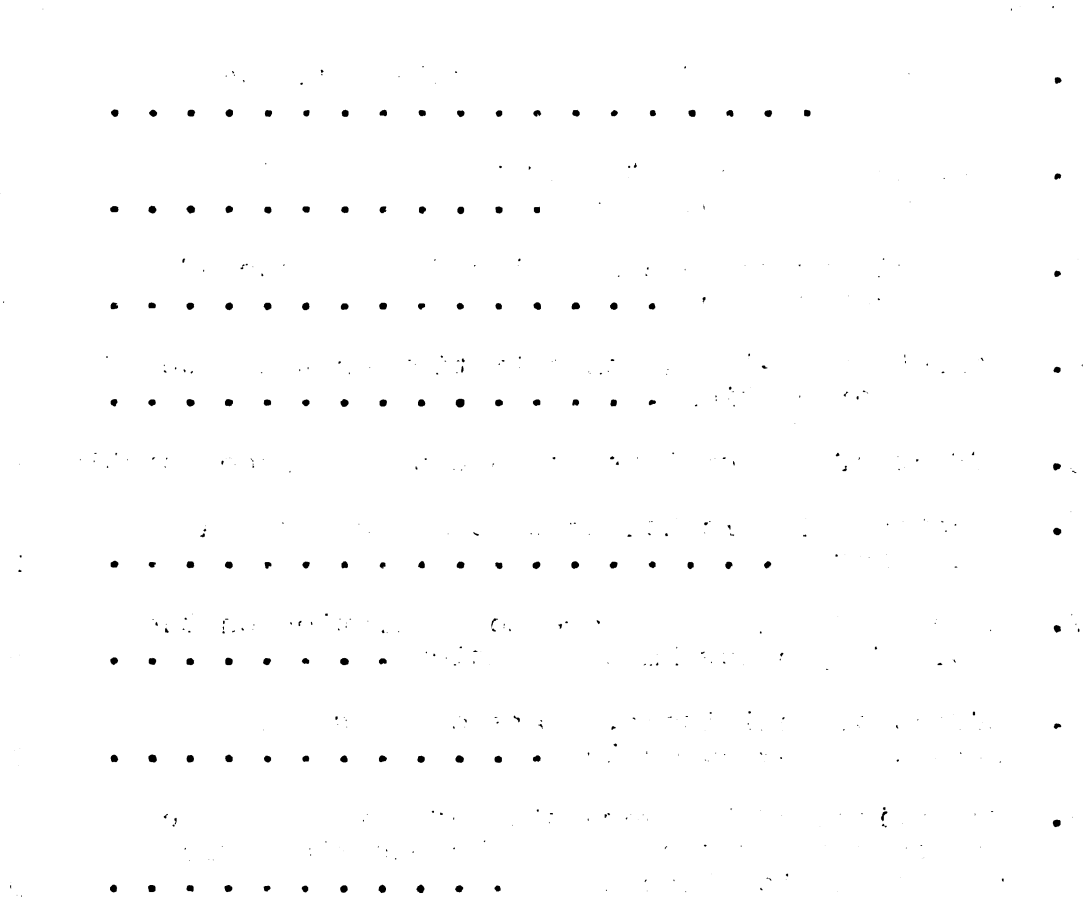

INTRODUCTION

Bacterial RNA synthesis has been one of the general areas of interest in this laboratory. An avenue of approach to the investigation of RNA metabolism is the study of the enzymes which are responsible for the synthesis and modification of RNA. An important enzyme which catalyzes the incorporation of nucleoside monophosphates into RNA is DNA-dependent RNA polymerase.

$$\left.\begin{matrix}\text{ATP}\\ \text{GTP}\\ \text{CTP}\\ \text{UTP}\end{matrix}\right\} \xrightarrow[\text{DNA Template}]{Mg^{++} \text{ and/or } Mn^{++}} \text{RNA} + PP_1$$

The present study presents the results of the purification and characterization of the partially purified DNA-dependent RNA polymerase from P. putida A.3.12. The results presented here do not represent a complete study of the most highly purified enzyme possible, but are only a partial characterization of the enzyme at the level of purification achieved to this time. A more complete study of the physical and chemical characteristics of the enzyme awaits further purification.

DNA-dependent RNA polymerase has been purified from many sources (see Elson, 1965). The most extensive purifications have been performed with extracts of Micrococcus lysodeiktus and Escherichia coli (Chamberlin and Berg, 1962;

Furth, Hurwitz and Anders, 1962; Nakamoto, Fox and Weiss, 1964; Stevens and Henry, 1964; Zillig, Fuchs and Millette, 1966). The specific activity of the most highly purified fraction from bacterial sources has reached 30 μ moles of nucleotide polymerized per hour per mg of protein.

RNA synthesis occurs in five steps:

(1) attachment of the enzyme to specific sites on the DNA template

(2) initiation

(3) polymerization

(4) termination of RNA synthesis at specific sites

(5) release of nacent RNA and enzyme from the template

The results obtained from studies performed with the bacterial enzymes have brought about some understanding of the first three steps. With regard to steps 1, 2 and 3, the following sequences of reactions can be written:

(1) attachment

$$\text{DNA} + \text{enzyme} \rightleftharpoons [\text{DNA–enzyme}]$$

The binding of RNA polymerase to DNA is reversible. Binding occurs at specific sites on the DNA duplex. For coliphage T_7 DNA there are approximately 50 binding sites for the E. coli RNA polymerase. The binding of the enzyme to DNA which occurs at low ionic strengths is inhibited at high ionic strengths (Berg, Kornberg, Pancher, and Dieckmann, 1965; Crawford, Crawford, Richardson and Slayter, 1965; Jones and Berg, 1966; Richardson, 1966; Steinberger and Stevens, 1966; Stead and Jones, 1967).

(2) initiation

$$[\text{DNA-enzyme}] + \text{purine nucleoside triphosphate} \longrightarrow [\text{DNA-enzyme-pppPu}]$$

The DNA-enzyme complex reacts preferentially with purine nucleoside triphosphates to form a complex which is stable in solutions of high ionic strength. The exact nature of the complex has not been established. (Anthony, Zeszotek and Goldthwait, 1966).

(3) polymerization

$$[\text{DNA-enzyme-pppPu}] + \text{NTP} \longrightarrow \text{DNA-enzyme-pppPu(pN)n} + PP_i$$

where NTP stands for ATP, GTP, CTP or UTP.

Polymerization occurs by the sequential addition of one nucleoside monophosphate to the 3′ end of the growing RNA chain (Bremer, Konrad, Gaines and Stent, 1965; Maitra and Hurwitz, 1965). The sequence of ribonucleotides incorporated into RNA is determined by the DNA strand which is transcribed. Transcription of DNA <u>in vitro</u> with several DNA species has been shown to be asymetric. With bacteriophage α DNA and with the duplex derivative of ϕX 174 DNA, asymetric transcription involves transcription of one strand exclusively. The strand that is copied <u>in vitro</u> is the same strand that is copied <u>in vivo</u> (Tocchini-Valentini, Stodolsky, Aurisicchio, Sarnat, Graziosi, Weiss and Geiduschek, 1963; Hayashi, Hayashi and Spiegelman, 1963). With bacteriophage λ DNA, transcription is also asymetric; however, portions of each

complementary strand are copied *in vitro* and *in vivo* (Cohen and Hurwitz, 1967; Taylor, Hradecna, and Szybalski, 1967).

With bacteriophage λ DNA as a template for the *E. coli* enzyme, transcription is further limited to the AT-rich half of the DNA duplex. *In vivo*, the AT-rich half programs the synthesis of early messenger RNA (Nano and Gros, 1966; Cohen, Maitra and Hurwitz, 1967). Transcription of T_4 DNA is also limited to the portion of the molecule which *in vivo* programs the synthesis of early messenger RNA (Geiduschek, Snyder, Cowill and Sarnat, 1966).

In vitro studies with the highly purified bacterial enzyme have not led to an understanding of the mechanism involved in steps 4 and 5. RNA, synthesized *in vitro* on T_4 or T_7 DNA templates by the *E. coli* RNA polymerase, remains attached to the template and enzyme (Bremer and Konrad, 1964; Richardson, 1966). Few, if any, RNA molecules are released from the complex during the course of the reaction. The relatively rapid decrease in the rate of RNA synthesis has been attributed to an interaction between the nascent RNA and the enzyme. Stent (1964) has suggested that ribosomes are involved in an active process by which nascent RNA is released from the DNA-enzyme-RNA complex.

The most highly purified preparations of RNA polymerase have been characterized with respect to the physical properties of the enzyme. The *E. coli* polymerase has a sedimentation coefficient of 21 when measured in buffers of low ionic

strength and 13 when measured at high ionic strengths (Richardson, 1966). The molecular weight of the 21 S species was estimated to be 8.8×10^5 and that of the 13 S species to be 4.4×10^5. However, in buffers of intermediate ionic strength, a variety of forms exist with sedimentation coefficients of 14, 16 and 19.5 S (Stevens, Emery, and Sternberger, 1966). Electron micrographs of the E. coli RNA polymerase have resulted in a model showing the enzyme to consist of six cylindrical subunits surrounding a hollow core in a hexagonal array forming a short hollow cylinder with dimensions of 125 Å in diameter and 95 Å in length (Zillig, Fuchs and Millette, 1966).

MATERIALS AND METHODS

Growth of Pseudomonas putida A.3.12

DNA-dependent RNA polymerase was extracted from Pseudomonas putida A.3.12 which was grown in a Fermacell, model F-130, 130 liter batch fermentor. The growth media contained the following in grams per liter: yeast extract, 5; glucose, 8; NaCl, 8; $(NH_4)_2HPO_4$, 6; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 1; and $FeCl_3$, 0.005. A 6 to 8 liter innoculum of an overnight culture was added to 100 liters of sterile media. The temperature of growth was 33°C. The areation rate was 6 to 8 cubic feet per minute, and stirring was maintained at 300 rev/min. Cell density was measured at 660 mμ. An optical density of 1.5 units/ml was equivalent to a cell density of 5×10^8 cells/ml. The doubling time for P. putida A.3.12 grown under these conditions was 45 minutes. When an optical density of the culture reached 3.0 units/ml, it was centrifuged in a Sharples continuous flow centrifuge, type A.3-12. The yield was 400 to 450 g wet weight of packed cells. After harvesting, the cells were stored at -20°C. For periods of 10 months no decrease in RNA polymerase activity was observed.

Growth and Purification of Bacteriophage gh-1

Bacteriophage gh-1 was grown on a 6 to 8 liter culture of P. putida A.3.12 in a Microferm Laboratory Fermentor. Growth and purification of the bacteriophage were performed according to the procedure of Lee and Boezi (1966). When the

cell density of the exponentially growing culture reached 5×10^8 cells/ml, bacteriophage gh-1, at a multiplicity of 5, was added. After 2 hours, the lysed culture was centrifuged at 4,000 x g for 10 minutes to remove cell debris followed by centrifugation at 16,000 x g for 2 hours to collect the bacteriophage. Following suspension of the viral pellet in tris (hydroxymethyl) aminomethane (Tris) adjusted to pH 8.0 with HCl, containing 0.2 M NaCl, the suspension was passed through a diethylaminoethyl (DEAE) cellulose column (3 by 20 cm) equilibrated with the same buffer. The fractions containing gh-1 were concentrated by centrifugation at 16,000 x g for 2 hours, and the resulting pellets were suspended in the buffer described above. Purified gh-1 was stored at 4°C.

