Release of the σ Subunit of *Pseudomonas putida* Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase†

Gary F. Gerard,‡ J. C. Johnson,§ and J. A. Boezi*

ABSTRACT: ^{35}S-Labeled *Pseudomonas 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 3.6–3.6 to 2.2–1.0, respectively. A study of the release of the σ 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 σ. 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 σ and core polymerase ($\alpha_2\beta\beta'$) but did not result in the release of σ. Binding of the ^{35}S-labeled enzyme to poly(A), poly(C), and to tRNA resulted in the release of σ. 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 σ. Release of σ subsequent to the binding of enzyme to poly(dC) and poly(U) occurred in the absence of manganese chloride but not in its presence. σ was released from the enzyme–polynucleotide complex during DNA-directed RNA synthesis. Within 3-min 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 σ. The other 40% of the enzyme molecules did not form a 200 mM KCl stable complex within 3 min. With longer times of incubation, these enzyme molecules could slowly form a 200 mM KCl stable complex but did not release σ. The sedimentation coefficient of *P. putida* σ released during DNA-directed RNA synthesis was 4.1–4.5 S.

Bacterial DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleoltidyl 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 σ subunit. The subunit structure of the core polymerase is $\alpha_2\beta\beta'$. The complex between core polymerase and σ 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 σ 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, σ 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 σ derived from holoenzyme to catalyze the σ-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 σ 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 four times the concentration of holoenzyme. In this regard, Travers (1971) has reported that core enzyme molecules can rapidly exchange σ. In the experiments on σ release during ϕ80 DNA-directed RNA synthesis, Travers and Burgess (1969) did not directly demonstrate the physical separation of σ from enzyme–polynucleotide complex nor did they measure the stoichiometry of σ release.

Krakow and von der Helm (1970) have presented evidence which can be taken to mean that there is a physical separation of σ 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 σ 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 electrophore-

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received October 18, 1971.* This work was supported in part by Grant GB-7914 from the National Science Foundation and by National Institutes of Health Training Grant GM-1091. Michigan Agriculture Experiment Station Article No. 5695.

‡ Present address: Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Mo. 63110.

§ Present address: Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, N. Y. 10461.

* To whom to address correspondence.

sis could influence the interaction between σ 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 σ 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 σ is released. The interaction between σ and core polymerase of *E. coli* and *A. vinelandii* appears to be weaker than the interaction between σ and core polymerase from *P. putida*. Dissociation of *E. coli* and *A. vinelandii* holoenzyme, but not *P. putida* holoenzyme, to σ 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 σ 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 σ, enzyme, and enzyme–polynucleotide complex. SDS[1]–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 σ from core enzyme–polynucleotide complex and also provides a means of measuring the stoichiometry of σ 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 A-1.5m) was obtained from Bio-Rad Laboratories. Rifampicin and streptolydigin were gifts from Gruppo Lepetit, Inc., Milan, Italy, and The Upjohn Co., respectively. Poly(dT), poly(dC), and poly(dA) were gifts from F. J. Bollum, University of Kentucky, Lexington, Ky. [^{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: 10.5×10^3 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). 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/mmole were added per 3 l. 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 min. The cells were harvested at the late logarithmic phase of growth, and stored at −20°. From a 3-l. culture, the yield of ^{35}S-labeled cells was 10 g (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 1 day after the ^{35}S-labeled cells were harvested.

Purification of ^{35}S-Labeled RNA Polymerase. Frozen ^{35}S-labeled *P. putida* (10 g) 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 × 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/20 g 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 to the figures. In the fractions collected from the sucrose gradients, the recovery of ^{35}S was at least 70% and in most cases 80–90% 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%.

[1] The only abbreviation used that is not listed in *Biochemistry* 9, 4022 (1970), is: SDS, sodium dodecyl sulfate.

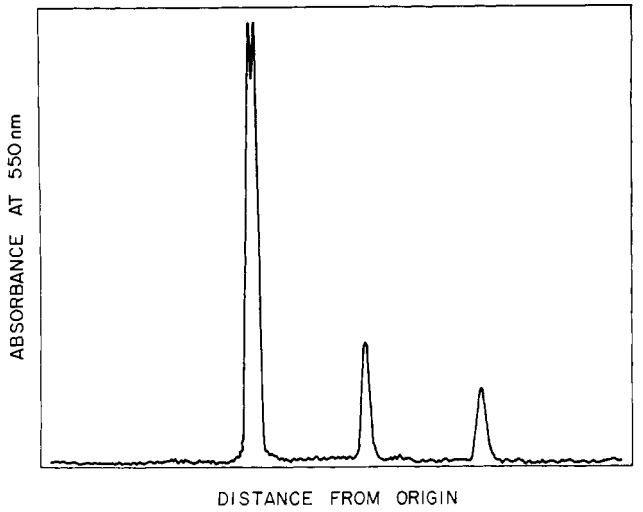
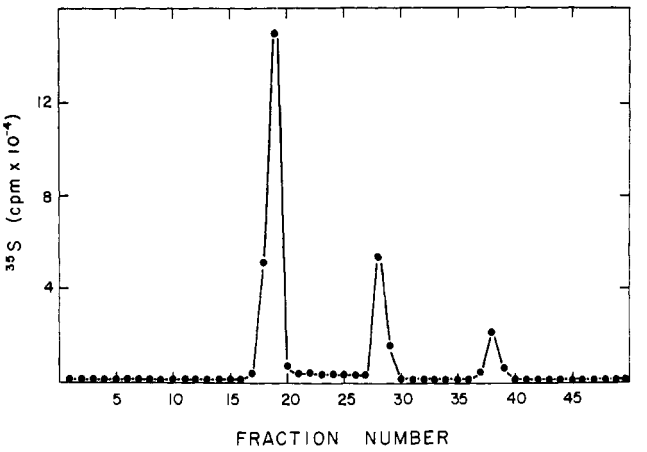


FIGURE 1: Densimetric tracing of a coomassie brilliant blue stained SDS–polyacrylamide gel of ^{35}S-labeled RNA polymerase. ^{35}S-Labeled RNA polymerase (30 μg) was incubated at 100° for 10 min in 60 μl of a solution containing 0.1 M sodium phosphate (pH 7.1), 1.0% SDS, 1.0% 2-mercaptoethanol, and 5% glycerol (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 hr 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.



