DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA: PURIFICATION AND CHARACTERIZATION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
JAMES CARL JOHNSON
1971

This is to certify that the

thesis entitled
DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA: PURIFICATION AND CHARACTERIZATION

presented by

JAMES CARL JOHNSON

has been accepted towards fulfillment
of the requirements for
Ph. D. degree in Microbiology

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Date April 1, 1971

ABSTRACT

DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA: PURIFICATION AND CHARACTERIZATION

By

James Carl Johnson

The objectives of this study were to obtain homogeneous DNA-dependent RNA polymerase from Pseudomonas putida, to determine the size and structure of the enzyme, and to study properties of the enzyme-mediated synthesis of RNA.

Two forms of RNA polymerase (nucleoside triphosphate RNA nucleotidyltransferase, EC 2.7.7.6) from P. putida were resolved by chromatography on phosphocellulose and subsequently purified to greater than 98 per cent of homogeneity. As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the polypeptide subunit structures of the two forms of the enzyme were $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$. The molecular weights of polypeptides α, β, β', and σ were 44,000, 155,000, 165,000, and 98,000, respectively. The sedimentation coefficients of $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ as determined by sucrose gradient centrifugation in 0.40 M potassium acetate were 13 S and 12 S, respectively,

but in 0.05 M potassium acetate, the sedimentation coefficients of the aggregate forms of $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ were 19 S and 25 S. Similar results were obtained by analytical ultracentrifugation. Polyacrylamide gel electrophoresis of $\alpha_2\beta\beta'\sigma$ or of $\alpha_2\beta\beta'$ resulted in several bands of protein. Each protein band was enzymatically active in the unprimed synthesis of poly A·poly U, thereby suggesting that the multiple bands represented aggregate forms of the enzyme.

^{35}S-labeled P. putida RNA polymerase $\alpha_2\beta\beta'\sigma$ was purified from labeled cells which had been grown on a minimal growth medium containing ^{35}S-labeled sulfate. As shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis the ^{35}S-labeled-enzyme was at least 98 per cent homogeneous. The specific radioactivity of the enzyme was 2.3×10^7 cpm per mg of protein. Analysis of ^{35}S content of each polypeptide subunit showed that the amount of ^{35}S of β', β, and σ relative to α was 3.6 to 3.6 to 2.2 to 1.0, respectively. The amount of β' plus β in the Initial Extract was 1.2 per cent of the total protein as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. From the amount of β' plus β relative to the total protein and the formula weight of $\alpha_2\beta\beta'\sigma$, it was calculated that there were approximately 5,000 molecules of $\alpha_2\beta\beta'\sigma$ per P. putida cell.

The reaction catalyzed by RNA polymerase was followed by measuring the incorporation of ^{3}H labeled-ribonucleoside monophosphates into RNA or by measuring the

formation of inorganic pyrophosphate. An enzymic method for the determination of inorganic pyrophosphate was developed. Inorganic pyrophosphate was quantitatively determined from the amount of NADPH formed via the action of UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The method for the determination of inorganic pyrophosphate was used for the assay of RNA polymerase by coupling the generation of inorganic pyrophosphate by RNA polymerase with NADPH formation. A mole of inorganic pyrophosphate released by RNA polymerase resulted in the reduction of a mole of $NADP^+$. By means of the radioactive assay of RNA polymerase or the assay for inorganic pyrophosphate, it was shown that $\alpha_2\beta\beta'\sigma$ was 4- to 5-fold more active in transcribing native bacteriophage gh-1 DNA than was $\alpha_2\beta\beta'$. With denatured DNA or poly d(A-T) as template, $\alpha_2\beta\beta'$ was twice as active as $\alpha_2\beta\beta'\sigma$. The rate of the DNA-directed polymerization reactions catalyzed by $\alpha_2\beta\beta'\sigma$ in the presence of pancreatic ribonuclease was increased 10 per cent, whereas that rate catalyzed by $\alpha_2\beta\beta'$ was increased 80 to 110 per cent.

DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF *PSEUDOMONAS PUTIDA*: PURIFICATION AND CHARACTERIZATION

By

James Carl Johnson

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1971

DEDICATION

To my parents who provided the
opportunity and encouragement
and to my wife who was blessed
with patience and understanding.

ACKNOWLEDGEMENTS

My sincere thanks and appreciation for the help and guidance in all phases of my graduate program are expressed to Dr. John A. Boezi. His insistance on clarity and simplicity in the design of experiments and in writing has been a great help to me. My thanks also go to Dr. Harold L. Sadoff whose efforts through the Department of Microbiology and Public Health enabled me to continue the research program with Dr. Boezi.

Sincere appreciation is also expressed to the other members of my Ph.D. guidance committee, Dr. Philipp Gerhardt, Dr. Ralph N. Costilow, and Dr. Robert R. Brubaker. I thank the other members of the Biochemistry and Microbiology and Public Health Departments with whom I have been associated for advice and discussions. Thanks are given to Gary Gerard, Bob Blakesley, Howard Towle, Kathy Rose, and Drs. Lucy Lee, Robert Armstrong, Ken Payne, and Seizen Toyama for discussions. I am indebted to Mrs. Monique DeBacker for technical assistance.

I thank the Departments of Biochemistry and Microbiology and Public Health and the National Science Foundation and the National Institutes of Health for financial support.

I especially thank the Department of Microbiology and Public Health for presenting a Graduate Office Scholarship to me in the spring of 1969.

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May 1, 1971

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GENERAL INTRODUCTION

The model for gene expression, developed nearly fifteen years ago, states that genetic information encoded in DNA in the form of deoxyribonucleotide sequences is transcribed into ribonucleotide sequences of RNA. Transcription is the process involving base pairing, whereby the genetic information contained in DNA is used to order a complementary sequence of bases in an RNA chain. Several species of RNA molecules which are transcribed from a DNA template have been described. These species include messenger RNA, ribosomal RNA, and transfer RNA. Each of these species of RNA is specifically involved in the synthesis of polypeptides. Ribosomal RNA is the structural nucleic acid component of ribosomes. Transfer RNA is the species of RNA that is able to accept and covalently combine with an amino acid and to hydrogen bond with a messenger RNA nucleotide triplet. Only messenger RNA, however, carries the information which specifies the primary structure of polypeptides. Translation of the information encoded in messenger RNA to form the primary structure of polypeptides is the second step in gene expression.

Transcription of DNA is the essential first step in gene function. Considering the importance of gene function in biology, one would like to know the details of control and mechanism of processes required for transcription. One approach to the study of transcription has been to search for the activities which are required for *in vitro* synthesis of RNA from DNA templates in cell-free extracts. This approach was adopted by several laboratories nearly ten years ago and led to the discovery of an activity which catalyzed the synthesis of RNA from a DNA template.

The enzyme that catalyzes transcription of DNA is RNA polymerase. It was first isolated from bacterial extracts but has recently been isolated from extracts of a variety of prokaryotic and eukaryotic cells. Genetic experiments have been used to demonstrate that the RNA polymerase prepared from bacteria is the major, if not the only, enzymatic activity responsible for the synthesis of messenger, ribosomal, and transfer RNA. Recently, an effort has been directed toward the determination of the subunit polypeptide structure of the purified enzyme. This approach has led to the discovery of protein factors which have the property of influencing the enzyme-mediated reaction *in vitro*. Until now these studies have been generally restricted to the RNA polymerase obtained from the extensively studied bacterium, *Escherchia coli*. Two varieties of RNA polymerase have been prepared from *E. coli*.

These varieties differed in structure by a single polypeptide chain, which was called sigma. Sigma was found to cause the RNA polymerase molecule to initiate synthesis at correct sites on the DNA templates used for *in vitro* synthesis of RNA.

When work was begun for this thesis, only a partial purification of the enzyme from *E. coli* had been reported. It was decided that a second example of RNA polymerase from bacterial sources should be provided for study. *Pseudomonas putida* was utilized as the bacteria from which RNA polymerase was purified because the bacteria was being used in related studies of RNA structure and methylation. In addition, a bacteriophage which was specific for *P. putida* was isolated. It was of interest to determine whether the host RNA polymerase would transcribe the bacteriophage DNA. The procedure described herein for the purification of RNA polymerase resulted in two forms of the enzyme which were nearly homogeneous. One of the forms contained 100 per cent of the amount of sigma capable of being associated with the enzyme. The other form of RNA polymerase was completely devoid of sigma.

This thesis is organized into four major sections. The first is a literature review in which much of the information on bacterial RNA polymerase structure and factors has been described. The second and third sections are composed of articles describing the assay, purification

and structure of the enzyme. These articles have been included in the form of reprints from the journals in which they were published. The fourth section consists of a manuscript which is to be published. It concerns the purification and properties of ^{35}S-labeled RNA polymerase from P. putida and the use of the radioactive enzyme to investigate the release of sigma factor following the initiation of RNA synthesis.

LITERATURE SURVEY

Introduction

The first reports of the detection, isolation, and purification of a DNA-dependent RNA polymerase from bacterial sources occurred in the early 1960's (1,2,3,4,5). As of now, a prodigious literature concerning bacterial RNA polymerase has accumulated. The extent and variety of topics treated in the literature are beyond the scope of this survey. The following reviews and symposia should be consulted for a comprehensive treatment of the literature (6,7,8,9,10,11).

RNA polymerase has been purified from the following bacterial sources: Escherchia coli (several strains) (1,2,5,12,13,14); Azotobacter vinelandii (3,15); Micrococcus luteus formerly Micrococcus lysodeiktus (4); Bacillus subtilis (16); and Pseudomonas indogophera (17). The most extensively purified enzymes, those obtained from the various strains of E. coli and the RNA polymerase from A. vinelandii, are homogeneous as determined from sedimentation and electrophoretic studies. E. coli RNA polymerase has been the subject of most of the kinetic and structural studies.

The Essential Role of RNA Polymerase in Vivo

Evidence for the role of DNA-dependent RNA polymerase was provided through a study of E. coli mutants having alterations in a structural gene(s) for RNA polymerase which rendered the cell temperature sensitive or resistant to the rifamycin antibiotics. A mutant of E. coli which rendered the cell temperature sensitive (growth at 30° but not at 42°) was unable to incorporate labeled uridine into RNA at the non-permissive temperature (18). RNA polymerase isolated from the mutant grown at 30° was active when tested in vitro at 30°, but was considerably less active relative to the wild type RNA polymerase when tested in vitro at 42°. Subsequent analysis indicated that the E. coli mutant had a structurally altered RNA polymerase molecule. The nature of the alteration has not been determined.

Rifamycin or its derivative rifampicin when added to a growing culture of E. coli inhibited growth (19,20,21). Wild type E. coli cells were not able to incorporate labeled uridine into RNA following addition of rifampicin to the culture. It was shown that rifampicin inhibited the DNA-directed synthesis of RNA by RNA polymerase in vitro (22,23). Other studies determined that rifampicin binds to RNA polymerase and blocks initiation of RNA synthesis (24,25). Rifampicin resistant mutants of E. coli have been isolated which synthesize an altered RNA polymerase molecule (21). Rifampicin did not bind to the altered enzyme. Consequently, the antibiotic did not inhibit RNA synthesis in vivo or in vitro.

The studies on temperature sensitive and rifamycin resistant mutants together with the extensive biochemical studies on the RNA polymerase lead to the conclusion that the RNA polymerase as isolated by the various purification procedures is the "genetic transcriptase" (26), that is, the essential enzyme responsible for most if not all RNA synthesis from a DNA template in the bacterial cell.

Bacterial RNA Polymerase Structure

The subunit structure of purified E. coli RNA polymerase has been investigated. SDS or urea polyacrylamide gel electrophoresis of RNA polymerase which was denatured by means of SDS or urea in the presence of reducing agents resolved either three or four polypeptide subunits (27,28). The RNA polymerase which had been chromatographed on phosphocellulose contained three polypeptide subunits designated β', β, and α, with molecular weights 165,000, 155,000, and 39,000 respectively. The RNA polymerase which was not purified by phosphocellulose chromatography contained β', β, α, and σ polypeptide subunits. Sigma (σ) has a molecular weight of 95,000 (28). The molar ratio of the polypeptides obtained from the phosphocellulose purified enzyme was 1β' : 1β : 2α. The minimal polypeptide subunit formula for this enzyme was $\alpha_2\beta\beta'$. The RNA polymerase which was purified by gel filtration and sucrose or glycerol gradient centrifugation, but not by phosphocellulose chromatography contained

β', β, α, and σ in a molar ratio of 1β' : 1β : 2α : 1σ. The minimal subunit formula for this enzyme was $\alpha_2\beta\beta'\sigma$. The minimal formula weights calculated for $\alpha_2\beta\beta'$ and $\alpha_2\beta\beta'\sigma$ were 3.98×10^5 and 4.93×10^5 respectively. These minimal formula weights correspond to the monomeric forms of the enzyme.

In addition to the β', β, σ, and α polypeptide subunits of $\alpha_2\beta\beta'$ and $\alpha_2\beta\beta'\sigma$, a polypeptide (omega, ω) of molecular weight 9,000 was variably associated with the purified enzyme (13,29). It is not known whether ω is a structural component of the enzyme or whether it has any function in RNA synthesis.

Sephadex G-200 chromatography of denatured $\alpha_2\beta\beta'$ in the presence of SDS or urea resulted in the separation of β plus β' from α (27). The β plus β' polypeptide subunits were separated on DEAE-cellulose in urea (27). The molecular weights of the β plus β' and the α subunits determined by SDS-polyacrylamide gel electrophoresis were found to be the same as those determined by sedimentation equilibrium studies with the isolated subunits.

Amino acid compositions of α and of β plus β' as well as the $\alpha_2\beta\beta'$ enzyme have been performed (27). Methionine is the only N-terminal amino acid of either α, β, or β'.

Prior to the establishment of the polypeptide subunit structure of RNA polymerase, studies of the sedimentation properties of the enzyme were confusing. Two,

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three, or more species of molecules were observed in sedimentation velocity experiments with highly purified RNA polymerase (30,31). A partial understanding of the effects of ionic strength, subunit composition, preferential hydration, and irreversible dissociation has helped to clarify much of the confusion. Berg and Chamberlin analyzed the two species of RNA polymerase, $\alpha_2\beta\beta'$ and $\alpha_2\beta\beta'\sigma$, by sedimentation velocity and equilibrium experiments (29). Apoenzyme ($\alpha_2\beta\beta'$) in buffers of ionic strength above 0.26 behaved as a single sedimenting species ($S^0_{20,w}$=12.6). The dependence of sedimentation coefficient on the protein concentration was slight. When apoenzyme was centrifuged in buffers with ionic strength below 0.26, a variety of aggregated species was observed with average sedimentation coefficients of 44 to 48 S. Holoenzyme ($\alpha_2\beta\beta'\sigma$) in buffers with ionic strength of 0.12 or higher sedimented as a single species with $S^0_{20,w}$=15.0. When $\alpha_2\beta\beta'\sigma$ was sedimented in low ionic strength buffers, the holoenzyme aggregated to a dimer with $S^0_{20,w}$=23.0. RNA polymerase with less than a full complement of σ sedimented as a mixture of apo- and holoenzyme at both low and high ionic strengths. The molecular weights determined by sedimentation velocity analysis were in agreement with those calculated from SDS-polyacrylamide gel electrophoresis studies. Molecular weight determinations of $\alpha_2\beta\beta'$ and $\alpha_2\beta\beta'\sigma$ by sedimentation equilibrium have not been definitive.

Sedimentation of E. coli RNA polymerase in increasing concentrations of urea indicated that the $\alpha_2\beta\beta'$ form could undergo large conformational changes or could dissociate into smaller species (32). RNA polymerase dissociates into its polypeptide subunits in high urea concentrations (27,33). The 9 S species derived from RNA polymerase in low urea concentrations may represent a mixture of $\alpha\beta$ and $\alpha\beta'$ (32). Renaturation of urea or lithium chloride dissociated enzyme to obtain a partially active enzyme which has the characteristics of the non-dissociated enzyme has been described (34,35).

The polypeptide subunit structure of the A. vinelandii and the B. subtilis RNA polymerase was found to be similar to the E. coli polymerase. The A. vinelandii RNA polymerase consists of an apoenzyme with polypeptide subunit formula $\alpha_2\beta\beta'$ and a holoenzyme $\alpha_2\beta\beta'\sigma$ (36). The molecular weights of α, β, β', and σ were virtually identical to those from E. coli RNA polymerase. B. subtilis RNA polymerase, although not totally purified, contained polypeptide subunits of molecular weight 155,000, 120,000 57,000 and 45,000 (37). Two polypeptides at molecular weight 155,000 were resolved. The molar ratio of the 155,000 dalton material to the 45,000 dalton material was 1:1. Chromatography of the enzyme on phosphocellulose removed the 57,000 dalton polypeptide from the enzyme and resulted in an enzyme activity with altered template

specificities. The 57,000 dalton polypeptide is therefore similar to E. coli RNA polymerase σ factor in function but not in size.

Infection of E. coli by T_4 bacteriophage results in modification of the host RNA polymerase subunit structure. The α polypeptide chains are modified apparently by the addition of 5' AMP in a T_4 phage-specific reaction (38,39). This modification occurs early in infection. Adenylation of RNA polymerase from E. coli by ATP with an activity from uninfected cells has also been reported (40). The adenylated enzyme is less active in RNA synthesis than is the non-adenylated form. Both the β and β' polypeptides are modified late in T_4 infection (39,41). The host σ subunit is replaced by a T_4-specific σ factor early in infection (42,43). The T_4-specific σ has the same molecular weight as the host σ, and it confers different template specificities to the apoenzyme. Late in T_4 infection this T_4-specific σ may be replaced by yet a different T_4-specific σ (41).

Infection of E. coli with bacteriophage T_7 results in the de novo synthesis of an entirely new type of RNA polymerase molecule (44). The T_7-specific RNA polymerase is a product of gene 1 and is distinct from the host polymerase in that its activity is not inhibited by rifamycin, streptolydigin, or by antibody specific for the host enzyme. The enzyme consists of a single polypeptide

subunit of molecular weight 110,000 as determined by SDS-polyacrylamide gel electrophoresis.

Structural alteration of B. subtilis RNA polymerase during sporulation has been reported (37). The sporulation RNA polymerase has a polypeptide of 110,000 daltons which is not found in the enzyme from vegetative cells. The sporulation enzyme was found to contain only one of the 155,000 dalton polypeptides found in the vegetative RNA polymerase.

Protein Factors Affecting RNA Synthesis

A series of sequential reactions serve to describe the steps in RNA synthesis by RNA polymerase from a DNA template. These steps in RNA synthesis are association, initiation, elongation, termination, and dissociation. During association, the RNA polymerase molecule interacts with and binds to sites on the DNA template. Initiation occurs with the formation of the first phosphodiester bond between the 5'-terminal and the second ribonucleotide. Elongation follows with the sequential addition of ribonucleoside monophosphates and the release of pyrophosphate. At termination the synthesis of RNA ceases. Dissociation follows termination with the release of the RNA product and the RNA polymerase from the DNA template.

Sigma

Several proteins have been discovered which have the property of affecting one or more of the steps in RNA

synthesis by bacterial RNA polymerase *in vitro*. One such protein is the sigma (σ) polypeptide subunit of *E. coli* RNA polymerase (28). The *E. coli* sigma subunit has been isolated from holoenzyme ($\alpha_2\beta\beta'\sigma$) by chromatography on phosphocellulose (28).

The efficiency by which a DNA template may be transcribed by RNA polymerase is affected by the presence or absence of σ. The efficiency by which RNA polymerase transcribes a particular DNA template *in vitro* is measured from the overall rate of RNA synthesis which reflects the rate limiting step in either association, initiation, elongation, termination or dissociation. The initiation reaction may be followed by means of the exchange reaction which occurs between inorganic pyrophosphate and the ribonucleoside triphosphates involved in initiation (45). Measurements of RNA synthesis and the pyrophosphate exchange reaction showed that transcription from *E. coli* bacteriophage T_4 was dependent upon the presence of sigma (46). It was concluded that σ was probably necessary for the formation of the first phosphodiester bond catalyzed by RNA polymerase.