Growth and Purification of Bacteriophage T_4

Bacteriophage T_4 was grown on E. coli B in Fernback flasks containing basal C medium (Roberts, Abelson, Cowie, Bolton and Britten, 1957) with 0.4% glucose. When the cell density of an exponentially growing culture reached 5×10^8 cells/ml, (an optical density of 1.0 unit/ml was equivalent to a cell density of 5×10^8 cells/ml) bacteriophage T_4 was added at a multiplicity of 3 to 6. Simultaneously, L-tryptophan was added to 20 µg/ml. Following lysis of the culture, the bacteriophage were purified by a series of differential centrifugations at 4,000 x g for 10 minutes and 16,000 x g for 2 hours. After each high speed centrifugation

the viral pellets were suspended in 0.01 M Tris-HCl, pH 7.4 containing 0.2 M NaCl. Purified T_4 were stored at 4°C.

Growth and Purification of $[^{14}C]$ Thymidine Labeled Bacteriophage T_7

Bacteriophage T_7 labeled with $[^{14}C]$ thymidine was a gift of Dr. Lucy F. Lee. It was grown on a thymidine requiring mutant of E. coli B, purified by differential centrifugation and stored at 4°C.

Purification of Bacteriophage DNA

Bacteriophage deoxyribonucleic acid (DNA) was purified according to the method of Abelson and Thomas (1966). The purified phage preparation was adjusted to a concentration having an absorbancy at 260 mμ of 8 to 20 units/ml. An equal volume of freshly distilled, water saturated phenol was added, and the mixture was rolled at 60 rev/min for 30 minutes at room temperature. Following centrifugation at 3,000 x g for 10 minutes, the phenol layer and material from the interface were removed from the aqueous layer with a Pasteur pipet. An equal volume of phenol was added and the aqueous layer was extracted an additional 30 minutes. Following centrifugation at 3,000 x g for 10 minutes, the aqueous layer was transferred to a dialysis sac for dialysis overnight against 5 liters of 0.01 M Tris, pH 8.0, containing 0.1 M NaCl. In the morning the dialysis sac was transferred to 5 liters of fresh buffer solution for an additional 24

hours of dialysis. The DNA obtained by this method was stored at 4°C. The specific activity of [^{14}C] thymidine labeled T_7 DNA was 6,500 counts/min per μg.

Purification of P. putida A.3.12 DNA

The DNA from P. putida A.3.12 was purified according to the procedure of Thomas, Berns and Kelley (1966). Frozen cells (1 g) were suspended in 50 ml of standard saline-citrate (SSC: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). Sucrose was dissolved in the suspension to 27% (w/v). A solution of pronase (10 mg/ml) was prepared by dissolving the lylophilized powder in 0.01 M sodium acetate buffer which had been adjusted to a pH of 5.0 with acetic acid. Both the pronase solution and cell suspension were incubated at 75°C for 10 minutes, followed by cooling to room temperature. The pronase solution was added to the cell suspension to a concentration of 1 mg/ml, followed by sodium dodecyl sulfate to 1% (w/v). The suspension was incubated at 37°C for 3 hours after which an additional 0.5 mg/ml of pronase solution was added and the suspension further incubated at 37°C for 3 hours. An equal volume of distilled, water saturated phenol was added, and the mixture was rolled at 60 rev/min for 30 minutes at room temperature. Following centrifugation at 3,000 x g for 10 minutes, the phenol layer and material from the interface were removed from the aqueous layer with a Pasteur pipet. The aqueous layer was transferred to a dialysis sac and dialyzed overnight against 0.01 M Tris,

pH 8.0, containing 0.1 M NaCl. Pancreatic ribonuclease (RNase) which was prepared by boiling a solution (1 mg/ml) for 10 minutes was added to the dialyzate to a concentration of 50 μg/ml. The mixture was incubated at 37°C for 2 hours. Following two additional phenol extractions of the aqueous phase using the techniques described above, the DNA was precipitated with 2 volumes of cold ethanol and collected on a glass rod. The precipitate was dissolved in 0.01 M Tris-HCl, pH 8.0 to which had been added 0.3 M sodium acetate. Isopropanol was added to this solution to 0.54 volume with continuous stirring. The DNA was collected on a glass rod and dissolved in 0.01 M Tris-HCl, pH 8.0, containing 0.1 M NaCl. Fibers of DNA which were not collected on the glass rod were collected by centrifugation at 3,000 x g for 10 minutes and dissolved in the buffer solution containing the precipitate from the glass rod. The solution was dialyzed overnight against 5 liters of the same buffer.

The yield of DNA from 1 g of frozen cells was 1.4 mg. Its ratio of absorbance at 260 mμ to that at 280 mμ was 1.55. The solution of DNA was stored at 4°C.

Preparation of [^{3}H] Uracil Labeled RNA

[^{3}H] uracil labeled ribonucleic acid (RNA) with a specific activity of 478 counts/min per μg was a gift of Dr. Robert L. Armstrong. It had been extracted and purified from *E. coli* B using the SDS-phenol method (Armstrong, 1966) and was stored at -20°C.

Determination of DNA, RNA and Protein Concentrations

DNA and RNA concentrations were calculated from the absorbancy at 260 mμ by use of an extinction coefficient of 20 cm^2/mg. Protein concentrations were determined by the method of Lowry (1951) using bovine serum albumen as a standard or was calculated from the absorbancy values at 280 and 260 mμ using the formula of Layne (1957):

$$\text{Protein concentration (mg/ml)} = 1.55\ A^{280} - 0.76\ A^{260}$$

Procedure for the Measurement of Radioactivity

TCA Insoluble Radioactivity. The sample was mixed with 5 ml of cold 10% (w/v) trichloroacetic acid (TCA) and 250 μg of carrier salmon sperm DNA. The precipitate that formed in 15 minutes at 0 to 4°C was collected by filtering the sample, using gentle suction applied by a water aspirator, through a nitrocellulose membrane filter. Each sample tube and filter was washed with three, 5 ml portions of cold 10% TCA. The filter was blotted free of excess liquid, placed in a liquid scintillation vial, and dried in a 95°C oven. After the filter had cooled to room temperature, 5 ml of a fluor containing 0.1 g of 1,4-bis- 2-(5-phenyloxazolyl) - benzene (POPOP) and 4.0 of 2,-5 diphenyloxazole (PPO) per liter of tolune was added. The sample was counted in a Packard Tri Carb, model 3003, liquid scintillation spectrometer with gain and window discriminator settings for the radioisotopes as follows: $[^3H]$ gain 58%, window discriminator 50-1000; $[^{14}C]$ gain 16%, window discriminator 50-1000.

Duplicate counts each of 10 minutes were taken for the sample and the numerical average calculated.

TCA Soluble Radioactivity. The sample was mixed with 0.5 ml of cold 10% (w/v) TCA and 250 µg carrier salmon sperm DNA. The precipitate that formed in 15 minutes at 0 to 4°C was removed by filtration through a nitrocellulose membrane filter. The filtrate was collected and duplicate 0.1 ml samples were transferred to liquid scintillation vials containing 5 ml of a fluor composed of 60 g naphtalene, 4.0 g PPO, 0.2 g POPOP, 100 ml MeOH and 20 ml ethylene glycol per liter of p-dioxane. The sample was counted as described in A above.

Radioactive Assay of RNA Polymerase

The radioactive assay measured the conversion of [^{3}H] CTP into a TCA insoluble form. The reaction mixture contained 20 µ moles Tris-HCl, pH 8.0, 2.0 µ moles $MgCl_2$; 0.5 µ mole $MnCl_2$; 1.5 µ mole 2-mercaptoethanol; 0.005 µ mole EDTA; 0-195 µg DNA from various sources; 0.215 µ mole each of ATP, GTP, UTP and [^{3}H] CTP; and 0-36 units of RNA polymerase in 0.5 ml total volume. [^{3}H] CTP had a specific activity of 1140 counts/min per mµ mole. During the incubation of the reaction mixture, 0.1 ml samples were withdrawn and 5 ml of cold 10% TCA were added to stop the synthesis of RNA. One unit of enzyme activity corresponds to the amount of enzyme necessary to incorporate 1 mµ mole of CMP per hour under the conditions described above using gh-1 DNA as the template.

The specific activity of the RNA polymerase is the number of units per mg of protein.

Spectrophotometric Assay of RNA Polymerase

The assay employed for determining the formation of pyrophosphate by RNA polymerase measured the formation of NADPH. NADPH synthesis was coupled to pyrophosphate formation through the enzymatic reactions illustrated below. (Reactions 1-4).

RNA polymerase catalyzes the synthesis of RNA and pyrophosphate (Reaction 1). For each mole of ribonucleoside

$$\begin{matrix}\text{ATP}\\+\\\text{GTP}\\+\\\text{CTP}\\+\\\text{UTP}\end{matrix} \xrightarrow[\text{RNA Polymerase}]{\text{DNA}} \text{RNA} + PP_i \qquad (1)$$

triphosphate incorporated into RNA as the ribonucleoside monophosphate, a mole of pyrophosphate is formed. Pyrophosphate in the presence of uridine diphosphoglucose (UDPG) and uridinediphosphoglucose pyrophosphorylase under proper conditions is converted to glucose-1-phosphate and UTP (Reaction 2). The available glucose-1-phosphate is changed to glucose-6-phosphate by phosphoglucomutase (Reaction 3).

$$PP_i + \text{UDPG} \underset{\text{UDPG pyrophosphorylase}}{\rightleftharpoons} \text{glucose-1-phosphate} + \text{UTP} \qquad (2)$$

$$\text{glucose-1-phosphate} \underset{\text{phosphoglucomutase}}{\rightleftharpoons} \text{glucose-6-phosphate} \qquad (3)$$

As glucose-6-phosphate is formed, it is acted upon by glucose-6-phosphate dehydrogenase in the presence of $NADP^+$ to give

6-phosphogluconate, H^+ and NADPH (Reaction 4).

glucose-6-phosphate + $NADP^+$ $\underset{\text{glucose-6-phosphate dehydrogenase}}{\rightleftharpoons}$ 6-phospho-gluconate + H^+ + NADPH (4)

From the reactions above it is observed that one mole of NADPH is synthesized for each mole of pyrophosphate formed. Calculation of the number of moles of NADPH synthesized was made using the molar extinction coefficient of 6.22×10^6 cm^2/mole at 340 mμ (Kornberg, 1955).

The change in absorbancy at 340 mμ was followed using a Beckman DU spectrophotometer equipped with a Gilford automatic sample changer and a Sargent recorder. The temperature of incubation was controlled using Beckman thermal plates and a Haake circulating water bath.

The reaction mixture contained 20 μ moles Tris-HCl, pH 8.0; 20 μ moles $MgCl_2$; 0.5 μ mole $MnCl_2$; 1.5 μ mole 2-mercaptoethanol; 0.005 μ mole EDTA; 0-100 μg gh-1 DNA; 0.215 μ mole each of ATP, GTP, UTP and CTP; 0.22 μ mole NADP; 0.20 μ mole UDPG; 10 μg glucose-6-phosphate dehydrogenase; 1 μg uridine diphosphoglucose pyrophosphorylase and 0-29 units of RNA polymerase in a total volume of 0.5 ml.

The reaction mixture minus RNA polymerase was incubated at the reaction temperature (30°C) for 40 minutes. During this incubation some reduction of $NADP^+$ which was not the result of the RNA polymerase reaction was observed. After the completion of this reaction, RNA synthesis and pyrophosphate formation was initiated by the addition of RNA polymerase.

