DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA: STUDIES ON THE MECHANISM OF ACTION

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This is to certify that the

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ABSTRACT

DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA: STUDIES ON THE MECHANISM OF ACTION

By

Gary Floyd Gerard

The objective of this research was to study the mechanism of action of DNA-dependent RNA polymerase from Pseudomonas putida. Three different aspects of the RNA polymerase catalyzed synthesis of RNA were examined. First, the requirement for a hydroxyl group at the 2'-position of a ribonucleoside triphosphate substrate of RNA polymerase was examined. Second, the requirement for a hydrogen or hydroxyl group at the 2'-position of a polynucleotide template of the enzyme was investigated. Third, the release of the sigma subunit of RNA polymerase during DNA directed RNA synthesis was studied using ^{35}S-labeled RNA polymerase.

P. putida RNA polymerase can use 2'-O-methyladenosine 5'-triphosphate (AmTP) as a substrate for RNA synthesis. In the DNA-directed synthesis of RNA with AmTP or an equimolar mixture of AmTP and adenosine 5'-triphosphate (ATP) as the

adenyl substrates, a small amount of 2'-O-methyladenosine 5'-monophosphate (AmMP) was incorporated into RNA after a 60 minute incubation. This amounted to 1 to 2 pmoles of AmMP for each pmole of RNA polymerase added to the reaction mixture. AmMP-containing RNA synthesized in reaction mixtures which contained AmTP as the only added adenyl substrate had a sedimentation coefficient of less than 4 S as determined by sucrose density gradient analysis. Abortive release of the AmMP-containing RNA from the DNA-RNA polymerase-nascent RNA ternary complex did not occur. AmMP-containing RNA synthesized in reaction mixtures which contained both AmTP and ATP as the adenyl substrates was heterogeneous in size with most of the RNA having a sedimentation coefficient of about 30 S. Degradation of the RNA product by alkaline hydrolysis followed by alkaline phosphatase digestion showed that 90% of the AmMP residues were located at the 3'-end of the RNA chain with the remaining 10% at the 5'-end or in the interior of the chain. The studies with AmTP lead to the following conclusions. (1) A free 2'-hydroxyl group is not required for binding of an adenyl substrate by P. putida RNA polymerase or for subsequent incorporation into RNA. (2) Following the incorporation of AmMP into the 3'-end of the nascent RNA chain, the rate of RNA chain growth is greatly reduced. The reduction in the rate of RNA chain growth results in the accumulation

of nascent RNA chains with AmMP at the 3'-end. The rate-limiting step in RNA synthesis becomes the addition of the next nucleotide to the nascent RNA chain.

P. putida RNA polymerase can use 2'-O-methyl-polyuridylic acid (poly[Um]) and 2'-O-methylpolycytidylic acid as templates for the synthesis of polyadenylic acid (poly[A]) and polyguanylic acid, respectively. No template activity was detected with the purine-containing 2'-O-methylated homopolymers, 2'-O-methyladenylic acid (poly[Am]) and 2'-O-methylinosinic acid. The poly(Am) strand of either poly(Am) · poly(U) or poly(Am)· poly(Um) was not a template for poly(U) synthesis, and did not prevent the poly(U) or poly(Um) strand of the duplex from serving as a template for poly(A) synthesis. The poly(Um) strand of the duplex poly(Am) · poly(Um) was an effective template for poly(A) synthesis, but the poly(Um) strand of poly(A)· poly(Um) was not.

^{35}S-labeled P. putida DNA-dependent RNA polymerase, $\alpha_2\beta\beta'\sigma$, was purified from cells that had been grown in a minimal medium containing sodium [^{35}S]sulfate. The amount of ^{35}S in β', β, and σ relative to α was 3.6 to 3.6 to 2.2 to 1.0, respectively. A study of the release of the sigma subunit of P. putida RNA polymerase was carried out following the binding of enzyme to polynucleotides and during DNA-directed RNA synthesis. Sucrose density gradient

centrifugation was the technique employed to assay for the release of ^{35}S-labeled sigma. The subunits of ^{35}S-labeled RNA polymerase present in protein peaks resolved on sucrose gradients were identified by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Binding of ^{35}S-labeled RNA polymerase to native DNA weakened the interaction between sigma and core polymerase ($\alpha_2\beta\beta'$) but did not result in the release of sigma. Binding of the ^{35}S-labeled enzyme to polyadenylic acid, polycytidylic acid, and to transfer RNA resulted in the release of sigma. Binding of ^{35}S-labeled RNA polymerase to an alternating copolymer of deoxyadenosine and thymidine (poly[d(A-T)]), denatured gh-1 DNA, polythymidylic acid, and to polydeoxyadenylic acid did not result in the release of sigma. Release of sigma subsequent to the binding of enzyme to polydeoxycytidylic acid and polyuridylic acid occurred in the absence of manganese chloride but not in its presence. Sigma was released from the enzyme-polynucleotide complex during DNA-directed RNA synthesis. Within 3 minutes of incubation, about 60% of the ^{35}S-labeled RNA polymerase molecules initiated RNA synthesis and formed a 200 mM KCl stable complex with DNA and nascent RNA. All or almost all of these enzyme molecules released sigma. The other 40% of the enzyme molecules did not form a 200 mM KCl stable complex within 3 minutes. With longer times of incubation,

these enzyme molecules could slowly form a 200 mM KCl stable complex but did not release sigma. The sedimentation coefficient of P. putida sigma released during DNA-direction RNA synthesis was 4.1 to 4.5 S.

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DEDICATION

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GENERAL INTRODUCTION

DNA-dependent RNA polymerase is one of the most complex enzymes, both structurally and functionally, yet to be studied. The complete enzyme or holoenzyme from Escherichia coli, Azotobacter vinelandii, and Pseudomonas putida has five polypeptide subunits: one beta prime (β') subunit, one beta (β) subunit, one sigma (σ) subunit, and two alpha (α) subunits. The subunits of P. putida holoenzyme ($\alpha_2\beta\beta'\sigma$) have the following molecular weights: β', 165,000; β, 155,000; σ, 98,000; and α, 44,000. In order for RNA polymerase to catalyze the synthesis of a defined RNA molecule from a DNA template, the enzyme must first initiate RNA synthesis at a specific site on the DNA, it must then faithfully copy one of the strands of the DNA cistron in accordance with the classic Watson-Crick rules of base pairing, and the enzyme must finally terminate synthesis at a second specific site on the DNA template. The sigma subunit of RNA polymerase has the ability or imparts the ability acting in concert with the other RNA polymerase subunits, which collectively are called core enzyme ($\alpha_2\beta\beta'$), to recognize the specific DNA nucleotide sequences which are signals for initiation of transcription. The core

enzyme alone is also capable of recognizing some terminating DNA sequences, but other protein factors are required for recognition by core enzyme of all termination signals.

The results presented in this thesis are concerned with answering three different questions about the mechanism of action of P. putida RNA polymerase. The first two questions deal with RNA polymerase substrate and template structural requirements. First, can RNA polymerase use a nucleoside triphosphate as substrate which has a bulky methoxy group rather than a hydroxyl group at the 2'-position of the sugar moiety? Second, can RNA polymerase use a polynucleotide as template which has a bulky methoxy group rather than a hydrogen or hydroxyl group at the 2'-position of each sugar moiety? The third question dealt with is outlined below.

A tenet regarding the RNA synthetic process which is accepted as fact in current scientific thought and communication, both in biochemical journals and textbooks, is that the sigma subunit of RNA polymerase is released during RNA synthesis in order to combine with other core enzyme molecules which may subsequently bind to DNA to initiate RNA synthesis. The initial and most frequently cited experiment upon which the concept of sigma release in vitro is based, however, is both indirect and open to other interpretations. No release of sigma in vivo has

as yet been demonstrated. We have attempted to answer the question of whether or not sigma is released *in vitro* during RNA synthesis with *P. putida* RNA polymerase by using techniques which allow the physical separation of released sigma from core enzyme and which permit identification and quantitation of released sigma in a reaction mixture.

This thesis is organized into four major divisions. The first division is a literature survey in which much of the current information on the mechanism of action of bacterial DNA-dependent RNA polymerase has been summarized. The second division is an article on 2'-*O*-methyladenosine 5'-triphosphate as a substrate for *P. putida* RNA polymerase. The article is presented in the form of a reprint from the journal in which it was published. The third and fourth divisions are articles presented in the format of a scientific paper, with its own *Abstract*, *Introduction*, *Materials and Methods*, *Results*, *Discussion*, and *References* sections. Article Two deals with a study of 2'-*O*-methylhomopolyribonucleotides as templates for *P. putida* RNA polymerase. The contents of Article Two have been submitted to *Biochemical and Biophysical Research Communications* for publication. A preliminary report on some of the data in this article was presented at the 69th Annual Meeting of the American Society for Microbiology

(Gary F. Gerard and J. A. Boezi, Bacteriological Proceedings, 69, 135 [1969]). Article Three is concerned with the characterization of ^{35}S-labeled RNA polymerase from P. putida and the use of the enzyme to study the release of sigma factor during DNA-directed RNA synthesis. The contents of this article have been submitted to Biochemistry for publication. A preliminary report on some of the data in Article Three was presented at the 62nd Annual Meeting of the American Society of Biological Chemists (Gary F. Gerard, J. C. Johnson, and J. A. Boezi, Federation Proceedings, 30, 1162 [1971]).

LITERATURE SURVEY

Introduction

In bacteria a single enzyme, DNA-dependent RNA polymerase, is the enzyme directly responsible for the synthesis of all types of cellular RNA. Genetic information encoded in DNA is transcribed by RNA polymerase into complementary RNA which may be translated into specific proteins or may become the RNA of ribosomes or transfer RNA. Since the first reports of the detection of DNA-dependent RNA polymerase in bacteria in 1960, a prodigious literature concerning the purification, the chemical, physical, and structural characterization, and the elucidation of the mechanism of action of bacterial RNA polymerase has accumulated. The extent and variety of topics treated in the literature are beyond the scope of this survey. A number of recent reviews and symposia concerning RNA polymerase and transcription are available and should be consulted for a comprehensive treatment of the literature (1-8).

RNA polymerase has been isolated and extensively purified from a number of bacterial sources (1,2). The enzymes from Escherichia coli (several strains) (9-11),

Azotobacter vinelandii (12-14), and Pseudomonas putida (15) have been purified to homogeneity as determined by analysis of protein using polyacrylamide gel electrophoresis. The subunit composition of the enzyme from all three sources has been shown to be essentially the same by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. RNA polymerase in the holoenzyme form has the following polypeptide chain subunits: one beta prime subunit, β'; one beta subunit, β; two alpha subunits, α; and one sigma subunit, σ. Holoenzyme from E. coli can be separated into two functional parts by phosphocellulose chromatography (16): a core enzyme ($\alpha_2\beta\beta'$) which is the basic machinery for carrying out transcription, and a sigma (σ) factor which enables core to initiate RNA synthesis efficiently at specific DNA sites. In addition to sigma factor, there are several other protein factors (transcriptional factors) which have a pronounced effect on transcription. These transcriptional factors do not bind tightly enough to core enzyme to purify with it.

This survey will be restricted to a discussion of the enzyme from E. coli since it is the most highly studied and consequently, the best known. In addition, emphasis will be placed primarily on a discussion of the mechanism of each step involved in the RNA synthetic process and the role that the subunits of RNA polymerase and the

transcriptional factors play in specific steps. The steps of RNA synthesis which will be delineated are: (1) binding of RNA polymerase to the DNA template, (2) initiation of RNA synthesis, which involves formation of the first phosphodiester bond between the 5'-terminal and the second ribonucleotide, (3) propagation of synthesis or RNA chain elongation, and (4) termination of synthesis and release of enzyme and nascent RNA from the DNA template. Before discussing the mechanism of RNA synthesis, a few more details on the structure of E. coli RNA polymerase will be outlined.

Structure of RNA Polymerase

E. coli RNA polymerase may be dissociated into its individual polypeptide chain subunits by treatment with denaturing agents such as sodium dodecyl sulfate (SDS) or 8 M urea (17,18). The subunits can be separated analytically by electrophoresis on polyacrylamide gels containing SDS or 8 M urea (17,18), or preparatively by electrophoresis on cellulose acetate slabs containing 8 M urea (19,21,24). The molecular weights of the subunits have been determined by comparing mobilities of the SDS-dissociated subunits on SDS-containing polyacrylamide gels with the mobilities of polypeptides of known molecular weight (20). The values obtained in several laboratories are: β', 150,000 to 165,000; β, 145,000 to 155,000;

σ, 85,000 to 95,000; and α, 39,000 to 41,000 (17,21,22). The stoichiometry of the subunits in holoenzyme determined by measuring relative band intensities on stained SDS-polyacrylamide gels is $\alpha_2\beta\beta'\sigma$ (22,23). Tryptic fingerprints for each subunit have been obtained and they indicate that β', β, σ, and α have different amino acid sequences (19,24). Core enzyme can be obtained from holoenzyme by phosphocellulose chromatography (16) and the stoichiometry of subunits in core is $\alpha_2\beta\beta'$ (17). The minimal formula weights which can be calculated for core enzyme and holoenzyme are 400,000 ± 10% and 490,000 ± 10%, respectively. The molecule weight of the protomer form of core enzyme as determined by sedimentation equilibrium is 380,000 (22), which is in good agreement with the calculated minimal formula weight. As determined by sedimentation velocity and equilibrium experiments (22), both holoenzyme and core enzyme exist as aggregates of their protomer forms at low ionic strengths. Holoenzyme forms a dimer of its protomer at ionic strengths below 0.12, and core enzyme forms a complex containing at least six core enzyme protomers at ionic strengths below 0.26. Above ionic strengths of 0.12 and 0.26, respectively, holoenzyme and core enzyme exist as protomers.

Binding

Initial Binding. The initial binding of RNA polymerase to DNA is rapid and reversible (25-27), dependent upon ionic strength (25,28,29), and inhibited by RNA (30) and by the polyanion heparin (31). The number of enzyme molecules which can be bound by a DNA template at low ionic strength seems to be limited only by the amount of space available on a particular template (32). Enzyme molecules bound in a densely packed array on DNA, however, are bound in a transcriptively nonproductive way (32,33). Experiments involving a number of DNA templates including T_4, T_7, λ, and doubly-closed circular polyoma and papilloma DNA's have demonstrated that under low ionic strength conditions (ionic strength ~ 0.1), enzyme molecules which can be bound in a transcriptively productive manner are located at comparatively widely spaced points along the DNA (25, 27,34,35). The quantities of enzyme bound correspond to approximately one enzyme molecule in the dimer form to every 2,000 to 2,500 Å of DNA or every 750 DNA base pairs, or 15 to 70 enzyme binding sites per DNA molecule depending upon the size of the particular DNA species. This number of binding sites is much larger than the actual number of specific initiation sites or promoter sites thought to exist on T_4, T_7, and λ DNA (36-42). The preparations of *E. coli* RNA polymerase used to study initial binding probably rarely contained more than 50% of the stoichiometric

content of sigma (11,24). Indeed, sigma is not required for the initial binding of RNA polymerase to DNA since both holoenzyme and core enzyme bind to DNA in this manner with equal facility (43).

Enzyme-DNA Preinitiation Complex Formation. Subsequent to the initial binding of holoenzyme to T_7 DNA, a highly stable preinitiation complex can be formed between holoenzyme and a small number of specific sites on the T_7 DNA (44). Temperatures above 20° C are required to form the complex, and at low ionic strength and 37° C the complex has a half-life of 60 hours. The complex is much less stable at higher ionic strength and lower temperatures. Holoenzyme has also been observed to form similar preinitiation complexes with T_4 DNA (24). Sigma is required for the formation of these specific preinitiation complexes, since binding complexes formed between core enzyme and T_7 DNA are much less stable and are not located at a small number of specific sites (44). The sites on the T_7 DNA at which the preinitiation complexes are formed are thought to be the same as the specific promoter sites on T_7 at which RNA synthesis is initiated *in vitro* and *in vivo* by *E. coli* RNA polymerase (44). A possible reason which might help to explain the descrepancy between the number of initial binding sites and the smaller number of promoter sites on bacteriophage DNA has been suggested by Burgess (6).

RNA chains initiated by both core enzyme and holoenzyme begin almost exclusively with purine nucleosides. The second nucleoside is predominantly a pyrimidine with holoenzyme, but with core enzyme the next nucleosides most frequently are a run of one or more purines (45,46). The polypurine runs with core enzyme suggest that it does not start transcription entirely at random, but prefers to bind to regions of the DNA template rich in pyrimidines. This might also be true for holoenzyme since there is a correlation between the bacteriophage DNA strand transcribed preferentially by holoenzyme *in vitro* and *in vivo* and the strand containing pyrimidine clusters (47,48). These clusters are dispersed throughout the genome and their spacing has been estimated as on the average one cluster 15 to 40 nucleotides long every 500 to 5000 DNA nucleotide pairs (48-50). If only a fraction of the pyrimidine clusters have specific initiation sequences contiguous to them (1,48), it could help to explain the presence of a larger number of initial binding sites than specific initiation or promoter sites on DNA.

The strong temperature dependence of the formation of the specific preinitiation complex between holoenzyme and T_7 DNA (44) or T_4 DNA (24) suggests that a melting out

of the DNA helix occurs simultaneous with complex formation. Sigma is thought to mediate this opening of the bound region of the DNA helix since sigma is required for the formation of the specific preinitiation complex. Indeed, core enzyme appears to require a single-stranded or partially single-stranded region in a DNA template at which to bind to allow initiation of RNA synthesis. That is, polynucleotides such as single-stranded DNA (16) and double-stranded DNA containing or treated to produce single-strand breaks (16,51), and open structure double-helical DNA's such as poly[d(A-T)] (11) and twisted closed-circular ϕX-174 RF (51) and fd RF (45) are the only DNA's which serve as efficient templates for core enzyme.

Rifampicin-Resistant Enzyme-DNA Complex Formation. Rifampicin is an antibiotic which inhibits RNA synthesis *in vivo* (52) and *in vitro* (53). The drug blocks a step in RNA synthesis which occurs after binding of enzyme to DNA but prior to chain initiation (5), and functions by binding specifically to the β subunit of RNA polymerase (54). Subsequent to formation of the stable preinitiation complex described earlier, holoenzyme undergoes a change, perhaps a conformational change, which results in the formation of a rifampicin-resistant complex. This has been shown to occur with T_2, T_4, and T_7 DNA (24,36,55,56). The two complexes can be distinguished because the stable

preinitiation complex can be formed in the presence of rifampicin (5) whereas formation of the rifampicin resistant complex is blocked by rifampicin (36,56). The decay of the rifampicin-resistant complex to rifampicin sensitivity in the presence of the drug is two to three orders of magnitude faster than the dissociation of the stable preinitiation complex at the same ionic strength (36). Sigma is necessary for the formation of the rifampicin-resistant complex with intact double-stranded phage DNA (36,56). Enzyme bound to rifampicin loses its ability to bind the initiating purine nucleoside triphosphates even in the absence of DNA (57), so that it has been suggested that the change in enzyme associated with formation of the rifampicin-resistant complex is necessary for correct binding of the initiating purine nucleoside triphosphate and subsequent phosphodiester bond formation with the second nucleoside triphosphate (6).

The Role of RNA Polymerase Subunits in Binding. The subunit of core enzyme which by itself carries the specificity required for binding to DNA is β' (24). This conclusion is based on the fact that β' can efficiently bind DNA to membrane filters whereas β, σ, and α cannot (58,59). There may be other DNA-binding sites, however, which require interaction with other subunits in order to be exposed. Although sigma alone does not bind to DNA (60), the factor is intimately involved in the selection of a specific

binding site by RNA polymerase and perhaps subsequent to binding is required for local melting of the bound region of the DNA helix.

Initiation

Nucleoside Triphosphate Binding Sites. RNA chain initiation is usually defined as the oriented binding of two nucleoside triphosphates to RNA polymerase followed by phosphodiester bond formation with the formation of a dinucleoside tetraphosphate and the elimination of inorganic pyrophosphate (5). It has been established for some time that *in vitro* RNA synthesis is initiated almost entirely with purine nucleotides (61-64), so that the first nucleoside triphosphate which binds to enzyme is a purine. The binding of nucleoside triphosphates to enzyme has been studied through the use of kinetic analysis (65), and by equilibrium dialysis and fluorescence quenching (66,67). Based on these studies, a model has been proposed which suggests the existence of at least two nucleoside triphosphate-binding sites on RNA polymerase (57,67). One site which has been designated as the initiation binding site strongly prefers the binding of purine triphosphates. This binding does not require divalent metal ion and is inhibited by rifampicin. The second binding site, which has been designated as the polymerization binding site, exhibits approximately an equal affinity for all nucleoside

triphosphates, requires divalent metal ion, and is not affected by rifampcin. The initiation site is a relatively weak binding site while the polymerization site is a relatively strong binding site. There is a tenfold difference between the values for apparent substrate Km at the two sites. Some question has been raised, however, concerning the tenfold difference in apparent Km's, since for poly [d(A-T)]-directed poly r(A-U) synthesis the apparent Km's for ATP and UTP are the same measured either for initiation or for polymerization (68). In this study, higher substrate concentrations were required for initiation than chain elongation because initiation was found to be a bimolecular reaction and the ratelimiting step in poly r(A-U) synthesis, while chain elongation was found to be first-order.

The Role of Sigma in Initiation. The sigma subunit of E. coli RNA polymerase has a dramatic influence on both the efficiency and specificity with which the enzyme initiates RNA synthesis. Initiation may be monitored by means of a pyrophosphate exchange reaction catalyzed by RNA polymerase (69) in which radioactive inorganic pyrophosphate is incorporated into the ribonucleoside triphosphates involved in initiation (13). Measurement of the pyrophosphate exchange reaction has shown that with T_4 DNA as the template, initiation of RNA synthesis is dependent upon the presence of sigma (70). It was concluded from

this study that sigma is probably necessary for the formation of the first phosphodiester bond catalyzed by RNA polymerase. In this regard, the apparent Km values for initiation of nucleoside triphosphates were found to be higher in the absence of sigma then in its presence (45), suggesting that sigma facilitates initiation. RNA chain initiation can also be assayed by monitoring the DNA-directed incorporation of γ-^{32}P-labeled nucleoside triphosphates into RNA, for only the first nucleotide incorporated into the growing RNA chain retains its triphosphate group (61,62). With intact double-stranded bacteriophage DNA's such as T_4, T_7, and λ as template, the number of RNA chains initiated by RNA polymerase is some 5- to 75-fold greater in the presence of sigma than in its absence (6).

Sigma is necessary for accurate initiation of RNA synthesis at specific sites on the DNA template. Initiation of RNA synthesis by E. coli RNA polymerase in vitro on T_4 DNA (71,72), as well as T_7 DNA (40,41), in the presence of sigma is restricted to sites on one strand of the DNA which are the sites utilized in vivo during the "early" period of viral infection. In the absence of sigma, initiation of RNA synthesis in vitro occurs at random sites on both strands of the viral DNA. This has been demonstrated by means of hydribization competition experiments with in vitro

and *in vivo* RNA transcripts and separated strands of viral DNA. Through the elegant work of Suguira, Okamoto, and Takanami (45,73), a more direct demonstration has been made of how sigma restricts initiation to specific sites on DNA. These workers analyzed size, asymmetry, and 5'-terminal sequences of RNA synthesized by *E. coli* RNA polymerase from fd phage replicative form DNA *in vitro*. Holoenzyme initiates primarily three different RNA chains of discrete size and initial sequence from one DNA strand. In contrast, core enzyme transcribes both strands and produces RNA which has many initial sequences and is very heterogeneous in size. These same analyses have been performed on RNA transcripts synthesized from ϕ80 DNA (73,74), and again the results indicate that sigma restricts initiation of RNA synthesis by *E. coli* RNA polymerase to specific sites on DNA.