The asymmetry of RNA synthesis from native DNA is affected by the presence of σ in the reaction. RNA synthesis *in vivo* is asymmetric and must initiate and terminate at specific sites on the DNA template. RNA synthesis *in vitro* using holoenzyme is asymmetric on

E. coli bacteriophage T_7 or λ DNA and only the early phage genes are transcribed (6,47). Also, E. coli holoenzyme catalyzes asymmetric transcription of DNA from B. subtilis bacteriophage SP01 (48), and animal viruses SV40 and adenovirus (49,50). In contrast, E. coli apoenzyme catalyzes symmetric transcription from a variety of templates (T_4, F1, and T_7 bacteriophage DNA) (47,51,52). RNA synthesis by apoenzyme is initiated at non-specific sites on the DNA molecules. Therefore, it may be concluded that σ affects a specific interaction between the enzyme and functional sites on the DNA. The nature of the functional sites which specify holoenzyme recognition may be the same or similar on a variety of DNA molecules from different sources. The functional sites for holoenzyme initiation may be equivalent to the promotor regions (53). Thus, sigma may direct the initiation of specific RNA chains by identifying specific DNA promotor regions to which the enzyme may bind.

The effect of σ on the binding of RNA polymerase to DNA has been studied by a nitrocellulose filter binding assay (54,55). It was found that σ was not required for formation of an RNA-polymerase-DNA complex. The stability of the complex was greatly enhanced, however, by the presence of σ. The formation of the DNA-RNA polymerase holoenzyme complex was temperature sensitive which suggests that a cooperative reaction was involved (39). No more

than 7 or 8 molecules of holoenzyme could tightly bind to T_7 DNA. These data suggest that σ is involved in the formation of a highly stable enzyme-promotor complex which may entail opening of the DNA helix in localized specific regions.

From the above studies it may be hypothesized that σ is required for recognition of promotor regions on DNA, or σ is required for tight binding of the apoenzyme to the DNA, or both. By the first hypothesis, the specific information for site recognition is located in the σ subunit. By the second, the site recognition information is located in the apoenzyme. Then, sigma would act as an allosteric effector for tight, site-specific binding. Sigma, in the absence of apoenzyme, will not bind to DNA (55).

At some stage of RNA synthesis by E. coli and A. vinelandii holoenzyme in vitro σ is apparently released from the enzyme (46,52,56,57). This has led to the postulation of the sigma cycle by Traverse and Burgess (56). The essential feature of the sigma cycle is that once RNA synthesis starts, sigma is released to bind to other apoenzyme molecules which may subsequently bind to DNA at specific promotor sites and initiate RNA synthesis. Release of σ has not been demonstrated in vivo and there has been no direct demonstration of the release of σ from holoenzyme in vitro until now (58 and unpublished observations). The rationale for the release of sigma from holoenzyme during RNA synthesis remains obscure.

The incubation of holoenzyme with a variety of ribopolymers in the absence of nucleoside triphosphates results in the apparent release of σ (36). This suggests that if σ release occurs *in vivo* it may be mediated by RNA. However, much of the RNA synthesized *in vivo* is bound to either ribosomes in the case of messenger RNA (57) or protein in the case of ribosomal RNA (60) prior to release from the DNA-enzyme-RNA complex and, thus, the RNA may be unavailable for mediation of σ release *in vivo*.

Psi

A class of proteins which act as positive control elements in transcription have been isolated. These proteins which act as secondary specificity determinants have been named psi (Ψ) (61). One such psi factor which is specific for the ribosomal RNA genes (Ψ_r) has been partially purified from *E. coli* (61). In the presence of Ψ_r, but not in its absence, *E. coli* RNA polymerase holoenzyme catalyzes the synthesis of ribosomal RNA *in vitro* using *E. coli* DNA as the template. The specificity of transcription by apoenzyme is not affected by Ψ_r.

Ψ_r activity has been found to be associated with purified Qβ replicase (61). The RNA replicating enzyme purified from bacteriophage Qβ infected *E. coli* contains a phage specific polypeptide chain of 69,000, and three host specific polypeptides of molecular weights 33,000,

47,000, and 74,000 (62,63). The Ψ_r activity is associated with the two small host specific polypeptides.

Another psi-like factor which has positive control over the lac operon is CAP (64). CAP is a protein which requires bound cyclic AMP for its activity. The cyclic AMP-CAP complex is necessary for the transcription of the β-galactosidase genes by holoenzyme *in vitro* (65).

Rho

RNA chain termination and release may, in part, be mediated by a protein isolated and extensively purified from *E. coli* ribosome-free extracts. This protein has been named rho (ρ) (66). The protein is probably composed of four subunits each with a molecular weight of 50,000 as determined by SDS-polyacrylamide gel electrophoresis and sedimentation velocity experiments. Analysis of the size of RNA transcription products formed by *E. coli* holoenzyme using λ DNA as the template in the absence of added ρ determined that most of the RNA products had sedimentation coefficients which were greater than 22 S. In the presence of added ρ, two classes of RNA having sedimentation coefficients of 7 or 12 S were formed. The 7 and 12 S RNA products made *in vitro* are the same size as the early λ-specific messenger RNA made *in vivo*. The 12 S RNA molecule is probably the messenger RNA of the N gene of λ DNA. The protein product of the N gene is required for transcription of the late λ genes *in vitro* by *E. coli* holoenzyme (67).

Thus, it has been suggested that the N gene protein is an anti-ρ factor, a protein which interferes with the activity of ρ and permits DNA transcription into the late λ genes (67,68).

M-factor

Psi and rho proteins were obtained from the supernatant fraction of E. coli cells. Another protein, M-factor, has been purified from the ribosomal fraction of the cell (69). Like sigma, M-factor is a protein which has a sedimentation coefficient of 4 S and increases the rate of RNA synthesis by E. coli RNA polymerase holoenzyme in vitro. M-factor binds to purified E. coli RNA polymerase holoenzyme. The available data suggest that M-factor participates in RNA synthesis in vitro by combining with holoenzyme and affecting an event which occurs during or close to initiation (70).

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ARTICLE 1

AN ENZYMIC METHOD FOR DETERMINATION OF INORGANIC PYROPHOSPHATE AND ITS USE AS AN ASSAY FOR RNA POLYMERASE

By

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Reprinted from Analytical Biochemistry, 26, 137 (1968)

Reprinted from ANALYTICAL BIOCHEMISTRY, Volume 26, Number 1, November 1968

ANALYTICAL BIOCHEMISTRY **26**, 137–145 (1968)

An Enzymic Method for Determination of Inorganic Pyrophosphate and Its Use as an Assay for RNA Polymerase

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Received February 13, 1968

Inorganic pyrophosphate (PP_i) is a product of many biosynthetic processes. In biopolymer formation PP_i arises (*a*) in protein synthesis at the stage of amino acid activation, (*b*) in nucleic acid synthesis at the polymerization step, (*c*) in polysaccharide synthesis and saccharide interconversion in the formation of the glycosyl donor, and (*d*) in lipid synthesis at the stage of fatty acid activation.

The usual method of PP_i analysis involves its hydrolysis to inorganic orthophosphate (P_i) followed by P_i determination by the Fiske and SubbaRow method (1). Both inorganic and organic phosphate compounds interfere in this analysis.

Albrecht, Bass, Seifert, and Hansen (2) reported the purification and crystallization of UDP-glucose pyrophosphorylase from calf liver. The availability of this crystalline enzyme has made possible the development of a specific enzymic method for the determination of PP_i. Using phosphoglucomutase and glucose-6-phosphate dehydrogenase—both of which are commercially available, and UDP-glucose pyrophosphorylase, PP_i can be quantitatively determined from the amount of NADPH formed in the following series of reactions:

$$PP_i + \text{UDP-glucose} \xrightarrow{\text{UDP-glucose pyrophosphorylase}} \text{UTP} + \text{glucose-1-P}$$

$$\text{glucose-1-P} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-P}$$

$$\text{glucose-6-P} + \text{NADP} \xrightarrow{\text{glucose-6-P dehydrogenase}} \text{gluconolactone-6-P} + \text{NADPH}$$

Under suitable assay conditions, one equivalent of NADPH will be formed per equivalent of PP_i.

This procedure, i.e., coupling the determination of PP_i with the forma-

tion of NADPH via the three enzyme-catalyzed reactions, can also be used as an assay for enzymes which generate PP_i as a reaction product. A description of the enzymic method for the determination of PP_i and the application of the method for the assay of DNA-dependent RNA polymerase is the subject of this report.

MATERIALS AND METHODS

NADP, UDP-glucose, glucose-6-phosphate dehydrogenase, and the 5′-phosphate derivatives of adenosine, guanosine, uridine, and cytidine were obtained from P-L Biochemicals, Inc. Calf thymus DNA, pancreatic RNase (type I-A), and phosphoglucomutase were purchased from Sigma Chemical Co. DNase I (electrophoretically purified) and inorganic pyrophosphatase were obtained from Worthington Biochemical Corp. [^{3}H]-ATP and [^{3}H]-CTP were purchased from Schwarz BioResearch, Inc. Actinomycin and nogalamycin (3) were gifts from Merck, Sharp, & Dohme, and The Upjohn Company, respectively.

UDP-glucose pyrophosphorylase was isolated from calf liver by the procedure of Albrecht, Bass, Seifert, and Hansen (2) and recrystallized twice. RNA polymerase was purified from *Pseudomonas putida* A.3.12 by a procedure that will be the subject of another communication (4). For the experiments reported here, ammonium sulfate fraction II (A.S.II) of RNA polymerase was used. No inorganic pyrophosphatase activity has been detected in this fraction. Using 100 μg/ml *P. putida* bacteriophage gh-1 DNA as template, A.S.II had a specific activity of 2500–3000 mμmoles CTP converted into a trichloroacetic acid insoluble form per hour per milligram protein (radioactive assay). In the spectrophotometric assay, A.S.II had a specific activity of 5000–5500 mμmoles NADPH formed per hour per milligram protein.

P. putida bacteriophage gh-1 DNA (5, 6) was purified by the method described by Thomas and Abelson (7). The concentrations of both gh-1 DNA and calf thymus DNA were determined from their ultraviolet absorptions using the extinction coefficient $E_{260}^{1\%} = 200$. Calf thymus DNA was denatured by heating at 100° for 10 min, followed by quick cooling.

For the determination of PP_i by the UDP-glucose pyrophosphorylase assay system, the reaction mixture contained, in 0.5 ml: 50 μmoles Tris acetate buffer (pH 8.0), 1 μmole magnesium acetate, 0.2 μmole NADP, 0.2 μmole UDP-glucose, excess phosphoglucomutase and glucose-6-phosphate dehydrogenase, from 5 to 20 mμmoles PP_i, and enough UDP-glucose pyrophosphorylase so that reaction was complete in 15 to 30 min. NADPH formation was measured at 340 mμ in a Beckman model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder (8). Calculation of the number of mμmoles of NADPH

formed was made using the molar extinction coefficient of 6.22×10^3 (9).

The reaction mixture employed for the formation of RNA complementary to DNA by RNA polymerase contained, in 0.5 ml: 10 μmoles Tris acetate buffer (pH 8.0), 1 μmole magnesium acetate, 0.25 μmole manganese acetate, 0.2 μmole each of ATP, GTP, CTP, and UTP, 0.1 μmole NADP, 0.1 μmole UDP-glucose, excess phosphoglucomutase, glucose-6-phosphate dehydrogenase, and UDP-glucose pyrophosphorylase, 65 μg gh-1 DNA, and 4 to 17 μg RNA polymerase. Before initiating RNA synthesis with RNA polymerase, a 20 min incubation was required to allow reaction of PP_i contaminating the nucleoside triphosphates. NADPH formation was measured at 340 mμ in the spectrophotometric assay. For the radioactive assay, [^{3}H]-CTP (5×10^6 cpm/μmole) or [^{3}H]-ATP (3×10^6 cpm/μmole) was used. Material, insoluble in 10% cold trichloroacetic acid, was collected on nitrocellulose membrane filters and assayed for radioactivity in a liquid scintillation spectrometer.

The reaction mixture for polyriboadenylate (poly A) synthesis by RNA polymerase contained, in 0.5 ml: 10 μmoles Tris acetate (pH 8.0), 1 μmole magnesium acetate, 0.25 μmole manganese acetate, 0.2 μmole ATP, 0.1 μmole NADP, 0.1 μmole UDP-glucose, excess phosphoglucomutase, glucose-6-phosphate dehydrogenase, and UDP-glucose pyrophosphorylase, 50 μg heat-denatured calf thymus DNA, and 70 μg RNA polymerase. For the radioactive assay, [^{3}H]-ATP (1×10^7 cpm/μmole) was used.

RESULTS

Determination of Inorganic Pyrophosphate by the Pyrophosphorylase Assay System. The results presented in Table 1 demonstrate the equivalence between PP_i added to the pyrophosphorylase assay system and NADPH formed. For the three experiments reported, using from 5 to 20 mμmoles of PP_i per assay mixture, an equivalent amount of NADPH was formed. An equivalence between PP_i and NADPH was also found when P_i, 5′-mono-, di-, or triphosphate derivatives of adenosine or uridine, a mixture of the four nucleoside triphosphates, RNA polymerase,

TABLE 1

Determination of Inorganic Pyrophosphate by Pyrophosphorylase Assay System

PP_i added, mμmoles			NADPH formed, mμmoles		
Expt. I	Expt. II	Expt. III	Expt. I	Expt. II	Expt. III
5.0	5.2	5.6	4.9	5.0	5.3
10.0	10.0	10.0	10.1	10.0	10.4
15.0	15.0	15.1	14.8	15.2	16.0
20.0	20.0	20.2	19.5	21.1	20.0

TABLE 2
Effect of Various Agents on Determination of Inorganic Pyrophosphate

Additions to pyrophosphorylase assay system	NADPH, mμmoles
15 mμmoles PP_i	15.0–15.5
+150 mμmoles P_i	15.5
+15,000 mμmoles P_i	14.8
+200 mμmoles AMP	15.0
+200 mμmoles ADP	16.2
+200 mμmoles ATP	15.9
+200 mμmoles UMP	14.5
+200 mμmoles UDP	15.3
+200 mμmoles UTP	14.5
+200 mμmoles each of ATP, GTP, UTP, and CTP	14.6
+35 μg RNA polymerase	16.1
+250 μg denatured calf thymus DNA	16.1

or denatured calf thymus DNA were added to the assay mixture (Table 2). In other experiments, it was shown that actinomycin or nogalamycin do not interfere in PP_i determination.

Spectrophotometric Assay for RNA Polymerase. The requirements for the reduction of NADP by the pyrophosphorylase assay system concomitant with the generation of PP_i by RNA polymerase were determined. The results are given in Tables 3 and 4. If RNA polymerase, gh-1 DNA, the four nucleoside triphosphates, the purine nucleoside triphosphates, or the pyrimidine nucleoside triphosphates were omitted from the reaction mixture, no NADPH was formed (Table 3). As shown in Table 4, when DNase, inorganic pyrophosphatase, actinomycin or nogalamycin was added to the reaction mixture, little or no NADPH was formed. However, when RNase was added to the reaction mixture, the formation of NADPH was stimulated. The stimulatory effect of RNase on PP_i formation by RNA polymerase has been reported by Krakow (10) and Maitra and Hurwitz (11).

In Figure 1 the relationship between the rate of NADPH formation

TABLE 3
Requirements for NADPH Formation Concomitant with Synthesis of RNA

Components of reaction mixture	NADPH, mμmole/min/ml
Complete	1.00
−RNA polymerase	0
−gh-1 DNA	0
−ATP, GTP, UTP, CTP	0
−ATP, GTP	0
−UTP, CTP	0

TABLE 4
Effect of Various Agents on NADPH Formation Concomitant with Synthesis of RNA

Components of reaction mixture	NADPH, mμmoles/min/ml
Complete	1.00
+4 μg/ml DNase	0.02
+4 μg/ml RNase	1.38
+12 units/ml inorganic pyrophosphatase	0.03
+4 μg/ml actinomycin	0.03
+5 μg/ml nogalamycin	0

and the amount of added RNA polymerase is given. Under the assay conditions employed, the relationship was linear to the formation of 2 mμmoles NADPH/min/ml.

The total amount of RNA synthesized is proportional to the amount of RNA polymerase used (12, 13). Likewise, the total amount of NADPH formed was proportional to the amount of RNA polymerase added to the assay system. For example, when 8.5, 17, 25.5, and 34 μg/ml RNA polymerase were used, 19, 34, 50, and 64 mμmoles/ml NADPH were formed, respectively.

The kinetics of NADPH formation as measured in the spectrophotometric assay and RNA synthesis as measured by the conversion of [^{3}H]-CTP and [^{3}H]-ATP into a trichloroacetic acid insoluble form are given in Figure 2. Following a short lag, the rate of NADPH formation was 2.4 mμmoles/min/ml. The rates of incorporation of CMP and AMP were 1.1 and 0.6 mμmoles/min/ml, respectively. At the plateau, approximately 60 mμmoles/ml of NADPH was present, and 16 and 9 mμmoles/ml of CMP and AMP had been incorporated into RNA.

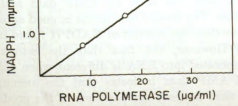

FIG. 1. Proportionality between rate of NADPH formation and amount of RNA polymerase.

For comparison of the results of the two assay procedures, it should be pointed out that the spectrophotometric assay measures the formation of NADPH as a consequence of the production of PP_i by RNA polymerase. PP_i is generated by RNA polymerase concomitant with the incorporation of each of the four nucleotides into the intrachain phosphodiester link of RNA. No distinction is made between the synthesis of trichloroacetic acid soluble or insoluble polyribonucleotides. The radioactive assay measures the incorporation of one of the four nucleotides into polynucleotides which are insoluble in trichloroacetic acid. Synthesis of oligo- or polynucleotides which are soluble in trichloroacetic acid is not detected.

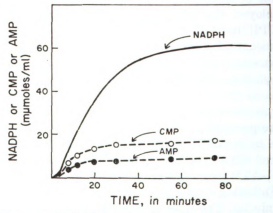

FIG. 2. Time course of NADPH formation and CMP and AMP incorporation into RNA.

The base composition of the RNA product has not been determined and is not necessarily identical to that of gh-1 DNA (mole % G + C = 57.0) which served as template (5). However, if it is assumed that the base composition of RNA is the same as the gh-1 DNA template, the total nucleotides incorporated into the TCA insoluble RNA product may be calculated from the amount of CMP and AMP incorporated. This amounts to 56 mμmoles/ml from the CMP data and 41 mμmoles/ml from the AMP data. The former estimate is in good agreement; the latter is somewhat lower than the amount of NADPH found in the spectrophotometric assay. However, the fact that the estimate of the total nucleotides incorporated into RNA is different when calculated from the CMP data and AMP data indicates that the assumption use in the calculation, i.e., that the base compositions of the product and template are identical, is not valid.

The spectrophotometric assay for RNA polymerase was used to meas-

ure NADPH formation concomitant with the generation of PP_i during the synthesis of polyriboadenylate (poly A). The kinetics of NADPH formation and [^{3}H]-AMP incorporation into poly A are presented in Figure 3. The initial rates of NADPH formation and AMP incorporation were equal. However, somewhat more NADPH was present near the end of the reaction—indicating the synthesis of some trichloroacetic acid soluble poly A or the incorporation of UTP, produced in the UDP-glucose pyrophosphorylase reaction, into polymer. No NADPH was formed when RNA polymerase was omitted from the reaction mixture or when unheated calf thymus DNA was used in place of heat-denatured calf thymus DNA.

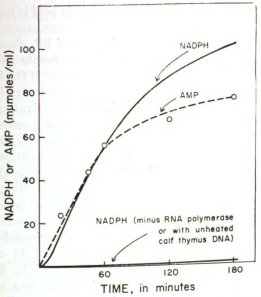

FIG. 3. Time course of NADPH formation and AMP incorporation into poly A.