Method for the Assay of Deoxyribonuclease

The time-dependent decrease in [^{14}C] thymidine labeled T_7 DNA which was insoluble in 10% TCA was employed as a measure of DNase activity. The reaction mixture used for the detection of DNase activity in the 60% Ammonium Sulfate Fraction II contained 6 μ moles $MgCl_2$; 3 μg [^{14}C] thymidine labeled T_7DNA; and 10 μg 60% Ammonium Sulfate Fraction II in a total volume of 0.6 ml. The reaction mixture was incubated at 37°C. Samples of 0.1 ml were periodically removed from the reaction mixture and mixed with 5 ml of cold 10% (w/v) TCA and 250 μg of carrier salmon sperm DNA. The precipitate which formed was collected according to the procedure described in part A of Procedure for Radioactivity Measurement.

Control experiments of two types were performed. The first followed the time dependent decrease in radioactivity of the TCA insoluble [^{14}C] labeled T_7DNA when no 60% Ammonium Sulfate Fraction II was added to the reaction mixture. The second experiment followed the parameter measured above when DNase at a final concentration of 0.017 and 0.17 μg/ml were added to reaction mixtures not containing 60% Ammonium Sulfate Fraction II.

Method for the Assay of Ribonuclease

The time dependent increase in TCA soluble [^{3}H] uracil labeled E. coli RNA radioactivity was employed as a measure of RNase activity. The reaction mixture used for the detection

of RNase activity in the 60% Ammonium Sulfate Fraction II contained 20 μ moles Tris-HCl, pH 8.0; 0.005 μ mole EDTA; 2 μ moles $MgCl_2$; 0.5 μ mole $MnCl_2$; 1.5 μ moles 2-mercaptoethanol; 14 μg [^{3}H] uracil labeled RNA and 10 μg of 60% Ammonium Sulfate Fraction II in a total volume 0.5 ml. The reaction mixture was incubated at 37°C. Samples of 0.1 ml were periodically removed over an hour of incubation and mixed with 0.5 ml of cold 10% TCA and 250 μg of carrier salmon sperm DNA. Radioactivity was measured in the filtrate according to part B of Procedure for Radioactivity Measurement.

Control experiments of two types were performed. The first followed the time dependent increase in TCA soluble [^{3}H] uracil labeled E. coli RNA radioactivity when no 60% Ammonium Sulfate Fraction II was added to the reaction mixture. The second experiment followed the time dependent increase in TCA soluble radioactivity when RNase at a final concentration of 0.4 μg/ml was added to the reaction mixture in the absence of 60% Ammonium Sulfate Fraction II.

Characterization of the RNA Synthesized by RNA Polymerase

The RNA product of RNA polymerase was characterized by its susceptibility to degradation by RNase and 0.3 M KOH and by its resistance to degradation by DNase.

A reaction mixture containing 80 μ moles Tris-HCl, pH 8.0; 8.0 μ moles $MgCl_2$; 2.0 μ moles $MnCl_2$; 6.0 μ moles 2-mercaptoethanol; 0.02 μ mole EDTA; 380 μg gh-1 DNA; 0.86 μ mole each of ATP, GTP, UTP and [^{3}H] CTP; and 4.2 units of

RNA polymerase (60% Ammonium Sulfate Fraction II) in a total volume of 2.0 ml was incubated at 30°C for one hour. Part of the reaction mixture (0.5 ml) was heated at 100°C for 10 minutes, then cooled in an ice bath to 0°C. The remaining 1.5 ml of reaction mixture was made 0.2% (w/v) with respect to SDS. The mixture was incubated at 37°C for an additional 10 minutes, then cooled to 0°C in an ice bath. The precipitate which formed overnight was removed by centrifugation at 5,000 x g for 10 minutes. The pellet was discarded and the supernatant fluid was extensively dialyzed against 0.01 M sodium acetate buffer adjusted to pH 5.2 with acetic acid.

Aliquots of the reaction mixture which had been heated at 100°C for 10 minutes were subjected to the following treatments:

(1) Incubation for 1 hour at 37°C in the presence of 1 µg/ml RNase.

(2) Incubation for 3 hours at 37°C in the presence of 10 µg/ml RNase.

(3) Incubation for 30 minutes at 37°C in the presence of 1 µg/ml DNase.

Aliquots of the reaction mixture which had been incubated with SDS were subjected to the following treatments:

(1) Incubation for 30 minutes at 37°C in the presence of 25 µg/ml RNase. The pH of the reaction was brought to 8.0 with Tris-HCl buffer.

(2) Incubation for 200 minutes at 37°C in 0.3 M KOH.

Following treatment as described above, the samples were mixed with cold 10% TCA. TCA insoluble radioactivity was determined.

General Methods and Materials

Glass beads, purchased from E. H. Sargent & Co., were prepared by washing successively in 0.5 M NaOH, water and 0.5 M HCl. They were rinsed free of traces of acid with water then dried. Streptomycinsulfate was purchased from Calbiochem. It was made to 10% (w/v) with water. Cellex D, a product of Bio Rad Laboratories, having an exchange capacity of 0.75 meq/g was prepared by washing extensively in 0.1 M NaOH followed by 0.1 M HCl. It was rinsed with water until traces of acid were removed. The fine particles were removed by decantation. Dialysis tubing was prepared by boiling in 5% sodium bicarbonate. When used for DNA preparations it was further treated by boiling in 0.001 M EDTA. RNase and electrophoretically purified DNase were purchased from Worthington Biochemicals Corporation. Pronase, B grade, was purchased from Calbiochem, Nogalamycin and Actinomycin D were gifts of the Upjohn Company and Merck and Company respectively. Salmon sperm DNA, type III, was purchased from Sigma Chemical Company. Bact-T-flex nitrocellulose membrane filters were obtained from Carl Schleicher and Schuell Co.

Unlabeled nucleoside triphosphates were purchased from the P. L. Laboratories Inc. $[^3H]$ CTP with a specific activity

of 1.2 c/m mole was obtained from Schwarz Bioresearch, Inc.

$NADP^+$, UDPG and the enzymes used in the assay of pyrophosphate were the generous gifts of Dr. R. G. Hansen. Phosphoglucomutase was prepared by dissolving an ammonium sulfate suspension in water to a final concentration of 1.0 mg/ml. One mg converted 50 μ moles glucose-1-phosphate to glucose-6-phosphate per minute at pH 7.4 and at 30°C. Glucose-6-phosphate dehydrogenase was prepared by diluting the crystalline suspension to 0.2 mg/ml in water. One mg converted 130 μ moles of $NADP^+$ to NADPH per minute at pH 7.4 at 25°C. UDPG-pyrophosphorylase was prepared by making a 1 to 100 dilution of the crystalline suspension (10 mg/ml) with 0.1 M Tris-acetate buffer, pH 8.0. One mg would convert 240 μ moles of pyrophosphate to glucose-1-phosphate and UTP per minute at pH 8.0 at 25°C.

Chromatography of $[^3H]$ CTP

Whatman #1, acid washed paper, was spotted 11 cm from the edge with $[^3H]$ CTP, CTP, CDP, and CMP. The unlabeled nucleoside triphosphates were applied at 90 to 100 μg per spot. $[^3H]$ CTP was spotted with approximately 3×10^5 counts/min. The chromatogram was developed with isobutyric acid, concentrated NH_4OH and water (66/1/33) using descending chromatography at 23°C. During 14 hours of development, the front moved 37 cm. The chromatogram was air dried and read by visual inspection of ultraviolet light quenching upon illumination with a Mineralight model SL, 2537 lamp. The

lanes containing [^{3}H] CTP were cut into 3/4 inch squares and placed in liquid scintillation vials to which was added 5 ml of the fluor described in part A of Procedure for Radioactivity Measurement. The radioactive content of the squares was determined. Between 80 and 85% of the radioactivity co-chromatographed with the CTP marker.

Purification of DNA-Dependent RNA Polymerase

Initial Extract. All of the following purification procedures were performed at 0 to 4°C unless otherwise stated. To prevent extensive loss of enzyme activity, it was necessary to avoid any delay in the completion of the purification.

Frozen cells (25 g) were mixed with glass beads (62.5 g) and disrupted by grinding using a mortar and pestle. Following rupture of the cells in 15 to 20 minutes, 62.5 ml of cold buffer A (0.01 M Tris-HCl, pH 8.0; 0.01 M $MgCl_2$; and 0.001 M EDTA) was added. After centrifugation at 25,000 x g for 15 minutes, the supernatant fluid was carefully decanted and saved. The pellet was suspended in 31 ml of cold buffer A and centrifuged as above. The combined 96 ml of supernatant fluid is referred to as the Initial Extract.

High Speed Supernatant Fraction. The Initial Extract was centrifuged at 150,000 x g for 90 minutes. The High Speed Supernatant Fraction (83 ml) was collected by decantation.

Streptomycin Precipitate. Streptomycin sulfate was added to the High Speed Supernatant Fraction at a final

concentration of 0.5% (w/v). The streptomycin sulfate solution was added slowly with continuous stirring. A fibrous precipitate which formed was collected on a glass rod, squeezed to remove excess liquid and added to 25 ml of buffer A containing 0.2 M $(NH_4)_2SO_4$. After 30 minutes the precipitate which was not collected with the glass rod was collected by centrifugation at 25,000 x g for 15 minutes. The pellet was added to the solution containing the fibrous precipitate. The combined precipitates were stirred slowly for 4 to 5 hours to dissolve the material.

30-60% Ammonium Sulfate Fraction I. DNase was added to the Streptomycin Precipitate at a final concentration of 1 µg/ml. The solution was dialyzed in the cold room (4°C) for 12 hours against 6 liters of a buffer solution prepared at 22 to 24°C containing 0.005 M 2-mercaptoethanol in buffer A. Approximately 40 to 50% of the initial absorbancy at 260 mµ was recovered following dialysis.

Ammonium sulfate (saturated at room temperature and adjusted to pH 7.0 with ammonium hydroxide) was added to a final concentration of 30% of saturation. The mixture was stirred 15 minutes and a precipitate was removed by centrifugation at 25,000 x g for 10 minutes. Saturated ammonium sulfate was added to the supernatant fluid at 60% of saturation. The mixture was stirred 30 minutes, and the precipitate was collected by centrifugation at 25,000 x g for 15 minutes. The precipitate was dissolved in 4.0 ml of buffer B (0.029 M Tris-HCl, pH 8.0; 0.000,09 M EDTA; 0.013 M $MgCl_2$; 0.001 M

$MnCl_2$; and 0.014 M 2-mercaptoethanol). The protein concentration was 4 to 6 mg/ml.

Peak DEAE-Cellulose Fraction. The 30-60% Ammonium Sulfate Precipitate was diluted to a protein concentration of about 2 mg/ml with buffer A containing 0.005 M 2-mercaptoethanol. A DEAE-cellulose column (1 x 12 cm) was equilibrated with the same buffer. The diluted solution was passed on to the column at a rate of 1 ml/min. The column was washed with buffer A containing 0.005 M 2-mercaptoethanol until the absorbancy of the effluant was less than 0.05 units/ml at 260 mμ. A second wash of 40 ml of buffer A containing 0.005 M 2-mercaptoethanol and 0.1 M NaCl was passed through the column. The enzyme was eluted from the column with buffer A containing 0.005 M 2-mercaptoethanol and 0.2 M NaCl. Enzyme activity appeared within 10 to 12 ml of the latter eluant. No appreciable activity was eluted with buffers containing higher salt concentrations. The flow rate was maintained at 1 ml/min throughout the chromatography.