Based mainly on the studies summarized in this survey on the role of sigma in binding and initiation, it has been hypothesized that sigma may play a dual role in the RNA synthetic process: one in stabilizing enzyme-DNA complexes at specific initiation (promoter) sites, and the other in facilitating initiation of the RNA chains at the specific sites (6). It is not known whether actual recognition of the initiation sequence is carried out by core enzyme or sigma. If the specific information

for site selection is localized in sigma, then sigma would direct the core polymerase to bind at the promoter site. On the other hand core polymerase could identify a specific nucleotide sequence and the role of sigma might be to act as an allosteric effector to promote tight, site-specific binding.

The Role of Positive Control Factors in Initiation. The existence of a class of protein factors in bacteria which function as positive control elements for initiation of transcription of different general classes of RNA has been hypothesized (76). These factors would act as secondary specificity determinants for promoter recognition by RNA polymerase, the primary specificity determinant being sigma. They would require the presence of sigma to function, and would be regulated by low molecular weight effectors (75). It has been suggested that in addition to the initiation site at which the sigma-directed interaction of holoenzyme with the DNA promoter takes place, there is a secondary site located in the promoter region which, if present, requires the binding of a positive-control element for initiation of RNA synthesis (6). To date, two such positive-control elements, ψr and CAP, have been isolated from E. coli.

A positive-control factor or psi (ψ) factor has been partially purified from E. coli which is specific for ribosomal RNA genes (75). This factor, ψr, stimulates

transcription of E. coli DNA by holoenzyme but not by core enzyme. In the absence of ψr with E. coli DNA as template, less than 0.2% of the RNA synthesized by E. coli holoenzyme in vitro is ribosomal RNA (75,76). The RNA synthesized in the presence of ψr by holoenzyme is 30 to 40% ribosomal RNA (75). Guanosine tetraphosphate, ppGpp, which is known to accumulate in stringent strains of E. coli during amino acid starvation (77), and which is thought to directly inhibit ribosomal RNA synthesis in vivo (78), has been tested for its effect on ψr-stimulated RNA synthesis. The nucleotide at a concentration of 0.4 mM inhibits ψr-stimulated RNA synthesis 50 to 65% while inhibiting RNA synthesis by holoenzyme alone by only 10% (79). No ψr-directed ribosomal RNA synthesis was detected at this ppGpp concentration.

A second positive control factor purified from E. coli, catabolite gene-activating protein (CAP) (80) or cyclic AMP receptor protein (CRP) (81), mediates the effect of cyclic AMP which is required for the expression of genes subject to catabolite repression (82). The action of CAP in regulating the transcription of lactose operon-containing DNA has been studied in a cell-free system (83,84). In a purified system consisting of lac operon-containing DNA and core polymerase, the synthesis of lac-specific messenger RNA is stimulated only if CAP, cyclic

AMP, and sigma are all present. If lac repressor is added to the complete system, about 80% of lac-specific transcription is blocked. This inhibition by repressor can be overcome with synthetic inducers of the lac operon. Both cyclic AMP and CAP are required for the formation of a rifampicin-resistant preinitiation complex (36) which can make lac messenger RNA, indicating that both cyclic AMP and CAP are required for binding of holoenzyme to lac promoter. Finally, it has been reported that CAP itself binds tightly to lac-containing DNA (84).

Chain Elongation

Growth of RNA chains occurs with 5' to 3' chemical polarity with the sequential addition of nucleoside monophosphates to the 3'-terminus of the nascent RNA chain (61,62). The process involves classical Watson-Crick base pairing between substrate ribonucleoside triphosphates and the DNA template to produce a complementary RNA copy of one of the two strands in any region of the DNA template. The overall rates of RNA chain elongation obtained with current enzyme preparations and assay conditions in vitro are comparable to those obtained in vivo (42,85).

A change occurs in the enzyme at some point after RNA chain initiation which makes the interaction between enzyme and DNA resistant to high ionic strength (25,86).

With poly[d(A-T)] as the template, ATP or UTP alone does not stabilize binding of holoenzyme to poly[d(A-T)] (87). The dinucleotide primer, ApU, by itself does not stabilize the complex against 0.1 M KCL and ATP must be added to form ApUpA before stabilization occurs (88).

At some stage in *in vitro* chain elongation, sigma is apparently released from the holoenzyme and core polymerase continues chain elongation (23). This has led to the postulation of the sigma cycle in which sigma functions in RNA synthesis only during initiation (23). What triggers sigma release and the rationel for its release are not clear. Several lines of evidence indicate that sigma may be released *in vivo* during RNA synthesis. RNA polymerase-DNA complexes actively involved in RNA synthesis have been isolated from *E. coli*, and it has been determined by SDS-polyacrylamide gel electrophoresis that the complexes as obtained do not contain sigma (89). RNA polymerase-λ DNA complexes have been isolated from λ-infected *E. coli* which apparently do not contain detectable amounts of sigma as determined by more indirect kinetic analyses (90).

Termination

There are three aspects of the process of termination which are of interest: (1) the cessation of RNA synthesis at a specific site on DNA , (2) the release of the completed

RNA chain from the ternary enzyme-DNA-RNA complex, and (3) the release of enzyme from the ternary complex. There are several kinds of termination which occur *in vitro* during RNA synthesis from double-stranded DNA, and one kind is mediated by a protein factor called rho (ρ).

Nonspecific termination occurs at low ionic strength ($\mu \leq 0.1$) and is probably due to inhibition of enzyme by the RNA product; termination occurs without release of either enzyme or RNA(91). Specific termination is observed at $\mu=0.1$ or higher with fd RF (45,73), φ80 (46,73), T_2 (93), T_4 (92-96), and T_7 DNA (95,96), leading to the release of RNA chains of discrete sizes which vary with the template. The RNA chains synthesized from T_4 and T_7 DNA have predominantly uridine as the 3'-OH terminal nucleoside (95,96). At $\mu=0.11$, RNA chains are released from T_4 DNA but enzyme does not reinitiate (94). At $\mu=0.2$ to 0.37, enzyme is released and reinitiates RNA synthesis repeatedly (92, 94-96).

The Role of Rho Factor in Termination. A second kind of specific termination requires the presence of the termination factor rho (ρ) (97). This protein factor has been extensively purified from *E. coli* and is thought to be a tetramer of molecular weight 200,000 (97). Rho-induced termination is not very sensitive to either RNA polymerase or DNA concentration, suggesting that rho is

not tightly bound at its receptor site (93). Rho does not bind appreciably to DNA or to RNA polymerase, but does bind to RNA (93). Rho is not a simple ribonuclease (93, 95). Termination in the presence of rho on T_4, T_7, λ, φ80, and fd RF DNA occurs at specific rho-dependent sites which usually precede the rho-independent sites discussed earlier (42,46,73,93,97). Rho is inactive at high ionic strengths ($\mu \geq 0.15$) (93), and release of active enzyme has not yet been observed after rho-induced termination (92,93).

REFERENCES

1. Geiduschek, E. P., and Haselkorn, K., Ann. Rev. Biochem., 38, 647 (1969).

2. Richardson, J. P., Progr. Nucl. Acid Res. Mol. Biol., 8, 75 (1969).

3. Yarus, M., Ann. Rev. Biochem., 38, 841 (1969).

4. Geiduschek, E. P., Brody, E. N., and Wilson, D., Molecular Association in Biology, Academic Press, New York, 1968, p. 163.

5. Chamberlin, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 851 (1970).

6. Burgess, R. R., Ann. Rev. Biochem., 41, 711 (1971).

7. Silvestri, L. (Editor), First Lepetit Colloquium on RNA Polymerase and Transcription, American Elsevier Publishing Company, Inc., New York, 1970.

8. Cold Spring Harbor Symposia on Quantitative Biology, 35, Cold Spring Harbor Laboratory, New York, 1970.

9. Burgess, R. R., J. Biol. Chem., 244, 6160 (1969).

10. Zillig, W., Zechel, K., and Halbwacks, H., Hoppe-Seyler's Z. Physiol. Chem., 351, 221 (1970).

11. Berg, D., Barrett, K., and Chamberlin, M., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. 21, Academic Press, New York, p. 506.

12. Krakow, J. S., and Ochoa, S., Biochemische Z., 338, 796 (1963).

13. Krakow, J. S., and Fronk, E., J. Biol. Chem., 244, 5988 (1969).

14. Krakow, J. S., and von der Helm, K., Cold Spring Harbor Symposia on Quantitative Biology, 35, 73 (1970).

15. Johnson, J. C., DeBacker, M., and Boezi, J. A., J. Biol. Chem., 246, 1222 (1971).

16. Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. F. K., Nature, 221, 43 (1969).

17. Burgess, R. R., J. Biol. Chem., 244, 6168 (1969).

18. Walter, G., Seifert, W., and Zillig, W., Biochem. Biophys. Res. Commun., 30, 240 (1968).

19. Rudloff, R., and Zillig, W., as referenced by Burgess, R. R., Ann. Rev. Biochem., 41, 711 (1971).

20. Shapiro, A. L., Vinuela, E., and Maizel, J. V. Jr., Biochem. Biophys. Res. Commun., 28, 815 (1967).

21. Zillig, W., Fuchs, E., Palm, P., Rabussay, D., and Zechel, K., First Lepetit Colloquium on RNA Polymerase and Transcription, 1970, p. 151.

22. Berg, D., and Chamberlin, M., Biochemistry, 9, 5055 (1970).

23. Travers, A. A., and Burgess, R. R., Nature, 222, 537 (1969).

24. Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W., Cold Spring Harbor Symposia on Quantitative Biology, 35, 47 (1970).

25. Richardson, J. P., J. Mol. Biol., 21, 83 (1966).

26. Stead, N. W., and Jones, O. W., J. Mol. Biol., 26, 131 (1967).

27. Sternberger, N., and Stevens, A., Biochem. Biophys. Res. Commun., 24, 937 (1966).

28. Fuchs, E., Millette, R. L., Zillig, W., and Walter, G., Eur. J. Biochem., 3, 183 (1967).

29. Jones, O. W., and Berg, P., J. Mol. Biol., 22, 199 (1966).

30. Stevens, A., Emery, A. J. Jr., and Sternberger, N., Biochem. Biophys. Res. Commun., 24, 929 (1966).

31. Walter, G., Zillig, W., Palm, P., and Fuchs, E., Eur. J. Biochem., 3, 194 (1967).

32. Pettijohn, D., and Kamiya, T., J. Mol. Biol., 29, 275 (1967).

33. Richardson, J. P., Proc. Natl. Acad. Sci. U.S.A., 55, 1616 (1966).

34. Crawford, L. V., Crawford, E. M., Richardson, J. P., and Slayter, H. S., J. Mol. Biol., 14, 593 (1965).

35. Freeman, E. J., and Jones, O. W., Biochem. Biophys. Res. Commun., 29, 45 (1967).

36. Bautz, E. K. F., and Bautz, F. A., Nature, 226, 1219 (1970).

37. Bautz, E. K. F., and Bautz, F. A., Cold Spring Harbor Symposia on Quantitative Biology, 35, 227 (1970).

38. Naono, S., and Tokuyama, K., Cold Spring Harbor Symposia or Quantitative Biology, 35, 375 (1970).

39. Summers, W. C., First Lepetit Colloquium on RNA Polymerase and Transcription, 1970, p. 110.

40. Summers, W. C., and Siegel, R. B., Nature, 223, 1111 (1969).

41. Goff, G. G., and Minkley, E. G., First Lepetit Colloquium on RNA Polymerase and Transcription, 1970, p. 124.

42. Davis, R. W., and Hymen, R. W., Cold Spring Harbor Symposia on Quantitative Biology, 35, 269 (1970).

43. di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A., and Tocchini-Valentini, G. P., Nature, 222, 533 (1969).

44. Hinkle, D. C., and Chamberlin, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 65 (1970).

45. Sugiura, M., Okamoto, T., and Takanami, M., Nature, 225, 598 (1970).

46. Okamoto, T., Suguira, M., and Takanami, M., Biochemistry, 9, 3533 (1970).

47. Szybalski, W., Kubinski, H., and Sheldrick, P., Cold Spring Harbor Symposia on Quantitative Biology, 31, 123 (1966).

48. Summers, W. C., and Szybalski, W., Biochim. Biophys. Acta, 166, 371 (1968).

49. Sheldrick, P., and Szybalski, W., J. Mol. Biol., 29, 217 (1967).

50. Summers, W. C., and Szybalski, W., Virology, 34, 9 (1968).

51. Vogt, V., Nature, 223, 845 (1969).

52. Lancini, G., Pallanza, R., and Silvestri, L. G., J. Bact., 97, 761 (1969).

53. Umezana, H., Mizuno, S., Yamazaki, H., and Nitta, K., J. Antibiotics, 21, 234 (1968).

54. Heil, A., and Zillig, W., FEBS Letters, 11, 165 (1970).

55. Lill, H., Lill, U., Sippel, A., and Hartmann, G., First Leppetit Colloquium on RNA Polymerase and Transcription, 1970, p. 55.

56. Sippel, A. E., and Hartman, G. R., Eur. J. Biochem., 16, 152 (1970).

57. Goldthwait, P. A., Anthony, D. A., and Wu, C. W., First Lepetit Colloquium on RNA Polymerase and Transcription, 1970, p. 10.

58. Sethi, V. S., Zillig, W., and Bauer, H., FEBS Letters, 8, 236 (1970).

59. Seifert, W., and Sethi, V. S., Progress in Biophys. Mol. Biol., 23, as quoted by Burgess, R. R., Ann. Rev. Biochem., 41, 711 (1971).

60. Darlix, J. L., Sentenac, A., Ruet, A., and Fromageot, P., Eur. J. Biochem., 11, 43 (1969).

61. Maitra, U., and Hurwitz, J., Proc. Natl. Acad. Sci. U.S.A., 54, 815 (1965).

62. Bremer, H., Konrad, M. W., Gaines, K., and Stent, G. S., J. Mol. Biol., 13, 540 (1965).

63. Maitra, U., Nakato, Y., and Hurwitz, J., J. Biol. Chem., 242, 4908 (1967).

64. Bremer, H., and Bremer, R., Mol. Gen. Genet., 101, 6 (1968).

65. Anthony, D. D., Wu, C. W., and Goldthwait, D. A., Biochemistry, 8, 246 (1969).

66. Wu, C. W., and Goldthwait, D. A., Biochemistry, 8, 4450 (1969).

67. Wu, C. W., and Goldthwait, D. A., Biochemistry, 8, 4458 (1969).

68. Downey, K. M., and So, A. G., Biochemistry, 9, 2520 (1970).

69. Furth, J. J., Hurwitz, J., and Anders, M., J. Biol. Chem., 237, 2611 (1962).

70. Dunn, J. J., and Bautz, E. K. F., Biochem. Biophys. Res. Commun., 36, 925 (1969).

71. Bautz, E. K. F., Bautz, F. A., and Dunn, J. J., Nature, 223, 1022 (1969).

72. Hall, B. O., Fields, K., and Hager, G., First Lepetit Colloquium on RNA Polymerase and Transcription, 1970, p. 148.

73. Takanami, M., Okamoto, T., and Suguira, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 179 (1970).

74. Okamoto, T., Sugiura, M., and Takanami, M., Biochemistry, 9, 3533 (1970).

75. Travers, A. A., Kamen, R. I., and Schleif, R. F., Nature, 228, 748 (1970).

76. Pettijohn, D. E., Clarkson, K., Kossman, C. R., and Stonington, O. G., J. Mol. Biol., 52, 281 (1970).

77. Cashel, M., J. Biol. Chem., 244, 3133 (1969).

78. Cashel, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 407 (1970).

79. Travers, A., Kamen, R., and Cashel, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 415 (1970).

80. Zubay, G., Schwartz, D., and Beckwith, J. P., Proc. Natl. Acad. Sci. U.S.A., 66, 104 (1970).

81. Emmer, M., De Crombrugghe, B., Pastan, I., and Perlman, R., Proc. Natl. Acad. Sci. U.S.A., 66, 480 (1970).

82. Pastan, I., and Perlman, R. L., Nature New Biology, 229, 5 (1971).

83. Eron, L., Arditti, R., Zubay, G., Connaway, S., and Beckwith, J. P., Proc. Natl. Acad. Sci. U.S.A., 68, 215 (1971).

84. De Crombrugghe, B., Chen, B., Anderson, W. Nissley, P., Gottesman, M., Pastan, I., and Perlman, R., Nature New Biology, 231, 139 (1971).

85. Bremer, H., Cold Spring Harbor Symposia on Quantitative Biology, 35, 109 (1970).

86. Richardson, J. P., J. Mol. Biol., 21, 115 (1966).

87. Sentenac, A., Simon, E. J., and Fromageot, P., Biochim. Biophys. Acta, 161, 299 (1965).

88. So, A. G., and Downey, K. M., as referenced by Burgess, R. R., Ann. Rev. Biochem., 41, 711 (1971).

89. Pettijohn, D. E., Stonington, O. G., and Kossman, C. R., Nature, 228, 235 (1970).

90. Gariglio, P., Roodman, S. T., and Green, M. H., Biochem. Biophys. Res. Commun., 37, 945 (1969).

91. Bremer, H., and Konrad, M. W., Proc. Natl. Acad. Sci. U.S.A., 51, 801 (1964).

92. Maitra, U., and Barash, F., Proc. Natl. Acad. Sci., U.S.A., 64, 779 (1969).

93. Richardson, J. P., Cold Spring Harbor Symposia on Quantitative Biology, 35, 127 (1970).

94. Millette, R. L., and Trotter, C. D., Proc. Natl. Acad. Sci. U.S.A., 66, 701 (1970).

95. Millette, R. L., Trotter, C. C., Herrlich, P., and Schweiger, M., Cold Spring Harbor Symposia on Quantitative Biology, 35, 135 (1970).

96. Maitra, U., Lockwood, A. H., Dubhoff, J. S., and Guha, A., Cold Spring Harbor Symposia on Quantitative Biology, 35, 143 (1970).

97. Roberts, J. W., Nature, 224, 1168 (1969).

ARTICLE 1

2'-O-METHYLADENOSINE 5'-TRIPHOSPHATE. A SUBSTRATE FOR DEOXYRIBONUCEIC ACID DEPENDENT RIBONUCLEIC ACID POLYMERASE OF PSEUDOMONAS PUTIDA

By

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2′-*O*-Methyladenosine 5′-Triphosphate. A Substrate for Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase of *Pseudomonas putida**

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ABSTRACT: 2′-*O*-Methyladenosine 5′-triphosphate (AmTP) was a substrate for deoxyribonucleic acid dependent ribonucleic acid polymerase of *Pseudomonas putida*. In the DNA-directed synthesis of RNA with AmTP or an equimolar mixture of AmTP and adenosine 5′-triphosphate as the adenyl substrates, a small amount of 2′-*O*-methyladenosine 5′-monophosphate (AmMP) was incorporated into RNA after 60-min incubation. This amounted to 1–2 pmoles of AmMP for each pmole of RNA polymerase added to the reaction mixture. AmMP-containing RNA synthesized in reaction mixtures which contained AmTP as the only added adenyl substrate had a sedimentation coefficient of less than 4 S as determined by sucrose density gradient analysis. Abortive release of the AmMP-containing RNA from the DNA–RNA polymerase–nascent RNA ternary complex did not occur. AmMP-containing RNA synthesized in reaction mixtures which contained both AmTP and ATP as the adenyl substrates was heterogeneous in size with most of the RNA having a sedimentation coefficient of about 30 S. Degradation of the RNA product by alkaline hydrolysis followed by alkaline phosphatase digestion showed that 90% of the AmMP residues were located at the 3′ end of the RNA chain with the remaining 10% at the 5′ end or in the interior of the chain. The studies with AmTP lead to the following conclusions. (1) A free 2′-hydroxyl group is not required for binding of an adenyl substrate by *P. putida* RNA polymerase or for subsequent incorporation into RNA. (2) Following the incorporation of AmMP into the 3′ end of the nascent RNA chain, the rate of RNA chain growth is greatly reduced. The reduction in the rate of RNA chain growth results in the accumulation of nascent RNA chains with AmMP at the 3′ end. The rate-limiting step in RNA synthesis becomes the addition of the next nucleotide to the nascent RNA chain.

A contribution to the understanding of the mechanism of the DNA-directed synthesis of RNA by RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6) has been made through studies of base-altered analogs of the four common ribonucleoside triphosphates. Knowledge of the extent and specificity of incorporation of base-altered analogs by *Escherichia coli* RNA polymerase has added to the understanding of the structural requirements for hydrogen bonding between complementary bases in the RNA synthetic process (Kahan and Hurwitz, 1962) and has led to a definition of the role of base-stacking interactions with nearest neighbors in RNA synthesis (Goldberg and Rabinowitz, 1961; Nishimura *et al.*, 1966; Slapikoff and Berg, 1967; Ikehara *et al.*, 1968).

The mechanism of RNA synthesis has also been studied through the use of substrate analogs altered in the ribose moiety. Studies of 3′-deoxyadenosine 5′-triphosphate (3′-dATP) have indicated that a 3′-hydroxyl group is not required for binding of a nucleoside to *E. coli* or *Micrococcus luteus* RNA polymerase or for incorporation of a nucleotide into the 3′ end of RNA by these enzymes (Shigeura and Boxer, 1964; Shigeura and Gordon, 1965; Sentenac *et al.*, 1968a,b). Once 3′-dAMP is incorporated into the 3′ end of a RNA chain, however, RNA chain growth is terminated. Another substrate analog of ATP, 3′-amino-3′-deoxyadenosine 5′-triphosphate, appears to function in a similar manner as a RNA chain growth terminator (Shigeura *et al.*, 1966).

Studies of analogs of nucleoside triphosphates altered at the 2′ position of the ribose moiety have not clearly defined the requirement for a 2′-hydroxyl group in reactions catalyzed by RNA polymerase. Experiments using 2′-deoxyribonucleoside 5′-triphosphates have indicated that these analogs probably do not bind appreciably to *E. coli* or *M. luteus* RNA polymerase nor are they incorporated into RNA in a native DNA-directed reaction (Weiss and Nakamoto, 1961; Chamberlin and Berg, 1962; Furth *et al.*, 1962; Anthony *et al.*, 1969). Other experiments using denatured calf thymus DNA (Chamberlin and Berg, 1964) and poly[d(A-T)] (Krakow and Ochoa, 1963) as templates indicated that the 2′-deoxyribonucleoside 5′-triphosphates may have substrate activity. Studies with 1-(β-D-arabinofuranosyl)cytosine 5′-triphosphate (ara-CTP)[1] have shown that no appreciable DNA-directed RNA synthesis by *E. coli* RNA polymerase occurred when ara-CTP was used in place of CTP (Cardeilhac and Cohen, 1964). These studies were not designed, however, to detect the direct incorporation of a small amount of labeled ara-CMP into RNA. Chu and Fischer (1968) reported that ara-C is incorporated into both terminal and internal positions of RNA of murine leukemic cells *in vivo* indicating that for this system an unaltered 2′ position on the ribose moiety of a nucleoside triphosphate is not required for incorporation into RNA.

This report describes the effects of 2′-*O*-methyladenosine 5′-triphosphate (AmTP) on the reactions catalyzed by RNA

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[1] The abbreviation used that is not listed in *Biochemistry* 9, 4022 (1970), is: ara-C, 1-(β-D-arabinofuranosyl)cytosine.

polymerase of *Pseudomonas putida*. The purpose of this investigation was twofold. First, we wished to establish whether or not AmTP was a substrate for RNA polymerase, and if so, what effect the incorporation of AmMP into the 3′ end of nascent RNA would have on further RNA chain growth. Second, we hoped to prepare 2′-*O*-methyl-containing RNA which was complementary to a DNA template. 2′-*O*-Methylribonucleoside diphosphates have been used to prepare various synthetic polymers using polynucleotide phosphorylase (Rottman and Heinlein, 1968; Rottman and Johnson, 1969). The results of the present studies demonstrated that AmTP is a substrate for *P. putida* RNA polymerase. Following the incorporation of AmMP into RNA, the rate of RNA chain growth was greatly reduced. Consequently, the synthesis of RNA chains containing more than a few AmMP residues was infeasible.