DISCUSSION

The determination of PP_i via the action of UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase has proved to be accurate, sensitive, and specific. The method is limited only by the sensitivity of the quantitative determination of NADPH and by the specificity of the enzymes. Neither P_i nor any of the organic phosphate compounds tested interfered with PP_i determination.

The enzymic method for measuring PP_i has been adapted for use as an assay for RNA polymerase. Using this method, the formation of

NADPH—as a consequence of the generation of PP_i by RNA polymerase—can be easily measured at a rate of 0.1–0.2 mμmole/min/ml. Furthermore, the enzymic assay procedure permits a continuous monitoring of the RNA polymerase reaction. Consequently, a study of the reaction kinetics under a variety of conditions can be conveniently performed. In addition to these attributes, the spectrophotometric assay has certain advantages over the radioactive assay. The amount of NADPH formed is a direct measure of the total nucleotides incorporated into the intrachain phosphodiester link of the polyribonucleotide product. In the radioactive assay, a knowledge of the base composition of the product is required in order to calculate the amount of polymer formed from the incorporation of one nucleotide. Furthermore, the solubility of the polymer product in trichloroacetic or perchloric acid is not a factor in the spectrophotometric assay as it is in the radioactive assay.

The enzymic method for the determination of PP_i should find application for the assay of many enzymes which generate PP_i as a reaction product. Some examples are DNA polymerase (14), RNA-dependent RNA polymerase (15) polyriboadenylate polymerase (16), the aminoacyl-sRNA synthetases (17), and the fatty acid activating enzymes (18).

Although PP_i is a product of many biosynthetic processes, the intracellular concentration of PP_i is low due, supposedly, to the action of ubiquitous inorganic pyrophosphatases (19). The hydrolysis of PP_i may be considered to be an energetically wasteful process since the energy of the anhydride bond is lost as heat. However, in the phosphorylation of glucose in liver microsomes (20) and in the formation of phosphoenolpyruvate in the propionic acid bacteria (21) PP_i serves as an energy source. Futhermore, PP_i has been implicated in photophosphorylation in *Rhodospirillum rubrum* (22) and in oxidative phosphorylation in *Acetobacter suboxydans* (23) and in *Escherichia coli* (24). Knowledge of the intracellular levels of PP_i in a variety of organisms might provide a clue to the metabolic fate of PP_i. Methods have not been available that are sufficiently sensitive or specific for the determination of PP_i in biological material. The use of this present procedure, which is limited only by the sensitivity of the quantitative determination of NADPH and by the specificity of the enzymes, could help clarify the function of PP_i in metabolism.

SUMMARY

1. An enzymic method for the determination of PP_i has been developed. PP_i can be quantitatively estimated from the amount of NADPH formed via the action of UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. Neither P_i nor

any of the organic phosphate compounds tested interfered in the PP_i determination.

2. The method has been used for the assay of DNA-dependent RNA polymerase—by coupling the generation of PP_i by RNA polymerase with NADPH formation. The RNA polymerase catalyzed syntheses of RNA and polyriboadenylate have been assayed by this procedure.

ACKNOWLEDGMENTS

The competent technical assistance of Madame M. DeBacker is acknowledged. Supported, in part, by National Institutes of Health Grant AM 11156. Michigan Agriculture Experiment Station Journal No. 4289.

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ARTICLE 2

DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA

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Reprinted from Journal of Biological Chemistry, 216, 1222 (1971)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 246, No. 5, Issue of March 10, pp. 1222–1232, 1971
Printed in U.S.A.

Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase of *Pseudomonas putida**

(Received for publication, June 5, 1970)

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SUMMARY

Two forms of RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) from *Pseudomonas putida* were resolved by chromatography in 50% glycerol on phosphocellulose and purified. As determined by sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis, the subunit structures of the two forms of the enzyme were $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$. The molecular weights of α, β, β', and σ were 44,000, 155,000, 165,000, and 98,000, respectively. The sedimentation coefficients of $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ as determined by sucrose gradient centrifugation in 0.40 M potassium acetate were 13 S and 12 S, respectively, but in 0.05 M potassium acetate, the sedimentation coefficients were 19 S and 25 S. Similar results were obtained by analytical ultracentrifugation. Multiple protein bands resulted from polyacrylamide disc gel electrophoresis of $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$. Each band was enzymatically active in the unprimed synthesis of poly A·poly U. RNA polymerase $\alpha_2\beta\beta'\sigma$ was 4- to 5-fold more active in transcribing *P. putida* bacteriophage gh-1 DNA and coliphage T_4 DNA than was $\alpha_2\beta\beta'$. With calf thymus DNA as a template, the two forms were equally active in RNA synthesis. With denatured DNA or poly d(A-T), $\alpha_2\beta\beta'$ was twice as active as was $\alpha_2\beta\beta'\sigma$. The rates of the DNA-directed polymerization reactions catalyzed by $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ were affected differently by added RNase. The initial rate of the reaction catalyzed by $\alpha_2\beta\beta'\sigma$ was increased 10%, whereas that catalyzed by $\alpha_2\beta\beta'$ was increased 80 to 110%.

DNA-dependent RNA polymerase (ribonucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) has been purified and characterized from a number of bacterial sources (1–6). The enzyme from *Escherichia coli* has been the most extensively studied. It has been purified by a variety of procedures (1, 7–16), and its catalytic and physical properties have been studied by a number of laboratories (9, 10, 12, 13, 17–21).

A method utilizing phosphocellulose chromatography for the purification of *E. coli* RNA polymerase has been described by Burgess (22). The subunit structure of enzyme prepared by this method, designated core enzyme, was $\alpha_2\beta\beta'$ (23). Burgess, Travers, Dunn, and Bautz (24) showed that σ, a subunit which is involved in the initiation of RNA synthesis, was separated from the core enzyme by phosphocellulose chromatography. The subunit structure of the holoenzyme or complete enzyme was $\alpha_2\beta\beta'\sigma$. At the present time, only the subunit structure of the *E. coli* enzyme has been documented in detail. Knowledge of the structural as well as the catalytic properties of RNA polymerase from other sources must be acquired before a generalized view of structure and function in the transcriptional process can be set forth.

This report describes the purification of RNA polymerase from *Pseudomonas putida*. Two forms of the enzyme have been resolved by phosphocellulose chromatography. Each form has been characterized with respect to its subunit composition and certain of its physical and catalytic properties.

EXPERIMENTAL PROCEDURE

Materials—Whatman DEAE-cellulose (DE-1) and phosphocellulose (P-1) were purchased from Reeve Angel, New York, New York. UDP-glucose, NADP, glucose 6-phosphate dehydrogenase, dithiothreitol, and the unlabeled 5′-phosphate derivatives of the ribonucleosides were obtained from P-L Biochemicals. ^{3}H-Labeled ribonucleotides were from Schwarz BioResearch. Calf thymus DNA, herring sperm DNA, pancreatic RNase (type I-A), and phosphoglucomutase were from Sigma. Pancreatic DNase I (electrophoretically purified) and catalase were obtained from Worthington. UDP-glucose pyrophosphorylase was isolated from calf liver (25) and recrystallized twice. RNA polymerase from *E. coli* K-12 was a gift from C. Scharrenberger and E. K. F. Bautz, Rutgers University. Poly d(A-T) was purchased from Miles Laboratories, Inc., Elkhart, Indiana. Actinomycin D and nogalamycin were gifts from Merck Sharpe and Dohme and The Upjohn Company, respectively. Rifampicin was purchased from Mann. Acrylamide and bis-acrylamide were from Canalco, Rockville, Maryland, and recrystallized according to the procedure of Loening (26). Ethidium bromide and Coomassie brilliant blue were obtained from Calbiochem and Colab Laboratories, Inc., Glenwood, Illinois, respectively. Diethyloxydiformate was purchased from Naftone, Inc., New York, New York. Nitrocellu-

* This work was supported in part by Grant GB-7914 from the National Science Foundation. This is Michigan Agriculture Experiment Station Article 5112.

lose membrane filters, type B-6, were from Schleicher and Schuell, Inc., Keene, New Hampshire.

Growth of P. putida—*P. putida* (the same or similar to ATCC 12633) was grown in 100-liter volumes in a New Brunswick Fermacell, model F-130. The growth medium 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. The cells were grown at 33° with aeration at 8 to 10 cu ft per min into the early stationary phase and then harvested in a Sharples centrifuge. The 1800 to 1900 g (wet weight) of packed cells were stored at −20°.

Preparation of DNA and RNA—*P. putida* bacteriophage gh-1 DNA (27, 28) and *E. coli* bacteriophage T_4 DNA were purified by the method of Thomas and Abelson (29). Calf thymus DNA was further purified by SDS[1]-phenol extraction. *P. putida* DNA and *E. coli* ^{3}H-DNA were prepared by the procedure of Thomas, Berns, and Kelly (30). After RNase treatment and phenol extraction, the DNA preparation was mixed with diethyloxydiformate and incubated at 22° for 30 min. Following phenol extraction, ethanol precipitation, and isopropyl alcohol fractionation, the DNA was dissolved in a buffer containing 0.01 M Tris acetate (pH 8.0), and 0.1 M sodium acetate and dialyzed for 10 hours. Denatured DNA was prepared by heating dilute solutions of DNA at 100° for 20 min, followed by quick cooling in ice water. *E. coli*-soluble and ribosomal ^{3}H-RNA were prepared as described by Payne and Boezi (31), Native DNA and RNA concentrations were determined spectrophotometrically based on the extinction coefficient $E^{1\%}_{260} = 200$.

Radioactive Assay of RNA Polymerase—The radioactive assay of RNA polymerase measured the incorporation of CMP into a form insoluble in trichloracetic acid. The reaction mixture contained 20 mM Tris acetate (pH 8.0), 4 mM magnesium acetate, 1 mM manganese acetate, 60 mM ammonium acetate, 0.4 mM each of ATP, GTP, CTP, and UTP, and 110 μg per ml of DNA and RNA polymerase. CTP was labeled with ^{3}H at 5 × 10^3 cpm per nmole. Incubation was at 30° for 10 min unless otherwise indicated. After incubation, 50- to 250-μl samples of the reaction mixtures were mixed with 100 μl of 0.1% SDS. Cold 10% trichloracetic acid (5 ml), which had been filtered through Celite, and 250 μg of herring sperm DNA were added. After 15 min at 0–4°, insoluble material was collected on a nitrocellulose membrane filter. The filter was washed with four 5-ml portions of cold 10% trichloracetic acid, dried, and then monitored for radioactivity in a liquid scintillation spectrometer. The scintillation fluid (5 ml) contained 4 g of 2,5 bis[2-(5-*tert*-butyl-benzoxazolyl)]thiophene per liter of toluene. One unit of RNA polymerase activity was defined as that amount of enzyme which catalyzed the incorporation of 1 nmole of CMP per hour at 30°. The specific activity was the number of units per mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (32) with bovine serum albumin as a standard.

Spectrophotometric Assay of RNA Polymerase—The spectrophotometric assay of RNA polymerase as described by Johnson *et al.* (33) coupled the formation of inorganic pyrophosphate to $NADP^+$ reduction. In addition to the components used in the radioactive assay, the reaction mixture contained 0.2 mM $NADP^+$, 0.2 mM UDP-glucose, and excess phosphoglucomutase, glucose 6-phosphate dehydrogenase, and UDP-glucose pyrophosphorylase. Incubation was at 30°. Calculation of the number of nanomoles of NADPH formed was made with the molar extinction coefficient of 6.2×10^3 (34).

Assay of Other Enzyme Activities—The assay for exo- and endo-DNase and RNase measured the solubilization of ^{3}H-DNA and ^{3}H-soluble RNA and ^{3}H-ribosomal RNA. An additional assay for endonuclease measured the change in sedimentation patterns of ^{3}H-DNA in alkaline sucrose gradients. Inorganic pyrophosphatase was determined by measuring the change in inorganic pyrophosphate by using the coupled UDP-glucose pyrophosphorylase assay system (33). ATP-AMP phosphotransferase was measured by the formation of ^{3}H-ADP from ^{3}H-ATP and AMP. Enzymes which hydrolyze the ribonucleoside triphosphates were measured by the formation of ribonucleoside mono- and diphosphates which were identified by paper chromatography in isobutyric acid-NH_4OH-H_2O (66:1:33). ATP-RNA adenylyltransferase was measured as described by Payne and Boezi (31). Catalase was determined spectrophotometrically by measuring the disappearance of H_2O_2 at 240 nm. UDP-glucose pyrophosphorylase was measured as described by Albrecht *et al.* (25).

Polyacrylamide Disc Gel Electrophoresis—Polyacrylamide gels were prepared according to the general procedures of Davis (35) and Ornstein (36). Clear gels were prepared from a solution which was 3.75% in acrylamide by mixing together 1 part water,

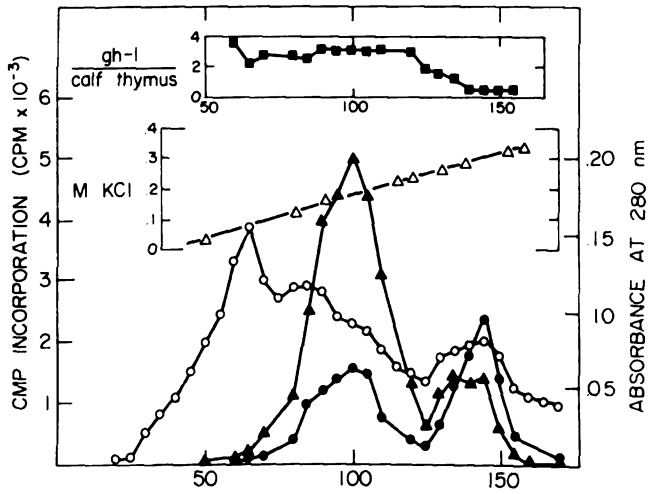

FIG. 1. Phosphocellulose chromatography of DEAE-cellulose fraction. Dialyzed DEAE-cellulose fraction (960 ml) was applied at 3 ml per min to a phosphocellulose column (5 × 40 cm) which had been equilibrated with phosphate-glycerol buffer (0.02 M potassium phosphate, pH 7.5, 0.005 M 2-mercaptoethanol, 0.0001 M EDTA, and 50% glycerol (v/v)). Following application of the enzyme fraction, the column was washed with 4 liters of the phosphate-glycerol buffer at a rate of 5 ml per min. A linear 4-liter gradient from 0.0 to 0.4 M KCl in phosphate-glycerol buffer was used to elute RNA polymerase. The column was developed at 6 ml per min and 170 fractions of 20 ml each were collected. ○——○, absorbance at 280 nm; ▲——▲, CMP incorporation with gh-1 DNA as the template; ●——●, CMP incorporation with calf thymus DNA as the template; △——△, KCl concentration as determined from conductivity measurements; ■——■ (in the *inset*), the ratio of CMP incorporation with gh-1 DNA as the template to that with calf thymus DNA as the template. For the assay of RNA polymerase, 10-μl samples of the fractions were assayed in 60-μl reaction mixtures according to the method described under "Radioactive Assay of RNA Polymerase." Fractions 93 through 115 (Phosphocellulose Fraction I) and 140 through 153 (Phosphocellulose Fraction II) were pooled.

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

1 part Solution A (36.3 g of Tris, 0.23 ml of *N*,*N*,*N'*,*N'*-tetramethylethylenediamine, 48 ml of 1 N HCl, and water to a total of 100 ml), 2 parts Solution B (15.0 g of acrylamide, 0.55 g of bisacrylamide, and water to 100 ml), and 4 parts Solution C (0.14 g of ammonium persulfate and water to 100 ml). Glass tubes with an internal diameter of 5 mm were filled with the gel mixture to a height of 7 cm. Water was layered on top of the gel mixture to form a level interface. Following polymerization, the gels were brought to the temperature of electrophoresis and then subjected to electrophoresis for 30 min at 5 ma per gel prior to sample application. Following electrophoresis, the protein was stained for 4 to 12 hours with 0.3% Coomassie blue in 10% trichloracetic acid containing 5% methanol. Unbound stain was removed by rinsing the gels with 10% trichloracetic acid.

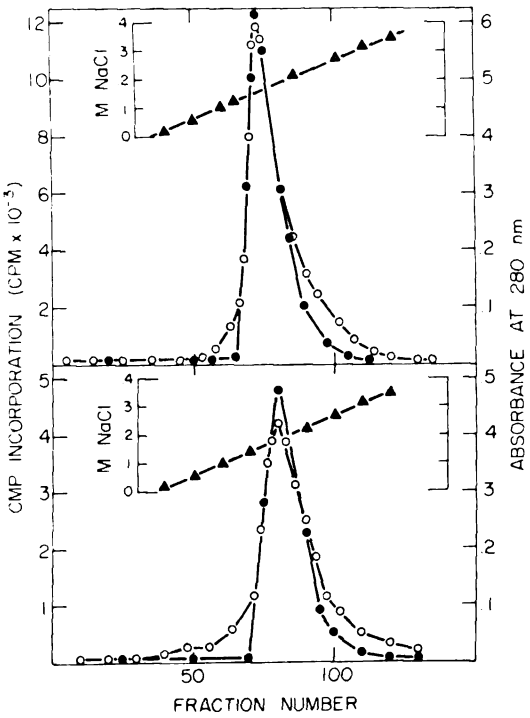

FIG. 2. DEAE-cellulose chromatography of Phosphocellulose Fractions I (*upper*) and II (*lower*). Dialyzed Phosphocellulose Fraction I (510 ml) was diluted to 1020 ml with Buffer ASH-glycerol (0.01 M Tris-HCl, pH 8.0, 0.005 M 2-mercaptoethanol, 0.01 M $MgCl_2$, 0.0001 M EDTA, and 50% glycerol (v/v)). The diluted enzyme fraction was applied at 3.3 ml per min to a DEAE-cellulose column (2.5 × 19 cm) which had been equilibrated with Buffer ASH-glycerol. The column was then washed with 90 ml of Buffer ASH-glycerol. A linear 400-ml gradient from 0.0 to 0.40 M NaCl in Buffer ASH-glycerol was used to elute RNA polymerase. The column was developed at 3.3 ml per min and 125 fractions of 3 ml each were collected (*upper*). ○——○, absorbance at 280 nm; ●——●, CMP incorporation with gh-1 DNA as the template; ▲——▲, NaCl concentration as determined from conductivity measurements. For the assay of RNA polymerase, 5-μl samples of the fractions were assayed in 60-μl reaction mixtures according to the method described under "Radioactive Assay of RNA Polymerase." Fractions 67 through 85 were pooled. Dialyzed Phosphocellulose Fraction II (200 ml) was diluted to 400 ml with Buffer ASH-glycerol and applied at 2.5 ml per min to a DEAE-cellulose column (2.0 × 17 cm). The column was then washed with 80 ml of Buffer ASH-glycerol. A linear 250-ml gradient from 0.0 to 0.40 M NaCl in Buffer ASH-glycerol was used to elute RNA polymerase. The column was developed at 2.7 ml per min and 130 fractions of 2 ml each were collected (*lower*). ○——○, absorbance at 280 nm; ●——●, CMP incorporation with gh-1 DNA as the template; ▲——▲, NaCl concentration as determined by conductivity measurements. For the assay of RNA polymerase, 10-μl samples of the fractions were assayed in 60-μl reaction mixtures as described under "Radioactive Assay of RNA Polymerase." Fractions 74 through 90 were pooled.