60% Ammonium Sulfate Fraction II. Immediately after elution, the enzyme was concentrated. A saturated ammonium sulfate solution was added to the fractions containing RNA polymerase activity at 60% of saturation. Following 15 minutes of precipitation during which the solution was occasionly stirred, the precipitate was collected by centrifugation at 37,000 x g for 30 minutes and dissolved in 0.5 ml of buffer B. The protein concentration of the resultant solution was 5 mg/ml.

RESULTS

Summary of the Purification Procedure

The summary of the purification procedure is presented in Table I. Results of the purification through the Streptomycin Precipitate fraction (step 3) are presented for one preparation of the enzyme. This Streptomycin Precipitate fraction was not purified further. A second purification was performed which was carried through the 60% Ammonium Sulfate Fraction II to obtain the results of steps 4, 5 and 6. The results of the first three steps of the second purification were not typical and are not presented in Table I. The assays of the first three steps of the second purification were not performed immediately following the isolation of the fraction. Since activity of RNA polymerase is lost upon storage of fractions 1, 2 and 3, the rate of CMP incorporation into RNA was less than that from freshly prepared fractions. The best estimate of the total purification was obtained by compiling the results of the two separate purifications.

The assays used for the compilation of Table I were of two types. The enzyme fraction was either assayed in the presence of 98 μg/ml gh-1 DNA(+) or was assayed in the absence of any exogenous DNA(-). CTP was converted to an acid insoluble form when DNA was omitted from reaction mixtures containing RNA polymerase from fractions 1 through 4. Evidently, there is sufficient DNA in the enzyme fraction to function as a template for RNA polymerase. Invariably, addition of DNA to

Table I. Summary of the purification procedure.
* Assays were performed either in the presence of 98 μg/ml of gh-1 DNA (+) or in its absence (-).
** Concentration of gh-1 DNA used in the assay was rate limiting.
Each enzyme fraction was assayed at 37°C in the standard 0.5 ml reaction mixture described in Materials and Methods. Samples (0.1 ml) were withdrawn at various times for radioactivity analysis. The total CMP incorporated at 5 minutes of incubation was used to calculate the units of enzyme.

TABLE I. Summary of the Purification

	Fraction	gh-1 DNA*	Units/ ml	Total Units	Protein mg/ml	Specific Activity	% Recovery	Fold Purification
1.	Initial Extract	-	624	61,700	18.2	34.3	100	-
		+	216	21,400	18.2	11.0		
2.	High Speed Supernatant Fraction	-	840	72,200	11.2	75.0	117	2.19
		+	264	22,700	11.2	23.6		
3.	Streptomycin Precipitate	-	1,340	36,900	1.85	726.0	59	21.3
			263	6,440	1.85	142.0		
4.	30-60% Ammonium Sulfate Fraction 1	-	12	48	5.90	2.0		
		+	840	3,360	5.90	142.0	5	11.8
5.	Peak DEAE-Cellulose Fraction	-	0	0	0.21	0.0		
		+	1,170	13,900	0.21	5,590.0	23	163
6.**	60% Ammonium Sulfate Fraction 11	-	0	0	5.17	0.0		
		+	19,400	11,000	5.17	3,760.0	18	110

any fraction before purification step 4 resulted in a decreased rate of RNA synthesis. However, the extent of RNA synthesis was greater in the presence of exogenous DNA than in its absence over long periods of incubation.

The highest specific activity reported for this purification was 5,590 mμ moles of CTP converted into a TCA insoluble form per hour per mg protein. Homogeneous RNA polymerase from E. coli has a specific activity of 7,000 mμ moles of CTP converted into an acid insoluble form per hour per mg (Richardson, 1966b).

Following DEAE-cellulose chromatography, it was necessary to concentrate the enzyme, since dilute enzyme solutions lost activity rapidly. Concentration by ammonium sulfate resulted in a relatively stable enzyme. The specific activity of the 60% Ammonium Sulfate Fraction II apparently decreases. This enzyme fraction was inadvertently assayed under conditions in which total RNA polymerase activity was not detected. The concentration of gh-1 DNA in this assay limited the rate of RNA synthesis by 40 to 50% (see Figure 4).

Purification of RNA polymerase from P. putida through the Peak DEAE-Cellulose Fraction has resulted in a 160 fold increase in specific activity with a 20% recovery of activity.

Properties of 60% Ammonium Sulfate Fraction II

Losses of 50% or more of the activity of RNA polymerase in the 60%. Ammonium Sulfate Fraction II were observed upon storage at 4°C for two weeks. No activity was detected after

freezing the fraction to -20°C and thawing to the assay temperature. The enzyme fraction could, however, be quickly frozen in a dry ice-acetone bath and stored in liquid nitrogen (-196°C) with only small losses in activity over a two month period. Repeated freezing to -196°C and thawing to 0°C did not significantly effect the activity of the enzyme.

Neither RNase nor DNase activities were observed in the 60% Ammonium Sulfate Fraction II as assayed according to the procedures described in Materials and Methods. The possibility that there are small amounts of these nucleases still associated with the enzyme fraction cannot be ruled out on the basis of this assay.

Comments Concerning the Purification Procedure

Further purification following the Streptomycin Precipitate (step 3) proved to be difficult. The usual procedures such as ammonium sulfate precipitation or DEAE-cellulose chromatography did not work in the presence of the high concentrations of nucleic acids found in the Streptomycin Precipitate fraction. Following the degradation of DNA by DNase, further purification proved possible.

The results of DEAE-cellulose chromatography are shown in Figure 1. Nearly all of the activity could be eluted from the column in one 10 ml fraction. The total recovery of absorbancy at 280 mμ from the column was 85%. The ratio of the absorbancy at 280 mμ compared to that at 260 mμ was 1.3 for the peak fraction. Using the data of Warburg and

Figure 1. Chromatography of 30-60% Ammonium Sulfate Fraction I on DEAE-cellulose. 10 ml fractions were collected. Bars drawn with dashed lines indicate the absorbancy of the eluant at 280 mμ. Bars drawn with solid lines represent CMP incorporation in mμ moles/ml of reaction mixture. Each 1 ml of reaction mixture contained 0.04 ml of the fraction and 98 μg/ml of gh-1 DNA. Incubation was 5 minutes at 37°C. TCA insoluble radioactivity was measured.

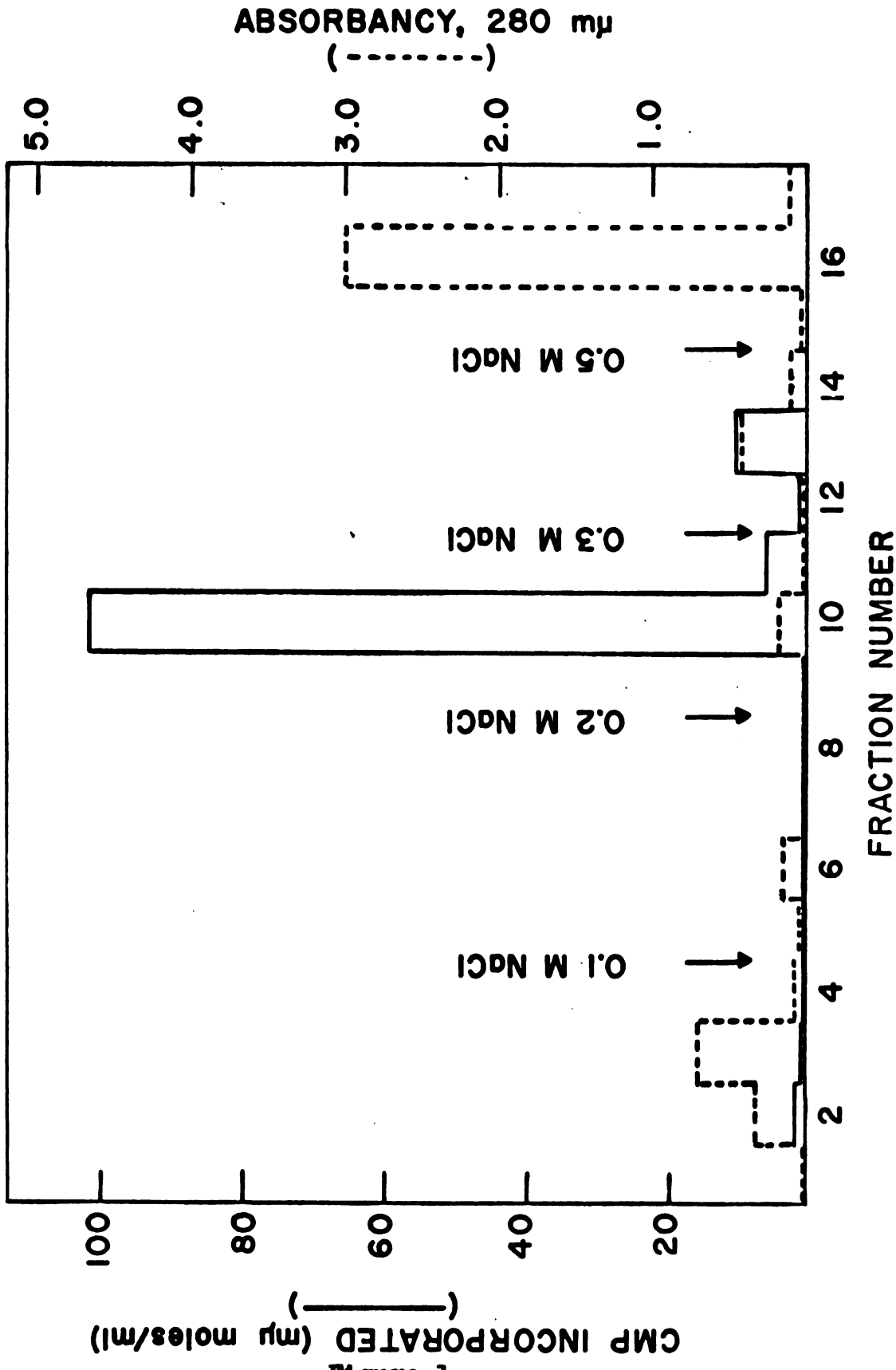
ABSORBANCY, 280 mμ
(--------)
5.0
4.0
3.0
2.0
1.0
0.5 M NaCl
0.3 M NaCl
0.2 M NaCl
0.1 M NaCl
2
4
6
8
10
12
14
16
FRACTION NUMBER
100
80
60
40
20
(————)
CMP INCORPORATED (mμ moles/ml)

Figure 1

Christian (1941), such a ratio indicates that the contamination with nucleic acids was less than 1.3%.

When DEAE-cellulose chromatography was performed in the absence of Mg^{++} or 2-mercaptoethanol or both, recovery of activity was poor.

Kinetics of the Synthesis of RNA by DNA-Dependent RNA Polymerase

The kinetics of the DNA-dependent synthesis of RNA by RNA polymerase (60% Ammonium Sulfate Fraction II) are presented in Figure 2. RNA synthesis was not observed when assayed in the absence of a DNA template. In the presence of exogenous DNA, RNA synthesis was linear for 5 minutes or less. By 30 minutes of incubation RNA synthesis had nearly stopped.

Effect of RNA Polymerase Concentration on Rate of Reaction

The rate of incorporation of CMP into RNA was linearly dependent upon enzyme concentration (Peak DEAE-Cellulose Fraction) within the range tested (Figure 3).

Effect of DNA Concentration on Rate of Reaction

The concentration of DNA in the reaction mixture strongly effects the initial rate of RNA synthesis (Figure 4). For these experiments approximately 220 µg of gh-1 DNA per ml of reaction mixture gave the maximum synthesis of RNA. Higher concentrations of DNA in the reaction mixture lowered the rate of RNA synthesis.