Materials and Methods

Materials. NADP, UDPG, glucose 6-phosphate dehydrogenase, and all the unlabeled 5′-phosphate derivatives of the ribonucleosides were purchased from P-L Biochemicals, Inc. Calf thymus DNA, herring sperm DNA, poly(A), poly(U), poly(C), and phosphoglucomutase were obtained from Sigma Chemical Co. Poly[d(A-T)] was from Miles Laboratories, Inc. *E. coli* alkaline phosphatase, electrophoretically purified, was from Worthington Biochemical Corp. [^{3}H]ATP and [^{3}H]adenosine were obtained from Schwarz BioResearch, Inc. Whatman DEAE-cellulose (DE-1) was from Reeve Angel. Nitrocellulose membrane filters (type B-6) were obtained from Schleicher & Schuell. Wheat germ RNA (Singh and Lane, 1964), *E. coli* B tRNA (Holley *et al.*, 1961), and *Pseudomonas putida* rRNA (Payne and Boezi, 1970) were prepared by published procedures. *P. putida* bacteriophage gh-1 DNA (Lee and Boezi, 1966, 1967) was prepared by the method of Thomas and Abelson (1966). *P. putida* RNA polymerase was purified by the procedure of Johnson *et al.* (1971). The preparations of RNA polymerase used in these experiments were at least 90% pure. UDPG pyrophosphorylase was isolated from calf liver (Albrecht *et al.*, 1966) and recrystallized twice.

Descending paper chromatography employed one of the following solvent systems: (a) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v), (b) isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2, v/v), and (c) isopropyl alcohol–concentrated NH_4OH–0.1 M boric acid (7:1:2, v/v).

AmTP was prepared by the procedure of Rottman and Heinlein (1968). AmTP was further purified by descending chromatography on acid-washed Whatman No. 3MM paper. Successive development of AmTP was carried out in solvent systems a and b, and in each case, development was followed by elution from the paper and lyophilization to concentrate the eluant. The purified AmTP contained a small amount of AmDP (about 5%) as determined by chromatography in solvent a.

The synthesis of [^{3}H]AmTP from [^{3}H]adenosine was carried out by methods similar to those described by Rottman and Heinlein (1968). The following modifications in procedure were made. (1) [^{3}H]AmMP prepared from [^{3}H]Am and UMP with wheat seedling phosphotransferase was purified by descending chromatography on Whatman No. 3MM paper in solvent a, and (2) [^{3}H]AmTP prepared from [^{3}H]-AmMP and ATP by treatment with rabbit muscle myokinase was purified in two steps. First, [^{3}H]AmTP and [^{3}H]AmDP were separated from [^{3}H]AmMP and the 5′-phosphate derivatives of adenosine by descending chromatography in solvent c. Second, [^{3}H]AmTP was separated from [^{3}H]-AmDP by electrophoresis in 0.05 M ammonium formate (pH 3.5) on acid-washed Whatman No. 3MM paper using a Hormuth pherograph (Brinkman Instruments, Inc.) (1800 V, 2 hr). The purified [^{3}H]AmTP contained a small amount of [^{3}H]AmDP (about 10%) as determined by chromatography in solvent a.

Analytical Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The concentrations of native gh-1 and calf thymus DNA were determined spectrophotometrically based on the extinction coefficient $E^{1\%}_{260}$ 200. The molar extinctions, ϵ(P), used to determine the homopolymer concentrations were: 10.5×10^3 at 257 nm, 9.2×10^3 at 260 nm, and 6.5×10^3 at 267 nm for poly(A), poly(U), and poly(C), respectively, in 0.1 M NaCl–0.05 M Tris-acetate (pH 7.5) (Ts'o *et al.*, 1962). For poly[d(A-T)], ϵ(P) = 6.7×10^3 at 260 nm and pH 7.5 was used (Radding and Kornberg, 1962).

Assay of RNA Polymerase. The reaction mixture for monitoring the gh-1 DNA-directed synthesis of RNA by radioisotope incorporation contained 20 mM Tris-acetate (pH 8.0), 5 mM 2-mercaptoethanol, 2 mM magnesium acetate, 0.5 mM manganese acetate, 60 mM ammonium acetate, 0.2 mM ATP, and/or 0.2 mM AmTP, 0.2 mM each of UTP, GTP, and CTP, 100 μg/ml of gh-1 DNA, and *P. putida* RNA polymerase. The concentrations of divalent metal ions (Mg^{2+} and Mn^{2+}), monovalent cation (NH_4^+), nucleoside triphosphates, and DNA used in the reaction mixture gave the optimal rate of RNA synthesis. ATP or AmTP was labeled with ^{3}H. RNA polymerase ($\alpha_2\beta\beta'\sigma$) contained a full compliment of σ factor. After incubation at 30°, the total reaction mixture or a sample from it was mixed with 100 μl of 0.1% sodium dodecyl sulfate. Cold 10% trichloroacetic acid–1% sodium pyrophosphate solution (5 ml) and 250 μg of herring sperm DNA were then added. After 15 min at 0–4°, the acid-insoluble product was collected on a membrane filter. Each filter was washed with four 5-ml portions of cold 10% trichloroacetic acid–1% sodium pyrophosphate, dried, and then monitored for radioactivity using liquid scintillation spectrometry. The scintillation fluid (5 ml) contained 4 g of 2,5 bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene/l. of toluene.

Reactions using the templates poly[d(A-T)], poly(U), poly(A), poly(C), and denatured calf thymus DNA were monitored using a spectrophotometric assay (Johnson *et al.*, 1968). The assay couples the formation of inorganic pyrophosphate from ribonucleoside triphosphate polymerization to NADP reduction. 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 ATP and UTP, 45 μg poly[d(A-T)], and 27 μg/ml of RNA polymerase. The reaction mixture with poly(U) contained 100 mM Tris-acetate (pH 8.0), 1 mM manganese acetate, 0.4 mM ATP, 23 μM poly(U), and 20 μg/ml of RNA polymerase. The reaction mixture with poly(A) contained 20 mM Tris-acetate (pH 8.0), 2.8 mM UTP, 3.6 μM poly(A), 20 μg/ml of RNA polymerase. The reaction mixture with poly(C) contained 100 mM Tris-acetate (pH 8.0), 1.5 mM manganese acetate, 1.2 mM GTP, 20 μM poly(C), and 2 μg/ml of RNA polymerase. The reaction mixture with denatured calf thymus DNA contained 20 mM Tris-acetate (pH 8.0), 2 mM magnesium acetate, 0.5 mM manganese acetate,

0.4 mM ATP, 94 μg/ml of denatured calf thymus DNA, and 93 μg/ml of RNA polymerase. In addition, all of the above reaction mixtures contained 0.4 mM NADP, 0.4 mM UDPG, and excess phosphoglucomutase, glucose 6-phosphate dehydrogenase, and UDPG pyrophosphorylase. For the concentration of divalent metal ion used in each type of reaction, the optimal concentrations of nucleoside triphosphate(s) and template were used. The amount of RNA polymerase used in each case was rate limiting.

Analysis of the Positions of the [³H]AmMP Residues in RNA. Reaction mixtures (0.5 ml) containing [³H]AmTP (97,000 cpm/nmole), or [³H]AmTP and ATP, 95 μg/ml of RNA polymerase, and the other components of the reaction mixture for the gh-1 DNA-directed synthesis of RNA listed above were incubated for 60 min. After incubation, a sample of each reaction mixture was removed and assayed for ³H-labeled trichloroacetic acid insoluble product. The remainder of each reaction mixture was then treated in the following manner. Trichloroacetic acid to 5% and 2 mg of wheat germ RNA were added. The resultant precipitate was collected by centrifugation and washed by resuspension in five 5-ml portions of cold 5% trichloroacetic acid, once with 5 ml of 95% ethanol, and once with 5 ml of ethanol–ether (3:1, v/v). The washed precipitate together with an additional 8 mg of wheat germ RNA was dissolved in 1 ml of 1.0 N KOH and incubated at room temperature for 90 hr. Cold 4 N $HClO_4$ (0.25 ml) was added and the mixture was centrifuged at 0°. The pellet was washed twice with 1 ml of cold 0.2 N $HClO_4$. The supernatant from the first centrifugation and the washings were combined, neutralized with KOH, and centrifuged to remove $KClO_4$. The neutralized solution which contained 200 A_{260} units was diluted to 6.0 ml and made 0.05 M in $(NH_4)_2CO_3$ (pH 8.5). Following the addition of 0.3 mg of *E. coli* alkaline phosphatase, the solution was incubated at 37° for 24 hr. After lyophilization, the phosphatase digest was dissolved in 1 ml of H_2O. Samples were removed to assay radioactivity by liquid scintillation spectrometry (Bray, 1960). The rest of the phosphatase digest was diluted to 100 ml and applied to a DEAE-cellulose column (1.2 × 15 cm) in the formate form at a flow rate of 1 ml/min. The nucleosides were eluted from the column with 0.005 M ammonium formate followed by the dinucleoside monophosphates with 0.5 M ammonium formate (Price and Rottman, 1970). After repeated lyophilization, samples from each fraction were monitored for radioactivity and for absorbancy at 260 nm. Based on the absorbancy at 260 nm of the nucleoside and dinucleoside monophosphate fractions, the 2′-*O*-methyl content of the carrier wheat germ RNA was 2.5%. The value reported by Singh and Lane (1964) for wheat germ RNA is 1.9%. The total recovery of A_{260} units from the DEAE-cellulose column for each type of reaction mixture was 97% of the A_{260} units present in the neutralized KOH hydrolysate.

Identification of the ³H-labeled material in each fraction was carried out in the following manner. The nucleoside fraction was chromatographed on acid-washed Whatman No. 3MM paper in solvent a. This procedure separated A and Am from G, U, and C. A and Am were then separated by chromatography in solvent c. The dinucleoside monophosphate fraction was subjected to electrophoresis on Whatman No. 1 paper in 0.1 M ammonium bicarbonate (pH 7.8) using a Spinco Model R instrument (400 V, 4 hr). Separation of dinucleoside monophosphates (AmpN) from any contaminating nucleosides is achieved by this procedure.

Sucrose Density Gradient Analysis of [³H]AmMP-Containing RNA and the gh-1 DNA·(RNA Polymerase)·(Nascent RNA) Complex. Reaction mixtures (0.25 ml) containing [³H]AmTP (70,000 cpm/nmole), or [³H]AmTP and ATP, 95 μg/ml of RNA polymerase, and the other components of the reaction mixture for the gh-1 DNA-directed synthesis of RNA as listed above were incubated for 60 min. After cooling to 4°, a sample (0.1 ml) was removed from each reaction mixture and mixed with 10 μl of 5% sodium dodecyl sulfate. The mixture was incubated at 37° for 5 min. After cooling to 4°, the mixture was centrifuged at 5000 rpm for 5 min to remove precipitated sodium dodecyl sulfate. Samples (0.1 ml) of the original reaction mixtures and the sodium dodecyl sulfate treated samples were layered on 4.8-ml 5–20% linear sucrose gradients prepared in 50 mM Tris-acetate (pH 8.0), 100 mM NaCl, and 1 mM dithiothrietol. After centrifugation for 260 min at 39,000 rpm in the Spinco SW39 rotor at 4°, fractions were collected from the bottom of the centrifuge tubes. To determine the position of [³H]AmMP-containing RNA in the gradients, each fraction or a sample of each was precipitated with cold 10% trichloroacetic acid–1% sodium pyrophosphate, collected on a membrane filter, and monitored for radioactivity.

The position of DNA in the gradients from the original reaction mixtures was determined by assaying samples (50 μl) of each fraction for template activity, measured as the incorporation of [¹⁴C]AMP into RNA in the presence of excess RNA polymerase. At 32 μg/ml of RNA polymerase, the rate of [¹⁴C]AMP incorporation into RNA was proportional to the DNA concentration between 0 and 3 μg per ml. In the sucrose density gradient analyses, *E. coli* B tRNA, *P. putida* rRNA, and gh-1 DNA were used as markers of known sedimentation coefficients.

Results

Incorporation of [³H]AmMP into RNA. [³H]AmMP was incorporated into RNA in a gh-1 DNA-directed reaction catalyzed by *Pseudomonas putida* RNA polymerase (Table I). In the two reaction mixtures which contained RNA polymerase, gh-1 DNA, GTP, CTP, UTP, and [³H]AmTP, 55 and 66 pmoles of [³H]AmMP per ml were incorporated into RNA. If ATP at a concentration equal to that of AmTP was included in the reaction mixture, approximately the same amount of [³H]AmMP was incorporated.

Relative to the amount of AMP incorporated into RNA, the amount of AmMP incorporated was small (Table I). In comparable reaction mixtures, one containing [³H]ATP, the other [³H]AmTP, approximately 200 times as much [³H]AMP (11,400 pmoles/ml) was incorporated into RNA as [³H]AmMP (55–66 pmoles/ml). In the reaction mixtures containing an equimolar mixture of ATP and AmTP, 77 times as much [³H]AMP (5800 pmoles/ml) as [³H]AmMP (75 pmoles/ml) was incorporated into RNA.

Sucrose Density Gradient Analysis of [³H]AmMP-Containing RNA. The sedimentation patterns of [³H]AmMP-containing RNA synthesized in gh-1 DNA-directed reactions with [³H]AmTP or an equimolar mixture of [³H]AmTP and ATP as the adenyl substrates were examined by sucrose gradient centrifugation. The results are presented in Figure 1. Most of the [³H]AmMP-containing RNA synthesized in the reaction mixture which contained [³H]AmTP as the adenyl substrate was small in size, as judged from a sedimentation coefficient of less than 4 S (Figure 1, lower). [³H]AmMP-containing RNA synthesized in the reaction mixture which contained both [³H]AmTP and ATP was of diverse size,

TABLE I: Incorporation of [³H]AmMP or [³H]AMP into RNA.[a]

Expt	Adenyl Substrate(s) Added to the Reaction Mix.	Reaction Mix. Component Omitted	Incubn Time (min)	[³H]AmMP or [³H]AMP Incorpd (pmoles/ml)
1	[³H]AmTP	None	0	0
	[³H]AmTP	None	30	66
	[³H]AmTP	RNA polymerase	30	0
	[³H]AmTP, ATP	None	30	75
	[³H]AmTP, ATP	gh-1 DNA	30	4
	AmTP, ATP	None: [³H]AmTP added immediately before reaction was terminated	30	0
2	[³H]AmTP	None	30	55
	[³H]AmTP, ATP	None	30	72
3	[³H]ATP	None	0	0
	[³H]ATP	None	30	11,400
	[³H]ATP	gh-1 DNA	30	57
	[³H]ATP, AmTP	None	30	5,800

[a] Reaction mixtures (0.05 ml) containing the components described in Materials and Methods for the gh-1 DNA-directed synthesis of RNA were incubated for the times indicated. After incubation, the amount of radioactivity in the trichloroacetic acid insoluble product was determined as described in Materials and Methods. [³H]AmTP and [³H]ATP where indicated were present at 70,000 and 15,000 cpm per nmole, respectively. RNA polymerase was at 63 μg/ml. The values for the incorporation of [³H]-AmMP and [³H]AMP were those obtained after the subtraction of 16 and 35 pmoles per ml, respectively, which were the amounts of ³H-labeled substrates which were trapped on the membrane filters from reaction mixtures terminated at zero time.

TABLE II: Position of the [³H]AmMP Residues in RNA.[a]

Expt	Adenyl Substrate(s) Added to the Reaction	Cl_3CCOOH-Insoluble RNA Product Formed (cpm)	Alkaline Phosphatase Digest (cpm)	Nucleoside Fraction (cpm)	Dinucleoside Monophosphate Fraction (cpm)
1	[³H]AmTP	11,000	500		
2	[³H]AmTP, ATP	10,000	9700	7700	900

[a] Reaction mixtures (0.5 ml) containing the components for the gh-1 DNA-directed synthesis of RNA described in Materials and Methods were incubated for 60 min, and were treated as described in Materials and Methods.

with most of the [³H]AmMP-containing RNA having a sedimentation coefficient of about 30 S (Figure 1, upper).

Position of the [³H]AmMP Residues in RNA. The RNA produced in gh-1 DNA-directed reactions with [³H]AmTP or [³H]AmTP and ATP as the adenyl substrates was washed with cold trichloroacetic acid to remove unreacted substrates and was then degraded with KOH followed by alkaline phosphatase. As a result of this procedure, AmMP residues at the 3′ end of the RNA chain were converted into Am, and those at the 5′ end or in the interior of the RNA chain were converted into dinucleoside monophosphates (AmpN). Separation of Am and AmpN was achieved by chromatography on DEAE-cellulose. The results are shown in Table II.

For the analysis of the RNA synthesized in the reaction mixture which contained [³H]AmTP as the adenyl substrate, only 5% of the radioactivity in the [³H]AmMP-containing RNA product was recovered after the washing procedure with trichloroacetic acid prior to KOH hydrolysis. As shown by the sucrose density gradient analysis (Figure 1, lower), [³H]AmMP-containing RNA produced in the reaction mixture with [³H]AmTP as the adenyl substrate was small in size. Consequently, it was lost during the repeated washing with cold trichloroacetic acid. Insufficient radioactivity was present in the alkaline phosphatase digest (Table II, expt 1) to permit analysis of the nucleoside and dinucleoside monophosphate content by DEAE-cellulose chromatography.

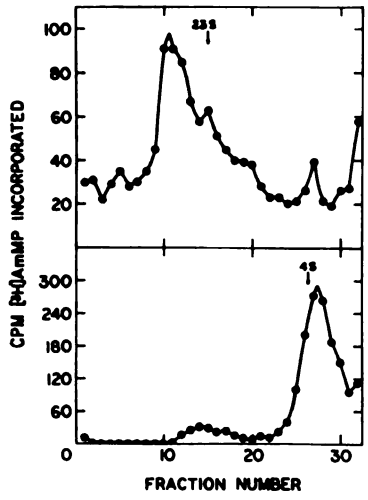


FIGURE 1: Sucrose density gradient analysis of the [³H]AmMP-containing RNA product. Reaction mixtures containing the components of the gh-1 DNA-directed synthesis of RNA with [³H]-AmTP (lower) or [³H]AmTP and ATP (upper) were treated with sodium dodecyl sulfate and analyzed by sucrose gradient centrifugation as described in Materials and Methods.

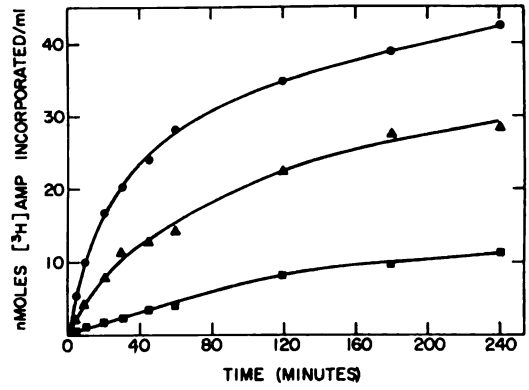


FIGURE 3: Effect of AmTP on the incorporation of [³H]AMP into RNA. The reaction mixtures contained, in a volume of 0.5 ml, those components for the gh-1 DNA-directed synthesis of RNA listed in Materials and Methods. RNA polymerase was present at 33 μg/ml and [³H]ATP at 15,000 cpm/nmole. At various times during the incubation, 50-μl aliquots were removed and assayed for radioactivity in the trichloroacetic acid insoluble product as described in Materials and Methods. Incorporation of [³H]AMP is shown in the absence of AmTP (●), and in the presence of 0.2 mM (▲) and 0.8 mM (■) AmTP.

For the analysis of the RNA synthesized in the reaction mixture which contained [³H]AmTP and ATP as the adenyl substrates (Table II, expt 2), 97% of the radioactivity in the [³H]AmMP-containing RNA product was recovered in the alkaline phosphatase digest. The alkaline phosphatase digest was separated into nucleoside and dinucleoside monophosphate fractions by chromatography on DEAE-cellulose. The nucleoside fraction contained 90% of the radioactivity recovered from the DEAE-cellulose column and the dinucleoside monophosphate contained 10%. Analysis of the nucleoside fraction from the DEAE-cellulose column by paper chromatography confirmed that the radioactive material was [³H]Am. Analysis of the dinucleoside monophosphate fraction by paper electrophoresis showed that the fraction was not contaminated with labeled Am and that the radioactive material present had the same electrophoretic mobility as authentic dinucleoside monophosphates.

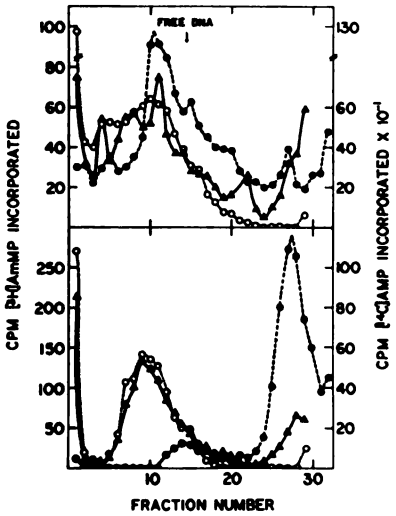


FIGURE 2: Sucrose density gradient analysis of the DNA·(RNA polymerase)·([³H]AmMP-containing RNA) ternary complex. Samples of reaction mixtures containing the components of the gh-1 DNA-directed synthesis of RNA with [³H]AmTP (lower) or [³H]-AmTP and ATP (upper) were analyzed by sucrose gradient centrifugation or treated with sodium dodecyl sulfate and then analyzed by sucrose gradient centrifugation. (●) [³H]AmMP-containing RNA of the samples that had been treated with sodium dodecyl sulfate. (▲) [³H]AmMP-containing RNA of samples that had not been treated with the detergent. (○) DNA, as monitored by template activity measured as the incorporation of [¹⁴C]AMP into RNA in the presence of an excess of RNA polymerase.

Nonrelease of [³H]AmMP-Containing RNA from the gh-1 DNA·(RNA Polymerase)·(Nascent RNA) Complex. The data presented in Table II led to the conclusion that most of the [³H]AmMP incorporated into RNA was at the 3′ end of RNA chains. A possible explanation for this conclusion is that the addition of an AmMP residue to the 3′ end of a nascent RNA chain induced the release of that chain from the DNA·(RNA polymerase)·(nascent RNA) ternary complex before RNA chain propagation could continue. To look for release of [³H]AmMP-containing RNA, sucrose density gradient analysis was used. After incubation, samples of reaction mixtures containing the components of gh-1 DNA-directed RNA synthesis with [³H]AmTP or [³H]AmTP and ATP as the adenyl substrates were treated with sodium dodecyl sulfate, and centrifuged through sucrose gradients. The position in the gradients of [³H]AmMP-containing RNA, that is [³H]AmMP-containing RNA released from the ternary complex, was determined. Other samples of the reaction mixtures, not treated with sodium dodecyl sulfate, were also centrifuged through sucrose gradients. The position in the gradients of the DNA·(RNA polymerase)·(nascent RNA) ternary complex was determined to be that position where [³H]AmMP-containing RNA and DNA cosedimented at speeds faster than that of free [³H]AmMP-containing RNA or free DNA. The results are presented in Figure 2. In the case of the analysis of the reaction mixture containing [³H]-AmTP as the adenyl substrate, the data show that approximately 85% of the [³H]AmMP-containing RNA cosedimented with DNA in the ternary complex. This result leads to the conclusion that release of [³H]AmMP-containing RNA from the ternary complex had not occurred to any significant degree. In the case of the analysis of the reaction mixture containing both [³H]AmTP and ATP as the adenyl substrates, it is not clear how much, if any, release of [³H]AmMP-

TABLE III: The Effect of AmTP on Several Reactions Catalyzed by RNA Polymerase.[a]

Expt	NTP Substrates Added	Polymer Added	AmTP (mM)	Initial Velocity: nmoles of NADPH Produced/min per ml	Inhibn (%)
1	ATP, UTP	Poly[d(A-T)]		2.6	
	ATP, UTP	Poly[d(A-T)]	0.4	1.5	42
	UTP	Poly[d(A-T)]		0.1	
	UTP	Poly[d(A-T)]	0.4	0.1	
	ATP, UTP			<0.1	
2	ATP	Poly(U)		1.0	
	ATP	Poly(U)	0.4	0.5	50
		Poly(U)	0.4	0	
	ATP			0	
3	ATP	dDNA[b]		1.4	
	ATP	dDNA[b]	0.4	0.2	86
		dDNA[b]	0.4	0	
	ATP			0	
4	UTP	Poly(A)		3.1	
	UTP	Poly(A)	0.2	3.1	0
	UTP	Poly(A)	1.0	3.3	0
		Poly(A)	2.8	0	
	UTP			0	
5	GTP	Poly(C)		4.5	
	GTP	Poly(C)	0.4	4.2	7
		Poly(C)	0.4	0	
	GTP			0	

[a] The reaction mixtures, in a volume of 0.25 ml, contained the components described in Materials and Methods. The production of NADPH was measured as described in Materials and Methods. [b] dDNA-denatured calf thymus DNA.

containing RNA from the ternary complex had occurred because of the difficulty in distinguishing between free RNA and RNA in the ternary complex.