SDS-polyacrylamide disc gel electrophoresis was performed according to a modification of the procedure of Shapiro, Viñuela, and Maizel (37). Acrylamide-SDS mixtures containing 3.75% acrylamide were prepared by the procedure described above with the exception that Solution A contained 0.8 M sodium phosphate (pH 7.1), 0.8% SDS, and 0.23 ml of *N*,*N*,*N'*,*N'*-tetramethylethylenediamine per 100 ml of solution. For acrylamide-SDS mixtures with 5% acrylamide, Solution B contained 20 g of acrylamide, 0.735 g of bisacrylamide, and water to 100 ml. For acrylamide-SDS mixtures with 3% acrylamide, Solution B contained 12 g of acrylamide, 0.44 g of bisacrylamide, and water to 100 ml. SDS-polyacrylamide gels 7 or 11 cm in height were prepared. Following electrophoresis, the SDS-polyacrylamide gels which contained 10 μg of protein or more were stained by the procedure described above. SDS-polyacrylamide gels which contained less protein were stained with 0.4% Coomassie blue in 10% trichloracetic acid and 20% methanol for 12 hours. Destaining was performed by first washing the gels 6 hours in 10% trichloracetic acid and 33% methanol and then rinsing the gels in 10% trichloracetic acid until areas of the gel without protein were colorless.

RESULTS

Purification of RNA Polymerase

The entire purification procedure was performed at 0-4°.

Initial Extract—A block of frozen *P. putida* cells, 990 g (wet weight), was cut into small pieces. Buffer ASH (0.01 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 0.0001 M EDTA, and 0.005 M 2-mercaptoethanol) was added (990 ml) and the mixture was stirred until a smooth cell suspension was obtained. The cell suspension was twice passed through the pressure chamber of a Manton-Gaulin lab homogenizer (38) which was maintained at 7,000 p.s.i. This process, which provided nearly complete rupture of the cells, resulted in a viscous cell extract. Pancreatic DNase I (0.5 μg per ml) was added to the cell extract and the mixture was stirred for 15 min. Following centrifugation at 12,000 × *g* for 30 min, the supernatant fraction was decanted and saved. The pellet fraction was suspended in 200 ml of Buffer ASH and recentrifuged. The resulting supernatant fraction was combined with the supernatant fraction from the first centrifugation (initial extract, 2,150 ml).

High Speed Supernatant Fraction—The initial extract was centrifuged at 78,000 × *g* for 90 min. The clear, straw-colored supernatant fraction was collected by decantation. The pellet fraction was suspended in 200 ml of Buffer ASH and recentrifuged. Supernatant fractions from the first and second centrifugations were combined (high speed supernatant fraction, 1,720 ml).

Ammonium Sulfate (60%) Fraction—The high speed supernatant fraction was diluted with buffer ASH to a protein concentration of 2.5 mg per ml. Solid ammonium sulfate (410 g) was added to the diluted high speed supernatant fraction (2,500 ml) to give 30% saturation. After stirring for 30 min, the suspension was centrifuged at 12,000 × *g* for 30 min. The supernatant fraction was brought to 60% saturation with the addition of 452 g of ammonium sulfate. After stirring for 30 min, insoluble

TABLE I

Summary of purification

The purification of RNA polymerase through the DEAE-cellulose fraction was conducted on material from 990 g (wet weight) of cells. After this, only one-half of the DEAE-cellulose fraction was processed. RNA polymerase activity was measured by the method described under "Radioactive Assay of RNA Polymerase." gh-1 DNA was used as the template.

Enzyme fraction	Protein (total)	Activity (total)	Specific activity
	mg	*units*	*units/mg*
Initial extract	13.0×10^4	99×10^4	8
High speed supernatant fraction	6.1×10^4	70×10^4	11
60% ammonium sulfate fraction	5.1×10^4	270×10^4	53
DEAE-cellulose fraction	1.6×10^4	330×10^4	210
Phosphocellulose Fraction I	73	16×10^4	2200
Phosphocellulose Fraction II	62	2.6×10^4	420
DEAE-cellulose Fraction PCI	30	9.6×10^4	3200
DEAE-cellulose Fraction PCII	22	1.4×10^4	640

material, which included RNA polymerase, was collected by centrifugation. Buffer ASH (800 ml) was added and the mixture was stirred. The resulting solution was dialyzed for 9 hours in 12 liters of Buffer ASH (60% ammonium sulfate fraction, 940 ml).

DEAE-cellulose Fraction—Acid- and base-washed DEAE-cellulose suspended in Buffer ASH was poured into a column and packed under air pressure of 1 to 2 p.s.i. The packed column (14.2 × 60.0 cm) was washed with Buffer ASH until the pH of the outflow was 7.0 to 7.2. The 60% ammonium sulfate fraction was diluted with Buffer ASH to a protein concentration of 8.5 mg per ml (6 liters) and applied to the column at a rate of 160 ml per min. Following application of the diluted enzyme fraction, the column was washed with 16 liters of Buffer ASH containing 0.1 M NaCl. RNA polymerase was eluted with Buffer ASH containing 0.2 M NaCl. Fractions containing RNA polymerase activity were pooled (DEAE-cellulose fraction, 5 liters).

Phosphocellulose Fractions I and II—Acid- and base-washed phosphocellulose was suspended in phosphate-glycerol buffer (0.02 M potassium phosphate (pH 7.5), 0.0001 M EDTA, 0.005 M 2-mercaptoethanol, and 50% glycerol (v/v)), poured into a column, and packed by gravity. The packed column (5 × 40 cm) was first washed with 750 ml of phosphate-glycerol buffer containing 0.5 M KCl and then with phosphate-glycerol buffer until chloride ion was no longer detected in the outflow. The pH of the outflow was 7.5.

The DEAE-cellulose fraction was dialyzed for 12 hours in 50 liters of a buffer containing 0.01 M Tris-HCl (pH 8.0), 0.0001 M EDTA, and 0.001 M 2-mercaptoethanol. Dialysis was continued for 20 hours with one buffer change in 20 liters of phosphate-glycerol buffer. During dialysis against phosphate-glycerol buffer, the DEAE-cellulose fraction was reduced in volume to 1620 ml.

Glycerol (300 ml) was added to the dialyzed DEAE-cellulose fraction. The fraction was divided into two equal parts. One part (960 ml) was stored at −20° for processing at a later date. The other part was applied to the phosphocellulose column at a rate of 3 ml per min. Following application of this enzyme fraction, the column was washed with 4 liters of phosphate-glycerol buffer at a rate of 5 ml per min. A linear gradient from 0.0 to 0.4 M KCl in phosphate-glycerol buffer was used to elute RNA polymerase (Fig. 1). Two peaks of RNA polymerase activity were resolved. The first peak eluted at 0.18 M KCl and the second eluted at 0.30 M KCl. Fractions of the first peak, which had a ratio of enzyme activity of 3.0 with gh-1 DNA as template to that with calf thymus DNA as template, were pooled (Fraction PCI, 480 ml). Fractions of the second peak, with an enzyme activity ratio of 0.5, were pooled (Fraction PCII, 200 ml).

DEAE-cellulose Fractions PCI and PCII—DEAE-cellulose

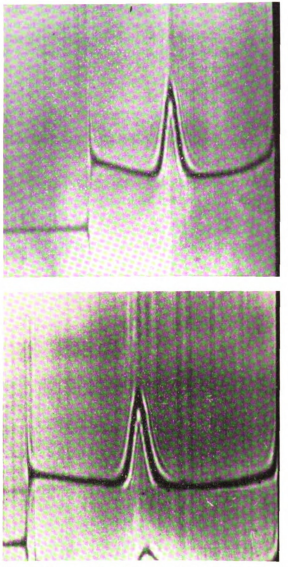

FIG. 3. Schlieren sedimentation pattern of Fractions PCI (*upper*) and PCII (*lower*). Fraction PCI at a concentration of 3.4 mg per ml in a buffer containing 0.05 M Tris acetate (pH 8.0), 0.01 M magnesium acetate, 0.001 M 2-mercaptoethanol, and 0.40 M potassium acetate was centrifuged at 4.6° in a Kel F centerpiece in the An-D rotor of the Spinco model E analytical ultracentrifuge. The picture was taken at 29 min after reaching a speed of 56,000 rpm. The schlieren optical system was used and the phase plate angle was 65°. Fraction PCII at a concentration of 3.3 mg per ml in a buffer containing 0.05 M Tris-HCl (pH 8.0), 0.01 M magnesium chloride, 0.001 M 2-mercaptoethanol, and 0.40 M potassium chloride was centrifuged at 20.1° in the Kel F centerpiece in the An-D rotor of the Spinco model E analytical ultracentrifuge. The picture was taken 38 min after reaching a speed of 56,000 rpm.

suspended in Buffer ASH-glycerol (Buffer ASH with 50% glycerol (v/v)) was used to prepare two columns. The columns, 2.5 × 19 cm and 2.0 × 17 cm, were first washed with 200 ml of Buffer ASH-glycerol containing 0.5 M NaCl, followed by 500 ml of Buffer ASH-glycerol.

In preparation for chromatography, Phosphocellulose Fractions I and II were each dialyzed for 10 hours in 10 liters of Buffer ASH-glycerol, and then were diluted 2-fold. Phosphocellulose Fraction I was applied to the larger DEAE-cellulose column and Fraction II to the smaller column. After each column was washed with a column volume of Buffer ASH-glycerol, RNA polymerase was eluted with a linear gradient of 0.0 to 0.4 M NaCl. The elution profiles are presented in Fig. 2. For both columns, the peak of RNA polymerase activity was eluted at 0.17 M NaCl. Peak fractions from each column were pooled and dialyzed for 10 hours in 2 liters of a solution at 75% saturation with ammonium sulfate. The white flocculent precipitate which formed was collected by centrifugation at 20,000 × *g* for 20 min and was dissolved in a buffer containing 0.05 M Tris acetate (pH 8.0), 0.01 M magnesium acetate, 0.0001 M EDTA, 0.001 M dithiothreitol, and 50% glycerol at a protein concentration in excess of 10 mg per ml. The enzyme fraction from each column was dialyzed for 12 hours in 200 ml of the same buffer (DEAE-cellulose Fraction PCI, 1.95 ml, and DEAE-cellulose Fraction PCII, 1.35 ml).

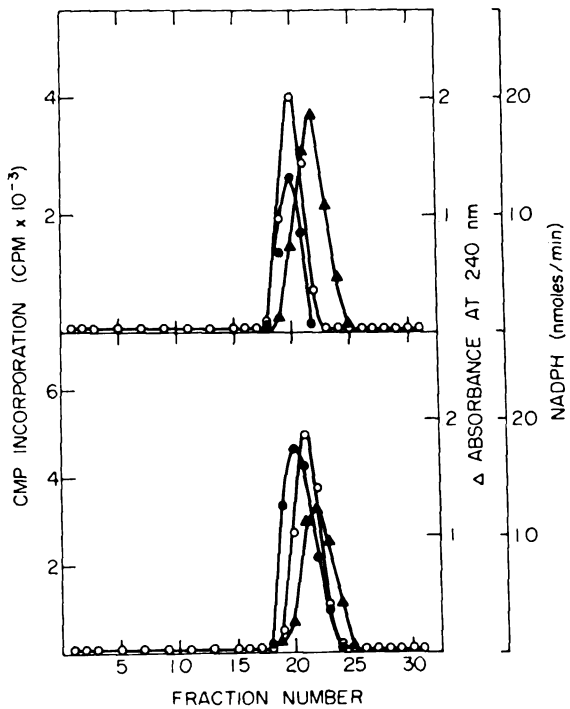

FIG. 4. Sucrose gradient centrifugation of Fractions PCI (*upper*) and PCII (*lower*) in 0.40 M potassium acetate. Fraction PCI (56 μg) or Fraction PCII (107 μg), catalase (79 μg), and UDP-glucose pyrophosphorylase (10 μg) were dissolved in 0.10 ml of a buffer containing 0.05 M Tris acetate (pH 8.2), 0.005 M 2-mercaptoethanol, 0.005 M magnesium acetate, and 0.40 M potassium acetate. The enzyme mixtures were layered on 5 to 20% linear sucrose gradients (4.8 ml) prepared in the above mentioned buffer and centrifuged in a Spinco model L-2 centrifuge at 34,000 rpm in the SW 39 rotor for 9 hours at 3°. After centrifugation, a hole was punched in the bottom of each centrifuge tube and 31 fractions per gradient were collected. Catalase and UDP-glucose pyrophosphorylase were assayed on 30- and 5-μl samples, respectively, as described under "Assay of Other Enzyme Activities." RNA polymerase was assayed on 15-μl samples in 125-μl reaction mixtures with gh-1 DNA as described under "Radioactive Assay of RNA Polymerase" except that reaction mixtures were incubated for 20 min. Recovery of Fraction PCI activity was 96% and that for Fraction PCII was 100%. ▲——▲, catalase as measured by the change in absorbance at 240 nm; ●——●, UDP-glucose pyrophosphorylase as measured by NADPH formation; ○——○, Fraction PCI (*upper*) or Fraction PCII (*lower*) as measured by CMP incorporation.

Summary and Comments on Purification—A summary of the purification is presented in Table I. The determination of RNA polymerase activity in the enzyme fractions through the DEAE-cellulose fraction is complicated by the presence of several contaminating enzyme activities, such as DNase and RNase, and by fragments of *P. putida* DNA.

P. putida cell rupture by means of the Manton-Gaulin lab homogenizer was easy and efficient. One kilogram of frozen cells could be broken in a few minutes and cell rupture was essentially complete as judged from the direct microscopic observation of the broken cell suspension.

Phosphocellulose chromatography was performed in 50% glycerol to stabilize the enzyme and to prevent the conversion of Fraction PCI to Fraction PCII. When purified Fraction PCI was chromatographed on phosphocellulose in 50% glycerol, it remained intact and eluted as a single peak. When Fraction PCI was chromatographed in 5% glycerol, some conversion to Fraction PCII was observed.

Criteria of Purity—The purity of RNA polymerase Fractions PCI and PCII was evaluated by the following criteria. At the final step of the purification, chromatography on DEAE-cellulose, specific enzymatic activity was constant throughout the peak fractions (Fig. 2). In buffers containing 0.4 M salt, a single symmetrical schlieren pattern was observed in the analytical ultracentrifuge (Fig. 3). In polyacrylamide disc gel electrophoresis, comigration of protein and enzymatic activity were found. In SDS-polyacrylamide disc gel electrophoresis, only subunits which were derived from RNA polymerase were observed. Data for the last two criteria are given below.

Fractions PCI and PCII were examined for the presence of contaminating enzymes which might interfere in the assay of RNA polymerase. No DNase and RNase activities were detected. When 5 to 25 times the amount of protein ordinarily used in the assay for RNA polymerase was incubated for 4 hours with ^{3}H-DNA or with ^{3}H-soluble or ^{3}H-ribosomal RNA, no radioactivity was solubilized. Also, the alkaline sucrose gradient profile of ^{3}H-DNA was unchanged after incubation with Fraction PCI or with Fraction PCII. Other activities which were not detected were inorganic pyrophosphatase, ATP-AMP phosphotransferase, ATP-RNA adenylyltransferase, and enzymes which hydrolyze the ribonucleoside triphosphates to their mono- or diphosphate derivatives.

A few of the preparations of Fractions PCI and PCII were contaminated with materials that sedimented at 5 S and at 25 S. These contaminants amounted to 5 to 15% of the total and were removed by rechromatography on phosphocellulose or by sucrose or glycerol gradient centrifugation.

Characterization of Fractions PCI and PCII

Storage—Fractions PCI and PCII were stored in liquid nitrogen at protein concentrations greater than 10 mg per ml in a buffer containing 0.05 M Tris acetate (pH 8.0), 0.01 M magnesium acetate, 0.0001 M EDTA, 0.001 M dithiothreitol, and 50%

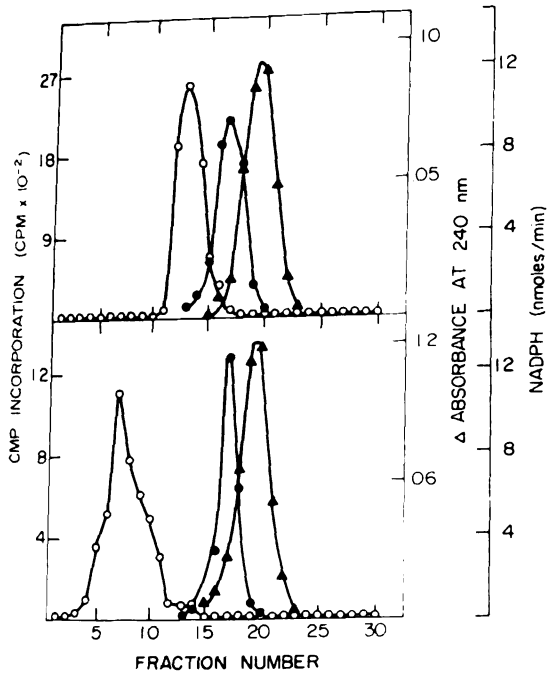

FIG. 5. Sucrose gradient centrifugation of Fractions PCI (*upper*) and PCII (*lower*) in 0.05 M potassium acetate. Fraction PCI (77 μg) or Fraction PCII (80 μg), catalase (59 μg), and UDP-glucose pyrophosphorylase (8 μg) were dissolved in 0.10 ml of a buffer containing 0.05 M Tris acetate (pH 8.2), 0.005 M 2-mercaptoethanol, 0.005 M magnesium acetate, and 0.05 M potassium acetate. The enzyme mixtures were layered on 5 to 20% linear sucrose gradients (4.8 ml) prepared in the above mentioned buffer and centrifuged in a Spinco model L-2 centrifuge at 34,000 rpm in the SW 39 rotor for 9 hours at 3°. After centrifugation, a hole was punched in the bottom of each centrifuge tube and 30 fractions per gradient were collected. Enzyme assays were performed as described in the legend to Fig. 4. Recovery of Fraction PCI activity was 83% and that for Fraction PCII was 59%. ▲——▲, catalase as measured by the change in absorbance at 240 nm; ●——●, UDP-glucose pyrophosphorylase as measured by NADPH formation; O——O, Fraction PCI (*upper*) or Fraction PCII (*lower*) as measured by CMP incorporation.

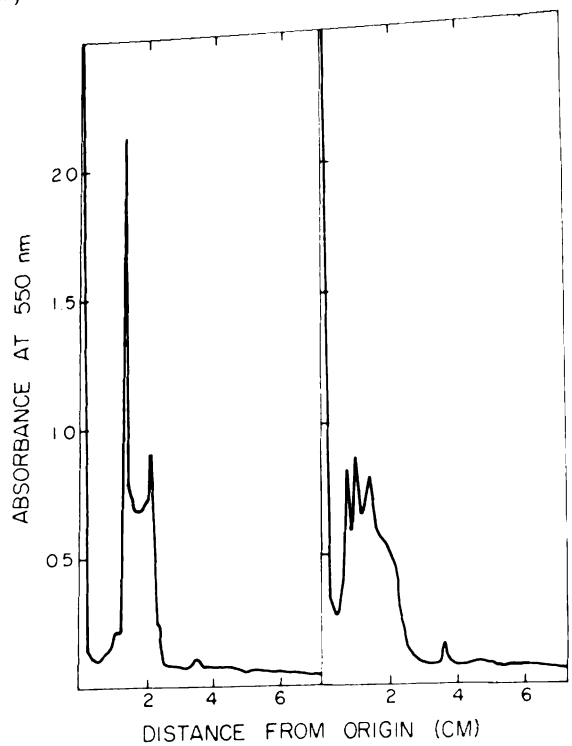

FIG. 6. Polyacrylamide disc gel electrophoresis of Fractions PCI (*left*) and PCII (*right*). Polyacrylamide gels containing 3.75% acrylamide were prepared as described under "Polyacrylamide Disc Gel Electrophoresis." Fraction PCI (20 μg) or Fraction PCII (20 μg) in 10 μl of a buffer containing 0.01 M Tris acetate (pH 8.0), 0.005 M magnesium acetate, 0.005 M 2-mercaptoethanol, and 5% glycerol was layered on a gel. Electrophoresis was performed at 5 ma per gel for 50 min at 0 to 4° in a buffer containing 0.025 M Tris-0.2 M glycine, pH 8.9. Following electrophoresis, the protein contained within the gels was stained with Coomassie blue. Each gel was then scanned at 550 nm with a Gilford linear transport.

glycerol. No loss of activity over a period of several months was observed, even after repeated freezing and thawing. Fractions PCI and PCII were also stored at −20° for several weeks without loss of activity.