The possibility that the apparent decrease in RNA synthesis at high concentrations of DNA was caused by a

Figure 2. Kinetics of the synthesis of RNA by DNA-dependent RNA polymerase. 60% Ammonium Sulfate Fraction II (260 µg) was added to the standard 0.5 ml reaction mixture containing either no exogenous DNA (O——O) or 45 µg of gh-1 DNA (●——●). During incubation at 37°C, 0.1 ml samples were withdrawn for radioactive analysis.

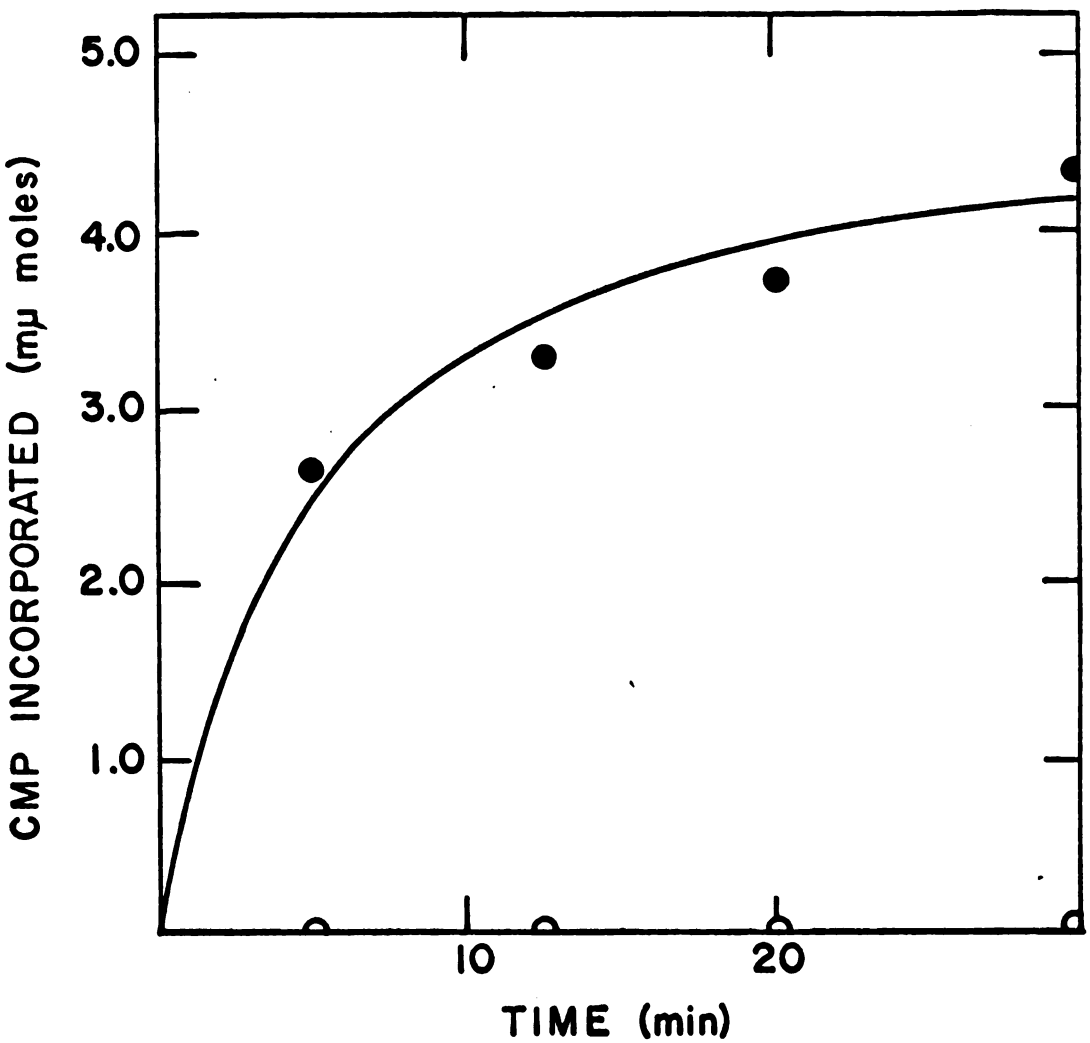

Figure 2

Figure 4. Effect of gh-1 DNA concentration on the rate of CMP incorporation. 60% Ammonium Sulfate Fraction II (2 µg) was added to a reaction mixture containing 4 µ moles Tris-HCl, pH 8.0; 0.4 µ mole $MgCl_2$; 0.1 µ mole $MnCl_2$; 0.3 µ mole 2-mercaptoethanol; 0.001 µ mole EDTA; 0.043 µ mole each of ATP, GTP, UTP and [^{3}H] CTP; and varying concentrations of gh-1 DNA in a total volume of 0.1 ml. Incubation was 5 minutes at 37°C. The results are plotted as CMP incorporation in mµ moles/ml of reaction mixture versus gh-1 DNA concentration in µg/ml.

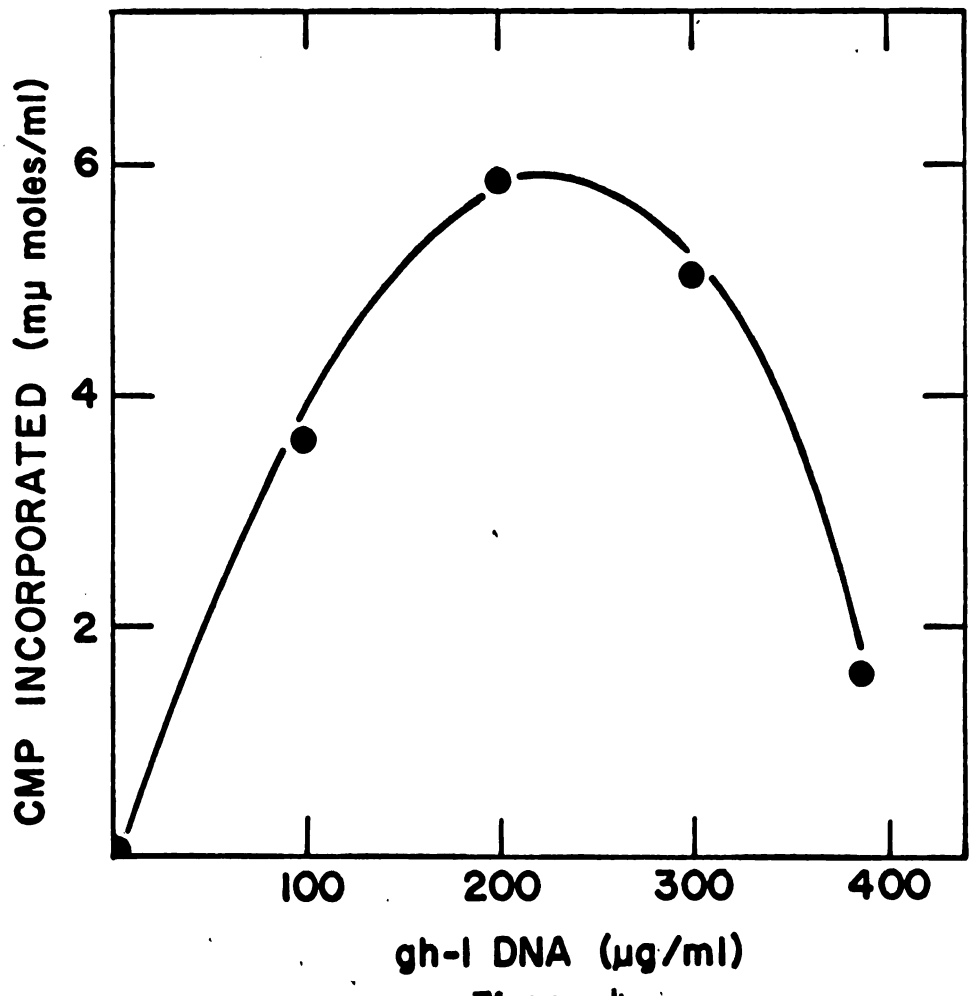

Figure 4

self-adsorption phenomena was considered. It would be expected that the self-adsorption of radioactivity in a precipitate would increase with increasing amounts of material. However no decrease in radioactivity was observed when up to 500 µg of gh-1 DNA was mixed with [^{3}H] uracil labeled RNA, precipitated with cold 10% TCA, and assayed as described in Materials and Methods.

Effect of pH on RNA Synthesis

The effect of pH on the rate of incorporation of CMP into RNA has been studied using Peak DEAE-Cellulose Fraction enzyme (Table II). The reaction has a broad pH optimum between pH 8 and 9 in the Tris-HCl buffer. When assayed below pH 7.0, no RNA synthesis was observed.

Effect of Temperature on RNA Synthesis

The kinetics of RNA synthesis using the 60% Ammonium Sulfate Fraction II enzyme at various reaction temperatures are presented in Figure 5. A lag in RNA synthesis observed at 25°C and 20°C was not observed at 30°C, 37°C or 42°C. Once the lag was over, the rate of RNA synthesis at 20°C was half that at 30°C. The extent of RNA synthesis was greater at 30°C through 30 minutes of incubation than at any other incubation temperature used.

Preincubation of the 60% Ammonium Sulfate Fraction II with the reaction mixture minus ribonucleoside triphosphates for 11 minutes at 25°C before initiation of the reaction with the ribonucleoside triphosphates, eliminated the lag that occurred at 25°C.

Table II. Effect of pH on the rate of CMP incorporation. Standard 0.5 ml reaction mixtures modified to contain 20 μ moles of Tris-HCl buffer at various pH values were prepared. Each reaction mixture contained Peak DEAE-Cellulose Fraction enzyme (10.5 μg) and T_4 DNA (15 μg). Samples (0.2 ml) were removed at 5 and 10 minutes as the mixtures were incubated at 37°C. The TCA insoluble radioactivity was determined.

TABLE II. Effect of pH on the Rate of CMP Incorporation

pH of Reaction Mixture	CMP Incorporation (mμ moles)
6.5	0.0
7.0	0.01
7.5	0.34
7.9	0.62
8.5	0.69
8.9	0.61
9.1	0.56

Figure 5. Effect of temperature on rate of CMP incorporation. The standard 0.5 ml reaction mixtures containing 95 µg of gh-1 DNA and 10 µg of 60% Ammonium Sulfate Fraction II were incubated at 20°C (●——●); 30°C (●——●); 37°C or 42°C (○——○). The reaction mixture used to obtain the curve at 25°C (●——○) was modified to a final volume of 0.75 ml. The concentration of the components were identical to the 0.5 ml reaction mixture. Samples (0.1 ml) were removed at various times for radioactivity analysis.