Absence of Synthesis of Trichloroacetic Acid Soluble Oligonucleotides. In all of the experiments described above in which AmTP was the adenyl substrate, only the formation of trichloroacetic acid insoluble RNA was monitored. In order to determine if trichloroacetic acid soluble oligonucleotides were being synthesized, the reaction was monitored by measuring inorganic pyrophosphate formation using a spectrophotometric assay (Materials and Methods). In a reaction mixture containing 50 μg/ml of RNA polymerase, 100 μg/ml of gh-1 DNA, 0.2 mM each of GTP, CTP, UTP, and AmTP, and the components of the coupled assay system, no inorganic pyrophosphate formation was observed. Thus, within the limits of sensitivity of the assay (a rate of formation of 0.02 nmole of inorganic pyrophosphate/min per ml of reaction mixture or greater), no DNA-directed reaction was detected, indicating that RNA polymerase was not repeatedly initiating the synthesis and release of trichloroacetic acid soluble oligonucleotides.

AmTP as an Inhibitor of RNA Polymerase Catalyzed Reactions. Although the presence of ATP at an equimolar concentration to that of [^{3}H]AmTP did not decrease the amount of [^{3}H]AmMP incorporated into RNA (see Table I), the presence of AmTP in reaction mixtures containing [^{3}H]ATP decreased the amount of [^{3}H]AMP incorporated. In reaction mixtures containing RNA polymerase, gh-1 DNA, and 0.2 mM each of GTP, CTP, UTP, and [^{3}H]ATP, the initial rate of [^{3}H]AMP incorporation into RNA was inhibited by 63 and 90% in the presence of 0.2 and 0.8 mM AmTP, respectively (Figure 3). The extent of [^{3}H]AMP incorporation at 240 min was reduced by 30 and 72%, respectively, by the two concentrations of AmTP.

The polymerization of ATP and UTP directed by poly[d(A-T)], and the polymerization of ATP directed by poly(U) or denatured calf thymus DNA were inhibited by AmTP (Table III). AmTP had little or no effect on the poly(A)-directed polymerization of UTP or the poly(C)-directed polymerization of GTP. These results indicate that AmTP was an inhibitor of only those reactions which used ATP as a substrate.

Discussion

AmTP was a substrate for the gh-1 DNA-directed synthesis of RNA by *Pseudomonas putida* RNA polymerase. By virtue of its structure and the fact that it inhibited only those RNA polymerase catalyzed reactions which used ATP as a substrate, AmTP may be regarded as a substrate analog of ATP. Thus, a free 2'-hydroxyl group is not required for binding of an adenyl substrate by *P. putida* RNA polymerase or for subsequent incorporation into RNA. However, in both gh-1 DNA-directed reactions studied, the amount of AmMP incorporated was small. With either AmTP or an equimolar mixture of AmTP and ATP as the adenyl substrates, approximately 1–2 pmoles of AmMP was incorporated in 60 min-pmole of enzyme added to the reaction mixtures. The pmoles of RNA polymerase was calculated using mol wt 5×10^5 (Johnson *et al.*, 1971).

AmMP-containing RNA synthesized in the reaction mixture which contained AmTP as the only added adenyl substrate was small in size, as judged from a sedimentation coefficient of less than 4 S. AmMP-containing RNA synthesized in the reaction mixture which contained AmTP and ATP was heterogeneous in size with most of the RNA having a sedimentation coefficient of 30 S. Under similar assay conditions, RNA synthesized in gh-1 DNA-directed reactions with the four common nucleoside triphosphates (GTP, CTP, UTP, and ATP) can achieve an even larger size, as judged from a sedimentation coefficient of 45 S (G. F. Gerard and J. A. Boezi, unpublished results).

AmMP incorporated into RNA in the reaction mixture which contained an equimolar mixture of AmTP and ATP was found both at the 3′ end of the RNA chain, and at the 5′ end or in the interior of the chain. Most of the AmMP was at the 3′ end of the chain. The presence of some AmMP at the 5′ end or in the interior of the RNA chain shows that nascent RNA chain growth can continue following the incorporation of AmMP. Thus, AmTP does not function as a chain terminator as is the case with 3′-dATP (Shigeura and Boxer, 1964).

The location of AmMP incorporated into RNA in the reaction mixture which contained AmTP as the only added adenyl substrate was not determined. Since there was no significant RNA chain release from the ternary complex, each enzyme molecule could have initiated the synthesis of only one RNA chain. Because there was only 1–2 pmoles of AmMP incorporated per pmole of enzyme, there was therefore probably only 1–2 AmMP residues/RNA chain. Most of the AmMP-containing RNA synthesized in this reaction mixture was estimated to have a sedimentation coefficient of somewhat less than 4 S. As estimated from the sucrose gradient profile, the sedimentation coefficient was approximately 3 S which corresponds to a chain length of 50–75 nucleotides (Madison, 1968). In order to explain the synthesis of RNA of this chain length containing only 1–2 AmMP residues, two alternatives are suggested. First, the RNA had an unusual base composition being very low in adenyl content. Second, the RNA had a base composition which reflected the overall base composition of the gh-1 DNA template (Lee and Boezi, 1966) with a mole per cent adenyl groups equal to 22%, but the additional adenyl groups were from ATP present as a contaminant in the reaction mixtures. Consistent with the second alternative is the observation that a slow rate of RNA synthesis (approximately 0.5% of the control rate) was detected in reaction mixtures which contained CTP, GTP, and UTP, but no added ATP (G. F. Gerard and J. A. Boezi, unpublished results). This observation suggests that one of the stock nucleoside triphosphates was contaminated with a small amount of ATP.

The results presented in this paper lead us to the following understanding of the mechanism of incorporation of AmMP into RNA by *P. putida* RNA polymerase. The substrate, AmTP, binds on the enzyme surface in place of ATP, and is incorporated into the 3′ end of the nascent RNA chain. The rate of incorporation of AmMP into the 3′ end of RNA is probably slower than that of AMP. This reduced rate is due to a lower affinity of the enzyme for AmTP than for ATP and/or a slower rate of phosphodiester-bond formation with AmTP as substrate than with ATP. The rate of initiation of synthesis of RNA chains is probably also slower with AmTP than with ATP. If ATP is present in the reaction mixture, competition between AmTP and ATP for binding on the enzyme results. Following the incorporation of AmMP into the 3′ end of the nascent RNA chain, the rate of RNA chain growth is greatly reduced. The reduction in rate of RNA chain growth results in the accumulation of nascent RNA chains with the AmMP at the 3′ end. The bulky 2′-*O*-methyl group at the 3′ end of the nascent RNA chain could impede translocation of RNA polymerase relative to the DNA template and nascent RNA chain and/or could hinder phosphodiester-bond formation with the incoming nucleoside triphosphate. Whatever the reason, the addition of the next nucleotide to the chain becomes the rate-limiting step in RNA synthesis. Abortive release of the RNA from the ternary complex does not occur. Following the addition of the next nucleotide, the rate of RNA chain growth may return to the normal *in vitro* rate, but perhaps not until the AmMP residue in the nascent RNA chain leaves the enzyme surface.

References

Albrecht, G. J., Bass, S. T., Seifert, L. L., and Hansen, R. G. (1966), *J. Biol. Chem. 241*, 2968.

Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8*, 246.

Bray, G. A. (1960), *Anal. Biochem. 1*, 270.

Cardeilhac, P. T., and Cohen, S. S. (1964), *Cancer Res. 24*, 1595.

Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.

Chamberlin, M., and Berg, P. (1964), *J. Mol. Biol. 8*, 708.

Chu, M. Y., and Fischer, G. A. (1968), *Biochem. Pharmacol. 17*, 753.

Furth, J. J., Hurwitz, J., and Anders, M. (1962), *J. Biol. Chem. 237*, 2611.

Goldberg, I. H., and Rabinowitz, M. (1961), *Biochem. Biophys. Res. Commun. 6*, 394.

Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merrill, S. H. (1961), *J. Biol. Chem. 236*, 200.

Ikehara, M., Murao, K., Harada, E.., and Nishimura, S. (1968), *Biochim. Biophys. Acta 155*, 82.

Johnson, J. C., DeBacker, M., and Boezi, J. A. (1971), *J. Biol. Chem. 246*, 1222.

Johnson, J. C., Shanoff, M., Bass, S. T., Boezi, J. A., and Hansen, R. G. (1968), *Anal. Biochem. 26*, 137.

Kahan, F. M., and Hurwitz, J. (1962), *J. Biol. Chem. 237*, 3778.

Krakow, J. S., and Ochoa, S. (1963), *Biochem. Z. 338*, 796.

Lee, L. F., and Boezi, J. A. (1966), *J. Bacteriol. 92*, 1821.

Lee, L. F., and Boezi, J. A. (1967), *J. Virol. 1*, 1274.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Madison, J. T. (1968), *Annu. Rev. Biochem. 37*, 131.

Nishimura, S., Harada, F., and Ikehara, M. (1966), *Biochim. Biophys. Acta 129*, 301.

Payne, K. J., and Boezi, J. A. (1970), *J. Biol. Chem. 245*, 1378.

Price, A. R., and Rottman, F. (1970), *Biochim. Biophys. Acta 199*, 288.

Radding, C. M., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2877.

Rottman, F., and Heinlein, K. (1968), *Biochemistry 7*, 2634.

Rottman, F., and Johnson, K. L. (1969), *Biochemistry 8*, 4354.

Sentenac, A., Ruet, A., and Fromageot, P. (1968a), *Eur. J. Biochem. 5*, 385.

Sentenac, A., Simon, E. J., and Fromageot, P. (1968b), *Biochim. Biophys. Acta 161*, 299.

Shigeura, H. T., and Boxer, G. E. (1964), *Biochem. Biophys, Res. Commun. 17*, 758.

Shigeura, H. T., Boxer, G. E., Meloni, M. L., and Sampson, S. D. (1966), *Biochemistry 5*, 994.

Shigeura, H. T., and Gordon, C. N. (1965), *J. Biol. Chem. 240*, 806.

Singh, H., and Lane, B. G. (1964), *Can. J. Biochem. 42*, 1011.

Slapikoff, S., and Berg, P. (1967), *Biochemistry 6*, 3654.

Thomas, C. A., and Abelson, J. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, S. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 553.

Ts'o, P. P., Helmkamp, G. K., and Sander, C. (1962), *Biochim. Biophys. Acta 55*, 584.

Weiss, S. B., and Nakamoto, T. (1961), *J. Biol. Chem. 236*, 618.

ARTICLE 2

TEMPLATE ACTIVITY OF 2'-O-METHYLPOLYRIBONUCLEOTIDES WITH PSEUDOMONAS PUTIDA DNA-DEPENDENT RNA POLYMERASE

By

Gary F. Gerard

ABSTRACT

Pseudomonas putida RNA polymerase can use the single stranded 2'-O-methylpolyribonucleotides, poly(Um) and poly(Cm), as templates for the synthesis of poly(A) and poly(G), respectively. No template activity was detected with the purine-containing 2'-O-methylated homopolymers, poly(Am) and poly(Im). The poly(Am) strand of either poly(Am)·poly(U) or poly(Am)·poly(Um) was not a template for poly(U) synthesis, and did not prevent the poly(U) or poly(Um) strand of the duplex from serving as a template for poly(A) synthesis. The poly(Um) strand of the duplex poly(Am)·poly(Um) was an effective template for poly(A) synthesis, but the poly(Um) strand of poly(A)·poly(Um) was not.

INTRODUCTION

Bacterial DNA-dependent RNA polymerase (EC 2.7.7.6) can use single- and double-stranded polydeoxyribonucleotides and polyribonucleotides as templates for the synthesis of RNA *in vitro* (1-5). A given polydeoxyribonucleotide is, however, a more effective template than a comparable polyribonucleotide. Thus, RNA polymerase does not have an absolute requirement for a template which has a hydrogen at the 2'-position, but can use polynucleotides that have a hydroxyl group at the 2'-position. Polynucleotides with substituents at the 2'-position other than a hydrogen or a hydroxyl group have not been tested as templates for RNA polymerase.

As part of a general study of the structure and mechanism of action of *Pseudomonas putida* RNA polymerase (6-8), we have undertaken a study to determine whether or not 2'-*O*-methylhomopolyribonucleotides can serve as templates for this enzyme. These 2'-*O*-methylated homopolymers have a bulkier substituent at the 2'-position than do polydeoxyribonucleotides or polyribonucleotides. In this report we present the results of a study of the template activity of both single- and double-stranded 2'-*O*-methylhomopolyribonucleotides.

MATERIALS AND METHODS

RNA polymerase was purified from Pseudomonas putida according to the procedure of Johnson, DeBacker, and Boezi (6) with the modifications reported by Johnson (8). The preparation of enzyme used in these experiments was at least 98% pure and contained one equivalent of sigma (σ) per equivalent of core enzyme ($\alpha_2\beta\beta'$).

The synthetic polyribonucleotides used in these studies were obtained from Miles Laboratories, Inc. The 2'-O-methylpolyribonucleotides were prepared according to published procedures (9-11), with the exception of poly(Im) (12). The synthetic polydeoxyribonucleotides were gifts from Dr. F. J. Bollum, University of Kentucky, Lexington, Kentucky. The mean value of the sedimentation coefficient determined by centrifugation through sucrose density gradients (pH 8.0) for each synthetic polyribo- and polydeoxyribonucleotide was between 4.0 and 6.0 S. For poly(Am), poly(Im), poly(Um), and poly(Cm), the mean sedimentation coefficients were 12.4, 16.0, 10.1 and 5.1 S, respectively. The double-stranded homopolynucleotides used in this study were prepared by incubating an equimolar mixture of the complementary single-stranded polynucleotides

at room temperature in the presence of 0.01 M NaCl-0.01 M Tris-HCl, pH 8.0, for 24 to 36 hours (13-15).

For the radioactive assay of RNA polymerase, the standard reaction mixture (0.8 ml) for monitoring polyribonucleotide synthesis contained 100 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 15 µg/ml RNA polymerase, 50 µM of the appropriate polynucleotide expressed in terms of nucleotide phosphate, 2 mM manganese chloride, and one of the following nucleoside triphosphates: 0.4 mM ^{3}H-ATP, 1.4 mM ^{3}H-UTP, 1.2 mM ^{3}H-GTP, or 0.4 mM ^{3}H-CTP. The specific radioactivity of the ^{3}H-labeled nucleoside triphosphates was usually 5 to 10 x 10^{3} cpm/nmole. The nucleoside triphosphate and the polynucleotide template used in each type of reaction mixture were present at saturating concentrations. Aliquots (50 µl) were removed from the reaction mixture at various times during 100 minutes of incubation at 30° C. The trichloroacetic acid insoluble radioactivity in each aliquot was then determined as previously described (7).

For the spectrophotometric assay of RNA polymerase (16), the reaction mixture contained, in addition to the components used in the radioactive assay, 0.4 mM $NADP^{+}$, 0.4 mM UDP-glucose, and excess phosphoglucomutase, glucose-6-phosphate dehydrogenase, and UDP-glucose pyrophosphorylase.

RESULTS

Single-stranded homopolynucleotides as templates. Poly(Um) was a template for poly(A) synthesis catalyzed by P. putida RNA polymerase. The time course of the reaction is shown in Figure 1a. A linear rate of poly(A) synthesis was observed during 100 minutes of incubation. For comparison, the time courses for the poly(dT)- and the poly(U)-directed synthesis of poly(A) are presented. The initial rates of AMP incorporation into trichloroacetic acid insoluble poly(A) were 3.5, 1.5, and 0.4 nmoles/minute per ml of reaction mixture for poly(dT), poly(U), and poly(Um), respectively. Thus, the initial rate of poly(A) synthesis with poly(Um) as the template was 27% of that observed with poly(U) as the template. No poly(Um)-directed poly(A) synthesis was observed if 2 mM magnesium chloride was used in place of 2 mM manganese chloride in the standard reaction mixture.

No poly(U) synthesis as measured by the incorporation of UMP into trichloroacetic acid insoluble polymer was detected with poly(Am) as the template (Figure 1b). In experiments in which the incorporation of as little as 0.02 nmole UMP/ml of reaction mixture could be measured, no poly(Am)-directed poly(U) synthesis was detected, even

after 100 minutes of incubation. If 2 mM magnesium chloride was used in place of 2 mM manganese chloride in the standard reaction mixture, again, no poly(Am)-directed poly(U) synthesis was detected. P. putida RNA polymerase can use poly(dA) and poly(A) as templates for poly(U) synthesis (Figure 1b). The initial rates of UMP incorporation into trichloroacetic acid insoluble poly(U) were 3.2 and 1.3 nmoles/minute per ml of reaction mixture for poly(dA) and poly(A), respectively.

Even though P. putida RNA polymerase did not catalyze the synthesis of trichloroacetic acid insoluble poly(U) with poly(Am) as the template, the possibility exists that the enzyme might catalyze the poly(Am)-directed synthesis of oligonucleotides of UMP which would be soluble in trichloroacetic acid. In order to determine if trichloroacetic acid soluble oligonucleotides of UMP were being synthesized, the spectrophotometric assay for RNA polymerase which monitors inorganic pyrophosphate was employed. With poly(Am) as the template and UTP as the substrate, no inorganic pyrophosphate formation was observed over 100 minutes of incubation. Thus, within the limits of sensitivity of the assay (a rate of formation of 0.02 nmole of inorganic pyrophosphate/minute per ml of reaction mixture or greater), no poly(Am)-directed reaction was detected, indicating that each RNA polymerase

molecule was not repeatedly initiating the synthesis and release of trichloroacetic acid soluble oligonucleotides of UMP.

P. putida RNA polymerase can bind poly(Am), even though the enzyme can not use the 2'-O-methylated polymer as a template. If the standard reaction mixture contained equimolar amounts of poly(Am) and poly(A), the initial rate of poly(U) synthesis was 50% of that observed in the presence of poly(A) alone. If the poly(A)-directed synthesis of poly(U) was allowed to proceed for 3 minutes before poly(Am) was added to the reaction mixture, no inhibition in the rate of poly(U) synthesis was observed.

The results obtained with the cytidine- and inosine-containing polynucleotides are shown in Table 1. The rate of reaction for each polynucleotide was determined from a time course similar to those presented in Figure 1. Poly(Cm) was a template for poly(G) synthesis. The rate of poly(G) synthesis with poly(Cm) as the template was slow, however, being only 3.7% of that observed with poly(C) as the template. Within the limits of sensitivity of the assay, no poly(Im)-directed synthesis of poly(C) was detected. Poly(I) itself was a poor template for P. putida RNA polymerase.

Double-stranded homopolynucleotides as templates. Duplexes of adenosine- and uridine-containing homopolyribonucleotides and 2'-O-methylhomopolyribonucleotides

were tested as templates for P. putida RNA polymerase. The results are presented in Table 2. As was the case with single-stranded poly(Am), no poly(U) synthesis was detected with the poly(Am) strand of poly(Am)·poly(U) or poly(Am)·poly(Um) as the template. The poly(Am) strand of the duplex did not, however, prevent either poly(U) or poly(Um) from serving as a template for poly(A) synthesis.

In the case of poly(Um) in a duplex, the poly(Um) strand of poly(Am)·poly(Um) was an effective template for poly(A) synthesis, but the poly(Um) strand of poly(A)·poly(Um) was not. The rate of AMP incorporation with poly(Am)·poly(Um) as the template was the same as that observed with single-stranded poly(Um) as the template. The rate of AMP incorporation with poly(A)·poly(Um) was only 12% of that observed with either poly(Am)·poly(Um) or single-stranded poly(Um) as the template.

DISCUSSION

P. putida RNA polymerase can use the single-stranded 2'-*O*-methylhomopolyribonucleotides, poly(Um) and poly(Cm), as templates. Of the two pyrimidine-containing polymers, poly(Um) was the more effective template. No template activity was detected with the purine-containing 2'-*O*-methylated homopolymers, poly(Am) and poly(Im). As a general rule, for a particular group of single-stranded homopolynucleotides having the same base, the order of template effectiveness for *P. putida* RNA polymerase was: polydeoxyribonucleotides > polyribonucleotides > 2'-*O*-methylpolyribonucleotides.

The finding that there was no detectable template activity with poly(Am) but that poly(Um) and poly(Cm) were templates is unexpected in light of the fact that poly(A), like poly(U) and poly(C), is an effective template. No poly(Im)-directed synthesis of poly(C) was detected, but this finding is not unexpected since poly(I) itself was a poor template for poly(C) synthesis. The inability of poly(Am) to serve as a template for poly(U) synthesis is probably not due to some anomaly in the gross secondary structure of the 2'-*O*-methylated polymer.

First, P. putida RNA polymerase can bind to poly(Am). Second, there is no significant difference at 30° C and neutral pH in the single-stranded conformation of poly(Am) and poly(A) as measured by ultraviolet and circular dichroism spectroscopy (13). Third, even in the duplex structures, poly(Am)·poly(U) and poly(Am)·poly(Um), poly(Am) does not serve as a template, but its duplex partners do. The explanation for why poly(Am) is not a template for poly(U) synthesis awaits further experimentation.

The poly(Um) strand of the duplex, poly(A)·poly(Um), was not an effective template for poly(A) synthesis, whereas the poly(Um) strand of poly(Am)·poly(Um) was. In addition, for the duplex poly(A)·poly(Um), the poly(A) strand was a effective template even though the poly(Um) strand was not. These patterns of transcription are difficult to explain. Perhaps, a knowledge of the three-dimensional structure of these duplexes will provide the key for interpreting the observed patterns of template activity. In this regard, x-ray diffraction studies of the duplexes are in progress (17).