Ultraviolet Absorbances—In several different preparations of Fractions PCI and PCII, the $A_{280}:A_{260}$ ratios were 1.5 to 1.6. The λ_{max} for each was 278 nm. For either Fractions PCI or PCII, a protein concentration of 1 mg per ml as determined by Lowry protein analysis gave an absorbance of 0.67 at 280 nm.

Sedimentation Coefficients—The sedimentation coefficients of Fractions PCI and PCII were greatly influenced by the ionic strength of the solvent. The results demonstrate that both Fractions PCI and PCII aggregate in solvents of low ionic strength. When measured with the analytical ultracentrifuge in a buffer containing 0.05 M Tris acetate, 0.01 M magnesium acetate, 0.001 M 2-mercaptoethanol, and 0.40 M potassium acetate (pH 8.2), the sedimentation coefficient of Fraction PCI was 12.8 ($s^{0}_{20,w}$) and that of Fraction PCII was 11.8 ($s^{0}_{20,w}$). When measured in the above buffer containing 0.05 M potassium acetate rather than 0.40 M potassium acetate, the sedimentation coefficient of Fraction PCI was 19.9 ($s^{0}_{20,w}$) and that of Fraction PCII was 26.5 ($s^{0}_{20,w}$),

Fractions PCI and PCII were also analyzed by sucrose gradient centrifugation in buffers containing 0.40 M and 0.05 M potassium acetate. The results are presented in Figs. 4 and 5. In the buffer with 0.40 M potassium acetate, Fraction PCI cosedimented with the UDP-glucose pyrophosphorylase marker (Fig. 4). Under the same conditions, Fraction PCII sedimented somewhat more slowly than the UDP-glucose pyrophosphorylase marker, but slightly faster than the catalase marker. With catalase as the reference ($s^{0}_{20,w}$ = 11.3), the sedimentation coefficient of Fraction PCI was 13 S and that of Fraction PCII was 12 S. In the buffer containing 0.05 M potassium acetate, the faster sedimenting forms, *i.e.* the aggregates of Fractions PCI and PCII, were observed (Fig. 5). Again, with catalase as the reference, the sedimentation coefficient of Fraction PCI was 19 S and that of Fraction PCII was 25 S.

Polyacrylamide Disc Gel Electrophoresis—Multiple protein bands (probably due to aggregation) were observed for Fractions PCI and PCII in polyacrylamide gels (Fig. 6). The proteins contained within duplicate gels which had not been stained with Coomassie blue were tested for enzymatic activity by an assay *in situ* (39). Following electrophoresis, the gels were incubated in reaction mixtures containing ATP, UTP, and Mn^{2+} to promote the unprimed synthesis of poly A·poly U. After incubation, the polynucleotides were stained with ethidium bromide. Polynucleotides were formed in all regions of the gels which contained protein, including the protein at the origin and that

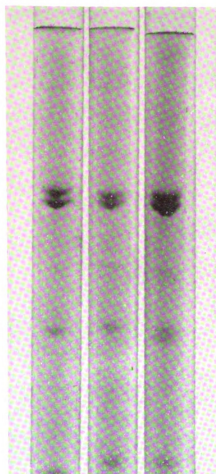

FIG. 7. SDS-polyacrylamide disc gel electrophoresis of RNA polymerase. *Escherichia coli (left)*, *Pseudomonas putida* Fraction PCI *(center)*, and combined *E. coli* and *P. putida* Fraction PCI *(right)*. Reaction mixtures containing 75 μg of *E. coli* (holoenzyme) or *P. putida* Fraction PCI or 75 μg of each were incubated in 150 μl of a mixture containing 0.1 M sodium phosphate (pH 7.1), 1.0% SDS, 1.0% 2-mercaptoethanol, and 5% glycerol for 3 hours at 37°. The mixtures (3 μl) were layered upon SDS-polyacrylamide gels (11 cm) prepared from an acrylamide-SDS mixture containing 3.75% acrylamide. Electrophoresis was performed at 25° for $4\frac{1}{2}$ hours at 8 ma per gel in 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. The protein was stained with Coomassie blue.

small amount which had migrated about 3.5 cm from the origin. Gels incubated in reaction mixtures from which ATP, UTP, or Mn^{2+} were omitted did not stain with ethidium bromide.

Subunit Structures of Fractions PCI and PCII—The subunit structures of Fractions PCI and PCII were examined by the SDS-polyacrylamide disc gel electrophoresis technique (37, 40). By means of this technique, the constituent subunits were identified, their molecular weights were estimated, and the subunit structural formulas were determined.

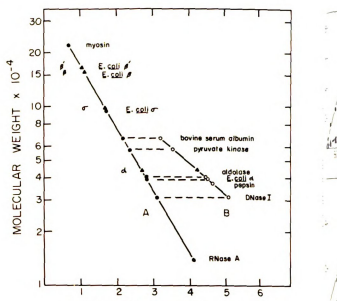

FIG. 8. Molecular weight determination by SDS-polyacrylamide disc gel electrophoresis. *Curve A*, each protein was separately incubated in a mixture containing 0.1 M sodium phosphate, (pH 7.1), 1% SDS, and 1% 2-mercaptoethanol for 3 hours at 37°. In the case of myosin, the incubation mixture also contained 6 M urea. After incubation, each protein was layered on a SDS-polyacrylamide gel prepared from acrylamide-SDS mixtures containing 5% acrylamide. Electrophoresis was performed at 7.5 ma per gel for 3 hours at 22° in 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. *Curve B*, proteins were incubated together in a mixture containing 0.1 M sodium phosphate (pH 7.1), 1% SDS, 1% 2-mercaptoethanol, and 5% glycerol for 3 hours at 37°. After incubation, the mixture was layered on a SDS-polyacrylamide gel prepared from an acrylamide-SDS mixture containing 5% acrylamide. Electrophoresis was performed at 12 ma per gel for 3 hours at 25° in 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. The proteins were stained with Coomassie blue and the distances of migration from the origin were measured. The molecular weights of the polypeptides used as standards were 220,000 for myosin (47), 165,000 for *E. coli* β' (23), 155,000 for *E. coli* β (23), 95,000 for *E. coli* σ (24), 68,000 for bovine serum albumin (48), 57,000 for pyruvate kinase (49), 40,000 for aldolase (50), 39,000 for *E. coli* α (23), 37,000 for pepsin (51), 31,000 for pancreatic DNase I (52), and 13,700 for pancreatic RNase, type 1-A (53). ▲, subunits of *P. putida* RNA polymerase; ● (*Curve A*) and ○ (*Curve B*), polypeptides used as standards.

Fraction PCI was incubated at 37° for 3 hours in 0.1 M sodium phosphate (pH 7.1), 1% SDS, and 1% 2-mercaptoethanol. The resulting subunits were separated by electrophoresis on SDS-polyacrylamide gels. Four subunits were resolved (Fig. 7, *center gel*). For comparison, the subunits derived from *E. coli* RNA polymerase (holoenzyme) were analyzed in a similar manner (Fig. 7, *left gel*). The four *E. coli* subunits, which have been designated β', β, σ, and α in order of increasing mobility (23, 24), and the four *P. putida* subunits were similar. In keeping with the nomenclature used for the *E. coli* subunits, the *P. putida* subunits derived from Fraction PCI were designated β', β, σ, and α. By means of the same procedure, the β', β, and α subunits but not the σ subunit, were derived from Fraction PCII. The β', β, and α subunits of Fractions PCI and PCII had identical mobilities.

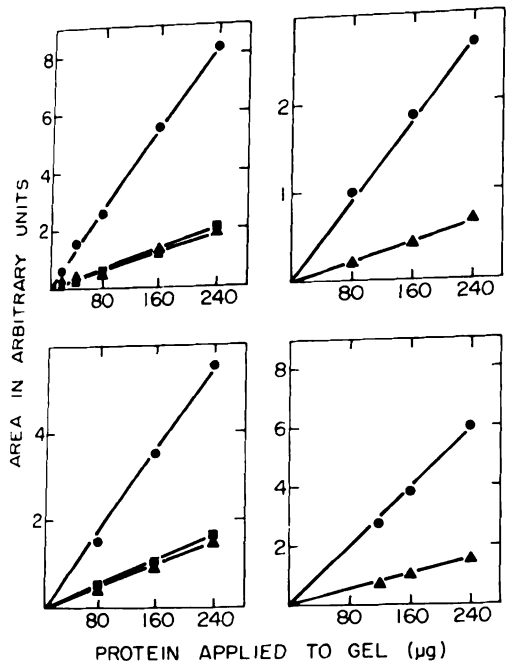

FIG. 9. Relationship between amount of each subunit and amount of protein applied to SDS-polyacrylamide gels. Fractions PCI or PCII was incubated in a buffer containing 0.1 M sodium phosphate (pH 7.1), 1% SDS, and 1% 2-mercaptoethanol for 3 hours at 37°. After incubation, 20 to 240 μg of Fraction PCI or 80 to 240 μg of Fraction PCII were layered on SDS-polyacrylamide gels prepared from a 5% acrylamide-SDS mixture. Electrophoresis was performed as described in Fig. 8 (*Curve A*). Following electrophoresis, the gels were either scanned at 280 nm *immediately* or at 550 nm after the proteins were stained with Coomassie blue. The areas under the subunit peaks were measured. ●—●, β′ plus β subunit; ▲—▲, α subunit; ■—■, σ subunit. *Upper left*, Fraction PCI, 550 nm scans; *upper right*, Fraction PCII, 550 nm scans; *lower left*, Fraction PCI, 280 nm scans; *lower right*, Fraction PCII, 280 nm scans.

The molecular weights of the *P. putida* subunits were determined from a standard curve which related the log of the molecular weight to the distance of migration from the origin (Fig. 8). The molecular weights of β′, β, σ, and α were estimated to be 165,000, 155,000, 98,000, and 44,000, respectively. The molecular weights of the β′ and β subunits of the *P. putida* enzyme were identical with those of the *E. coli* enzyme. The molecular weights of the σ and α *P. putida* subunits were somewhat greater than those of the *E. coli* enzyme (see Fig. 7, *right gel*).

In attempts to dissociate the subunits into smaller structures, Fraction PCI was heated at 50° for 3 hours or 100° for 10 min in 0.1 M sodium phosphate (pH 7.1), 1% SDS, and 1% 2-mercaptoethanol prior to application on a SDS-polyacrylamide gel. β′, β, σ, and α were the only structures detected. Fraction PCII was dialyzed for 15 hours in 0.1 M Tris-0.03 M citric acid (pH 7.6), 1% SDS, 8 M urea, 1% 2-mercaptoethanol, and 0.01% EDTA. After dialysis, one sample was applied directly to a SDS-polyacrylamide gel containing 8 M urea, a second sample was heated at 37° for 1 hour, and a third sample was heated at 75° for 1 hour prior to application on the gel. β′, β, and α were the only structures detected. When Fraction PCI or PCII was denatured in 7 M guanidine hydrochloride and 0.1 M dithiothreitol and then dialyzed in 0.1 M sodium phosphate (pH 7.1), 1% SDS, and 1% 2-mercaptoethanol at 40° for 15 hours prior to electrophoresis, only the β′, β, σ, and α bands of Fraction PCI and the β′, β, and α bands of Fraction PCII were observed.

TABLE II

Molar concentration of β′ + β and σ subunits relative to α subunit

Method used in quantitative determination	RNA polymerase fraction	Subunits	Relative amount of subunits	Relative molecular weight of subunits	Relative molar concentration of subunits
Gel scans at 550 nm (after Coomassie blue staining)	PCI	β′ + β	4.2	3.6	1.1
		σ	1.1	2.2	0.5
		α	1.0	1.0	1.0
	PCII	β′ + β	4.1	3.6	1.1
		α	1.0	1.0	1.0
Gel scans at 280 nm	PCI	β′ + β	3.8	3.6	1.0
		σ	1.1	2.2	0.5
		α	1.0	1.0	1.0
	PCII	β′ + β	3.9	3.6	1.0
		α	1.0	1.0	1.0

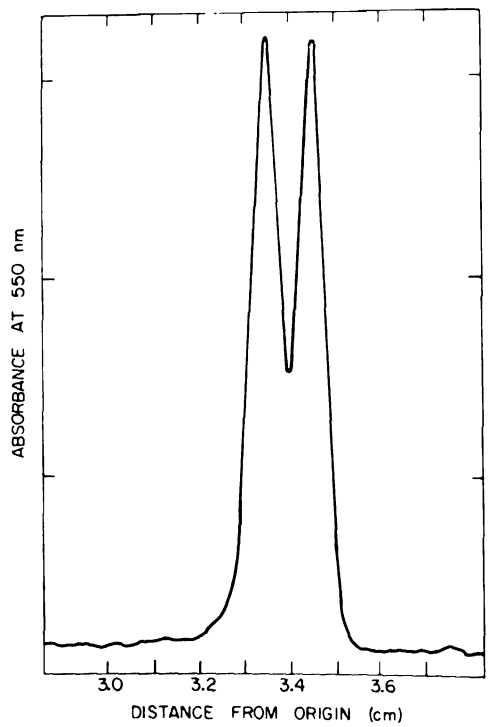

FIG. 10. Densimetric tracing of β′ and β region of SDS-polyacrylamide gel of Fraction PCI. Fraction PCI was denatured according to the procedure described in Fig. 7. A gradient SDS-polyacrylamide gel was prepared by the procedure of Margolis and Kenrick (54) using 3% and 5% acrylamide-SDS mixtures. The 5% acrylamide-SDS mixture was made 10% in glycerol to stabilize the acrylamide gradient during polymerization. Denatured Fraction PCI (1.5 μg) was layered on the gradient SDS-polyacrylamide gel. Electrophoresis was performed at 8.0 ma per gel for 4¾ hours at 25° in 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. The protein was stained with Coomassie blue. The densimetric tracing at 550 nm was made with a Gilford linear transport.

The amount of β′ plus β and of σ relative to α in Fraction PCI and the amount of β′ plus β relative to α in Fraction PCII was determined from measurements of the area under each subunit peak in densimetric tracings of SDS-polyacrylamide gels containing different amounts of the material. The area under each

TABLE III

Characteristics of DNA-dependent synthesis of RNA by Fractions PCI and PCII

The complete reaction mixture contained the components described under "Spectrophotometric Assay of RNA Polymerase." The concentration of Fraction PCI in the reaction mixture was 9 μg per ml, whereas that of Fraction PCII was 25 μg per ml. gh-1 DNA was used as the template.

Components of reaction mixture	NADPH formation in fraction	
	PCI	PCII
	nmoles/min/ml	
Complete	1.7	1.0
Minus RNA polymerase	0	0
Minus gh-1 DNA	0	0
Minus ATP, GTP, UTP, CTP	0	0
Minus ATP, GTP	0	0
Minus UTP, CTP	0	0
Plus 4 μg/ml DNase	0	0
Plus 4 μg/ml actinomycin	0	0
Plus 4 μg/ml nogalamycin	0	0

TABLE IV

Effects of DNA templates on rates of RNA synthesis by Fractions PCI and PCII

All reaction mixtures, with the exception of those containing poly d(A-T), contained the components of the reaction mixture described under "Radioactive Assay of RNA Polymerase." CMP incorporation into RNA was measured. The reaction mixture with poly d(A-T) contained 20 mM Tris acetate (pH 8.0), 1 mM manganese acetate, 0.4 mM each of UTP and ^{3}H-ATP (6×10^3 cpm per nmole), and poly d(A-T) at 0.4 optical density unit at 260 nm per ml of reaction mixture. AMP incorporation into poly r(A-U) was measured. Incubation was at 30° for 10 min for all reaction mixtures. For each template, ribonucleotide incorporation into polymer was measured at several different concentrations of Fractions PCI and PCII to make certain that the amount of incorporation was proportional to the amount of added enzyme.

DNA template	CMP or AMP incorporation into fraction		Activity ratio of Fraction PCI to Fraction PCII
	PCI	PCII	
	nmoles/hr/mg protein		
gh-1	3200	650	4.9
T_4	580	130	4.5
Calf thymus	1100	1200	0.9
Pseudomonas putida	370	290	1.3
Denatured gh-1	280	620	0.5
Denatured calf thymus	90	210	0.4
Poly d(A-T)	830	1400	0.6

subunit peak was measured on gels which had been scanned at 550 nm after they were stained with Coomassie blue or at 280 nm for those which were not stained. In these analyses, β′ and β were not resolved because of the large amount of protein applied to the SDS-polyacrylamide gels. The results are presented in Fig. 9. By either measurement, a linear relationship for the area under each subunit peak with respect to the amount of protein applied to the gel was established. From the slopes of the lines in Fig. 9, the amount of β′ plus β and σ relative to α for Fraction PCI and the amount of β′ plus β relative to α for Fraction PCII was determined. The results are tabulated in Table II. There were two β′ + β subunits and one σ subunit for every two α subunits in Fraction PCI and one β′ + β subunit for each α subunit in Fraction PCII.

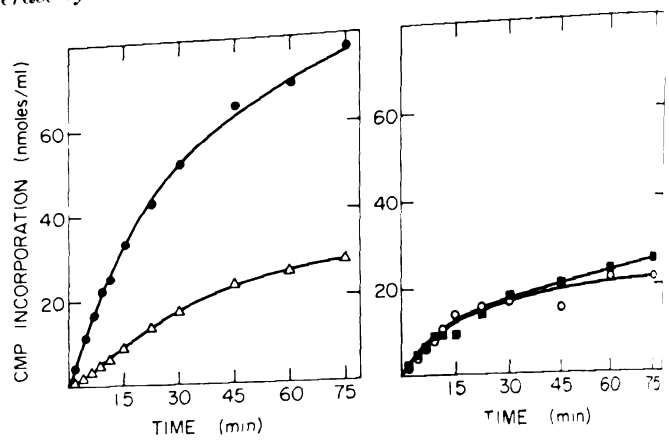

FIG. 11. Kinetics of RNA synthesis by Fractions PCI and PCII. The reaction mixtures contained the components described under "Radioactive Assay of RNA Polymerase." The concentration of Fraction PCI was 37 μg per ml and that of Fraction PCII was 38 μg per ml. CMP incorporation into RNA with gh-1 DNA as the template (*left*): ●——●, Fraction PCI; △- -△, Fraction PCII. CMP incorporation into RNA with calf thymus DNA as the template (*right*): ■——■, Fraction PCI; ○——○, Fraction PCII.

The amount of β′ relative to β in Fraction PCI and in Fraction PCII was determined by means of Coomassie blue-stained SDS-polyacrylamide gels. The amount of protein applied on these gels was small and β′ and β were resolved. For both Fractions PCI and PCII, the amount of β′ was equal to β. A densimetric tracing of the β′ and β region of a SDS-polyacrylamide gel of Fraction PCI is presented in Fig. 10.

The subunit structural formula for Fraction PCI was therefore assigned as $\alpha_2\beta\beta'\sigma$ with a molecular weight of 506,000 or an integral multiple of it. The sedimentation coefficient of 13 S indicated that the molecular weight of Fraction PCI was about 500,000. Thus, the subunit structural formula for Fraction PCI was $\alpha_2\beta\beta'\sigma$ with a molecular weight of 506,000. Based upon the size indicated by a sedimentation coefficient of 12 S and the subunit content, the structural formula for Fraction PCII was $\alpha_2\beta\beta'$ and its molecular weight was 408,000.

DNA-directed Synthesis of RNA by Fractions PCI and PCII

General Characteristics of RNA Synthesis—Some of the characteristics of the DNA-dependent RNA synthesis by Fractions PCI and PCII as determined with the spectrophotometric assay are presented in Table III. Rifampicin (data not shown) inhibited the synthesis of RNA by Fractions PCI and PCII, with a concentration of 0.03 μg per ml producing a 50% inhibition. This concentration of the antibiotic is similar to that required to give 50% inhibition of RNA synthesis by *E. coli* RNA polymerase (41, 42).