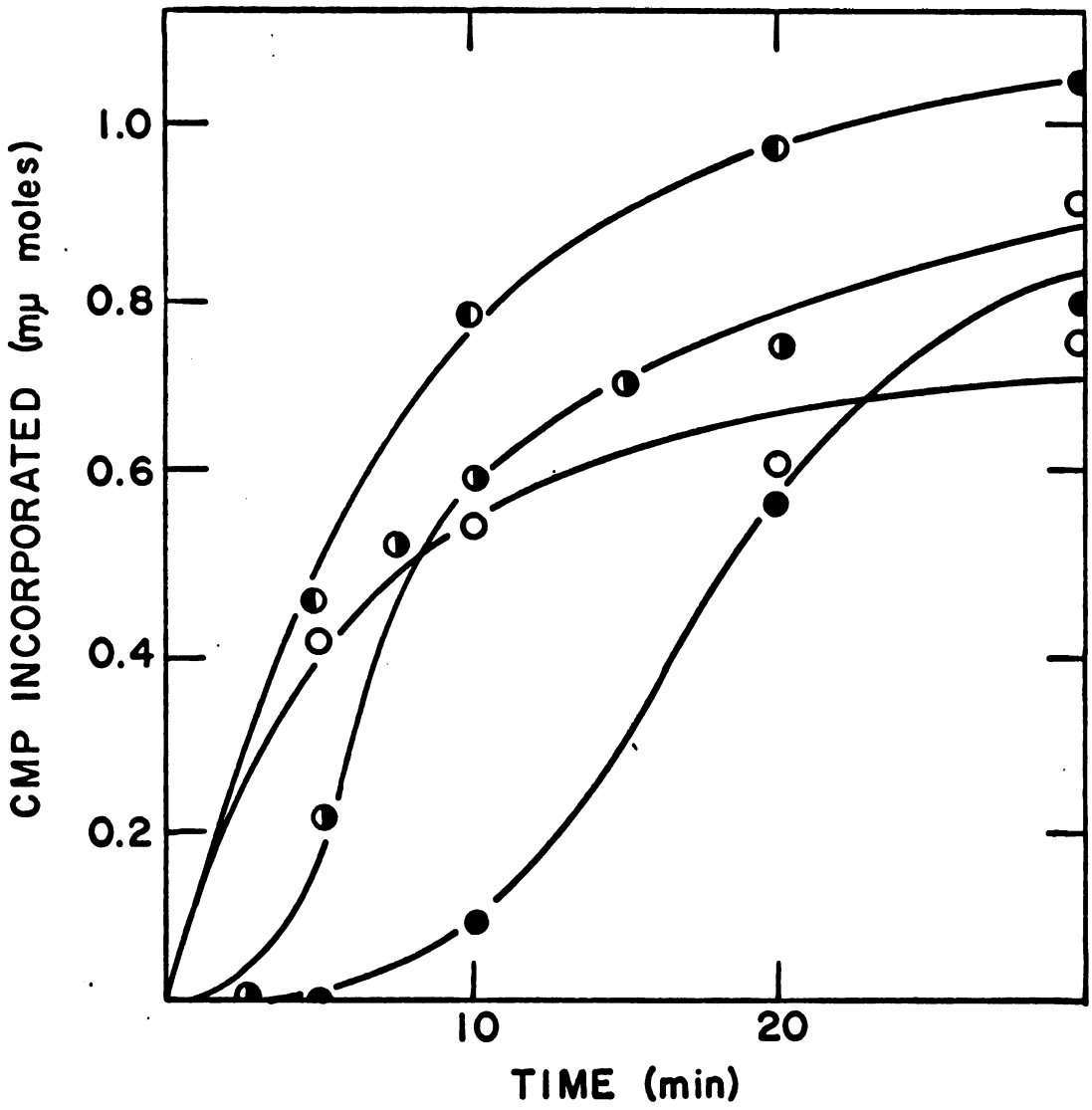

Figure 5

Effect of Mg^{++} and Mn^{++} Concentration on RNA Synthesis

Table III shows that Mg^{++} or Mn^{++} stimulate RNA synthesis. In the absence of divalent cation no synthesis of RNA was observed. The addition of both Mg^{++} and Mn^{++} to the reaction mixture at the concentrations used by Chamberlin and Berg (1962) was more effective in the stimulation of RNA synthesis than either of the two cations individually.

Effect of Substrate Concentration on RNA Synthesis

The results presented in Table IV show that maximal RNA synthesis was observed when the concentration of each triphosphate was 4.3×10^{-4} M. Inhibition of RNA synthesis was observed when concentrations of substrate greater than 4.3×10^{-4} M were used.

Effect of Various DNA Templates on RNA Synthesis

When DNA from various sources, at approximately the same concentration, was used as the template for RNA polymerase (Peak DEAE-Cellulose Fraction), differences in the total incorporation of monophosphates into RNA were observed (Table V). The total monophosphate incorporation into RNA was calculated using the CMP incorporation observed and the published GC-Content of each of the templates. Gh-1 DNA functions 2 fold better than either T_4 or P. putida DNA and over 5 fold better than salmon sperm DNA.

Kinetics of Pyrophosphate Formation by RNA Polymerase

The kinetics of pyrophosphate formation are presented in Figure 6. When RNA polymerase (60% Ammonium Sulfate Fraction

Table III. Effect of Mg^{++} and Mn^{++} concentration on the rate of CMP incorporation. Peak DEAE-Cellulose Fraction was dialyzed 4 hours against buffer A containing 0.005 M 2-mercaptoethanol to remove traces of Mg^{++} and Mn^{++}. This enzyme (21 µg) was added to the standard 0.5 ml reaction mixture modified to give the Mg^{++} and Mn^{++} concentrations shown. Each reaction mixture contained 68 µg of salmon sperm DNA. Samples (0.1 ml) were withdrawn periodically as the reaction mixtures were incubated at 37°C. TCA insoluble radioactivity was determined.

TABLE III. Effect of Mg^{++} and Mn^{++} on the Rate of CMP Incorporation

Concentration of Mg^{++} in the Reaction Mixture (mμ moles/ml)	Concentration of Mn^{++} in the Reaction Mixture (mμ moles/ml)	CMP Incorporated (mμ moles)
0.0	0.0	0.0
4.0	0.0	0.25
0.0	1.0	0.20
4.0	1.0	0.30

Table IV. Effect of substrate concentration on the rate of CMP incorporation. The reaction mixture was that of Figure 4 only each was modified to contain the various substrate concentrations shown and 19.8 µg of gh-1 DNA. The reaction mixtures contained 2 µg of 60% Ammonium Sulfate Fraction II. Each mixture was incubated 10 minutes and the TCA insoluble radioactivity was determined.

TABLE IV. Effect of Substrate Concentration on the Rate of CMP Incorporation

Concentration of Each Substrate in Reaction Mixture (M)	Rate of Incorporation of CMP into RNA (mμ moles/10 min)
0.0	0.0
0.86×10^{-4}	0.13
2.15×10^{-4}	0.25
4.30×10^{-4}	0.48
10.1×10^{-4}	0.16
20.1×10^{-4}	0.07

Table V. Effect of various DNA templates on the rate of synthesis of RNA. The standard 0.5 ml reaction mixture contained 10.5 μg of Peak DEAE-Cellulose Fraction and the stated concentration of each of the various DNA templates. Samples (0.1 ml) were withdrawn at various times from the reaction mixtures which were incubated at 37°C. TCA insoluble radioactivity was measured. From the CMP incorporation and the GC-content of the DNA template, the total nucleotide incorporation was calculated.

TABLE V. Effect of Various DNA Templates on the Rate of RNA Synthesis by RNA Polymerase

Source of DNA Template	DNA Concentration (µg/ml)	CMP Incorporated (mµ moles)	% GC	Factor	Nucleotides Incorporated (mµ moles)
Salmon sperm	135	0.15	41.2	4.85	0.73
P. putida	140	0.50	63.0	3.17	1.6
T_4	118	0.32	35.0	5.72	1.7
gh-1	129	1.1	57.0	3.50	3.8

Figure 6. Kinetics of pyrophosphate formation by RNA polymerase. To the standard 0.5 ml reaction mixture was added 99 µg of gh-1 DNA and 25.1 µg of 60% Ammonium Sulfate Fraction II (curve a). The complete reaction mixture enzyme (curve b); minus DNA (curve c); minus ribonucleoside triphosphates (curve d). The reaction mixtures were incubated at 30°C during the assay.

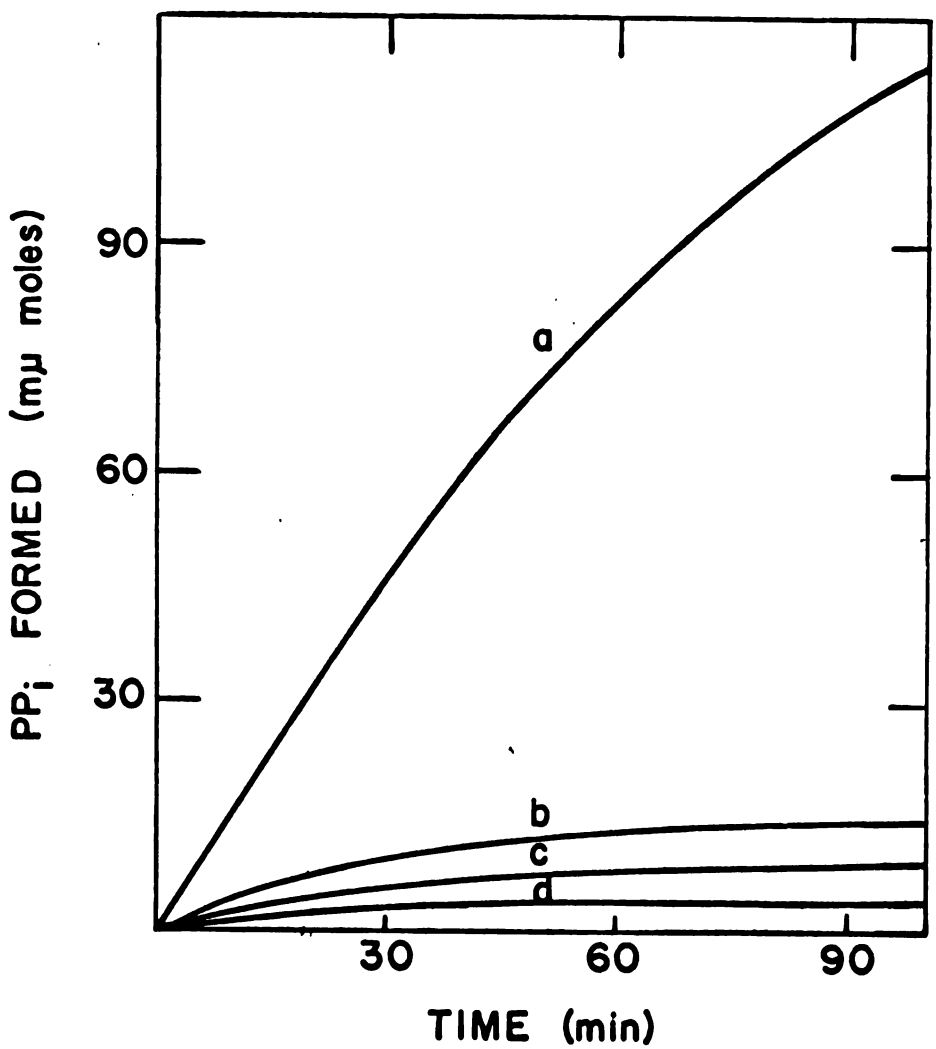

Figure 6

II), curve b; gh-1 DNA, curve c; or ribonucleoside triphosphates, curve d, are not included in the reaction mixture, little synthesis of pyrophosphate was observed. The kinetics of pyrophosphate formation in the complete reaction mixture are presented in curve a. The synthesis of pyrophosphate is linear for 30 minutes at 30°C.

Effect of RNA Polymerase Concentration on the Rate of Pyrophosphate Formation

The rate of pyrophosphate formation by RNA polymerase was linearly dependent upon the enzyme concentration within the range tested (Figure 7).

Effect of Inhibitors, RNase or DNase on Pyrophosphate Formation

Actinomycin D, nogalamycin or DNase (Figure 8, curve c) inhibit the formation of pyrophosphate by RNA polymerase. RNase stimulated the formation of pyrophosphate (curve a).