REFERENCES

1. Chamberlin, M., and Berg, P., Proc. Nat. Acad. Sci., 48, 81 (1962).

2. Krakow, J. S., and Ochoa, S., Proc. Nat. Acad. Sci., 49, 88 (1963).

3. Straat, P. A., Ts'o, P.O.P., and Bollum, F. J., J. Biol. Chem., 243, 5000 (1968).

4. Karstadt, M., and Krakow, J. S., J. Biol. Chem., 245, 746 (1970).

5. Fox, C. F., Robinson, W. S., Haselkorn, R., and Weiss, S. B., J. Biol. Chem., 239, 186 (1964).

6. Johnson, J. C., DeBacker, M., and Boezi, J. A., J. Biol. Chem., 246, 1222 (1971).

7. Gerard, G. F., Rottman, F., and Boezi, J. A., Biochemistry, 10, 1974 (1971).

8. Johnson, J. C., Ph. D. Thesis, Michigan State University, 1971.

9. Rottman, F., and Heinlein, K., Biochemistry, 7, 2634 (1968).

10. Rottman, F., and Johnson, K. L., Biochemistry, 8, 4354 (1969).

11. Dunlap, B. E., Friderici, K. H., and Rottman, F., Biochemistry, 10, 2581 (1971).

12. Rottman, F., unpublished results (1971).

13. Bobst, A. M., Rottman, F., and Cerutti, P. A., J. Mol. Biol., 46, 221 (1969).

14. Warner, R., Ann. N. Y. Acad. Sci., 69, 314 (1957).

15. Zmudzka, B., and Shugar, D., FEBS Letters, 8, 52 (1970).

16. Johnson, J. C., Shanoff, M., Bass, S. T., Boezi, J. A., and Hansen, R. G., Anal. Biochem., 26, 137 (1968).

17. Langridge, R., unpublished results (1971).

TABLE 1.--Single-stranded cytidine- and inosine-containing polynucleotides as templates for *P. putida* RNA polymerase.

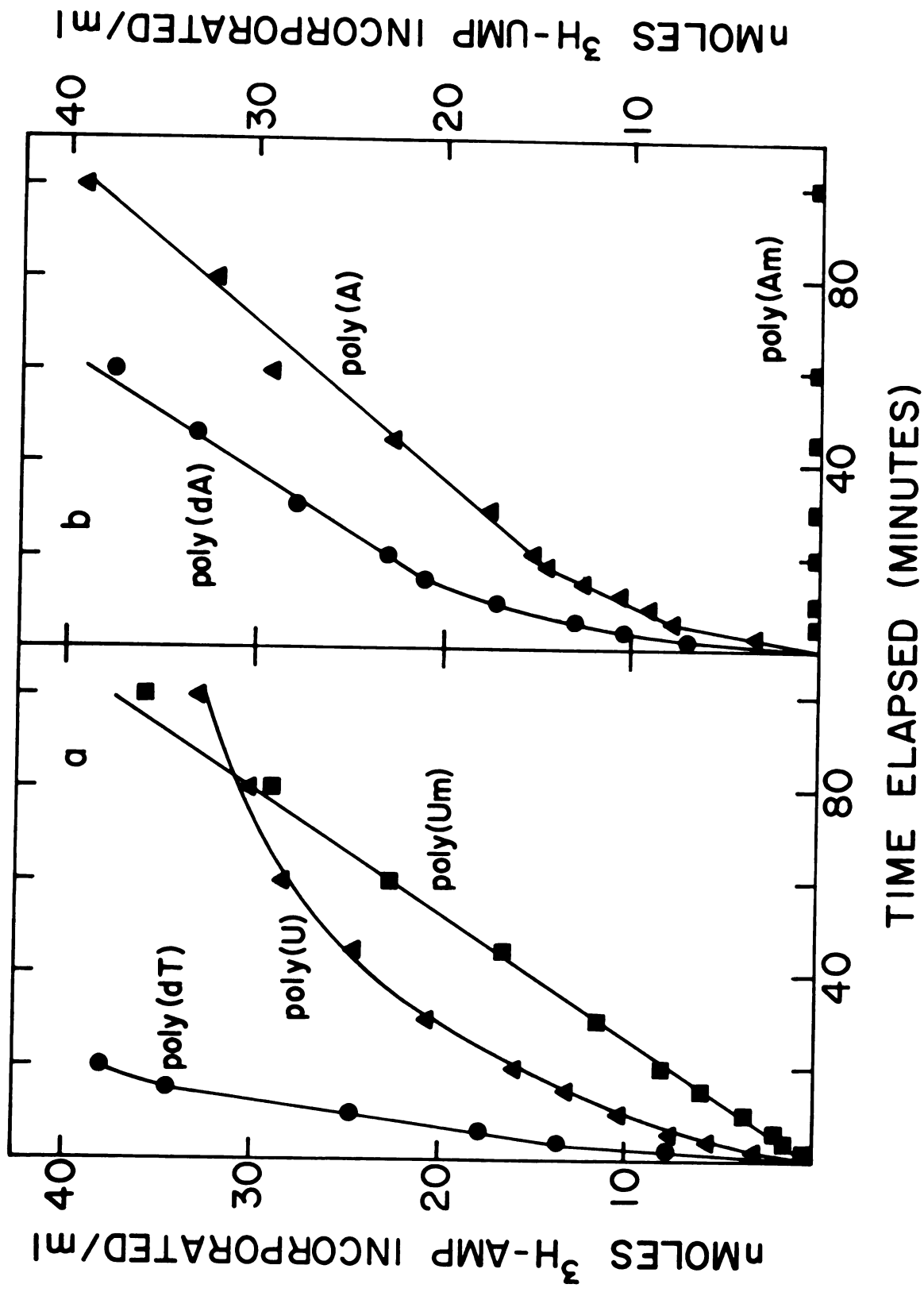
Polynucleotide Template	Nucleoside Triphosphate Substrate	Reaction Velocity: nmoles of nucleoside monophosphate incorporated/ minute per ml
poly(Cm)	GTP	0.1
poly(C)	GTP	2.7
poly(dC)	GTP	3.5
poly(Im)	CTP	< 0.003
poly(I)	CTP	0.03
poly(dI)	CTP	0.65

TABLE 2.--Double-stranded adenosine- and uridine-containing homopolyribonucleotides and 2'-O-methyl homopolyribonucleotides as templates for P. putida RNA polymerase.

Double-stranded Polynucleotide Template	Nucleoside Triphosphate Substrate	Reaction Velocity: nmoles of nucleoside monophosphate incorporated/ minute per ml
poly(Am)•poly(U)	UTP	< 0.005
	ATP	1.1
poly(Am)•poly(Um)	UTP	< 0.005
	ATP	0.4
poly(A)•poly(Um)	UTP	1.4
	ATP	0.05
poly(A)•poly(U)	UTP	0.9
	ATP	0.9

FIGURE 1.--

(a) Time course of poly(A) synthesis directed by poly(dT), poly(U), and poly(Um). (b) Time course of poly(U) synthesis directed by poly(dA), poly(A), and poly(Am).


nMOLES ³H-AMP INCORPORATED/ml
nMOLES ³H-UMP INCORPORATED/ml
TIME ELAPSED (MINUTES)
a
b
poly(dT)
poly(U)
poly(Um)
poly(dA)
poly(A)
poly(Am)
10
20
30
40
40
80

ARTICLE 3

RELEASE OF THE SIGMA SUBUNIT OF PSEUDOMONAS PUTIDA DEOXYRIBONUCEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE

By

Gary F. Gerard

ABSTRACT

^{35}S-labeled Pseudomonas putida deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase, $\alpha_2\beta\beta'\sigma$, was purified from cells that had been grown in a minimal medium containing sodium [^{35}S]sulfate. The amount of ^{35}S in β', β, and σ relative to α was 3.6 to 3.6 to 2.2 to 1.0, respectively. A study of the release of the sigma subunit of P. putida RNA polymerase was carried out following the binding of enzyme to polynucleotides and during DNA-directed RNA synthesis. Sucrose density gradient centrifugation was the technique employed to assay for the release of ^{35}S-labeled sigma. The subunits of ^{35}S-labeled RNA polymerase present in protein peaks resolved on sucrose gradients were identified by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Binding of ^{35}S-labeled RNA polymerase to native DNA weakened the interaction between sigma and core polymerase ($\alpha_2\beta\beta'$) but did not result in the release of sigma. Binding of the ^{35}S-labeled enzyme to poly(A), poly(C), and to tRNA resulted in the release of sigma. Binding of ^{35}S-labeled RNA polymerase to poly[d(A-T)], denatured gh-1 DNA, poly(dT), and to poly(dA) did not result in the release of sigma.

Release of sigma subsequent to the binding of enzyme to poly(dC) and poly(U) occurred in the absence of manganese chloride but not in its presence. Sigma was released from the enzyme-polynucleotide complex during DNA-directed RNA synthesis. Within 3 minutes of incubation, about 60% of the ^{35}S-labeled RNA polymerase molecules initiated RNA synthesis and formed a 200 mM KCl stable complex with DNA and nascent RNA. All or almost all of these enzyme molecules released sigma. The other 40% of the enzyme molecules did not form a 200 mM KCl stable complex within 3 minutes. With longer times of incubation, these enzyme molecules could slowly form a 200 mM KCl stable complex but did not release sigma. The sedimentation coefficient of P. putida sigma released during DNA-directed RNA synthesis was 4.1 to 4.5 S.

INTRODUCTION

Bacterial DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6) from Escherichia coli (Burgess, 1969; Burgess et al., 1969), Azotobacter vinelandii (Krakow and von der Helm, 1970), and Pseudomonas putida (Johnson et al., 1971) is composed of a core polymerase and a sigma subunit. The subunit structure of the core polymerase is $\alpha_2\beta\beta'$. The complex between core polymerase and sigma is referred to as holoenzyme. Both core polymerase and holoenzyme are able to catalyze the synthesis of RNA that is complementary to a DNA template. With holoenzyme as the catalyst, RNA synthesis in vitro is initiated with high efficiency at specific DNA promotor sites which function in vivo (Bautz et al., 1969; Sugiura et al., 1970). With core polymerase as the catalyst, initiation of RNA synthesis does not occur specifically at these promotor sites but occurs in a random manner. Consequently, the RNA synthesized in vitro by holoenzyme corresponds more closely to RNA synthesized in vivo than does that synthesized by core polymerase.

The sigma subunit functions either in the process by which holoenzyme recognizes the specific promotor

sites or in the process by which holoenzyme binds tightly to them or in both processes (Hinkle and Chamberlin, 1970; Zillig et al., 1970). Travers and Burgess (1969) have concluded that subsequent to initiation of DNA-directed RNA synthesis in vitro by E. coli RNA polymerase, sigma is released from the enzyme-polynucleotide complex leaving core polymerase to catalyze RNA chain elongation. The experimental basis for this conclusion rests on the observation that core polymerase molecules added to a reaction mixture in which ϕ80 DNA-directed RNA synthesis by holoenzyme was occurring, could use sigma derived from holoenzyme to catalyze the sigma-dependent transcription of T_4 DNA. The possibility exists, however, that Travers and Burgess were not observing the release of sigma from the holoenzyme-ϕ80 DNA-RNA complex caused by events which are part of the RNA synthetic process. They may have been observing the depletion of sigma from holoenzyme-ϕ80 DNA-RNA complex, from holoenzyme-ϕ80 DNA complex, or from holoenzyme itself caused by the establishment of an equilibrium exchange reaction with core enzyme which was present at 4 times the concentration of holoenzyme. In this regard, Travers (1971) has reported that core enzyme molecules can rapidly exchange sigma. In the experiments on sigma release during ϕ80 DNA-directed RNA synthesis, Travers and Burgess (1969) did not directly

demonstrate the physical separation of sigma from enzyme-polynucleotide complex nor did they measure the stoichiometry of sigma release.

Krakow and von der Helm (1970) have presented evidence which can be taken to mean that there is a physical separation of sigma from A. vinelandii holoenzyme following the initiation of poly(A-U) synthesis from a poly[d(A-T)] template. These workers used polyacrylamide gel electrophoresis to resolve sigma from the enzyme-polynucleotide complex. Experiments similar to those of Krakow and von der Helm (1970) were reported by Ruet et al. (1970) with E. coli holoenzyme and T_4 DNA as the template. The conclusions of both groups of workers can be questioned, however, since the electrical potential field present during polyacrylamide gel electrophoresis could influence the interaction between sigma and core polymerase-polynucleotide complex. In the studies of Krakow and von der Helm (1970) and Ruet et al. (1970), as was the case in the study of Travers and Burgess (1969), the stoichiometry of sigma release was not measured.

DNA-dependent RNA polymerase of P. putida is the object of study in our laboratory. The P. putida holoenzyme differs from the E. coli and A. vinelandii holoenzyme in a property which may have some relevance to the question of whether or not sigma is released. The interaction between sigma and core polymerase of E. coli and

A. vinelandii appears to be weaker than the interaction between sigma and core polymerase from P. putida. Dissociation of E. coli and A. vinelandii holoenzyme, but not P. putida holoenzyme, to sigma and core polymerase occurs during phosphocellulose chromatography (Burgess et al., 1969; Johnson et al., 1971; Krakow and von der Helm, 1970).

We have undertaken a study to determine whether or not sigma is released from P. putida holoenzyme during DNA-directed RNA synthesis and following the binding of holoenzyme to a variety of polynucleotides. In this study, P. putida holoenzyme labeled with ^{35}S was used. Sucrose density gradient centrifugation was employed as a technique to resolve sigma, enzyme, and enzyme-polynucleotide complex. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was used to identify the subunits of RNA polymerase present in protein peaks resolved on the sucrose gradients. The use of these techniques provides a means of demonstrating directly the physical separation of sigma from core enzyme-polynucleotide complex and also provides a means of measuring the stoichiometry of sigma release. In this report, we present the results of this study.

MATERIALS AND METHODS

Materials. Sodium [^{35}S]sulfate and Omnifluor were purchased from New England Nuclear. Agarose (Bio-Gel A1.5m) was obtained from Bio-Rad Laboratories. Rifampicin and streptolydigin were gifts from Gruppo Lepetit, Inc., Milan, Italy, and The Upjohn Company, respectively. Poly(dT), poly(dC), and poly(dA) were gifts from F. J. Bollum, University of Kentucky, Lexington, Kentucky. [^{3}H]-Poly(U) was from Miles Laboratories, Inc. Rabbit hemoglobin was a gift from A. J. Morris of this department. All other materials were obtained from sources previously described (Johnson et al., 1971; Gerard et al., 1971).

Analytical Methods. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The concentration of Pseudomonas putida bacteriophage gh-1 DNA was determined spectrophotometrically based on the extinction coefficient $E_{260}^{1\%}$ =200. The molar extinctions, ε(P), used to determine polyribonucleotide concentrations were: 10.5 x 10^3 at 257 nm, 9.2 x 10^3 at 360 nm, and 6.5 x 10^3 at 267 nm for poly(A), poly(U), and poly(C), respectively, in 0.1 M NaCl-0.05 M Tris acetate (pH 7.5) (Ts'o et al., 1962).

The molar extinctions, ε(P), for polydeoxyribonucleotides were 6.7×10^3 at 260 nm and pH 7.5 for poly[d(A-T)] (Radding and Kornberg, 1962) and 8.1×10^3, 5.3×10^3, and 9.7×10^3 at 260 nm for poly(dT), poly(dC), and poly(dA), respectively, in 0.001 M Tris-HCl (pH 8.0) (Bollum, 1966). P. putida RNA polymerase was assayed as previously described (Johnson et al., 1971).

Characterization of Synthetic Polynucleotides. The sedimentation velocity coefficients of the synthetic polynucleotides used in these experiments were determined by centrifugation through sucrose density gradients prepared in 0.1 M KCl-0.01 M Tris-HCl (pH 8.0) with E. coli tRNA as the standard. Each synthetic polynucleotide had a sedimentation coefficient with a mean value between 4.5 and 6.0 S. Each of the synthetic polynucleotides was an efficient template for P. putida RNA polymerase in the presence of manganese chloride.

Growth of Pseudomonas Putida. P. putida (the same or similar to ATCC 12633) was grown in a medium which 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, and 0.005 each of $CaCl_2$, $FeCl_3 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$ and $Mn(C_2H_3O_2)_2 \cdot 4H_2O$. For the production of ^{35}S-labeled cells, 50 mCi of sodium [^{35}S]sulfate with a specific activity of 845 mCi per mmole were added per

three liters 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. Doubling time for the culture was 100 minutes. The cells were harvested at the late logarithmic phase of growth, and stored at -20°. From a three-liter culture, the yield of ^{35}S-labeled cells was 10g (wet weight). Approximately 20 mCi of [^{35}S]sulfate had been incorporated during growth of the cells. The purification of ^{35}S-labeled RNA polymerase was begun one day after the ^{35}S-labeled cells were harvested.

Purification of ^{35}S-labeled RNA Polymerase. Frozen ^{35}S-labeled P. putida (10g) and an equal amount of frozen unlabeled P. putida cells were mixed with washed glass beads and ground in a mortar with a pestle. After cell rupture, RNA polymerase was purified through phosphocellulose chromatography by the method described by Johnson et al. (1971) except that ASH buffer was replaced with 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) and phosphocellulose chromatography was performed using buffers which contained 15% glycerol rather than 50% glycerol (v/v). After phosphocellulose chromatography, Phosphocellulose Fraction I was further purified by chromatography on an Agarose column (1.5 x 85 cm) developed with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA,

0.5 mM dithiothreitol, 500 mM KCl, and 5% glycerol (v/v), followed by centrifugation through a linear 10 to 30% glycerol gradient prepared in 50 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol, and 200 mM KCl. The yield of RNA polymerase was 500 µg of protein per 20g wet weight of cells. The time elapsed between purification of a given ^{35}S-labeled enzyme preparation and the use of that preparation for the experiments described in this report did not exceed two weeks.

Sucrose Density Gradient Centrifugation. The detailed experimental procedures used for the sucrose density gradient centrifugation of various mixtures of ^{35}S-labeled enzyme and polynucleotides are given in the legends of the figures. In the fractions collected from the sucrose gradients, the recovery of ^{35}S was at least 70% and in most cases 80% to 90% of the ^{35}S which had been layered on the sucrose gradients prior to centrifugation. In the experiments in which mixtures of ^{35}S-labeled enzyme and single stranded synthetic polynucleotides were centrifuged through sucrose gradients which contained manganese chloride, the recovery of ^{35}S was less than 50% due to the fact that the ^{35}S-labeled enzyme and ^{35}S-labeled enzyme-polynucleotide complexes adhered to the sides of the centrifuge tubes. This was true for both cellulose and polyallomer tubes (Beckman Instrument Co.). Addition

of bovine serum albumin to the sucrose gradients increased the recovery of ^{35}S to at least 90%.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using a modification of the procedure of Shapiro et al. (1967) as described by Johnson et al. (1971). SDS-polyacrylamide gels 11 cm in length were prepared from a polymerization mixture which was 3.75% in acrylamide. For the gels that were used in the analysis of fractions from the sucrose gradients, the polymerization mixture contained, in addition to the ingredients previously described, 12.5% glycerol (v/v). Following electrophoresis, the gels were immersed in 10% trichloroacetic acid. Protein was stained with 0.4% coomassie brilliant blue.

^{35}S analysis was performed on both stained and unstained gels. The gels were cut into 2 mm or 4 mm transverse fractions using a stainless steel support and cutting guide. Each fraction was placed in a scintillation vial and 0.2 ml of 30% H_2O_2 was added. After incubating at 70° for 9 hours or at 100° for 2 hours, 5 ml of a mixture containing 6 parts of Omnifluor solution (18.1g of Omnifluor per gallon of toluene) and 7 parts of Triton X-100 were added to each scintillation vial (Tishler and Epstein, 1968). The vials were capped, shaken, and monitored for their ^{35}S content in a liquid scintillation

spectrometer. The recovery of ^{35}S in the gel fractions was in most cases at least 70% of that which had been layered on the gel prior to electrophoresis.

RESULTS

Characterization of Pseudomonas Putida ^{35}S-Labeled RNA Polymerase. The specific enzymatic activity of P. putida ^{35}S-labeled RNA polymerase was 7800 nmoles of CMP incorporated per hour per mg of protein at 30° using P. putida bacteriophage gh-1 DNA as the template. The specific radioactivity of the ^{35}S-labeled enzyme was 2.3×10^7 cpm per mg of protein. As judged by SDS-polyacrylamide gel electrophoresis (see Figures 1 and 2) and sucrose density gradient centrifugation (see upper diagram of Figures 3 and 4), the ^{35}S-labeled enzyme was at least 98% pure.

A densimetric tracing of a Coomassie brilliant blue stained SDS-polyacrylamide gel of ^{35}S-labeled RNA polymerase is presented in Figure 1. As calculated from the relative amounts of the subunits and their relative molecular weights, there was one equivalent of sigma per equivalent of $\alpha_2\beta\beta'$ for the ^{35}S-labeled enzyme.

^{35}S analysis of a SDS-polyacrylamide gel of the ^{35}S-labeled enzyme is presented in Figure 2. The amount of ^{35}S in β' plus β and in σ relative to α was 7.2 to 2.2 to 1.0, respectively. The amount of ^{35}S of β' was found to be equal to that of β in three experiments in which β'

and β were separated by SDS-polyacrylamide gel electrophoresis. In these three experiments, the time of electrophoresis was 6 hours rather than the 4.75 hours reported for the experiment presented in Figure 1.

The Release of the Sigma Subunit of RNA Polymerase During gh-1 DNA-Directed RNA Synthesis: Analysis by Centrifugation through Sucrose Density Gradients Containing 50 mM KCl. Reaction mixtures containing ^{35}S-labeled enzyme, ^{35}S-labeled enzyme plus gh-1 DNA, and ^{35}S-labeled enzyme plus gh-1 DNA and the four common nucleoside triphosphates were incubated for 3 minutes. After incubation, sucrose gradient centrifugation was used to detect free ^{35}S-labeled sigma. If the sigma subunit is released from the holoenzyme during the incubation period prior to centrifugation, it would be expected to be found near the top of the sucrose gradient well resolved from free enzyme and enzyme-polynucleotide complex. The results are presented in Figure 3. ^{35}S-labeled RNA polymerase that had been incubated in the reaction mixture which lacked gh-1 DNA and the nucleoside triphosphates sedimented as a single symmetrical peak (upper diagram). ^{35}S-labeled RNA polymerase that had been incubated with gh-1 DNA sedimented much faster than free enzyme, indicating that it was bound to gh-1 DNA (middle diagram). Little or no free ^{35}S-labeled protein was observed near the top of the sucrose

gradient. Most of the ^{35}S-labeled RNA polymerase that had been incubated with gh-1 DNA and the nucleoside triphosphates sedimented much faster than free enzyme (lower diagram). This fast sedimenting material is a complex which contained gh-1 DNA, ^{35}S-labeled enzyme, and RNA. RNA was determined to be present in the complex by experiments monitoring radioactive RNA synthesized from ^{3}H-labeled nucleoside triphosphates. A small amount of ^{35}S-labeled enzyme, which was not bound to gh-1 DNA was observed. A ^{35}S-labeled protein peak was detected near the top of the sucrose gradient (fraction numbers 24-28). This protein peak was identified as sigma by SDS-polyacrylamide gel electrophoresis (see below), indicating that the sigma subunit of RNA polymerase had been released during gh-1 DNA-directed RNA synthesis. All of the RNA polymerase molecules, however, had not released sigma. Based on the amount of free ^{35}S-labeled sigma and the amount of ^{35}S-labeled enzyme in the complex which contained gh-1 DNA and RNA, about 60% of the enzyme molecules in the complex had released sigma.

Identification of the ^{35}S-labeled subunits of RNA polymerase present in each of the peak fractions of the sucrose gradients was carried out by means of SDS-polyacrylamide gel electrophoresis of comparable fractions of duplicate sucrose gradients. In these SDS-polyacrylamide

gel electrophoresis experiments, β' and β were not resolved from each other. Free RNA polymerase (inset, upper diagram of Figure 3) contained the β'β, σ, and α subunits in the same relative amounts as those found in enzyme that had not been centrifuged through a sucrose gradient, i.e., one equivalent of sigma per equivalent of $\alpha_2\beta\beta'$. RNA polymerase bound to gh-1 DNA (inset, middle diagram) contained β'β, σ, and α, but the amount of σ relative to α and β'β was about 70% of that found in free enzyme. RNA polymerase bound in the complex that contained gh-1 DNA, enzyme, and RNA (inset A, lower diagram) contained β'β, σ and α. The amount of σ was about 35% of that found in free enzyme. The ^{35}S-labeled protein peak near the top of the sucrose gradient was identified as σ (inset B, lower diagram). Although the SDS-polyacrylamide gel data is not shown, the enzyme not bound to gh-1 DNA (lower diagram) contained β'β, σ and α in the same relative amounts as that found in enzyme that had not been centrifuged through a sucrose gradient.