The rate of RNA synthesis as measured by the rate of incorporation of CMP into a form insoluble in trichloracetic acid was proportional to the amount of enzyme used. For example, when 1.5, 4.5, and 7.5 μg of Fraction PCI were added to 125-μl reaction mixtures with gh-1 DNA as the template, 0.81, 2.5, and 3.8 nmoles of CMP, respectively, were incorporated in 10 min. When 1.5, 4.5, and 7.5 μg of Fraction PCII were used, 0.19, 0.55, and 0.72 nmole of CMP, respectively, were incorporated in 10

TABLE V

Effect of RNase on DNA-dependent reaction

The reaction mixtures contained the components listed under "Spectrophotometric Assay of RNA Polymerase." The concentration of Fraction PCI was 9 μg per ml in the reaction mixtures which contained gh-1 DNA and 49 μg per ml in the reaction mixtures which contained calf thymus DNA. The concentration of Fraction PCII was 25 μg per ml in the reaction mixtures which contained gh-1 DNA and 51 μg per ml in the reaction mixtures which contained calf thymus DNA. RNase, which had been heated to 100° for 10 min, was at a concentration of 4 μg per ml.

RNA polymerase	DNA template	NADPH formation		Percentage of increase in rate by RNase
		Plus RNase	Minus RNase	
		nmoles/min/ml		
Fraction PCI	gh-1	1.4	1.3	10
	Calf thymus	2.5	2.3	10
Fraction PCII	gh-1	2.7	1.3	110
	Calf thymus	4.4	2.4	80

min. The concentrations of DNA, the four nucleoside triphosphates, divalent metal ions (Mg^{2+} and Mn^{2+}), and monovalent cations (NH_4^+ or K^+) which gave the fastest rate of RNA synthesis by Fraction PCI were the same as those which gave the fastest rate of synthesis by Fraction PCII. The concentrations of the reagents were those used in the standard radioactive assay mixture.

Effects of DNA Templates on Rates of RNA Synthesis—The rates of Fraction PCI- and Fraction PCII-catalyzed RNA synthesis with a number of DNA templates were determined. These results are presented in Table IV. With *P. putida* bacteriophage gh-1 DNA or coliphage T_4 DNA as template, the specific activity of Fraction PCI (enzyme with σ) was 4 to 5 times greater than that of Fraction PCII (enzyme without σ). With denatured DNA or poly d(A-T) as template, the specific activity of Fraction PCI was half that of Fraction PCII. The kinetics of RNA synthesis by Fractions PCI and PCII, with gh-1 DNA and calf thymus DNA as the template, is presented in Fig. 11. Although differing in slope and in extent, the shapes of the curves were similar.

Effect of RNase—The rate of the DNA-directed polymerization of the nucleoside triphosphates is increased by the addition of RNase to the reaction mixture. This increased rate of reaction, monitored by the increased rate of inorganic pyrophosphate formation, has been reported by Maitra and Hurwitz (13), Johnson *et al.* (33), and Krakow (43). The RNA polymerase used in these published studies was not defined with respect to its complement of σ.

The effect of RNase on the DNA-directed reaction catalyzed by Fraction PCI (enzyme with σ) and by Fraction PCII (enzyme without σ) was investigated. The results are presented in Table V. The initial rates of the reactions catalyzed by Fraction PCII were increased 80 to 110%, whereas those catalyzed by Fraction PCI were increased only 10%.

DISCUSSION

Two forms of the enzyme RNA polymerase, $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$, have been purified from *P. putida*. Chromatography on phosphocellulose in 50% glycerol separated the enzyme into the two forms, with $\alpha_2\beta\beta'\sigma$ (Fraction PCI) eluting at 0.18 M KCl and $\alpha_2\beta\beta'$ (Fraction PCII) eluting at 0.30 M KCl. Each form of the enzyme was purified and characterized with respect to its subunit composition and to certain of its physical and catalytic properties. Some preparations of $\alpha_2\beta\beta'\sigma$ were contaminated with a small amount of a yellow-colored material which was removed by sucrose or glycerol gradient centrifugation. This material which sedimented at 25 S has been identified as an α-keto acid-dehydrogenation enzyme complex.

The β', β, σ, and α subunits of the *P. putida* enzyme probably are polypeptide chains rather than aggregates of polypeptide chains. Only these polypeptide subunits were detected by SDS-polyacrylamide disc gel electrophoresis for enzyme that had been denatured by a number of procedures. These procedures included denaturation of $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ at elevated temperatures in SDS, urea, or guanidine hydrochloride in the presence of the sulfhydryl reagents 2-mercaptoethanol or dithiothreitol. It is not known whether the two α polypeptide chains of the *P. putida* enzyme are identical. Furthermore, it is not known whether the σ of $\alpha_2\beta\beta'\sigma$ is a unique polypeptide chain or a mixture of polypeptide chains, each chain having a molecular weight of 98,000. The role of *E. coli* σ as an initiating factor of RNA synthesis suggests that there might be more than one type of σ polypeptide chain per cell (44).

The subunit structure of *P. putida* RNA polymerase is similar to but not identical with that of the *E. coli* enzyme. The subunit structure of the *E. coli* core enzyme is $\alpha_2\beta\beta'$ and that of the holoenzyme, or complete enzyme, is $\alpha_2\beta\beta'\sigma$ (23, 24). The molecular weights of the α, β, β', and σ subunits of *E. coli* RNA polymerase are 39,000, 155,000, 165,000, and 95,000 respectively. These subunits probably are polypeptide chains rather than aggregates. Only these subunits were observed by SDS-polyacrylamide disc gel electrophoresis for enzyme that had been denatured by a number of procedures (23). The molecular weights of a mixture of *E. coli* β' and β and of α were determined by equilibrium centrifugation in urea and in guanidine hydrochloride and shown to be essentially the same as those determined by SDS-polyacrylamide disc gel electrophoresis (23).

The molecular weights of the β' and β subunits of the *P. putida* enzyme were identical with those of the *E. coli* enzyme measured by SDS-polyacrylamide disc gel electrophoresis. The molecular weights of the σ and α *P. putida* subunits were somewhat greater than those of the *E. coli* enzyme. In all preparations of the *E. coli* enzyme, except those fractionated on hydroxyapatite and possibly those fractionated on phosphocellulose by means of a shallow KCl gradient, a small protein, ω (molecular weight 9000), was present (23). No ω has been detected in preparations of the *P. putida* enzyme.

Both $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ of *P. putida* transcribed a number of DNA templates, but with different efficiencies. With *P. putida* bacteriophage gh-1 DNA as the template, $\alpha_2\beta\beta'\sigma$ was 4 to 5 times more active in RNA synthesis than was $\alpha_2\beta\beta'$. Although differing in rate and in extent, the kinetics of gh-1 DNA-directed RNA synthesis by $\alpha_2\beta\beta'\sigma$ and by $\alpha_2\beta\beta'$ is similar.

The initial rates of DNA-directed polymerization reactions catalyzed by $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ were affected differently by RNase. The rate of the reaction catalyzed by $\alpha_2\beta\beta'\sigma$ was increased 10%, whereas that catalyzed by $\alpha_2\beta\beta'$ was increased 80 to 110%. The increased rate of polymerization in the presence of RNase was probably due to a relief of RNA product inhibition (45, 46). Apparently, the RNA synthetic process originated by $\alpha_2\beta\beta'$ was

more susceptible to RNA product inhibition than that originated by $\alpha_2\beta\beta'\sigma$. It is likely that this difference in susceptibility to product inhibition for $\alpha_2\beta\beta'\sigma$ and $\alpha_2\beta\beta'$ was due to differences in the structure of the enzymes of the DNA-enzyme-nascent RNA complexes involved in RNA chain elongation rather than in the structures of the enzymes of the DNA-enzyme complex involved in the initiation process. For *E. coli* RNA polymerase, σ is released following initiation, leaving the core enzyme ($\alpha_2\beta\beta'$) to catalyze chain elongation (44). An analogous process probably occurs with the *P. putida* enzyme. Thus, for the *P. putida* enzyme, the conformation of $\alpha_2\beta\beta'$ which catalyzes chain elongation must depend on whether or not σ was involved in the initiation process.

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ARTICLE 3

PURIFICATION AND PROPERTIES OF ^{35}S-LABELED RNA POLYMERASE OF PSEUDOMONAS PUTIDA

By

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(Manuscript to be published)

SUMMARY

^{35}S-labeled Pseudomonas putida RNA polymerase ($\alpha_2\beta\beta'\sigma$) was purified from labeled cells which had been grown on a minimal growth medium containing ^{35}S-labeled sulfate. As shown by SDS-polyacrylamide gel electrophoresis, the ^{35}S-labeled enzyme was at least 98 per cent pure. The specific enzymatic activity of the enzyme was 7,800 nmoles of CMP incorporated per hour per mg of protein at 30° using gh-1 DNA as the template. The specific radioactivity of the enzyme was 2.3×10^7 cpm per mg of protein. As shown by analysis of the isolated polypeptide subunits, the amount of ^{35}S of β', β, and σ relative to α was 3.6 to 3.6 to 2.2 to 1.0, respectively. The amount of β' plus β in the Initial Extract was 1.2 per cent of the total protein as determined by SDS-polyacrylamide gel electrophoresis.

INTRODUCTION

Radioactive RNA polymerase of high specific radioactivity would be a useful reagent in certain studies of enzyme structure and function. A radioactive assay of ^{3}H-, ^{14}C-, or ^{35}S-labeled protein could be used as a method of protein quantitation in place of the usual colorimetric assays. The colorimetric assay of Lowry which is the most commonly used method of protein quantitation and one of the most sensitive can not be used in many instances because of interference caused by such common biochemical reagents as Tris and mercaptoethanol (1).

^{3}H-, ^{14}C-, and ^{35}S-labeled Escherichia coli RNA polymerase apoenzymes ($\alpha_2\beta\beta'$) have been prepared (2,3,4). Labeled E. coli holoenzyme ($\alpha_2\beta\beta'\sigma$) or labeled RNA polymerase from other sources has not been prepared. The specific radioactivities of the ^{3}H- and ^{14}C-labeled E. coli enzymes were low. Consequently, the usefulness of the preparations was limited. This study describes the preparation and characterization of ^{35}S-labeled Pseudomonas putida RNA polymerase ($\alpha_2\beta\beta'\sigma$) of high specific radioactivity and high specific enzymatic activity.

EXPERIMENTAL PROCEDURE

Unless otherwise stated, the EXPERIMENTAL PROCEDURES used in these studies were the same as those described in the preceding chapter of the thesis.

Materials. ^{35}S-labeled sodium sulfate with specific activity 845 mCi per mmole and Omnifluor were purchased from New England Nuclear. Triton X-100 was from Sigma. Agarose (Bio-Gel A-1.5 m) was obtained from Bio-Rad Laboratories.

Growth of Pseudomonas putida. The growth medium contained the following in grams per liter: glucose, 20; NH_4Cl, 2; Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 8; $MgCl_2 \cdot 6H_2O$, 0.08; $Na_2SO_4 \cdot 10H_2O$, 0.03; $Mn(C_2H_3O_2)_2 \cdot 4H_2O$, 0.005; $CaCl_2$, 0.005; $FeCl_3 \cdot 6H_2O$, 0.005; and $Na_2MoO_4 \cdot 2H_2O$, 0.005. For the production of ^{35}S-labeled cells, 15 mCi of ^{35}S-labeled sodium sulfate were added per liter of growth medium. The cells were grown at 33° on a gyrorotatory shaker in 2.8-l Fernbach flasks containing 500 ml of growth medium. The doubling time for the culture was 100 minutes. When the cells reached the late logarithmic phase of growth, they were harvested and stored at -20°. From a three liter culture of ^{35}S-labeled cells which had been harvested at an optical density (660 nm) of 1.3 units per ml, the yield of cells (wet weight) was 9.7 g.

Assay of ^{35}S-labeled Proteins in polyacrylamide gels. Polyacrylamide gels were cut into 2 mm transverse fractions using a stainless steel support and cutting guide. The fractions were placed in scintillation vials. After the addition of 0.2 ml of 30% H_2O_2 to each vial, they were incubated at 65 to 70° for 9 hours or at 100 to 110° for 2 hours. Following incubation, 5 ml of a mixture containing 6 parts of Omnifluor solution (18.1 g of Omnifluor per gallon of toluene) and 7 parts of Triton X-100 were added to each vial (5). The vials were capped, shaken, and assayed for their ^{35}S-content in a scintillation spectrometer.

RESULTS

Purification of RNA Polymerase

The purification of ^{35}S-labeled RNA polymerase was performed at 0 to 40°.

Initial Extract. Frozen ^{35}S-labeled Pseudomonas putida (9.7 g) and frozen unlabeled P. putida (11.7 g) were mixed with washed glass beads (40 g) and ground in a mortar with a pestle. After cell rupture had occurred, 30 ml of buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 200 mM KCl, and 15% glycerol (v/v)) were added. The mixture was stirred until a smooth suspension was obtained. Pancreatic DNase I was added to a final concentration of 2.5 µg/ml. The mixture was stirred for 15 minutes, then centrifuged at 12,000 × g for 30 minutes. The supernatant fraction was decanted and saved. The pellet fraction was suspended in 30 ml of the above buffer and recentrifuged. The resulting supernatant fraction was combined with the supernatant fraction from the first centrifugation. (Initial Extract, 63 ml.)

High Speed Supernatant Fraction. The Initial Extract was diluted to 80 ml with the above buffer, then centrifuged at 78,000 × g for 90 minutes. The supernatant fraction was collected by decantation. (High Speed Supernatant Fraction, 64 ml.)

60% Ammonium Sulfate Fraction. Solid ammonium sulfate (23.2 g) was added to the High Speed Supernatant Fraction to give 60% of saturation. After the mixture was stirred for 15 minutes, the suspension was centrifuged at 12,000 × g for 15 minutes. The pellet fraction was dissolved in 15 ml of the above buffer and dialyzed against 2 liters of the buffer for 15 hours. (60% Ammonium Sulfate Fraction, 26 ml.)

DEAE-Cellulose Fraction. The 60% Ammonium Sulfate Fraction was diluted 4-fold into buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 15% glycerol (v/v)) and applied to a DEAE-cellulose column (5 by 15 cm) at a flow rate of 8 ml/min. The column was then washed with 500 ml of the above buffer containing 0.1 M NaCl. RNA polymerase was eluted with the above buffer containing 0.2 M NaCl. The fractions giving the bulk of the absorbance readings at 280 nm were pooled (230 ml). The pooled fractions were brought to 60% of saturation with solid ammonium sulfate (83.2 g). After the mixture was stirred for 15 minutes, the suspension was centrifuged at 12,000 × g for 15 minutes. The pellet fraction was dissolved in 11 ml of phosphate buffer (20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, and 15% glycerol (v/v)) containing 0.2 M KCl and dialyzed against 2 liters of the phosphate buffer with 0.2 M KCl for 15 hours (DEAE-cellulose Fraction, 11 ml).

Phosphocellulose Fraction I. The DEAE-cellulose Fraction was diluted to 31 ml with phosphate buffer and applied to a phosphocellulose column (2 by 7 cm) at a flow rate of 1 ml/min. The column was washed with 150 ml of phosphate buffer at a flow rate of 2 ml/min. A linear gradient from 0.1 to 0.5 M KCl in phosphate buffer was used to elute RNA polymerase. Fractions (2 ml) were collected at a flow rate of 2 ml/min. Fractions which contained RNA polymerase activity that had eluted at 0.18 M KCl were pooled. The pooled fractions were dialyzed against 2 liters of 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 200 mM KCl and 50% glycerol (v/v) for 5 hours and then against 2 liters of ammonium sulfate solution (75% of saturation, pH 7.5) for 12 hours. Insoluble material was collected by centrifugation at 12,000 × g for 30 minutes, then dissolved in 10 mM Tris acetate, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, 500 mM KCl and 7.5% glycerol (v/v). (Phosphocellulose Fraction I, 0.83 ml.)

Agarose Fraction I. Agarose beads were suspended in water, settled to remove fine material, and equilibrated in 10 mM Tris acetate, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, 500 mM KCl and 5% glycerol (v/v). A column (1.5 by 85 cm) was packed by gravity and washed with the above buffer at a flow rate of 0.2 ml/min. Phosphocellulose Fraction I was layered on the top of the column

and washed through at a flow rate of 0.2 ml/min. Fractions (1.5 ml) were collected. The elution profile is presented in Figure 1. Fractions 38 through 46 were pooled, dialyzed against 2 liters of 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 200 mM KCl, and 50% glycerol (v/v) for 5 hours, then against 2 liters of ammonium sulfate solution (75% of saturation, pH 7.5) for 12 hours. Insoluble material was collected by centrifugation, dissolved in 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 200 mM KCl, and 2% glycerol (v/v), and dialyzed against 200 ml of this buffer for 12 hours. (Agarose Fraction I, 0.2 ml.)

Glycerol Gradient Fraction I. Agarose Fraction I was layered on a 10 to 30% linear glycerol gradient (4.8 ml) prepared in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol and 200 mM KCl and centrifuged in the SW 39 rotor of the Spinco model L-2 centrifuge at 35,000 rpm for 19.1 hours at 4°. After centrifugation, a hole was punched in the bottom of the centrifuge tube and twenty fractions were collected. The ^{35}S-content of each fraction was measured (Figure 2). Fractions 5 through 9 were pooled and dialyzed against 500 ml of 50 mM Tris acetate, pH 8.0, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 200 mM KCl, and 50% glycerol (v/v) for 8 hours. (Glycerol Gradient Fraction I, 0.58 ml.) Glycerol Gradient Fraction I was stored at -20° at a protein concentration of 1 mg/ml.

FIGURE 1.--Agarose Gel Filtration of Phosphocellulose Fraction I

Phosphocellulose Fraction I (0.83 ml) was applied at a flow rate of 0.2 ml/min to a 1.5 by 85 cm Agarose column which had been equilibrated in 10 mM Tris acetate, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, 500 mM KCl, and 5% glycerol. The column was developed at 0.2 ml/min and 80 fractions of 1.5 ml each were collected. ^{35}S-labeled protein (o——o) was determined on 5-µl samples by liquid scintillation spectrometry (6). RNA polymerase activity (▲——▲) was determined on 5-µl samples in 125 µl reaction mixtures according to the method described in "Experimental Procedure (Radioactive assay of RNA polymerase)" of Article I. Fractions 38 through 46 were pooled.