The extent of RNase stimulation of pyrophosphate formation by RNA polymerase varies with the concentration of RNase (Table VI). The greatest observed stimulation of pyrophosphate formation occurred with RNase at a concentration of 2 µg/ml of reaction mixture.

Comparison of the Radioactive Assay and the Spectrophotometric Assay

Figure 9 presents the kinetics of the formation of pyrophosphate as followed by the spectrophotometric assay and as calculated from the GC-content of the DNA template and the CMP incorporation observed when followed by the radioactive assay.

Figure 7. Effect of RNA polymerase concentration on the rate of pyrophosphate formation. The standard 0.5 ml reaction mixture contained 99 µg of gh-1 DNA and various concentrations of 60% Ammonium Sulfate Fraction II. RNA polymerase was added following a 40 minute preincubation of the reaction mixture at 30°C. The assay was performed at 30°C.

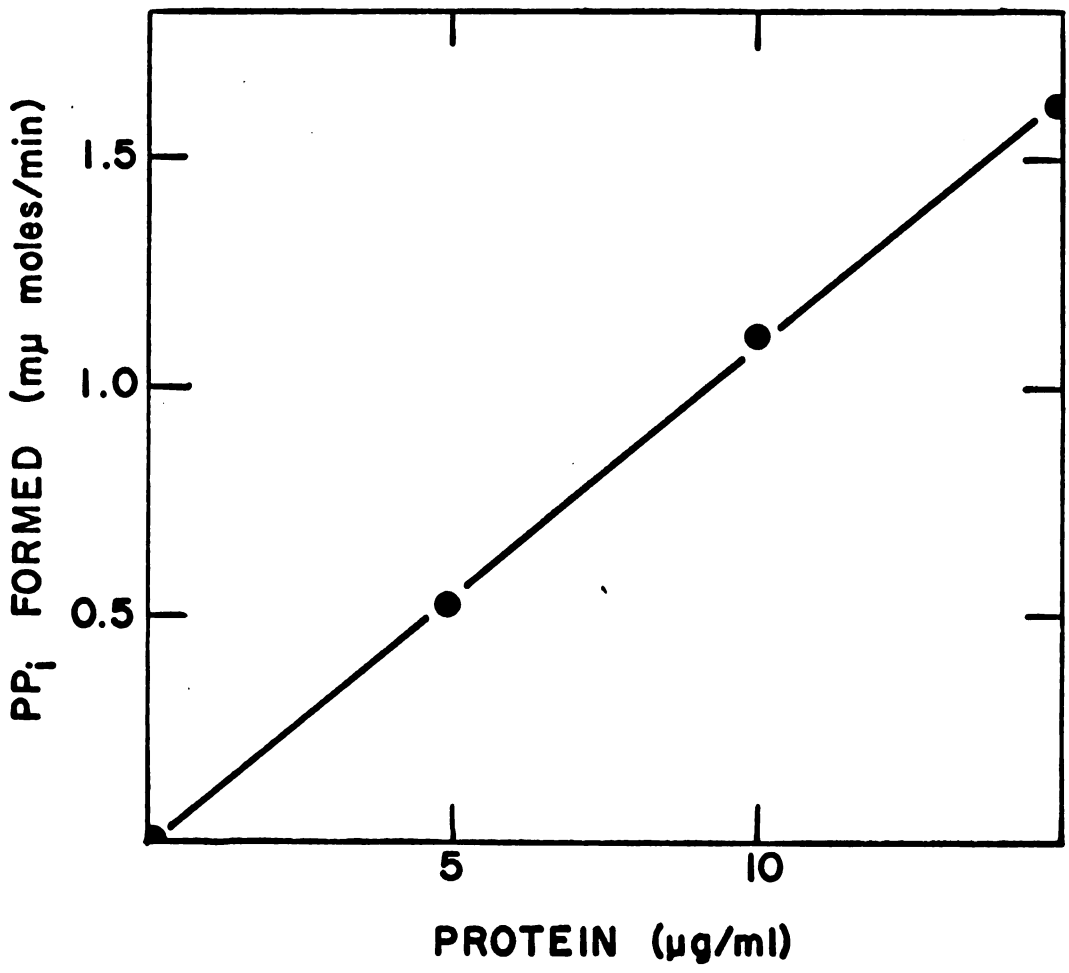

Figure 7

Figure 8. Effect of inhibitors, RNase or DNase on pyrophosphate formation. The standard 0.5 ml reaction mixture contained 99 µg of gh-1 DNA and 25.1 µg of 60% Ammonium Sulfate Fraction II (curve b). The results of the addition of 2.5 µg of nogalamycin or 2.0 µg of actinomycin D or 2 µg of DNase are presented in curve c. The result of the addition of 2 µg of RNase to the complete reaction mixture are shown in curve a. All reaction mixtures were incubated 40 minutes at 30°C before addition of the RNA-polymerase. The assay temperature was 30°C.

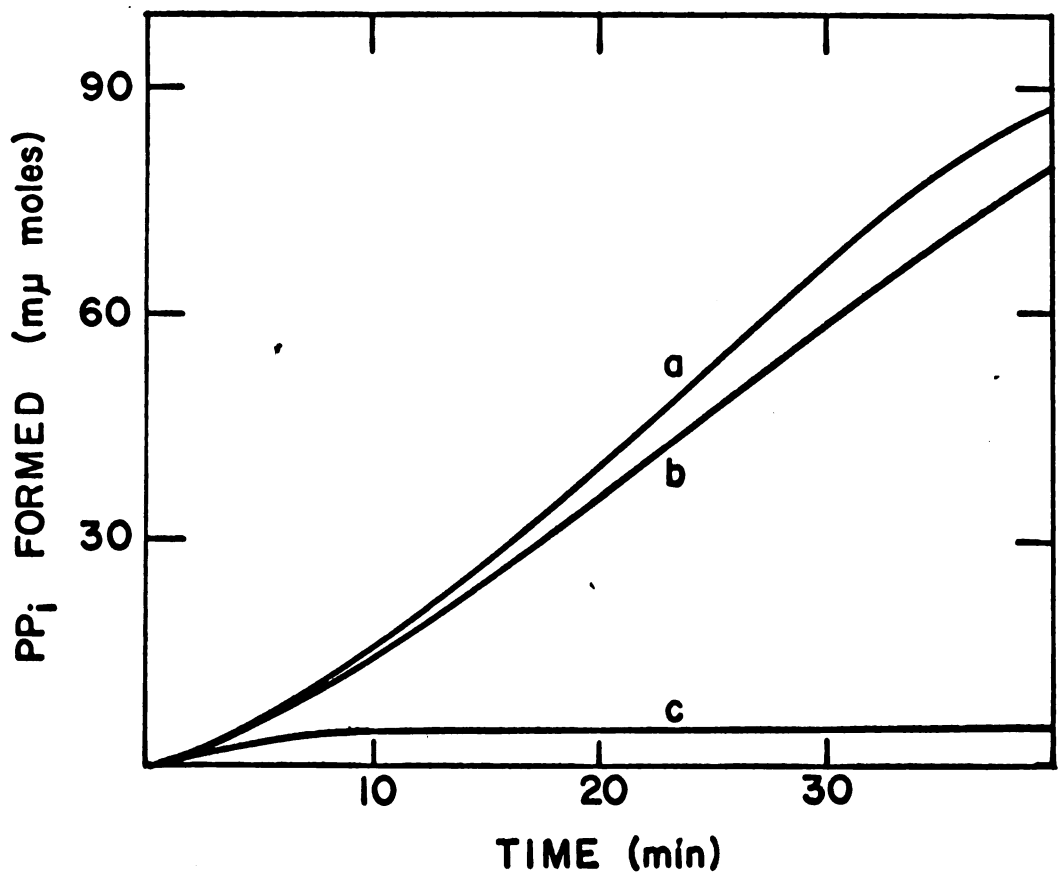

Figure 8

Table VI. Effect of ribonuclease concentration on the rate of pyrophosphate formation. The standard 0.5 ml reaction mixture was prepared with 99 µg/ml of gh-1 DNA and 5 µg of 60% Ammonium Sulfate Fraction II. RNase was added to the mixtures at the stated concentration. Following 40 minutes of preincubation, RNA polymerase was added. The assay was conducted at 30°C.

TABLE VI. Effect of Pancreatic Ribonuclease Concentration on Rate of Pyrophosphate Formation

Concentration of Pancreatic Ribonuclease in the Reaction Mixture (µg/ml)	Pyrophosphate Formation (% on Control)
0.00	100
0.01	113
0.10	122
1.00	130
2.00	142
20.0	113

Figure 9. Comparison of the formation of pyrophosphate as measured by the spectrophotometric assay (——) with the calculated formation of pyrophosphate as determined by the radioactive assay (●——●). Two standard 0.5 ml reaction mixtures containing the same concentration of each component were prepared. Each mixture contained 99 µg of gh-1 DNA and 10 ug of 60% Ammonium Sulfate Fraction II. The mixture which was used for the determination of CMP incorporation contained [^{3}H] CTP. The reaction mixtures were preincubated 40 minutes before addition of the RNA polymerase fraction. The assays were conducted at 30°C. Pyrophosphate formation as observed by the spectrophotometric assay and calculated from the GC-content of gh-1 DNA and the observed CMP incorporation were plotted.