The Effect of Binding ^{35}S-Labeled RNA Polymerase to gh-1 DNA on the Interaction between Core Polymerase and the Sigma Subunit. After centrifugation, all of the ^{35}S-labeled RNA polymerase molecules which had sedimented as free enzyme contained sigma (upper diagram of Figure 3), but only 70% of the RNA polymerase molecules which had

sedimented as enzyme-gh-1 DNA complex contained the subunit (middle diagram of Figure 3). Sigma which had dissociated from 30% of the RNA polymerase molecules of the enzyme-gh-1 DNA complex was not detected as a peak near the top of the sucrose gradient. This suggests that dissociation of sigma occurred during centrifugation as the enzyme-gh-1 DNA complex moved through the gradient. Accordingly, sigma should be found trailing behind the enzyme-gh-1 DNA complex throughout the sucrose gradient.

The concentration of KCl in the sucrose gradients affected the dissociation of sigma from the enzyme-gh-1 DNA complex. When 0, 50, or 100 mM KCl was present in the gradients, the enzyme-gh-1 DNA complex was stable, but sigma dissociated during centrifugation from 60, 30, and 10%, respectively, of the enzyme molecules of the enzyme-gh-1 DNA complex. When 200 mM KCl was present in the gradient, the enzyme-gh-1 DNA complex was not stable and dissociation of the complex occurred (see middle diagram of Figure 4). In the experiment in which no KCl was present in the sucrose gradients, SDS polyacrylamide gel analysis showed that sigma trailed behind the enzyme-gh-1 DNA complex throughout the gradient. When 0, 50, 100, or 200 mM KCl was present in the gradients, sigma did not dissociate during centrifugation from RNA polymerase molecules which had not been reacted with gh-1 DNA.

The Release of the Sigma Subunit of RNA Polymerase during gh-1 DNA-Directed RNA Synthesis: Analysis by Centrifugation through Sucrose Density Gradients Containing 200 mM KCl. Based on the amount of free ^{35}S-labeled sigma found near the top of the sucrose gradient which contained 50 mM KCl (lower diagram of Figure 3) and the amount of sigma found in the complex that contained gh-1 DNA, enzyme, and nascent RNA (inset A, lower diagram of Figure 3), only about 60% of the enzyme molecules in the complex released sigma. The other 40% of the enzyme molecules in the complex did not release sigma. Presumably, the enzyme molecules which released sigma were engaged in RNA synthesis and those which did not release sigma were not. To distinguish between enzyme molecules engaged in RNA synthesis and those not engaged in RNA synthesis, centrifugation through sucrose gradients containing 200 mM KCl was used. The ternary complex between RNA polymerase, DNA and nascent RNA is stable in solutions of high ionic strength, but the binary complex between RNA polymerase and DNA is not (Richardson 1966a; 1966b).

Reaction mixtures containing ^{35}S-labeled enzyme, ^{35}S-labeled enzyme plus gh-1 DNA, and ^{35}S-labeled enzyme plus gh-1 DNA and the four common nucleoside triphosphates were incubated for 3 minutes and then analyzed by centrifugation through sucrose density gradients containing

200 mM KCl. The results are presented in Figure 4. ^{35}S-labeled RNA polymerase that had been incubated in the reaction mixture which lacked gh-1 DNA and the nucleoside triphosphates sedimented as a symmetrical peak with a sedimentation coefficient of 13 S (Johnson et al., 1971) (upper diagram). ^{35}S-labeled RNA polymerase that had been incubated with gh-1 DNA to promote binding of the enzyme, indicating that the enzyme dissociated from gh-1 DNA as it moved into the sucrose gradient containing 200 mM KCl (middle diagram). About 40% of the ^{35}S-labeled RNA polymerase molecules that had been incubated with gh-1 DNA and the nucleoside triphosphates sedimented as free enzyme at 13 S and the remaining 60% sedimented in a 200 mM KCl stable complex that sedimented faster than 13 S. Free ^{35}S-labeled sigma whose identification is described below was observed near the top of the gradient.

Identification of the ^{35}S-labeled subunits of RNA polymerase present in each of the peak fractions was carried out using SDS-polyacrylamide gel electrophoresis. Both free enzyme and enzyme that had been bound to gh-1 DNA but dissociated from it in the sucrose gradient contained a full compliment of sigma, i.e., one equivalent of sigma per equivalent of $\alpha_2\beta\beta'$ (inset, upper and middle diagram of Figure 4). ^{35}S-labeled enzyme found in the 200 mM KCl stable complex contained only about 10% of the sigma found

in free enzyme (inset A, lower diagram). Free ^{35}S-labeled enzyme at the 13 S position of the sucrose gradient contained a full compliment of sigma (inset B, lower diagram). The ^{35}S-labeled protein near the top of the gradient was identified as sigma (inset C, lower diagram). The amount of the free ^{35}S-labeled sigma near the top of the gradient was equivalent to the amount of sigma released from the enzyme in the 200 mM KCl stable complex.

The 200 mM KCl stable complex contained ^{35}S-labeled RNA polymerase molecules that were engaged in gh-1 DNA-directed RNA synthesis. Radioactive RNA synthesized from ^{3}H-labeled nucleoside triphosphates cosedimented with the complex but not with the ^{35}S-labeled RNA polymerase molecules that sedimented as free enzyme. No 200 mM KCl stable complex was formed if either gh-1 DNA or the four common nucleoside triphosphates were omitted from the reaction mixture. If the reaction mixture contained ATP rather than all four of the nucleoside triphosphates, only about 8% of the enzyme molecules formed a 200 mM KCl stable complex (upper left diagram of Figure 5). If rifampicin was added to the complete reaction mixture, about 8% of the enzyme molecules formed a 200 mM KCl stable complex (upper right diagram of Figure 5).

The effects of the addition of streptolydigin and RNase to the complete reaction mixture on the formation of

the 200 mM KCl stable complex were examined. Streptolydigin is an antibiotic which inhibits RNA synthesis by RNA polymerase by inhibiting the rate of phosphodiester bond formation (Cassani et al., 1971). If streptolydigin was added to a complete reaction mixture, about 18% of the enzyme molecules formed a 200 mM KCl stable complex after 3 minutes of incubation (lower left diagram of Figure 5). If RNase was added to the complete reaction mixture, about 50% of the enzyme molecules formed a 200 mM KCl stable complex and released sigma (lower right diagram of Figure 5). This result is similar to that obtained in the absence of RNase (see lower diagram of Figure 4). Thus, hydrolysis by RNase of the nascent RNA chain protruding from the enzyme surface did not effect the formation of the 200 mM KCl stable complex or the release of sigma.

The effects of varying the concentration of RNA polymerase and the time of incubation on the amount of enzyme in the 200 mM KCl stable complex and the amount of free sigma found near the top of the sucrose gradient were examined. When 8, 25, and 50 µg of ^{35}S-labeled RNA polymerase per ml of reaction mixture was used, the amount of enzyme in the 200 mM KCl stable complex and the amount of free sigma increased proportionately. In this concentration range, the rate of gh-1 DNA-directed RNA synthesis is proportional to enzyme concentration. When the complete

reaction mixture was incubated 10 and 20 minutes rather than the standard 3 minute incubation, the amount of enzyme in the 200 mM KCl stable complex increased from 60% of the total enzyme to 70% and 80%, respectively. No increase in the amount of free sigma found near the top of the sucrose gradient was detected with the longer times of incubation.

Determination of the Sedimentation Coefficient of the Sigma Subunit of RNA Polymerase Released during gh-1 DNA-Directed RNA Synthesis. The sedimentation coefficient of sigma was determined using centrifugation through sucrose density gradients containing 50 mM KCl. Escherichia coli alkaline phosphatase with a sedimentation coefficient of 6.3 S (Garen and Levinthal, 1960) and rabbit hemoglobin with a sedimentation coefficient of 4.2 S (Chiancone et al., 1966) were used as markers. The results are presented in Figure 6. Free sigma sedimented as a nearly symmetrical peak with a sedimentation coefficient of 4.1 to 4.5 S.

The Effect of Binding ^{35}S-Labeled RNA Polymerase to Polyribonucleotides on the Release of the Sigma Subunit. The effect of binding ^{35}S-labeled RNA polymerase to several polyribonucleotides on the release of the sigma subunit was examined. The results with poly(U) are presented in Figure 7. ^{35}S-labeled RNA polymerase was incubated with [^{3}H]poly(U) in the presence of manganese chloride to promote the binding

of the enzyme to the polyribonucleotide, and was then centrifuged through a sucrose gradient which contained manganese chloride. No peak of sigma was detected near the top of the gradient (upper diagram of Figure 7), indicating that the binding of enzyme to poly(U) in the presence of manganese chloride did not result in the release of the sigma subunit. The binding of the enzyme to poly(U) in the absence of manganese chloride, however, resulted in the release of sigma. If binding and centrifugation were carried out in the absence of manganese chloride, free sigma was detected near the top of the sucrose gradient (lower diagram of Figure 7). About 70% of the enzyme molecules released sigma.

The binding of enzyme to poly(C), to poly(A), and to tRNA in either the presence or absence of manganese chloride resulted in the release of the sigma subunit. In these experiments, 40 to 60% of the enzyme molecules released sigma. In Figure 8, the results are presented for the binding of the enzyme to poly(C) in the presence and in the absence of manganese chloride (upper and middle diagram) and for poly(A) in the presence of manganese chloride (lower diagram). The data for tRNA are not presented. In the case of poly(A), SDS-polyacrylamide gel electrophoresis analysis showed that about 60% of the enzyme molecules in the main sedimentation peak and

in its fast moving shoulder had released sigma and that the ^{35}S-labeled protein that was detected near the top of the sucrose gradient was sigma (insets, lower diagram of Figure 8).

The Effect of Binding ^{35}S-Labeled RNA Polymerase to Polydeoxyribonucleotides on the Release of the Sigma Subunit. As already described, the binding of ^{35}S-labeled RNA polymerase to native gh-1 DNA did not result in the release of the sigma subunit, i.e., no peak of ^{35}S-labeled sigma was observed near the top of the sucrose gradient (middle diagram of Figure 3). The effect of binding ^{35}S-labeled RNA polymerase to other polydeoxyribonucleotides on the release of the sigma subunit was examined. The results are presented in Figure 9 for denatured gh-1 DNA (left diagram) and for poly[d(A-T)] (right diagram). In these sucrose density gradients, no peak of free sigma was observed, indicating that the binding of ^{35}S-labeled RNA polymerase to these polydeoxyribonucleotides did not result in the release of the sigma subunit. In the case of denatured gh-1 DNA, SDS-polyacrylamide gel electrophoresis analysis showed that RNA polymerase complexed to denatured gh-1 DNA and centrifuged through the sucrose gradient contained a full compliment of sigma (inset, left diagram of Figure 9).

In Figure 10 the results of the binding of ^{35}S-labeled RNA polymerase to poly(dT) (left diagram) and to poly(dC) (right diagram) are presented. In these experiments, manganese chloride was present in both the reaction mixtures and in the sucrose gradients. No release of sigma was observed. For poly(dC) but not for poly(dT), release of sigma was observed in the absence of manganese chloride (data not shown). The binding of the enzyme to poly(dA) in the presence or absence of manganese chloride did not result in the release of sigma (data not shown).

In the studies of the release of the sigma subunit during gh-1 DNA-directed RNA synthesis, the reaction mixture contained divalent metal ions, but the 50 mM and 200 mM KCl sucrose gradients did not (see Figures 3 and 4). The inclusion of manganese or magnesium chloride in the sucrose gradients, however, did not alter the amount of free sigma found near the top of the sucrose gradients.

DISCUSSION

^{35}S-labeled Pseudomonas putida RNA polymerase, $\alpha_2\beta\beta'\sigma$, of high specific radioactivity was purified from ^{35}S-labeled cells that had been grown on a minimal growth medium which contained sodium [^{35}S]sulfate. As a result of the high specific radioactivity of the enzyme, the assay of as little as 0.01 μg of protein was feasible by monitoring ^{35}S.

Analysis of the P. putida RNA polymerase subunits showed that the ^{35}S content of β', β, and σ relative to α was 3.6 to 3.6 to 2.2 to 1.0. Since the molar concentrations of β', β and σ relative to α are 0.5 to 0.5 to 0.5 to 1.0 (Johnson et al., 1971), there are 7.2 sulfur atoms in β' and in β and 4.4 sulfur atoms in σ for each sulfur atom in α. Consequently, in each 44,000g of β', of β, and of σ, there are twice as many sulfur-containing amino acids as in a gram molecular weight of α which is 44,000g. Burgess (1969) reported that for Escherichia coli RNA polymerase, in each 39,000g of β' plus β there are 1.3 times as many sulfur-containing amino acids as in a gram molecular weight of α which is 39,000g. The values for E. coli σ and for β' and β separately have not been reported.

Sigma was released from the enzyme-polynucleotide complex during gh-1 DNA-directed RNA synthesis. Within 3 minutes of incubation, about 60% of the ^{35}S-labeled RNA polymerase molecules initiated RNA synthesis and formed a 200 mM KCl stable complex with the gh-1 DNA template and the growing RNA chain. All or almost all of these enzyme molecules released sigma. The other 40% of the enzyme molecules did not form a 200 mM KCl stable complex within 3 minutes of incubation because, presumably, they did not initiate RNA synthesis. With longer times of incubation, these enzyme molecules could slowly form a 200 mM KCl stable complex but did not release sigma. These enzyme molecules are considered to be defective in their capacity to synthesize RNA because they initiate RNA synthesis very slowly. The enzyme molecules that finally do initiate RNA synthesis and form a 200 mM KCl stable complex with the longer times of incubation do not release sigma either because they have a direct impairment in the sigma release process itself, or because some event in RNA synthesis which triggers sigma release does not occur. Perhaps, sigma release is triggered once the synthesis of nascent RNA of a certain nucleotide length is achieved (Travers, 1971). If nascent RNA of this nucleotide length was not synthesized due to the fact that the defective enzyme molecules were impaired with respect to their ability to carry out phosphodiester bond formation, sigma would not be released.

The defective RNA polymerase molecules probably were not produced by the decay of ^{35}S. At the level of ^{35}S-labeling of RNA polymerase, only one enzyme molecule in every 50 to 100 contained a ^{35}S atom. Enzymatic activity of ^{35}S-labeled RNA polymerase was stable for 90 days when stored at -20° which is slightly longer than the 87.9 day half-life of ^{35}S. The defective enzyme molecules may have been damaged during the enzyme purification procedure. Although the specific enzymatic activity of the ^{35}S-labeled RNA polymerase preparation was comparable to most preparations of unlabeled P. putida RNA polymerase, enzyme preparations of higher specific enzymatic activity have been obtained by us.

The sedimentation coefficient of P. putida sigma released during gh-1 DNA-directed RNA synthesis was 4.1 to 4.5 S. The molecular weight of P. putida sigma is 98,000 (Johnson et al., 1971). Enzymes of globular conformation with molecular weights in the 100,000 range usually have sedimentation coefficients of 5.5 to 6.0 S (Holleman, 1966). Examples of enzymes involved in nucleic acid metabolism which are composed of single polypeptide chains of molecular weights in the 100,000 range and which have sedimentation coefficients of 5.5 to 6.0 S have been reported by Baldwin and Berg (1966), Jovin et al. (1969), Yaniv and Gros (1969), and Hayashi et al. (1970). The

relatively low sedimentation coefficient for P. putida sigma indicates that it does not have a globular conformation. The conformation of E. coli sigma probably is also not globular. Its molecular weight and sedimentation coefficient are 95,000 and 4.5 to 5.0 S, respectively (Burgess et al., 1969).

In the experiments in which the binding of P. putida ^{35}S-labeled RNA polymerase to polynucleotides was examined, the results obtained in the presence of manganese chloride should be emphasized since RNA synthesis is possible only in the presence of a divalent metal ion. Under this condition, binding of the enzyme to polydeoxyribonucleotides did not result in the release of sigma. Binding of the enzyme to polyribonucleotides on the other hand, with one exception, did result in the release of sigma.

Krakow and von der helm (1970) have reported that for Azotobacter vinelandii RNA polymerase, binding of the holoenzyme to single-stranded polydeoxyribonucleotides and polyribonucleotides caused the release of sigma. In their studies, Krakow and von der Helm used electrophoresis in polyacrylamide gels to assay for the release of sigma from the enzyme-polynucleotide complex. The effects that this method might have on the interaction between sigma and core-polynucleotide complex cannot be defined or controlled. For example, the effects of monovalent and

divalent cations which we have described in this study could not be appreciated using the polyacrylamide gel electrophoresis technique.

The mechanism by which sigma is released from the enzyme-polynucleotide complex following the binding of enzyme to polyribonucleotides is not understood. We do not know where on the enzyme surface the polyribonucleotides bind to affect the release of sigma. They could bind to the enzyme at the template binding site usually occupied by DNA or at the nascent RNA binding site occupied by the growing RNA chain during DNA-directed RNA synthesis. In the experiments in which the binding of P. putida RNA polymerase to synthetic polyribonucleotides was shown to result in the release of sigma, in fact, usually only about 60% of the enzyme molecules released sigma. Perhaps, the enzyme molecules which did not release sigma on binding to the synthetic polyribonucleotides were the defective enzyme molecules which could not release sigma during gh-1 DNA-directed RNA synthesis. Further experimentation is required before the mechanism of sigma release following the binding of the enzyme to polynucleotides and during DNA-directed RNA synthesis is understood.

REFERENCES

Baldwin, A., and Berg, P., J. Biol. Chem., 241, 831 (1966).

Bautz, E. K. F., Bautz, F. A., and Dunn, J. J., Nature, 223, 1022 (1969).

Bollum, F. J., in Procedures in Nucleic Acid Research, S. L. Cantoni and D. R. Davies (Editors), Harper and Row, New York, 1966, p. 579.

Bray, G. A., Anal. Biochem., 1, 270 (1960).

Burgess, R. R., J. Biol. Chem., 244, 6168 (1969).

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F., Nature, 221, 43 (1969).

Cassani, G., Burgess, R. R., Goodman, H. M., and Gold, L., Nature New Biology, 230, 197 (1971).

Chiancone, E., Vecchini, P., Forlani, L., Antonini, E., and Wyman, J., Biochim. Biophys. Acta, 127, 549 (1966).

Garen, A., and Levinthal, C., Biochim. Biophys. Acta, 38, 470 (1960).

Gerard, G. F., Rottman, F., and Boezi, J. A., Biochemistry, 10, 1974 (1971).

Hayashi, H., Knowles, J. R., Katze, J. R., Lapointe, J., and Soll, D., J. Biol. Chem., 245, 1401 (1970).

Hinkle, D. C., and Chamberlin, M., Cold Spring Harbor Symp. Quant. Biol., XXXV, 65 (1970).

Holleman, W. H., Ph.D. Thesis, Michigan State University, 1966.

Johnson, J. C., DeBacker, M., and Boezi, J. A., J. Biol. Chem., 246, 1222 (1971).

Jovin, T. M., Englund, P. T., and Bertsch, L. L., J. Biol. Chem., 244, 2996 (1969).

Krakow, J. S., and von der Helm, K., Cold Spring Harbor Symp. Quant. Biol., XXXV, 73 (1970).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

Radding, C. M., and Kornberg, A., J. Biol. Chem., 237, 2877 (1962).

Richardson, J. P., J. Mol. Biol., 21, 83 (1966a).

Richardson, J. P., J. Mol. Biol., 21, 115 (1966b).

Ruet, A., Sentenac, A., and Fromageot, P., FEBS Letters, 11, 169 (1970).

Shapiro, A. L., Vinuela, E., and Maizel, J. V., Biochem. Biophys. Res. Commun., 28, 815 (1967).

Sugiura, M., Okamoto, T., and Takanami, M., Nature, 225, 598 (1970).

Tishler, P. V., and Epstein, C. J., Anal. Biochem., 22, 89 (1968).

Travers, A., Nature New Biology, 229, 69 (1971).

Travers, A. A., and Burgess, R. R., Nature, 222, 537 (1969).

Ts'o, P. P., Helmkamp, G. K., and Sander, C., Biochim. Biophys. Acta, 55, 584 (1962).

Yaniv, M., and Gros, F., J. Mol. Biol., 44, 1 (1969).

Zillig, W., Zeckel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W., Cold Spring Harbor Symp. Quant. Biol., XXXV, 47 (1970).

FIGURE 1.--Densimetric Tracing of a Coomassie Brilliant Blue Stained SDS-Polyacrylamide Gel of ^{35}S-Labeled RNA Polymerase.

^{35}S-labeled RNA polymerase (30 µg) was incubated at 100° for 10 minutes in 60 µl of solution containing 0.1 M sodium phosphate (pH 7.1), 1.0% SDS, 1.0% 2-mercaptoethanol, and 5% glycerol (v/v). A 4-µl sample of the mixture (2 µg of protein) was layered on a 11 cm SDS-polyacrylamide gel. Electrophoresis was performed at 25° for 4.75 hours at 8 ma/gel. The protein was stained with Coomassie brilliant blue and the densimetric tracing was made at 550 nm using a Gilford linear transport. The subunits are from left to right β', β, σ and α.

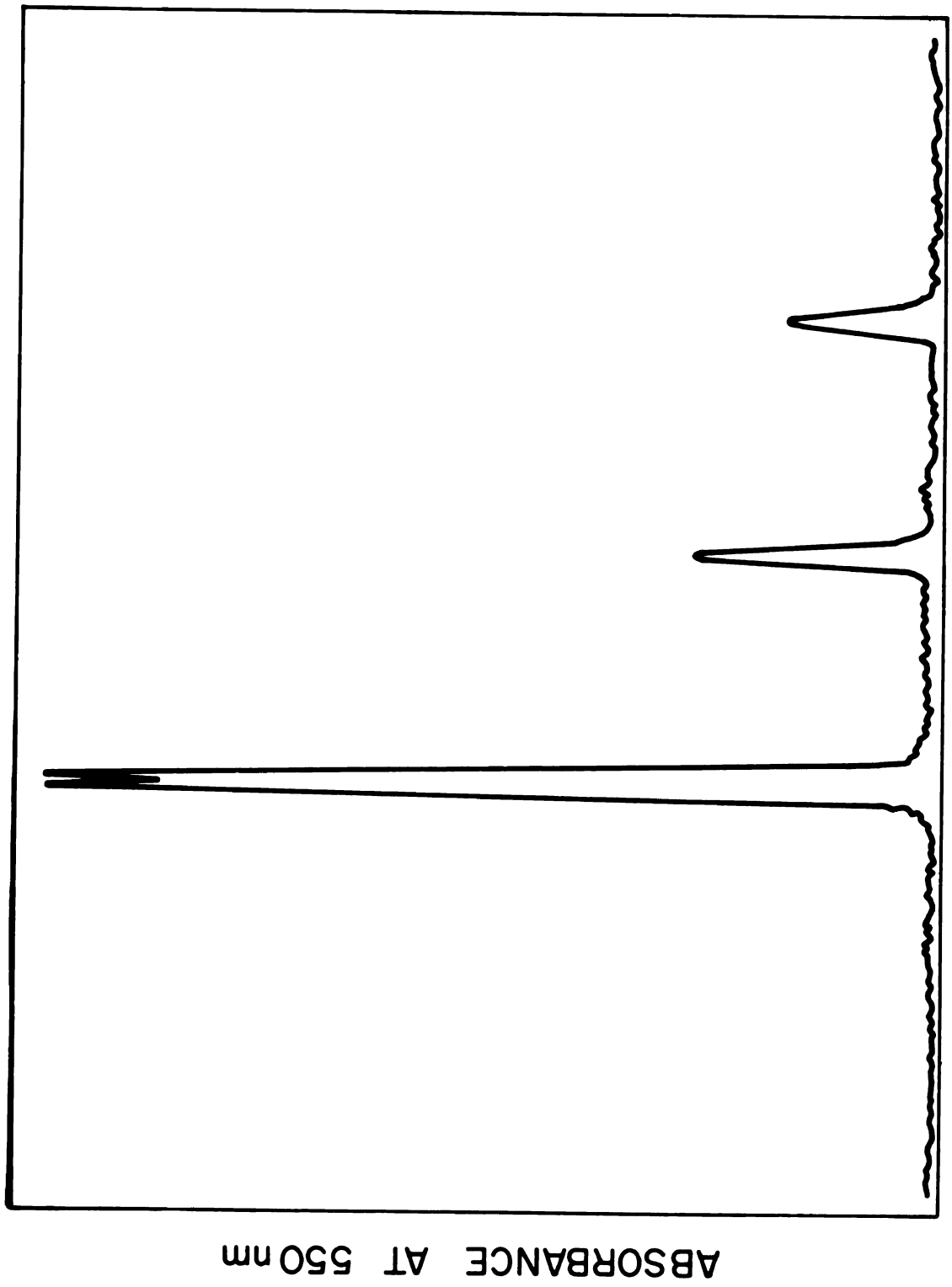

ABSORBANCE AT 550 nm
DISTANCE FROM ORIGIN

FIGURE 2.--^{35}S Analysis of a SDS-Polyacrylamide Gel of ^{35}S-Labeled RNA Polymerase.