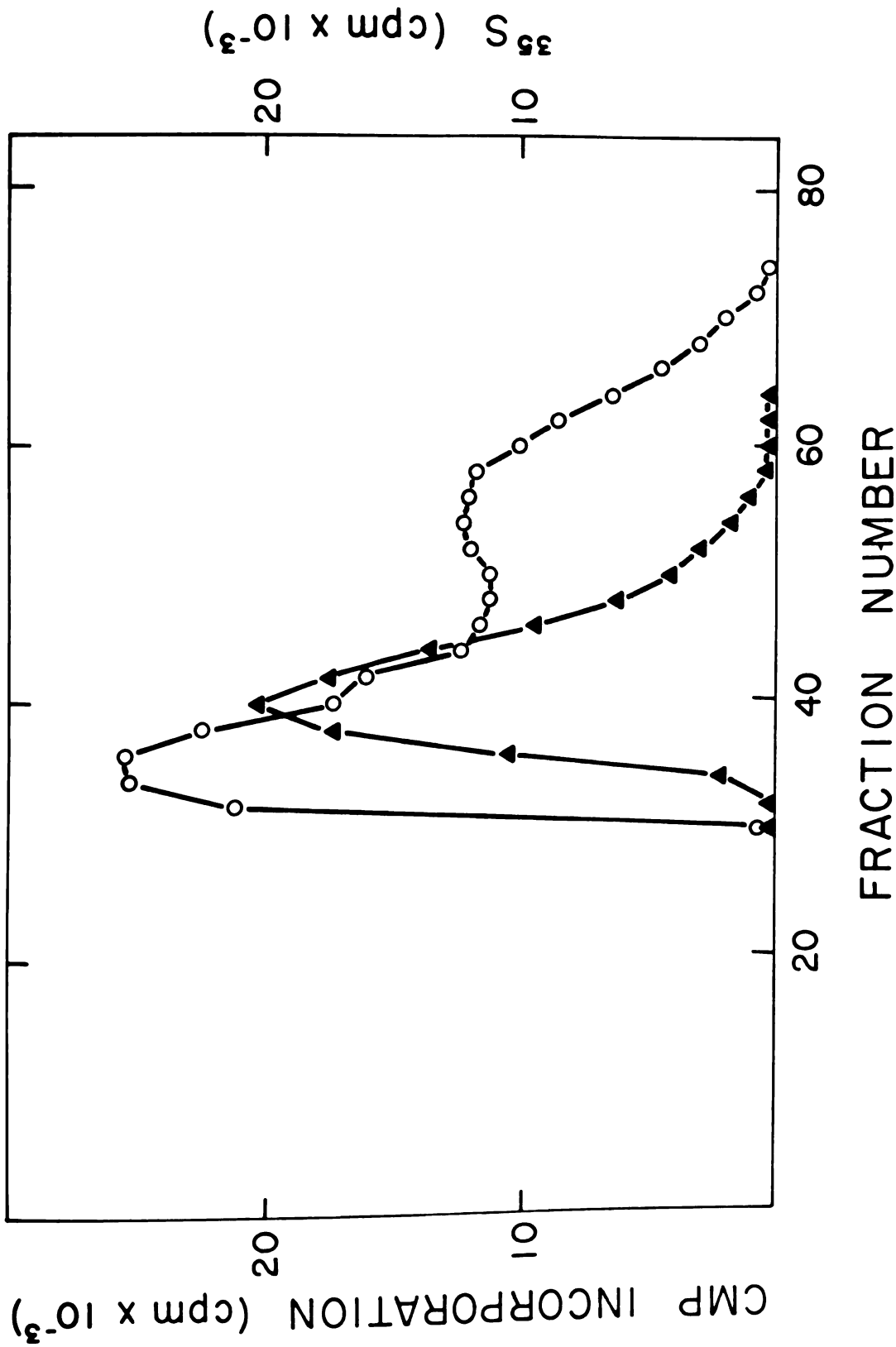
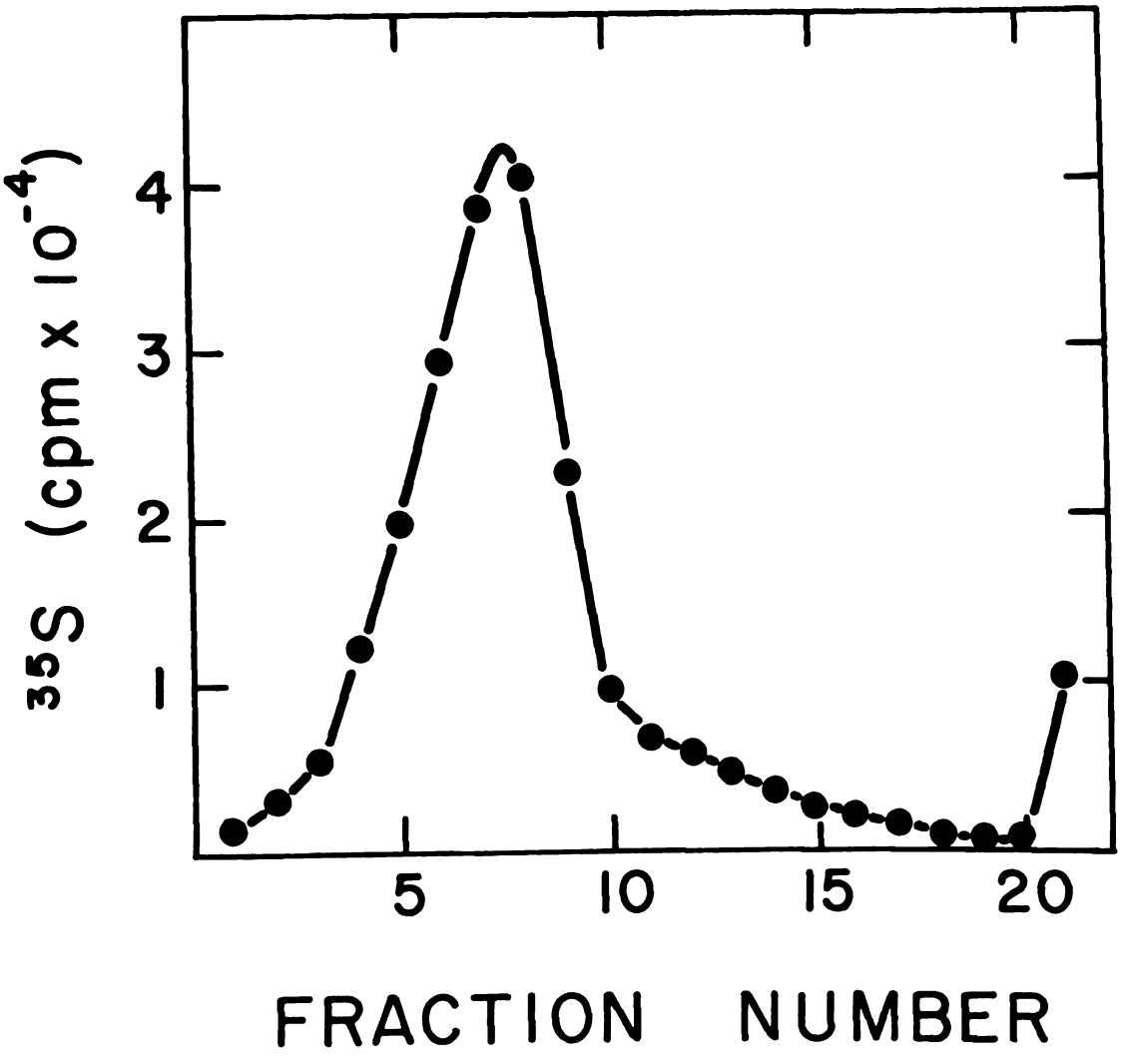

CMP INCORPORATION (cpm x 10⁻³)
20
10
FRACTION NUMBER
20
40
60
80
35S (cpm x 10⁻³)
20
10

FIGURE 2.--Glycerol Gradient Centrifugation of Agarose Fraction I

Agarose Fraction I (0.2 ml) was layered on a 10 to 30% linear glycerol gradient (4.8 ml) prepared in 50 mM potassium phosphate, pH 7.5, 1.0 mM dithiothreitol, and 200 mM KCl and centrifuged at 4° for 19.1 hours at 35,000 rpm in the SW 39 rotor of the Spinco model L-2 centrifuge. A hole was punched in the bottom of the tube and twenty fractions were collected. A sample (2 µl) of each fraction was assayed for ^{35}S by liquid scintillation spectrometry (6). Fractions 5 through 9 were pooled.


^{35}S (cpm x 10^{-4})
1
2
3
4
5
10
15
20
FRACTION NUMBER

Summary and Comments on the Purification. A summary of the purification in terms of the radioactivity recovered in each enzyme fraction of the purification procedure is presented in Table 1. Of the total ^{35}S-labeled material in the Initial Extract, 0.05% was recovered in Glycerol Gradient Fraction I. The amount of protein of the Glycerol Gradient Fraction I was 570 μg. As judged by sucrose gradient centrifugation (data not shown) and by SDS-polyacrylamide gel electrophoresis (see Figures 3, 5, 6, and 8) the ^{35}S-labeled RNA polymerase (Glycerol Gradient Fraction I) was at least 98% pure. SDS-polyacrylamide gel electrophoresis of the material of each enzyme fraction of the purification procedure was performed. The results are presented in Figure 3.

A densimetric tracing of a Coomassie blue stained SDS-polyacrylamide gel of the Initial Extract is presented in Figure 4. Polypeptide chains which have the same mobilities as the β' and β subunits of RNA polymerase were well separated from the majority of the polypeptide chains. Since these polypeptide chains are equal in amount and radioactive content (data not shown), and because of their unusual size, they are considered to be predominately if not exclusively the β' and β subunits of RNA polymerase. As calculated from the area occupied by β' plus β relative to the total area of the densimetric tracing of the SDS-polyacrylamide gel, β' plus β amounted to 1.2% of the total protein of the Initial Extract.

TABLE 1.--Summary of Purification

Enzyme Fraction	^{35}S (Total cpm)	^{35}S Remaining (%)
Initial Extract	2.83×10^{10}	100.0
High Speed Supernatant Fraction	2.38×10^{10}	84.2
60% Ammonium Sulfate Fraction	1.45×10^{10}	51.3
DEAE-cellulose Fraction	3.20×10^{9}	11.3
Phosphocellulose Fraction I	1.69×10^{8}	0.60
Agarose Fraction I	3.60×10^{7}	0.13
Glycerol Gradient Fraction I	1.32×10^{7}	0.05

The ^{35}S content of each enzyme fraction was determined by liquid scintillation spectrometry (6).

FIGURE 3.--SDS-polyacrylamide Gel Electrophoresis of Enzyme Fractions of the Purification Procedure

Samples of the various enzyme fractions were incubated at 100° for 10 minutes in 0.1 M sodium phosphate, pH 7.1, 1.0% SDS, 1.0% 2-mercaptoethanol and 5% glycerol. The samples (10 µg) were layered on 11 cm SDS-polyacrylamide gels prepared from an acrylamide-SDS mixture containing 3.75% acrylamide and 12.5% glycerol. Electrophoresis was performed at 25° for 2.5 hours at 8 ma/gel in a buffer containing 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. The protein was stained with Coomassie blue. The gels are from left to right: Initial Extract, High Speed Supernatant Fraction, 60% Ammonium Sulfate Fraction, DEAE-cellulose Fraction, Phosphocellulose Fraction I, Agarose Fraction I, and Glycerol Gradient Fraction I.

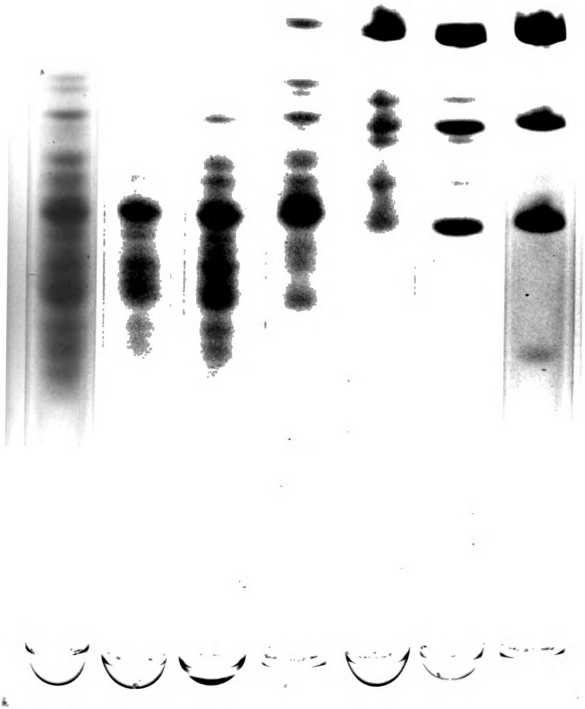
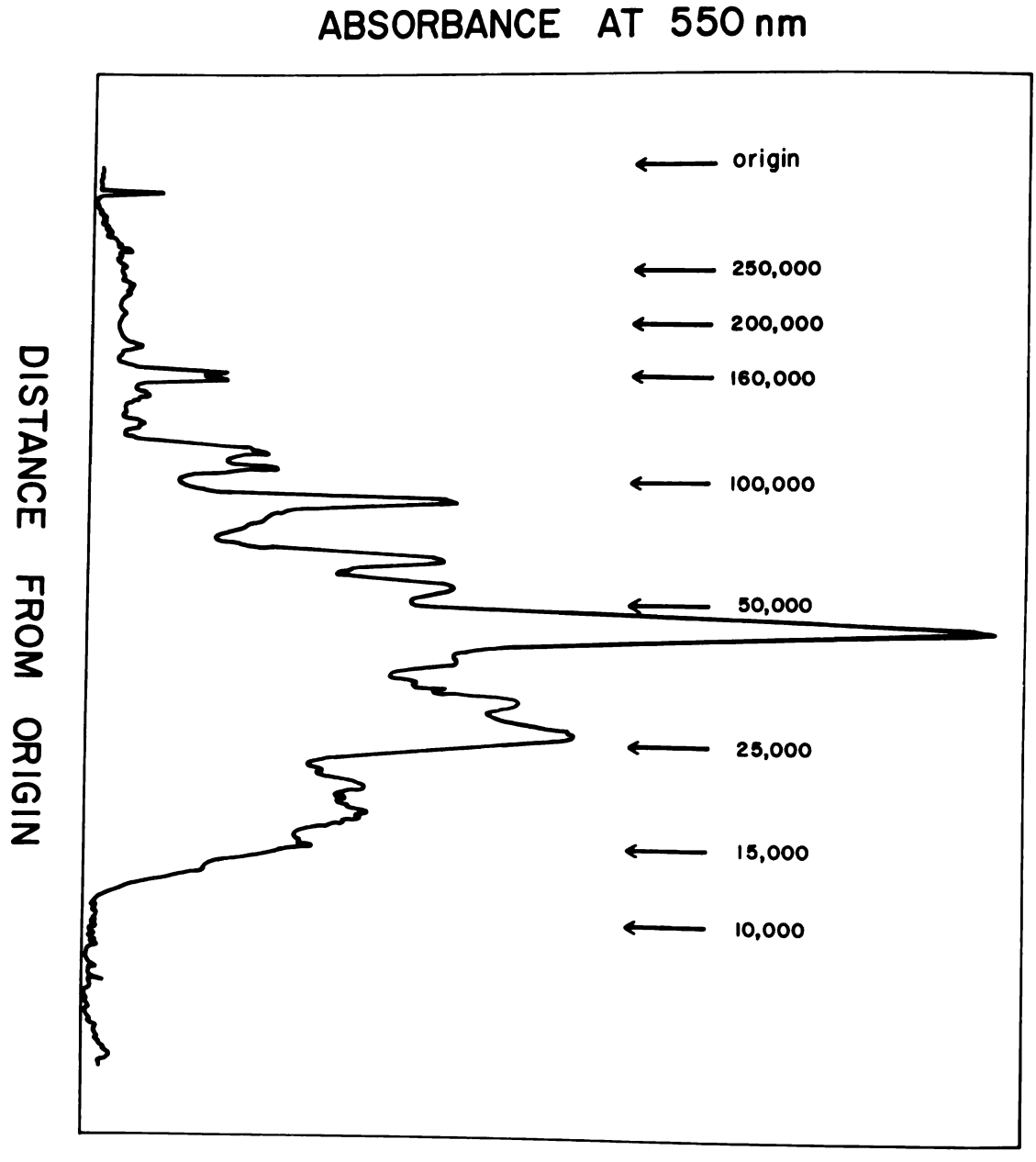


FIGURE 4.--Densimetric Tracing of an SDS-polyacrylamide Gel of Initial Extract

A mixture containing 100 µg protein of Initial Extract was incubated at 100° for 10 minutes in 50 µl of a solution containing 0.1 M sodium phosphate, pH 7.1, 1.0% SDS, 1.0% 2-mercaptoethanol, and 5% glycerol. A 5-µl sample of the mixture (10 µg protein) was layered on a 11 cm SDS-polyacrylamide gel prepared from an acrylamide-SDS mixture containing 3.75% acrylamide and 12.5% glycerol. Electrophoresis was performed at 25° for 2.5 hours at 8 ma/gel in a buffer containing 0.1 M sodium phosphate, pH 7.1, and 0.1% SDS. The protein was stained with Coomassie blue. Following destaining, the gel was scanned at 550 nm. The position of the polypeptide chains of the various molecular weights indicated by the arrows were determined using standards of known molecular weights.


ABSORBANCE AT 550 nm
DISTANCE FROM ORIGIN
origin
250,000
200,000
160,000
100,000
50,000
25,000
15,000
10,000

^{35}S analysis of sections of this gel (Figure 5) also led to the conclusion that β' plus β amounted to 1.2% of the total protein of the Initial Extract. Since β' plus β are 63% (by weight) of the holoenzyme ($\alpha_2\beta\beta'\sigma$), the holoenzyme could amount to 2% of the total protein of the Initial Extract. Accordingly, the yield of RNA polymerase in the Glycerol Gradient Fraction I was 2.5% of the estimated amount present in the Initial Extract.

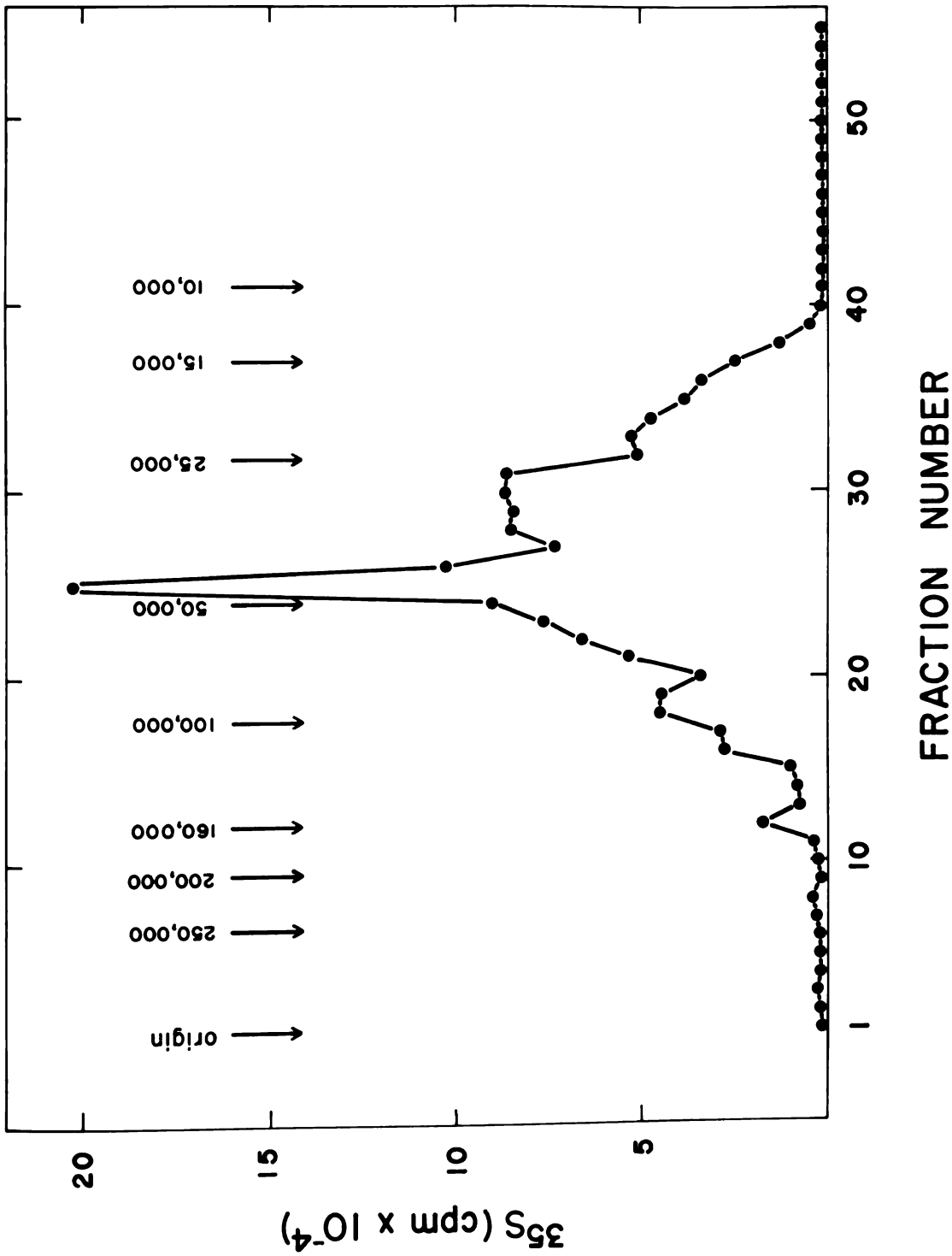
An estimation of the percent by weight in the Initial Extract of polypeptide chains of various molecular weights can be made from Figures 3 and 4. The per cent of polypeptide chains that have molecular weights greater than 25,000, 50,000, and 100,000 was 75, 31, and 9% respectively. One-half of the polypeptide chains have molecular weights greater than 36,000.