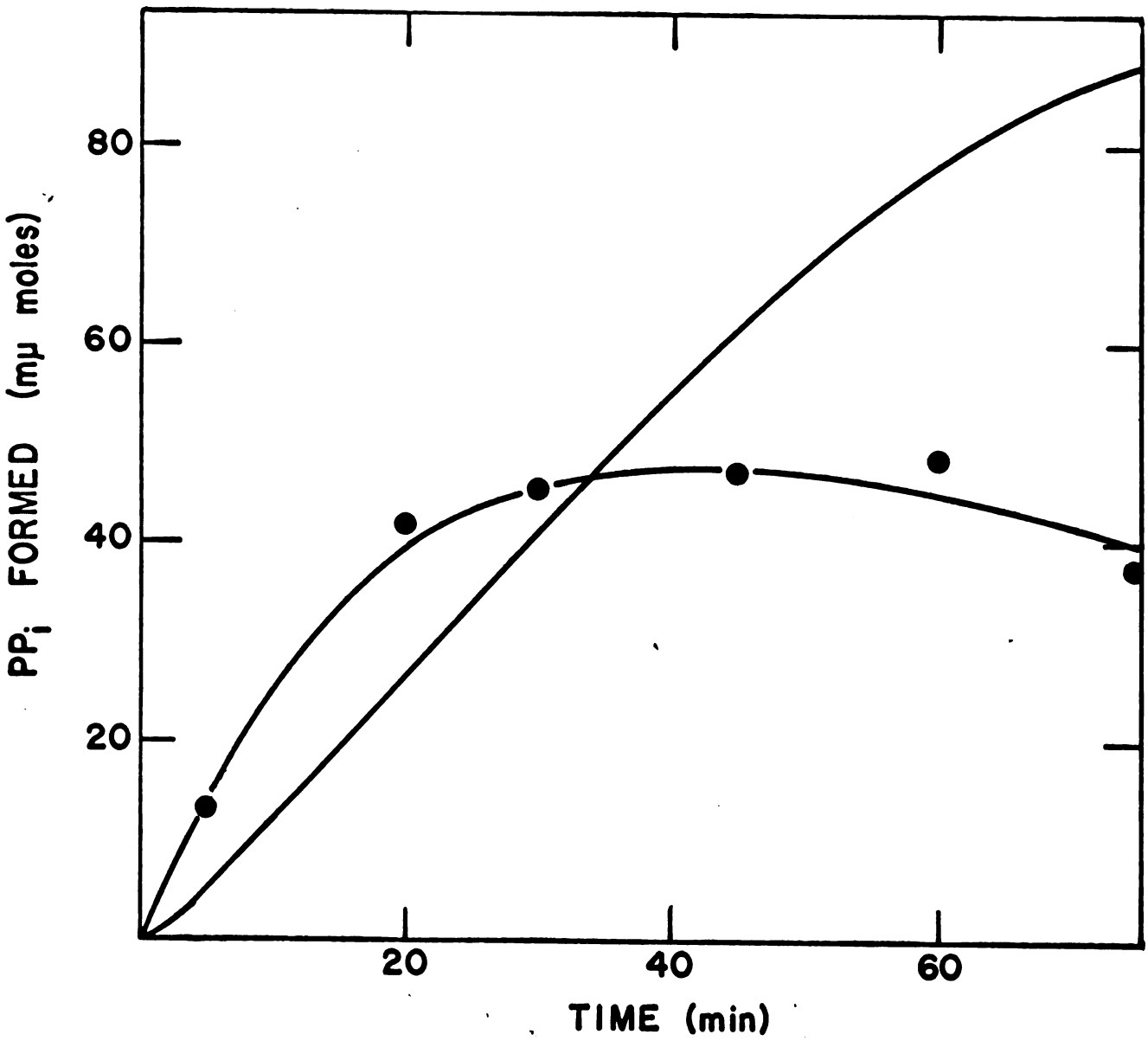

Figure 9

It is observed that the initial rate of the radioactive assay is greater, but within a factor of 2, than the steady state rate of the spectrophotometric assay. The extent of pyrophosphate formation is much greater than that calculated from the radioactive assay. The total amount of radioactivity detected after 40 minutes of reaction decreases. This is reflected in the lowered calculated quantities of pyrophosphate.

Characterization of the RNA Synthesized by RNA Polymerase

It is observed in Table VI that the RNA product from the *in vitro* reaction with RNA polymerase is completely resistant to DNase degradation and is greater than 96% susceptible to KOH digestion. The product RNA was only 80% susceptible to the action of RNase. However, treatment of the reaction mixture following the synthesis of RNA with SDS as described, resulted in RNA which was approximately 96% susceptible to RNase.

TABLE VII. Characterization of the RNA Synthesized by DNA-Dependent RNA Polymerase

Treatment	% of the [^{3}H] RNA Susceptible to the Treatment
Heat treated reaction mixture	
(1) RNase, 1 hour, 1 µg/ml	80.6
(2) RNase, 3 hours, 10 µg/ml	78.9
(3) DNase, 0.5 hour, 1 µg/ml	0.0
SDS treated reaction mixture	
(1) RNase, 0.5 hour, 25 µg/ml	96.4
(2) KOH, 3.3 hours, 0.3 M	96.5

DISCUSSION

This investigation has been concerned with the purification and characterization of DNA-dependent RNA polymerase from Pseudomonas putida A.3.12. The enzyme has been purified to a specific activity of 5,590 mμ moles of CTP converted to TCA insoluble form per hour per mg of protein. The increase in specific activity was 160 fold over that of the Initial Extract and the recovery of activity was 20%. The $A_{280}:A_{260}$ ratio of the Peak DEAE-Cellulose Fraction indicated that there was 1.3% (w/w) contamination by nucleic acids.

The kinetics of RNA synthesis exhibited by DNA-dependent RNA polymerase from P. putida were of the type described by Bremer and Konrad (1964) for the E. coli enzyme. In the absence of exogenous DNA there was no RNA synthesis. In the presence of exogenous DNA, RNA synthesis occurred. For both the E. coli and P. putida enzymes RNA synthesis was linear for a relatively short period of the assay. RNA synthesis stopped because of the suspected formation of a complex [DNA-enzyme-RNA] which inhibited further reaction.

It was observed that the kinetics of the reaction changed during the purification. The early fractions catalyzed the synthesis of RNA in a linear manner for nearly 15 minutes and the synthesis of RNA continued for 1.5 to 2.0 hours. It is possible that purification of the enzyme removed a factor which prevented or postponed the formation of the suspected complex. Such factors have been shown to exist. Revel and

Gros (1967) have found *in vitro* DNA-dependent RNA synthesis to be stimulated by ribosomes when a protein factor was present. Some RNA was observed to have been released from the template in the presence of this factor. A second possibility is that the exogenous DNA template which had been purified by phenol extraction did not have the same properties as did the endogenous *P. putida* DNA used for assay of the early fractions.

The effect of DNA concentration on the rate of RNA synthesis was investigated. It was observed that at high concentrations of DNA there was a marked inhibition of RNA synthesis. Theoretically, the binding of RNA polymerase to DNA should be favored at high DNA concentrations. The rate of synthesis of RNA would increase until all of the available enzyme was bound to DNA and was functioning. Any further addition of DNA to the reaction mixture would not cause an increased rate of RNA synthesis.

The possibility that Mg^{++} and Mn^{++} were removed from the reaction mixture by binding to nucleic acids, thus preventing the synthesis of RNA was considered. Binding of divalent cations to DNA has been demonstrated by equilibrium dialysis experiments and conductivity studies (Zubay and Doty, 1958). It is thought that binding involves the phosphate group and that each phosphate may bind one divalent cation. When gh-1 DNA was added at 390 µg/ml of reaction mixture, the molar concentration of nucleotide phosphate was

about 11.8 x 10^{-4} M. The molar concentration of Mg^{++} and Mn^{++} was 40 x 10^{-4} and 10 x 10^{-4} respectively. If all of the DNA phosphate was capable of binding Mg^{++} or Mn^{++} cations, there would still be an excess of free cations to perform other functions. However, Mg^{++} and Mn^{++} can be chelated by the nucleoside triphosphates (Lowenstein, 1958). The concentration of the nucleoside triphosphates in the reaction mixtures used for determining the effect of DNA was 17.2 x 10^{-4} M. Assuming each triphosphate was capable of chelating a Mg^{++} or Mn^{++} cation, the total calculated concentration of bound divalent metal ions would be 29 x 10^{-4} M (11.8 x 10^{-4} plus 17.2 x 10^{-4}). This brings the total bound metal ion concentration to within a factor of 2 of the divalent cation concentration initially added to the reaction mixture. However, not all of the triphosphates or DNA phosphates may bind Mg^{++} or Mn^{++}.

The inhibition RNA synthesis at high ribonucleoside triphosphate concentrations was observed. This phenomenon may also be explained on the basis of Mg^{++} and Mn^{++} binding to both DNA phosphate and the triphosphates. The concentration of DNA phosphate in these reaction mixtures was 7 x 10^{-4} M. The concentration of the triphosphates at which inhibition was first observed was 40 x 10^{-4} M. Since the concentration of divalent metal ions was only 50 x 10^{-4} M, it must be suspected that the Mg^{++} and Mn^{++} concentrations were insufficient and rate limiting.

When the 60% Ammonium Sulfate Fraction II was assayed at temperatures below 30°C, a lag phase in RNA synthesis was

observed. Zillig, Fuchs and Millette (1966) have observed a lag in the synthesis of RNA when the E. coli enzyme was assayed at 20°C. When the P. putida enzyme was preincubated with DNA before addition of the triphosphates, the lag phase was reduced. These results suggest that either the binding or the initiation reaction was affected by low temperatures. Richardson (1966) has observed that binding of T_4 DNA to E. coli RNA polymerase was rapid and complete at 0 to 4°C. Therefore, the initiation reaction is the process which is probably affected by temperature.

In vitro synthesis of RNA does not proceed equally with each DNA template. This observation may be explained on the basis that there are physical as well as chemical differences among the DNA templates. T_4 and gh-1 DNA have smaller *in vivo* molecular weights than does P. putida and probably salmon sperm DNA. It is expected that the large DNA molecules would be more susceptible to fragmentation during the isolation procedure. Fragmentation of gh-1 DNA does not occur during its purification. Furthermore, the purification of the bacteriophage DNA yielded a template having a greater A_{260}: A_{280} ratio than did P. putida. Both T_4 and gh-1 DNA are linear. However, T_4 contains the unusual glucosylated hydroxymethyl cytosine, whereas, gh-1 DNA contains no unusual bases (Lee and Boezi, 1966).

The assay employed for the measurement of the rate of pyrophosphate formation is unique. Its development was aided by the fortuitous facts that none of the enzymes in the assay have a pH optimum far from that of RNA polymerase and that

none of the components in the assay of RNA polymerase inhibit the coupling enzymes at the concentrations employed in the assay. The assay permits the kinetic study of the formation of pyrophosphate in the spectrophotometer where the results are observed and plotted as an infinite series of points.

Using the spectrophotometric assay it was shown that the RNA polymerase catalyzed formation of pyrophosphate was DNA dependent and required the nucleoside triphosphates for activity. Furthermore, pyrophosphate formation was shown to be inhibited by nogalamycin and actinomycin D, two specific inhibitors of the synthesis of RNA. When DNase was added to the reaction mixture, no pyrophosphate formation was observed. Finally, the rate of pyrophosphate formation was linearly dependent upon the concentration of the enzyme.

The advantage of the spectrophotometric assay in the study of RNA polymerase is that the reaction may be studied under conditions such that neither RNA nor pyrophosphate accumulate, i.e. in the presence of RNase. When the experiment was performed, it was found that RNase stimulated the reaction by up to 1.4 fold. Krakow measured $[^{32}PP_i]$ release from γ-^{32}P labeled ribonucleoside triphosphates during the reaction of RNA polymerase. It was observed that when pancreatic ribonuclease and T_1 ribonuclease were added to DNA-dependent RNA polymerase reactions, there was a stimulation of $[^{32}PP_i]$ release and its formation was linear for relatively longer periods. The results were consistent with the hypothesis that RNA formed during the course of the

reaction inhibited the reaction.

It was apparent from the comparison of the two methods of assay that pyrophosphate formation was linear for longer periods and was of greater extent than was the observed RNA synthesis. Two possible explanations are considered. The first implicates the action of RNase to explain the results. That RNase is present in the 60% Ammonium Sulfate Fraction II was demonstrated by the reduction in TCA insoluble radioactivity following 40 minutes of reaction. A steady state between synthesis and degradation of RNA probably existed at 40 minutes of reaction. As the RNA polymerase reaction began to stop, the RNase degraded more RNA than was synthesized. The total RNA in the reaction mixture decreased as was observed. The spectrophotometric assay, however, measured the formation of pyrophosphate. The presence of RNase, as has been shown, stimulated the reaction and prevented the formation of the complex which would stop further RNA synthesis. Thus, pyrophosphate would occur long after the steady state rate of RNA synthesis had been achieved.

It has been demonstrated that high RNase activities do not exist in the 60% Ammonium Sulfate Fraction II. Therefore, the question of whether or not sufficient RNase activity is present to account for the above explanation must be answered.

The second possibility suggests that following the formation of the complex which prevents further synthesis of RNA, the nucleoside triphosphates continue to be cleaved to the

monophosphates and pyrophosphate by a pyrophosphorylase-like activity of the bound enzyme. The bound enzyme, in effect, would be uncoupling RNA synthesis from pyrophosphate formation.

A choice between the two possibilities presented above awaits studies on more highly purified fractions which can be demonstrated to be free of RNase. Further purification and characterization of DNA-dependent RNA polymerase will be performed by this investigator as a part of his Ph. D. program.

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