The Coomassie brilliant blue stained SDS-polyacrylamide gel of ^{35}S-labeled RNA polymerase which was analyzed by the densimetric tracing presented in Figure 1 was cut into transverse fractions of 2 mm each. The ^{35}S content of each fraction was analyzed using the method described in Materials and Methods.

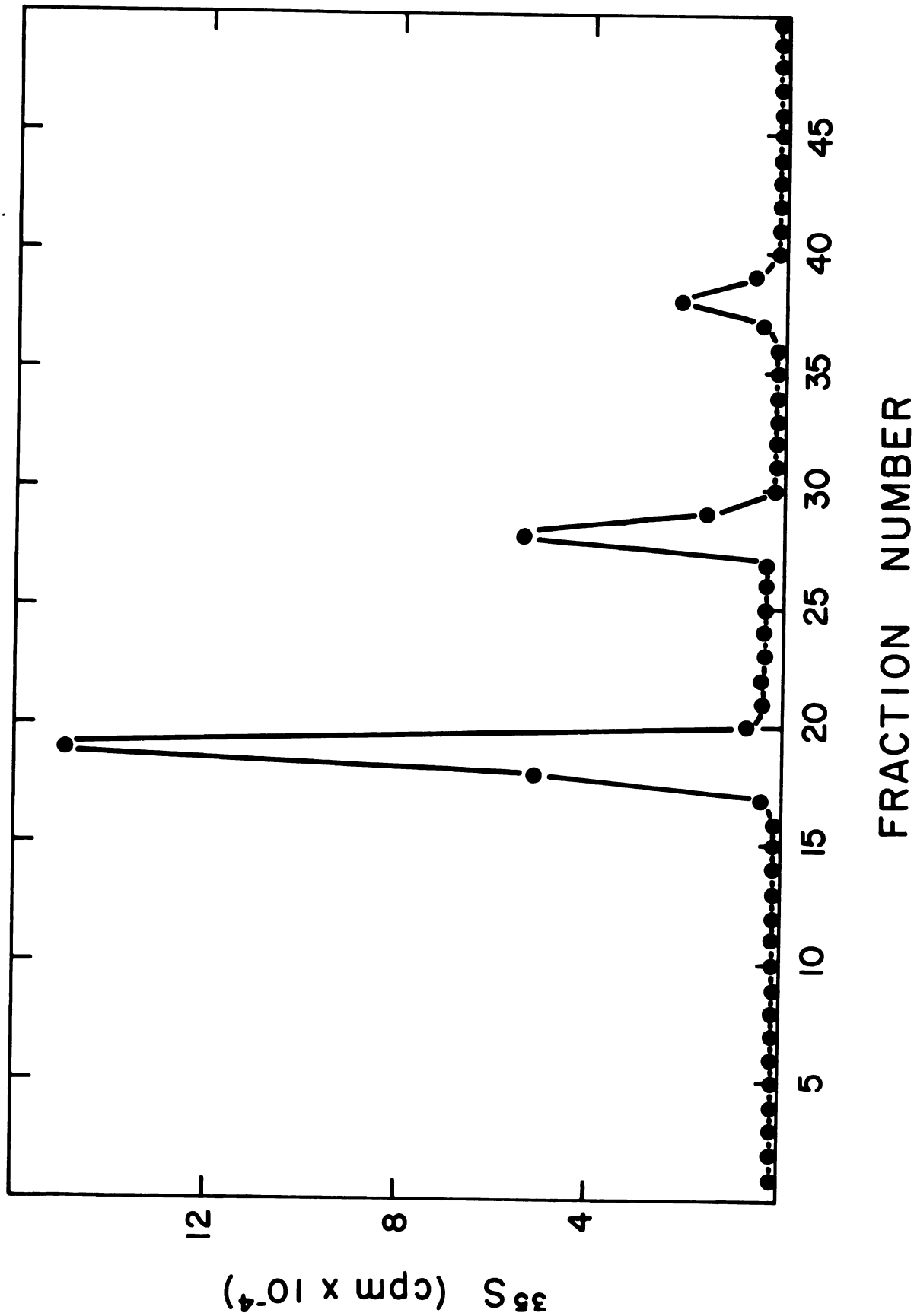

^{35}S (cpm x 10^{-4})
12
8
4
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

FIGURE 3.--The Release of the Sigma Subunit of RNA Polymerase during gh-1 DNA Directed RNA Synthesis: Analysis by Centrifugation through Sucrose Density Gradients Containing 50 mM KCl and by SDS-Polyacrylamide Gel Electrophoresis.

Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, and 25 µg/ml ^{35}S-labeled RNA polymerase (upper diagram), or the above constituents plus 100 µg/ml gh-1 DNA (middle diagram) or plus 100 µg/ml gh-1 DNA and 0.4 mM each of ATP, GTP, CTP, and UTP (lower diagram) were incubated at 30° for 3 minutes. After incubation, the reaction mixtures were cooled to 4°. A sample (0.10 ml) from each reaction mixture was then layered on a sucrose density gradient prepared by layering a 4.2 ml 5 to 20% linear sucrose gradient on top of 0.8 ml of 50% sucrose. The sucrose was prepared in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1 mM dithiothreitol. After centrifugation at 4° for 7 hr at 35,000 rpm in the Spinco SW39 rotor, fractions (0.16 ml) were collected from the bottom of the centrifuge tubes. Each fraction was then assayed for ^{35}S by liquid scintillation spectrometry (Bray, 1960). Comparable fractions from duplicate sucrose density gradients were made 200 mM in KCl and were incubated at 100° for 15 min in 200 µl of a solution containing 0.1 M sodium phosphate (pH 7.1), 1.0% SDS, and 1.0% 2-mercaptoethanol. The total SDS-treated mixtures were layered on 11 cm SDS-polyacrylamide gels. Electrophoresis was then performed at 25° at 4 ma/gel for the first 15 min, and then at 8 ma/gel for the remainder of the time. Total time of electrophoresis was 4.25 hr for sucrose density gradient fraction number 18 (inset, upper diagram) and for fraction number 27 (inset B, lower diagram) and was 5.25 hr for fraction number 6 (inset, middle diagram) and fraction number 4 (inset A, lower diagram). After electrophoresis, the gels were cut into 4 mm transverse fractions and the ^{35}S content of each gel fraction was analyzed according to the procedure described in Materials and Methods. For each inset, the ordinate is expressed as ^{35}S-CPM x 10^{-2} and the abscissa is expressed as Gel Fraction Number. The concentration of sucrose in the SDS-treated mixtures which were layered on the gels affected the electrophoretic mobilities of the ^{35}S-labeled subunits of RNA polymerase. Identification of the RNA polymerase subunits in the sucrose gradient fractions was made by comparison to the subunits of enzyme that had not been centrifuged through a sucrose gradient, but otherwise subjected to SDS-polyacrylamide electrophoresis in an identical manner.

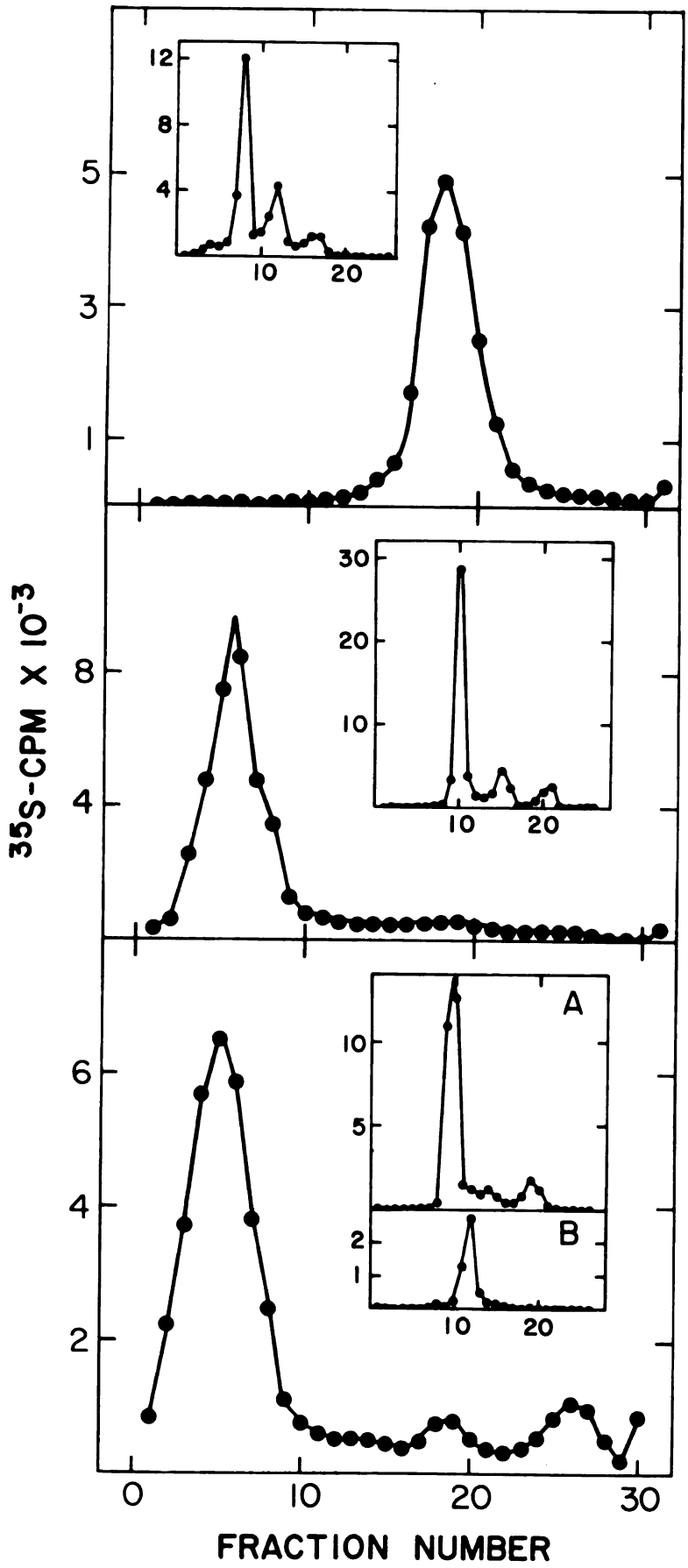

^{35}S-CPM X 10^{-3}
FRACTION NUMBER
A
B

FIGURE 4.--The Release of the Sigma Subunit of RNA Polymerase during gh-1 DNA-Directed RNA Synthesis: Analysis by Centrifugation through Sucrose Density Gradients Containing 200 mM KCl and by SDS-Polyacrylamide Gel Electrophoresis.

Reaction mixtures identical to those described for the upper, middle, and lower diagrams in the legend to Figure 3 were incubated at 30° for 3 min. After cooling, a sample (0.10 ml) from each reaction mixture was layered on a sucrose density gradient prepared by layering a 4.2 ml 5 to 20% linear sucrose gradient on top of 0.8 ml of 50% sucrose. The sucrose was prepared in 50 mM Tris-HCl (pH 8.0), 200 mM KCl, and 1 mM dithiothreitol. After centrifugation at 4° for 7 hr at 35,000 rpm, fractions were collected from the bottom of the centrifuge tubes and assayed for ^{35}S by liquid scintillation spectrometry (Bray, 1960).

Peak fractions from duplicate sucrose gradients were denatured in SDS as described in the legend to Figure 3 and then layered on 11 cm SDS-polyacrylamide gels. Electrophoresis was at 25° at 4 ma/gel for the first 15 min, and then at 8 ma/gel for the remainder of the time. Total time of electrophoresis was 4.25 hr for sucrose gradient fraction 20 (inset, upper diagram), fraction number 18 (inset, middle diagram), and fraction number 18 (inset B, lower diagram), and was 5.25 hr for fraction number 6 (inset A, lower diagram) and fraction number 27 (inset C, lower diagram). After electrophoresis, the gels were cut into 4 mm transverse fractions and the ^{35}S content of each gel fraction was analyzed according to the procedure described in Materials and Methods. For each inset, the ordinate is expressed as ^{35}S-CPM x 10^{-2} and the abscissa as Gel Fraction Number.

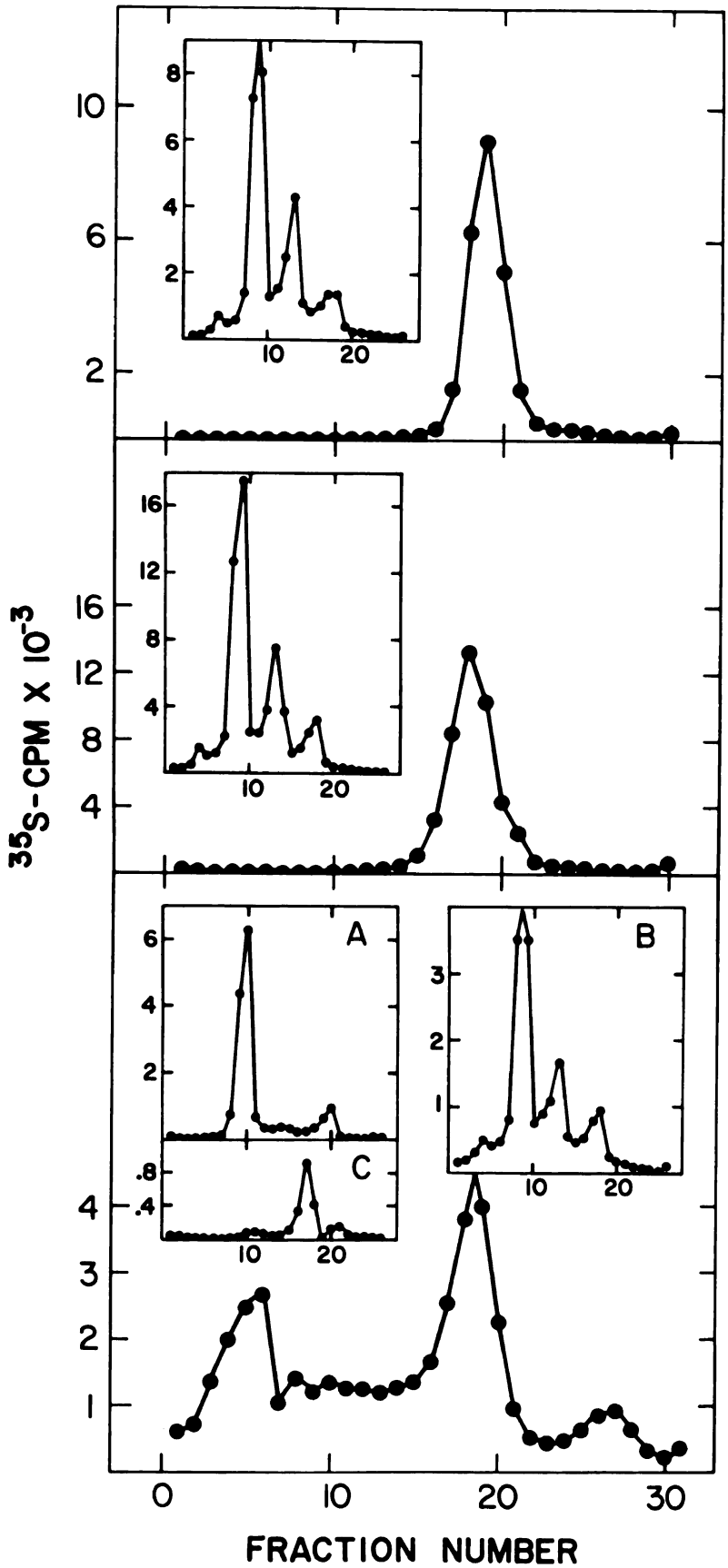

^{35}S-CPM X 10^{-3}
FRACTION NUMBER
A
B
C

FIGURE 5.--The Effect of ATP, Rifampicin, Streptolydigin, and RNase on the Formation of a 200 mM KCl Stable Complex.

Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, 25 µg/ml ^{35}S-labeled RNA polymerase, 100 µg/ml gh-1 DNA, and 0.4 mM ATP (upper left diagram), or 0.4 mM each of ATP, GTP, CTP, and UTP plus 1 µg/ml rifampicin (upper right diagram), plus 0.33 mM streptolydigin (lower left diagram), or plus 6 µg/ml pancreatic RNase (lower right diagram) were incubated at 30° for 3 min. After incubation, a sample (0.10 ml) of each reaction mixture was analyzed on sucrose gradients containing 200 mM KCl as described in the legend to Figure 4. The rate of the gh-1 DNA-directed synthesis of RNA as measured by the incorporation of [^3H]CMP into RNA was decreased by 98% over the control rate in the presence of either 1 µg/ml rifampicin or 0.33 mM streptolydigin in the reaction mixture.

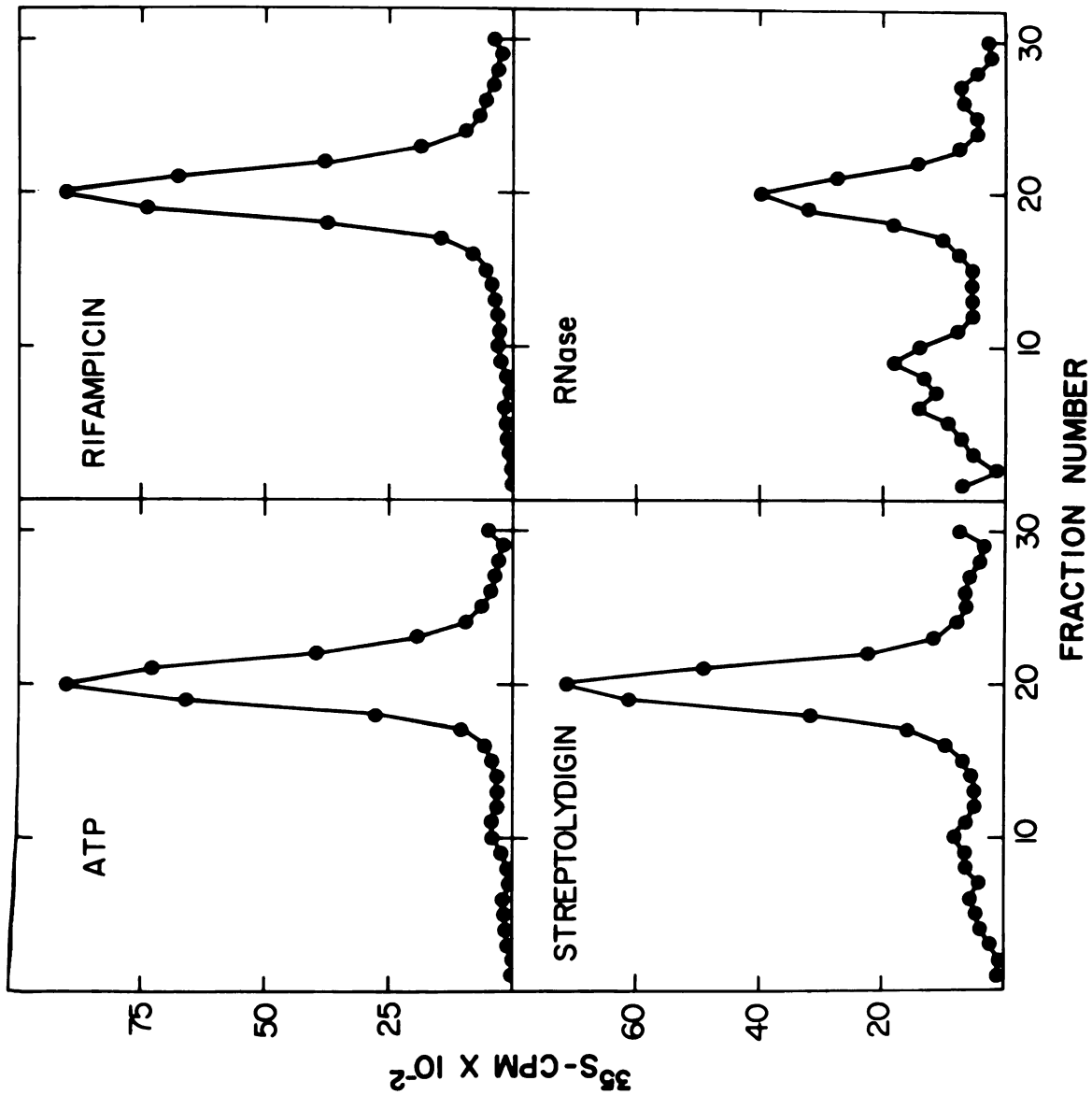

RIFAMPICIN
RNase
ATP
STREPTOLYDIGIN
75
50
25
60
40
20
10
20
30
FRACTION NUMBER
35S-CPM X 10-2

FIGURE 6.--Determination of the Sedimentation Coefficient of the Sigma Subunit of RNA Polymerase Released during gh-1 DNA-Directed RNA Synthesis.

Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, 250 µg/ml bovine serum albumin, and either 0.4 mM each of ATP, GTP, UTP, and CTP, 100 µg/ml gh-1 DNA, and 10 µg/ml ^{35}S-Labeled RNA polymerase, or 420 µg/ml E. coli alkaline phosphatase, or 25 A_{540} units/ml rabbit hemoglobin were incubated for 3 minutes at 30° and then chilled. A sample (0.10 ml) from each reaction mixture was layered on a sucrose gradient prepared by layering a 3.8 ml 5 to 20% linear sucrose gradient on top of 0.4 ml of 50% sucrose which had been layered on top of 0.8 ml of 75% sucrose. The sucrose was prepared in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, and 100 µg/ml bovine serum albumin. After centrifugation at 4° for 9 hr at 50,000 rpm in a Spinco SW50L rotor, fractions (0.16 ml) were collected from the bottom of the centrifuge tubes. Each fraction from the gradient containing ^{35}S-labeled RNA polymerase was assayed for ^{35}S (●) by liquid scintillation spectrometry (Bray, 1960). E. coli alkaline phosphatase was assayed spectrophotometrically at 410 nm using the substrate p-nitrophenyl phosphate (▲). The position of rabbit hemoglobin was determined by measuring absorbance at 540 nm (■).