Characterization of ^{35}S-labeled RNA Polymerase

Specific enzymatic activity and specific radioactivity of ^{35}S-labeled RNA polymerase. The specific enzymatic activity of ^{35}S-labeled RNA polymerase (Glycerol Gradient Fraction I) was 7,800 mnoles of CMP incorporated per hour per mg of protein at 30° using gh-1 DNA as the template. With calf thymus DNA as the template, the specific enzymatic activity was 3,300. The specific radioactivity of ^{35}S-labeled RNA polymerase was 2.3×10^7 cpm per mg of protein.

FIGURE 5.--^{35}S Analysis of an SDS-polyacrylamide Gel of Initial Extract

The Coomassie blue stained SDS-polyacrylamide gel of Initial Extract which was analyzed by the densimetric tracing presented in Figure 4 was cut into 55 fractions (2 mm each). The ^{35}S content of each fraction was analyzed according to the method described in "Experimental Procedure (Assay of ^{35}S-labeled proteins in polyacrylamide gels)."


origin
250,000
200,000
160,000
100,000
50,000
25,000
15,000
10,000
20
15
10
5
^{35}S (cpm x 10^{-4})
1
10
20
30
40
50
FRACTION NUMBER

Stability of ^{35}S-labeled RNA polymerase. Another preparation of ^{35}S-labeled RNA polymerase which had been purified through the Agarose gel filtration step was used in the study of the stability of ^{35}S-labeled enzyme. This preparation of enzyme had a specific enzymatic activity of 8,700 nmoles of CMP incorporated per hour per mg of protein using gh-1 DNA as the template. The specific radioactivity was 7×10^6 cpm per mg of protein. The preparation of enzyme was stored at a protein concentration of 6 mg/ml at -20° in 50 mM Tris acetate, pH 8.0, 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 200 mM KCl, and 50% glycerol (v/v). After 90 days which is slightly more than the 87.9 day half-life of ^{35}S, 80 to 90% of the enzymatic activity was retained.

SDS-polyacrylamide gel electrophoresis of ^{35}S-labeled RNA polymerase. The β', β, σ and α subunits of the ^{35}S-labeled enzyme were resolved by SDS-polyacrylamide gel electrophoresis (Figure 6). A densimetric tracing of a Coomassie blue stained SDS-polyacrylamide gel is presented in Figure 7. As determined from measurements of the area under each subunit peak, the amount of β' plus β and of σ relative to α was 3.7 to 1.1 to 1.0, respectively. The amount of β' was equal to the amount of β (Figure 8). As calculated from the relative amounts of the subunits and their relative molecular weights, the subunit structural formula for the ^{35}S-labeled enzyme was $\alpha_2\beta\beta'\sigma$.

FIGURE 6.--SDS-polyacrylamide Gel Electrophoresis of Glycerol Gradient Fraction I

A mixture containing 30 µg protein of Glycerol Gradient Fraction I was incubated at 100° for 10 minutes in 60 µl of a solution containing 0.1 M sodium phosphate, pH 7.1, 1.0% SDS, 1.0% 2-mercaptoethanol, and 5% glycerol. A 4-µl sample of the mixture (2 µg protein) was layered on an 11 cm SDS-polyacrylamide gel prepared from an acrylamide-SDS mixture containing 3.75% acrylamide. Electrophoresis was performed at 25° for 4.75 hours at 8 ma/gel in a buffer (0.1 M sodium phosphate, pH 7.1, and 0.1% SDS). The protein was stained with Coomassie blue.

[redacted]

[redacted]

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FIGURE 7.--Densimetric Tracing of an SDS-polyacrylamide Gel of Glycerol Gradient Fraction I

A 2-μg sample of Glycerol Gradient Fraction I was analyzed by SDS-polyacrylamide gel electrophoresis as described in the legend to Figure 6. The protein was stained with Coomassie blue. The densimetric tracing was made at 550 nm using a Gilford linear transport.

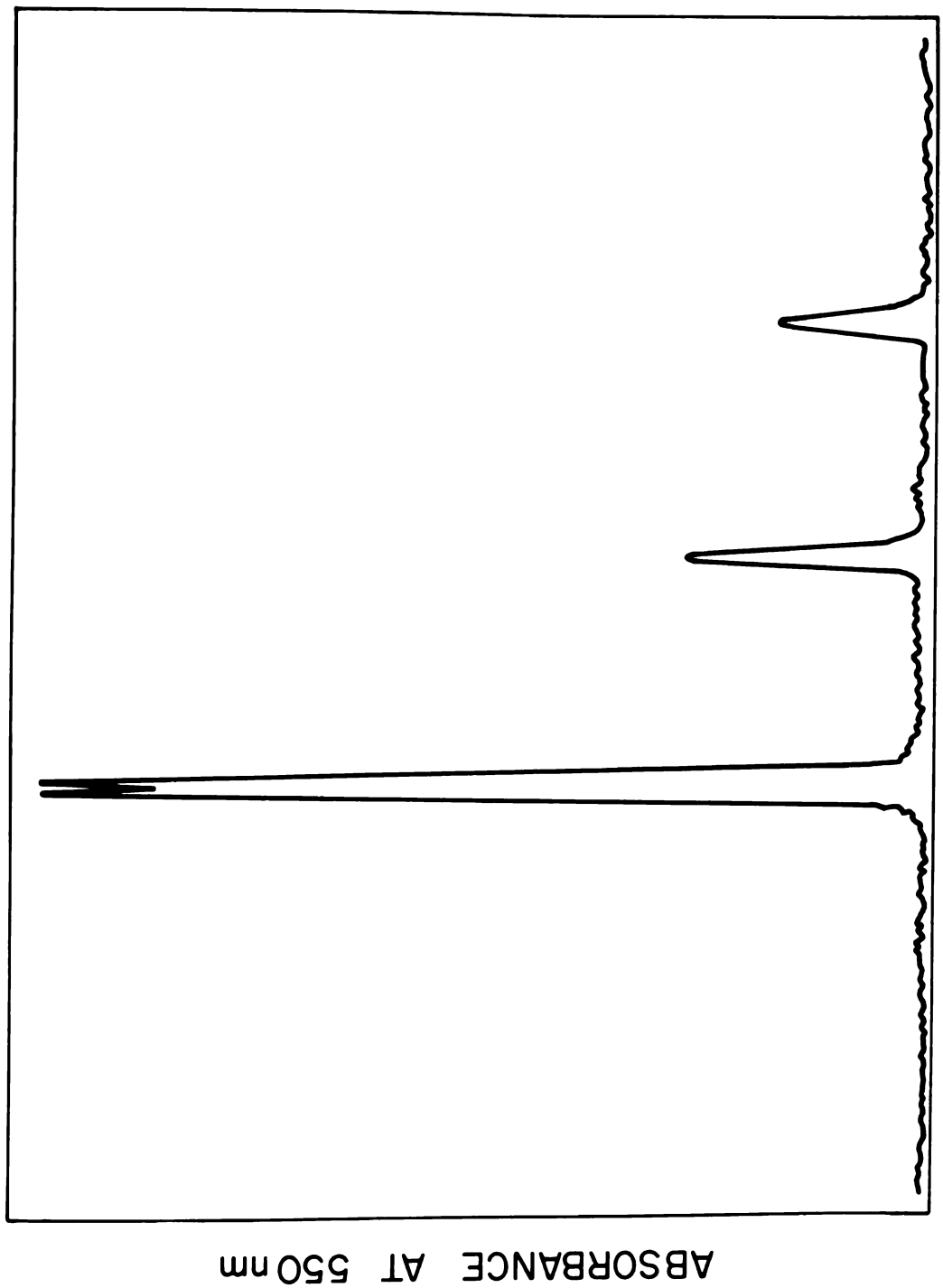
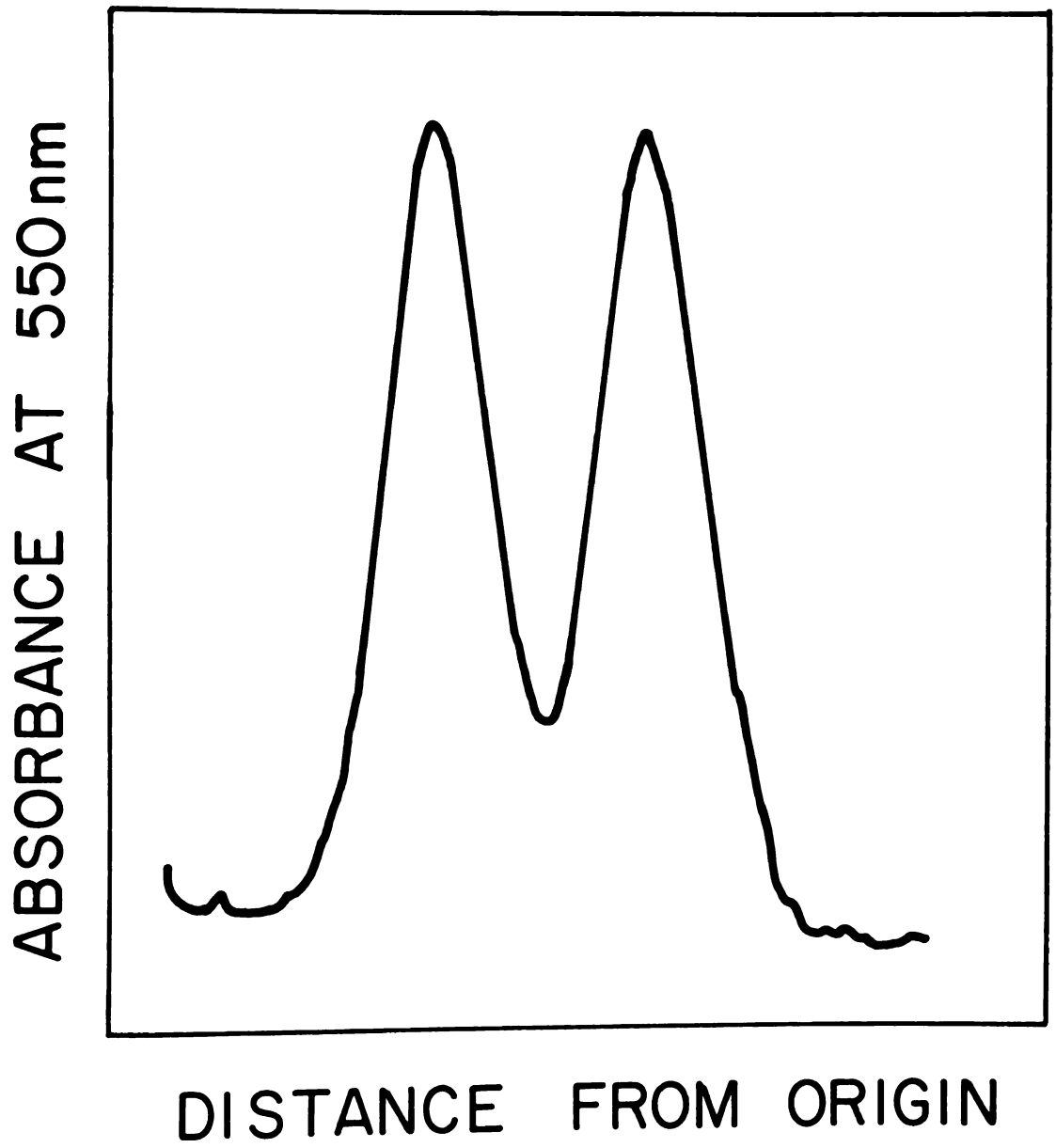

ABSORBANCE AT 550 nm
DISTANCE FROM ORIGIN

FIGURE 8.--Densimetric Tracing of the β' and β Region of an SDS-polyacrylamide Gel of Glycerol Gradient Fraction I

A 2-μg sample of Glycerol Gradient Fraction I was analyzed by SDS-polyacrylamide gel electrophoresis according to the legend to Figure 6 except that electrophoresis was for 6 hours. The gel was stained with Coomassie blue and scanned using a Joyce-Loebel Microdensitometer.


ABSORBANCE AT 550nm
DISTANCE FROM ORIGIN

Analysis of the ^{35}S content of sections of an SDS-polyacrylamide gel is presented in Figure 9. The amount of ^{35}S of β' plus β and σ relative to α was 7.2 to 2.2 to 1.0, respectively. In other experiments, the amount of ^{35}S of β' relative to β was determined. As shown in three different experiments, the ^{35}S contents of β' and β were equal (Table 2). Table 3 summarizes the data concerning the ^{35}S content of the subunits of RNA polymerase.

FIGURE 9.--^{35}S Analysis of an SDS-polyacrylamide Gel of Glycerol Gradient Fraction I

The Coomassie blue stained SDS-polyacrylamide gel of Glycerol Gradient Fraction I which was analyzed by the densimetric tracing presented in Figure 8 was cut into 50 fractions (2 mm each). The ^{35}S content of each fraction was analyzed according to the method described in "Experimental Procedure (Assay of ^{35}S-labeled proteins in polyacrylamide gels)."

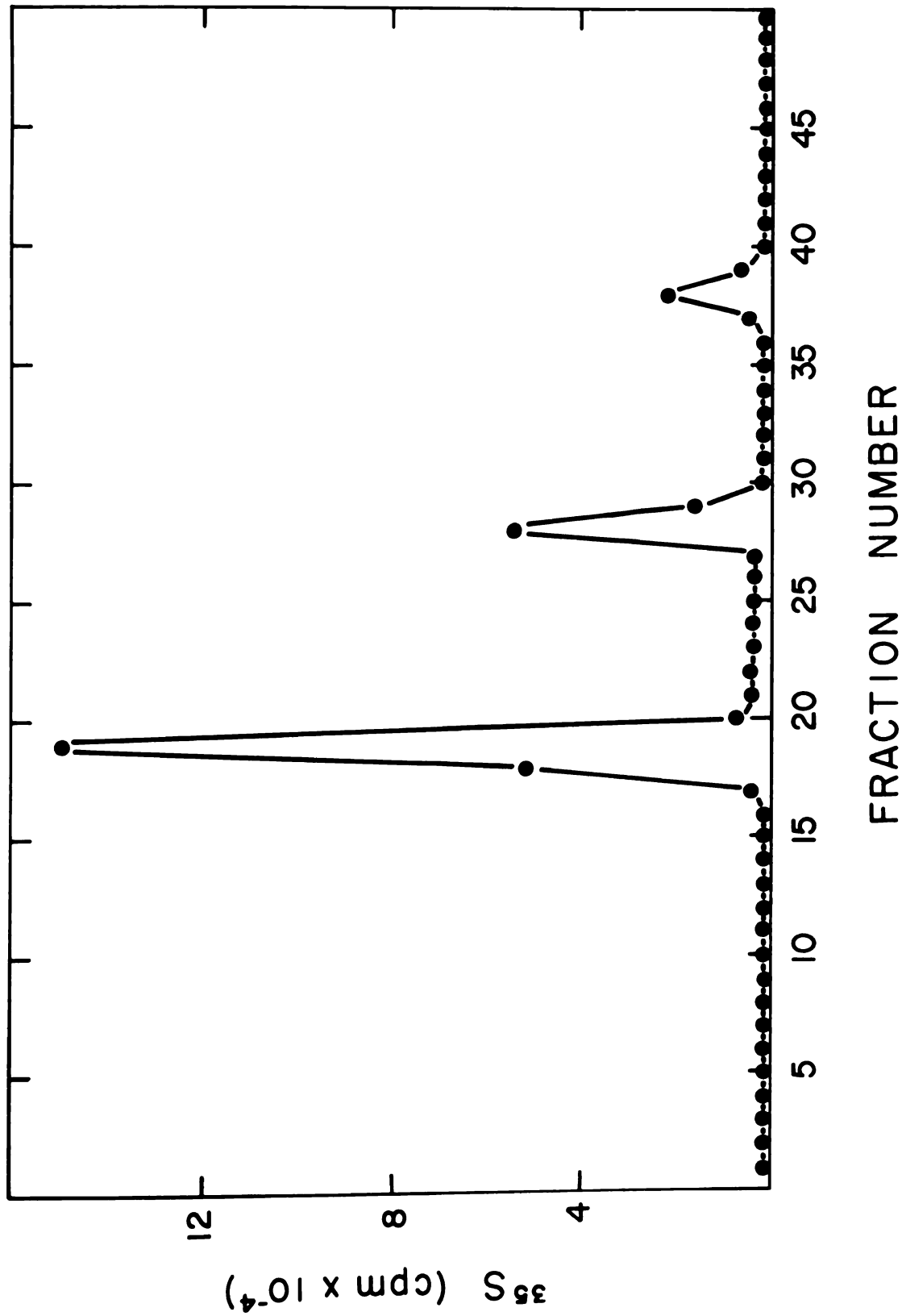

^{35}S (cpm x 10^{-4})
12
8
4
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

TABLE 2.--^{35}S Content of β' Relative to β

Experiment	^{35}S in β' (cpm)	^{35}S in β (cpm)	^{35}S Content of β' Relative to β
1	800	790	1.01
2	1490	1380	1.08
3	1510	1650	0.92

TABLE 3.--^{35}S Content of β', β, and σ Relative to α

Polypeptide Subunit	Relative Amount by Weight of Subunits	Relative ^{35}S Content of Subunits	Relative ^{35}S Content per 44,000 g of Subunits	Relative ^{35}S Content per Subunit Polypeptide Chain
β'	1.8	3.6	2.0	7.2
β	1.8	3.6	2.0	7.2
σ	1.1	2.2	2.0	4.4
α	0.5	0.5	1.0	1.0
α	0.5	0.5	1.0	1.0

DISCUSSION

^{35}S-labeled Pseudomonas putida RNA polymerase ($\alpha_2\beta\beta'\sigma$) was purified from labeled cells grown on minimal growth medium containing ^{35}S-labeled sulfate. The specific radioactivity of the pure enzyme was 2.3×10^7 cpm per mg protein. As a result of this high specific radioactivity, the radioactive assay used for protein quantitation was sensitive to 0.01 µg protein. As calculated from the known specific radioactivity of the ^{35}S-labeled sulfate used in the growth medium, one sulfur atom in approximately 20,000 was radioactive. If each RNA polymerase molecule contains 400 sulfur atoms, one enzyme molecule in every 50 contains a ^{35}S atom.

The amount of β' plus β in the Initial Extract was 1.2% by weight as determined by SDS-polyacrylamide gel electrophoresis. From the percent by weight of $\alpha_2\beta\beta'\sigma$ in the Initial Extract, the molecular weight of $\alpha_2\beta\beta'\sigma$ (5.06×10^5 daltons), and assuming that the Coomassie blue stain reacts with the polypeptides of the Initial Extract equivalently it was calculated that each bacterial cell contains approximately 5,000 molecules of $\alpha_2\beta\beta'\sigma$.

This preparation of ^{35}S-labeled RNA polymerase is being used in the study of the release of the σ subunit

from the holoenzyme ($\alpha_2\beta\beta'\sigma$) during the RNA synthetic process (7 and unpublished results). The results of this study have shown that σ is released following the binding of the holoenzyme to poly(A), poly(U), or to native P. putida DNA. The σ subunit is not released following the binding of the holoenzyme to poly[d(A-T)], native gh-1 DNA, denatured gh-1 or denatured P. putida DNA. Following the initiation of RNA synthesis on native gh-1 DNA as the template, σ is released from the enzyme. This result provides the first direct experimental evidence for the in vitro release of σ as described in the sigma cycle proposed by Travers and Burgess (8).

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