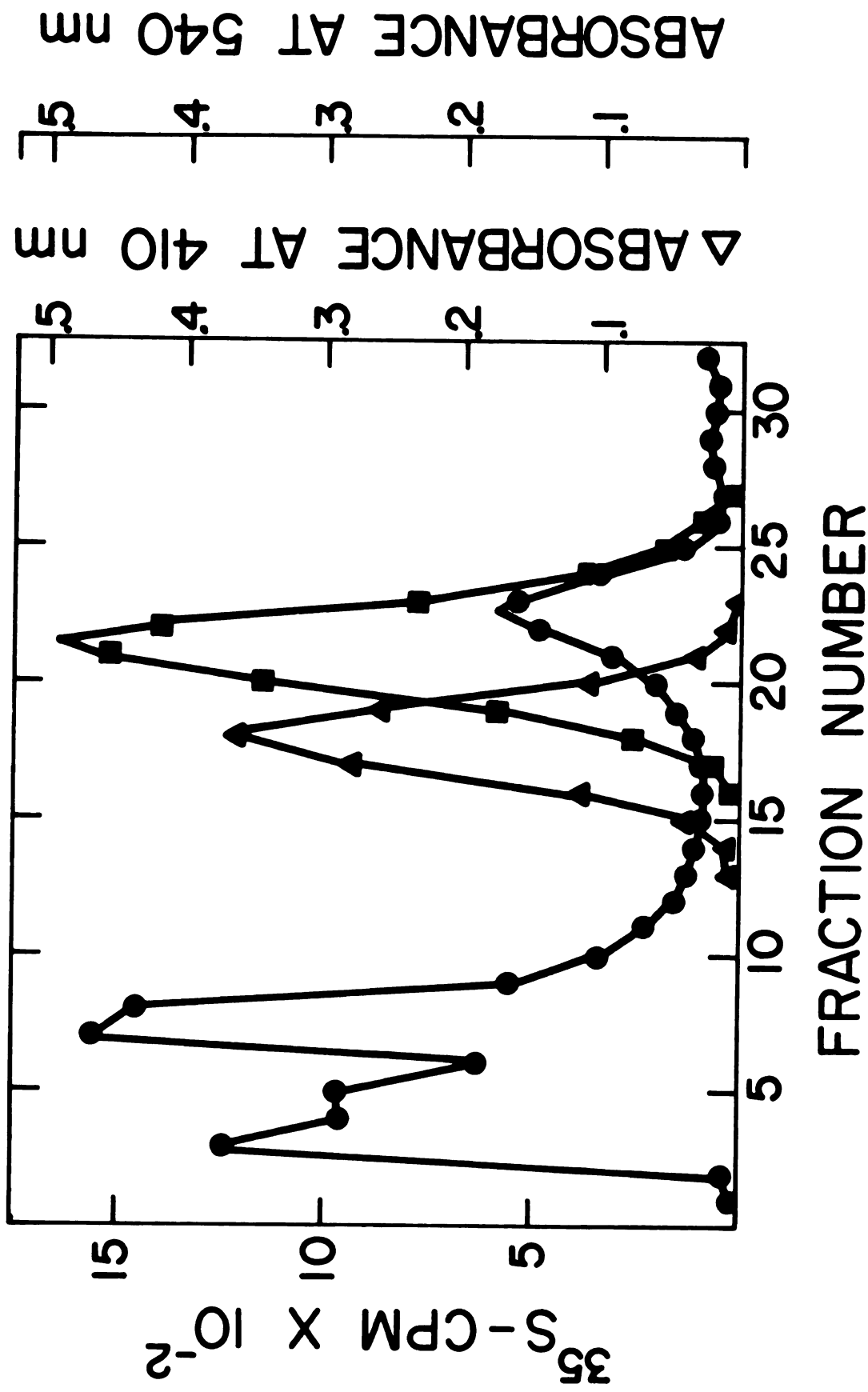

^{35}S-CPM X 10^{-2}
15
10
5
5
10
15
20
25
30
FRACTION NUMBER
Δ ABSORBANCE AT 410 nm
5
4
3
2
1
ABSORBANCE AT 540 nm
5
4
3
2
1

FIGURE 7.--The Effect of Binding ^{35}S-Labeled RNA Polymerase to [^{3}H]Poly(U) on the Release of the Sigma Subunit or RNA Polymerase.

Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 100 µM [^{3}H]poly(U) (5.8 x 10^5 cpm/µmole), 250 µg/ml bovine serum albumin, 10 µg/ml ^{35}S-labeled RNA polymerase, and either 2 mM $MnCl_2$ (upper diagram) or no added $MnCl_2$ (lower diagram) were incubated at 30° for 3 minutes and cooled. A sample (0.10 ml) from each reaction mixture was layered on a sucrose gradient prepared by layering a 4.2 ml 5 to 20% linear sucrose gradient on top of 0.8 ml of 50% sucrose. The sucrose was prepared in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 100 µg/ml bovine serum albumin, and either 2 mM $MnCl_2$ (upper diagram) or no added $MnCl_2$ (lower diagram). After centrifugation at 4° for 5.2 hr at 50,000 rpm in the Spinco SW50L rotor, fractions (0.16 ml) were collected from the bottom of the centrifuge tubes. Each fraction was then assayed for ^{35}S (●) and ^{3}H (▲ or ■) by liquid scintillation spectrometry (Bray, 1960).

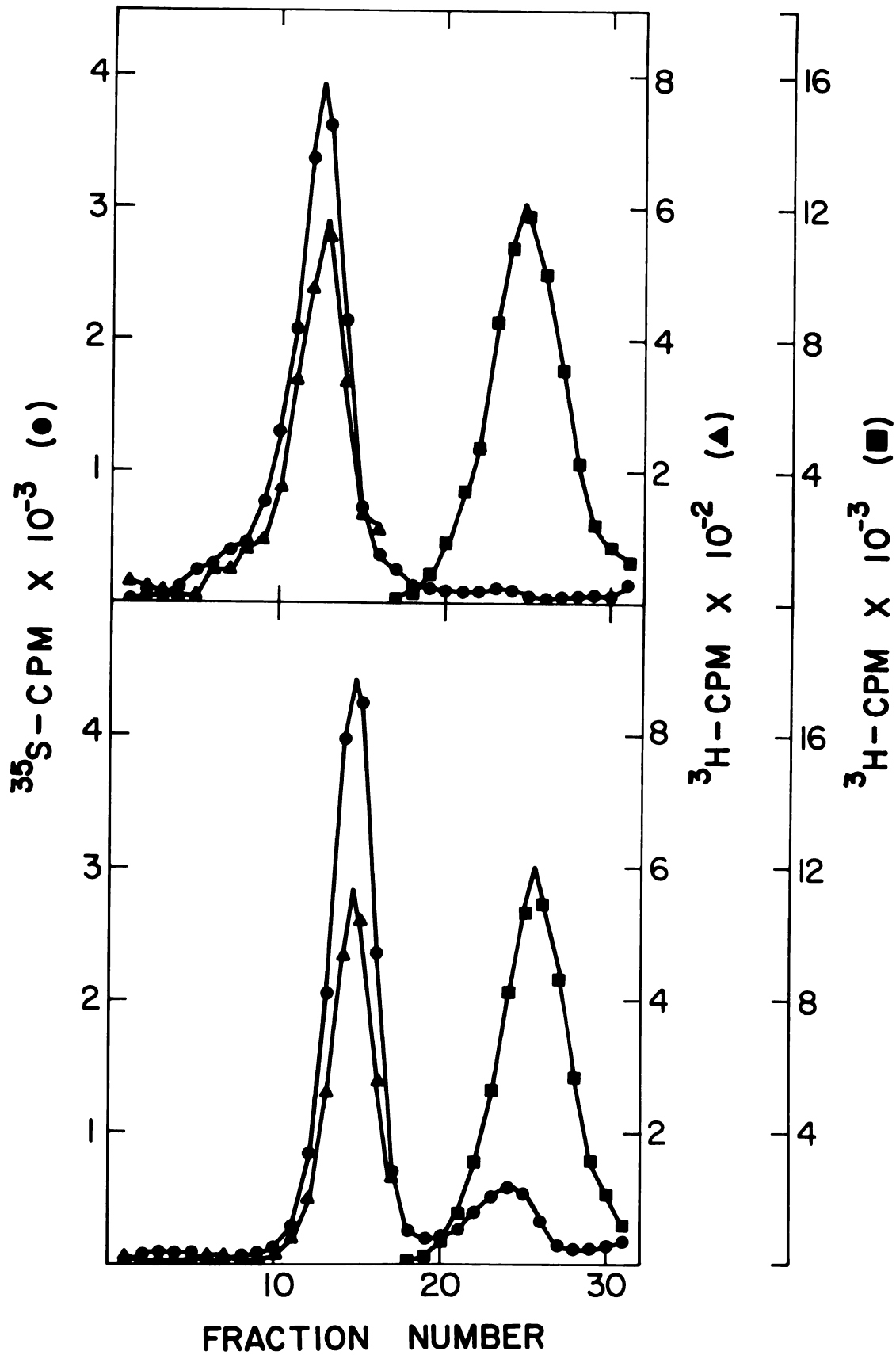

^{35}S-CPM X 10^{-3} (●)
^{3}H-CPM X 10^{-2} (▲)
^{3}H-CPM X 10^{-3} (■)
FRACTION NUMBER
10
20
30

FIGURE 8.--The Effect of Binding ^{35}S-Labeled RNA Polymerase to Poly(C) and Poly(A) on the Release of the Sigma Subunit of RNA Polymerase.

Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 µg/ml ^{35}S-labeled RNA polymerase and either 2 mM $MnCl_2$, 250 µg/ml bovine serum albumin, and 100 µM poly(C) (upper diagram), or 250 µg/ml bovine serum albumin and 100 µM poly(C) (middle diagram), or 2 mM $MnCl_2$ and 100 µM poly(A) (lower diagram), were incubated at 30° for 3 minutes and cooled. A sample (0.10 ml) from each reaction mixture was analyzed on sucrose gradients containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, and either 2 mM $MnCl_2$ and 100 µg/ml bovine serum albumin (upper diagram), or 100 µg/ml bovine serum albumin (middle diagram), or 2 mM $MnCl_2$ (lower diagram) as described in the legend to Figure 7.

Fraction number 11 (inset A), number 16 (inset B) and number 24 (inset C) from a duplicate sucrose gradient of a poly(A)-containing reaction mixture were heated with SDS as described in the legend to Figure 3 and then layered on 11 cm SDS-polyacrylamide gels. Electrophoresis was at 25° at 3.6 ma/gel for 10 hr. After electrophoresis, the gels were cut into 4 mm transverse fractions and the ^{35}S content of each fraction was analyzed according to the procedure described in Materials and Methods. For each inset, the ordinate is expressed as ^{35}S-CPM x 10^{-2} and the abscissa as Gel Fraction Number.

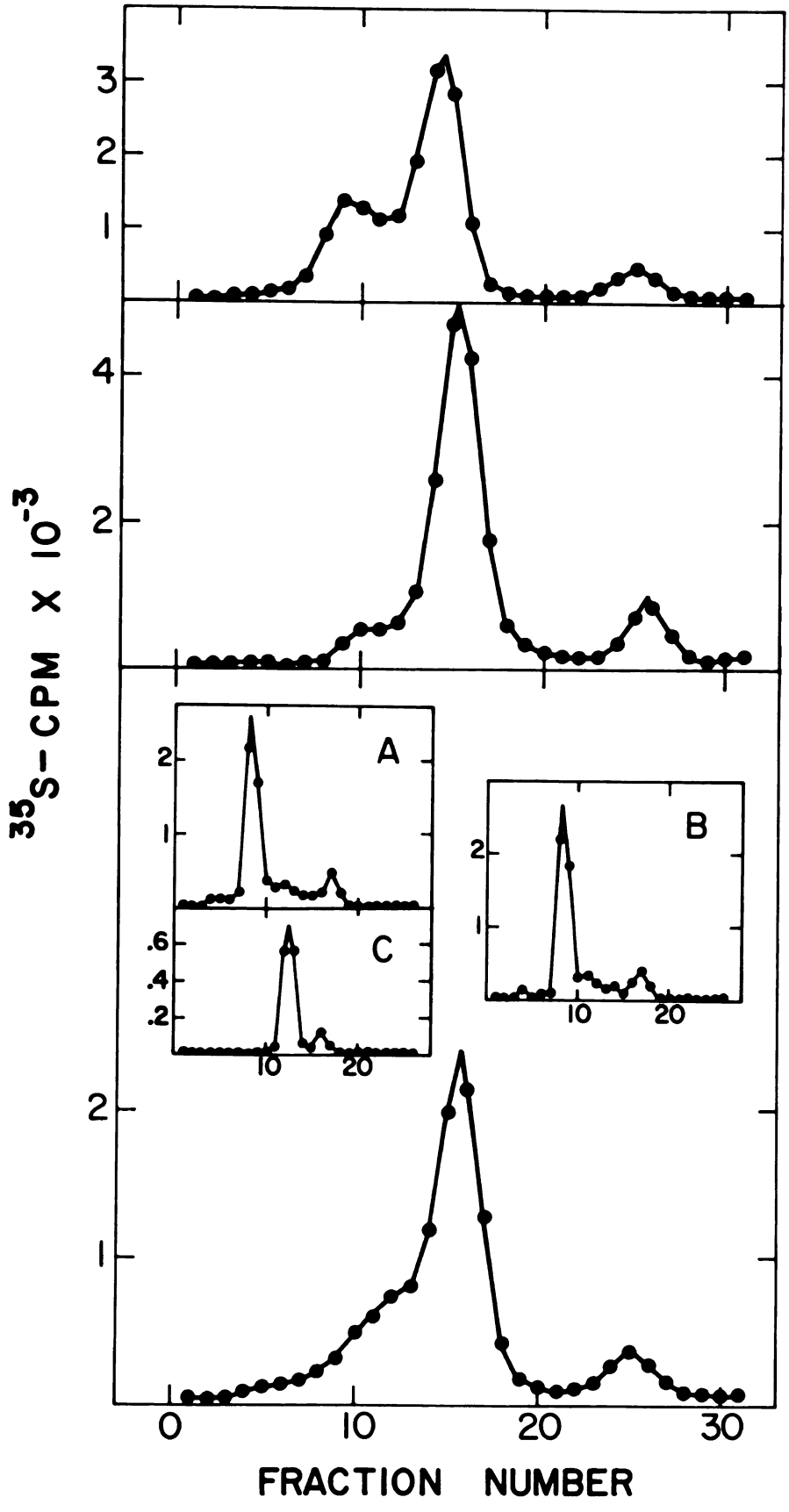

^{35}S-CPM X 10^{-3}
FRACTION NUMBER
A
B
C

FIGURE 9.--The Effect of Binding ^{35}S-Labeled RNA Polymerase to Denatured gh-1 DNA and to Poly[d(A-T)] on the Release of the Sigma Subunit of RNA Polymerase.

Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol and either 4 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM KCl, 100 µg/ml denatured gh-1 DNA and 25 µg/ml ^{35}S-labeled RNA polymerase (left diagram) or 2 mM $MnCl_2$, 100 µM poly[d(A-T)], and 17 µg/ml ^{35}S-labeled RNA polymerase (right diagram) were incubated at 30° for 3 minutes and then chilled. A sample (0.10 ml) from each reaction mixture was analyzed on sucrose gradients containing 50 mM KCl as described in the legend to Figure 3 with the exception that the sucrose gradient containing poly[d(A-T)] was centrifuged for 5.2 hr at 50,000 rpm in a Spinco SW50L rotor.

Fraction number 4 from a duplicate sucrose gradient of a denatured gh-1 DNA reaction mixture was treated with SDS as described in the legend to Figure 3 and then layered on an 11 cm SDS-polyacrylamide gel. Electrophoresis was at 25° at 4 ma/gel for 5.25 hr. After electrophoresis, the gel was cut into 4 mm transverse fractions and the ^{35}S content of each gel fraction was analyzed according to the procedure described in Materials and Methods. For the inset, the ordinate is expressed as ^{35}S-CPM x 10^{-2} and the abscissa as Gel Fraction Number.

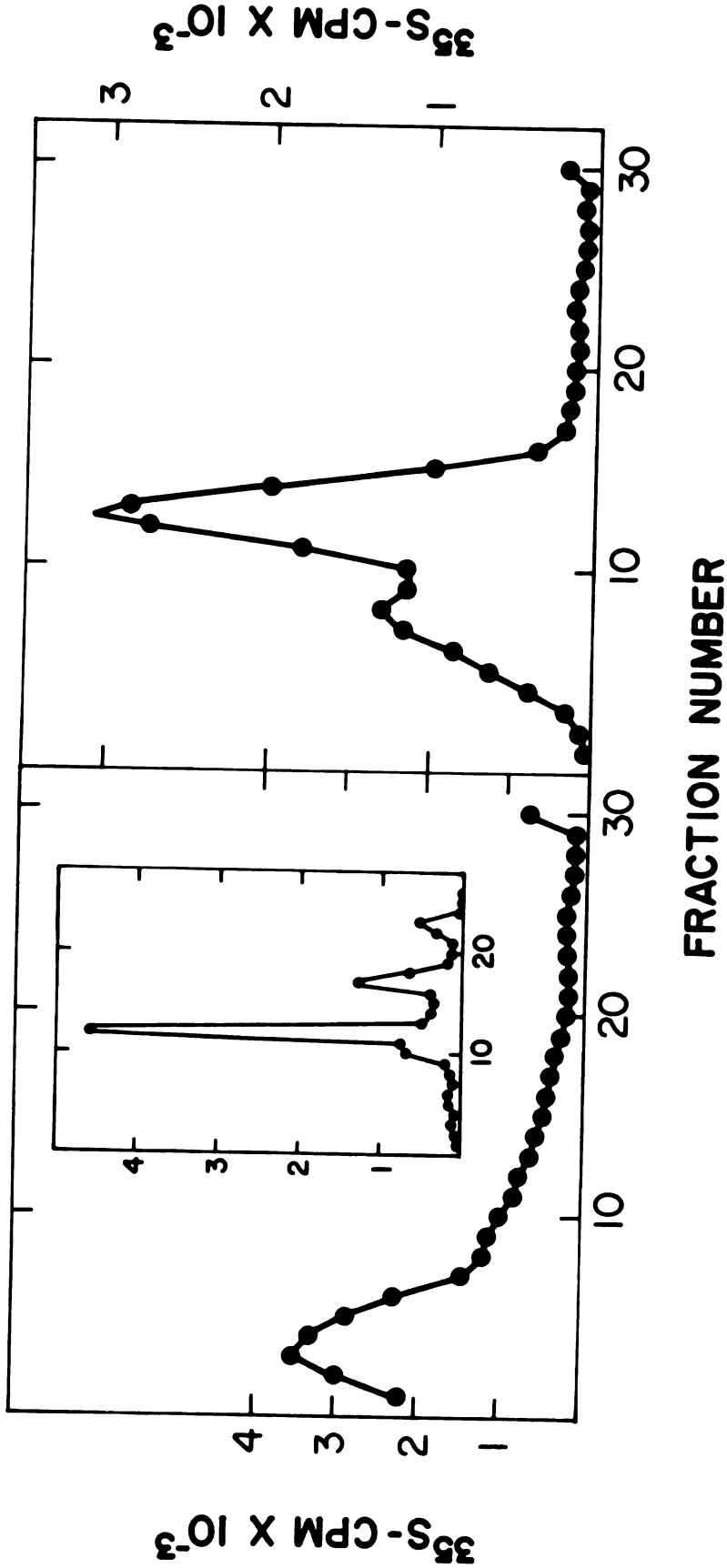
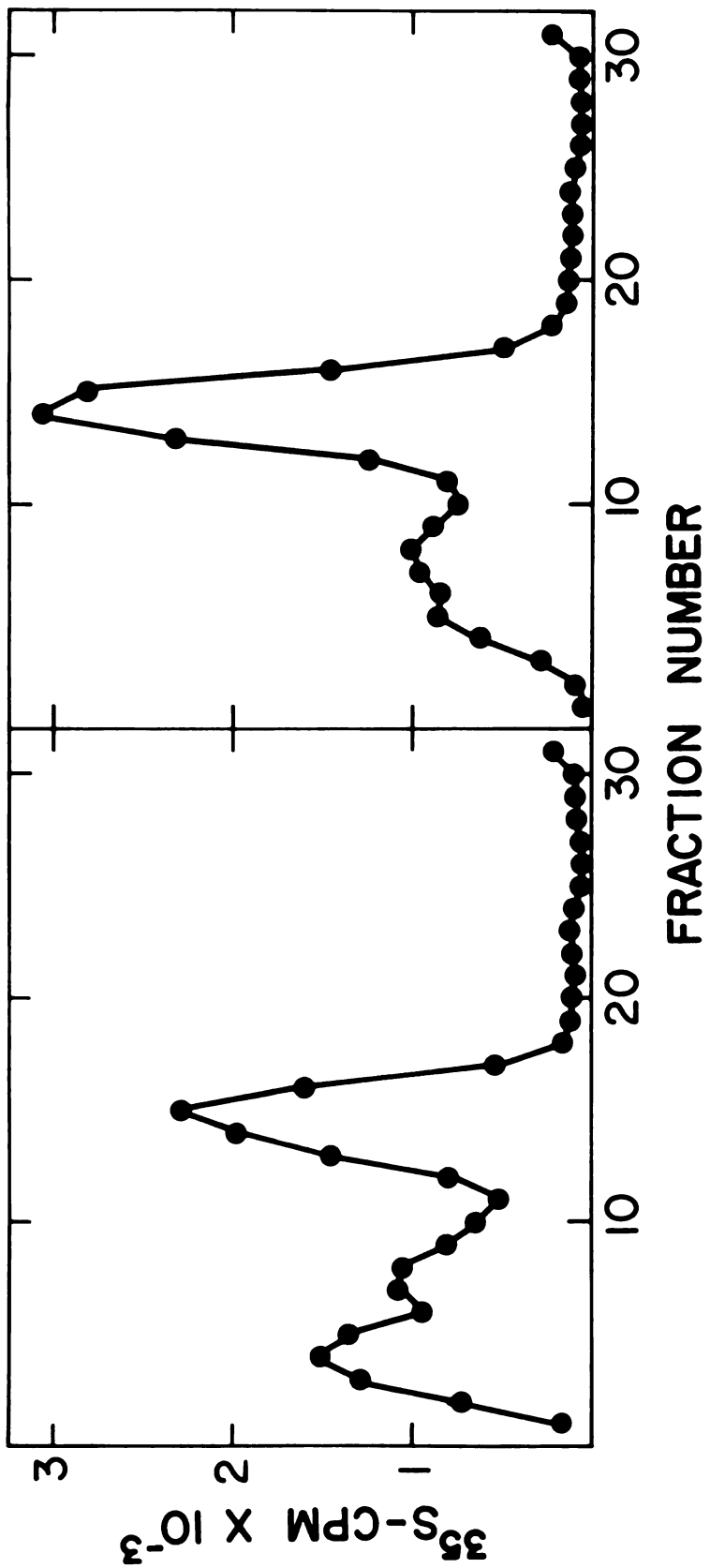

^{35}S-CPM X 10^{-3}
FRACTION NUMBER
^{35}S-CPM X 10^{-3}

FIGURE 10.--The Effect of Binding ^{35}S-Labeled RNA Polymerase to Poly(dT) and Poly(dC) on the Release of the Sigma Subunit of RNA Polymerase.

Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 2 mM $MnCl_2$, 250 µg/ml bovine serum albumin, 10 µg/ml ^{35}S-labeled RNA polymerase, and either 115 µM poly(dT) (left diagram) or 130 µM poly(dC) (right diagram) were incubated for 3 minutes at 30° and then chilled. A sample (0.10 ml) from each reaction mixture was layered on a sucrose gradient prepared by layering a 4.2 ml 5 to 20% linear sucrose gradient on top of 0.8 ml of 50% sucrose. The sucrose was prepared in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 2 mM $MnCl_2$, and 100 µg/ml bovine serum albumin. After centrifugation at 4° for 5.2 hr at 50,000 rpm in the Spinco SW50L rotor, fractions (0.16 ml) were collected from the bottom of the centrifuge tubes. Each fraction was then assayed for ^{35}S by liquid scintillation spectrometry (Bray, 1960).


^{35}S-CPM X 10^{-3}
1
2
3
10
20
30
FRACTION NUMBER