FIGHRE 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.

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- 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 hr or at 100° for 2 hr, 5 ml of a mixture containing six parts of Omnifluor solution (18.1 g of Omnifluor/gal. of toluene) and seven 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 P. 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 hr 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 hr rather than the 4.75 hr reported for the experiment presented in Figure 1.

Release of the σ 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 min. After incubation, sucrose gradient centrifugation was used to detect free ^{35}S-labeled σ. If the σ 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 monitor-

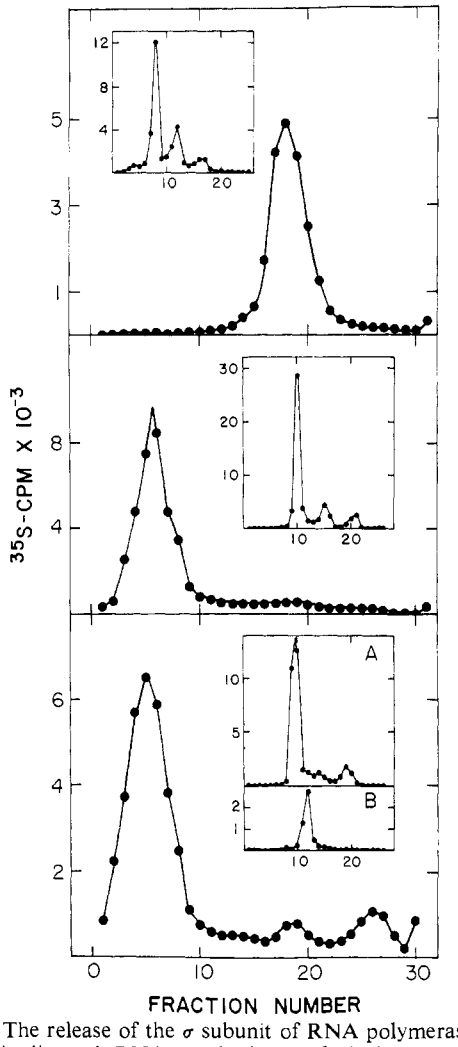


FIGURE 3: The release of the σ subunit of RNA polymerase during gh-1 DNA directed RNA synthesis. Analysis by centrifugation through sucrose density gradients containing 50 mM KCl and by SDS–polyacrylamide gel electrophoresis. Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, and 25 μg/ml of ^{35}S-labeled RNA polymerase (upper diagram), or the above constituents plus 100 μg/ml of gh-1 DNA (middle diagram) or plus 100 μg/ml of gh-1 DNA and 0.4 mM each of ATP, GTP, CTP, and UTP (lower diagram) were incubated at 30° for 3 min. 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 5–20% 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 18 (inset, upper diagram) and for fraction 27 (inset B, lower diagram) and was 5.25 hr for fraction 6 (inset, middle diagram) and fraction 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 $\times$ 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.

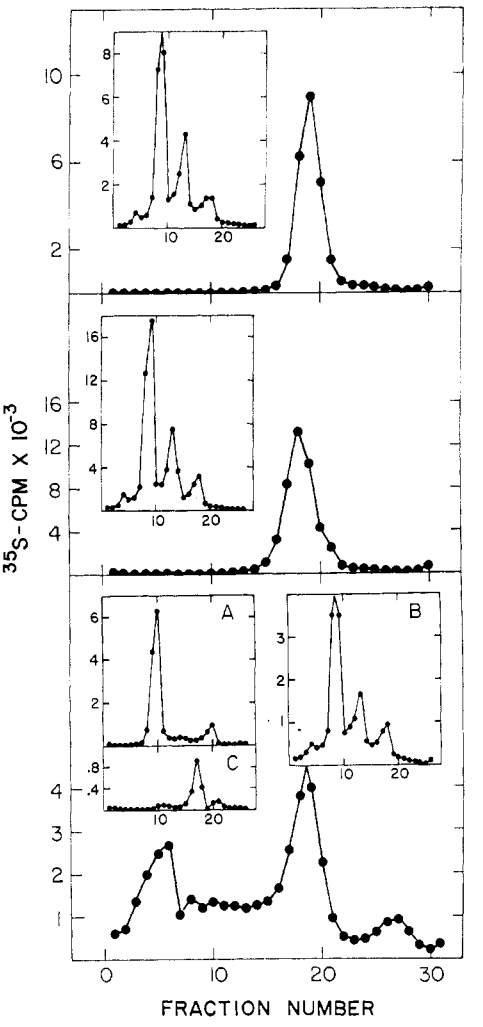


FIGURE 4: The release of the σ subunit of RNA polymerase during gh-1 DNA-directed RNA synthesis. Analysis by centrifugation through sucrose density gradients containing 200 mM KCl and by SDS–polyacrylamide gel electrophoresis. Reaction mixtures identical with those described for the upper, middle, and 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 5–20% 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 18 (inset, middle diagram), and fraction 18 (inset B, lower diagram), and was 5.25 hr for fraction 6 (inset A, lower diagram) and fraction 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 $\times$ 10^{-2} and the abscissa as gel fraction number.

ing 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 (fractions 24–28). This protein peak was identified as σ by SDS–polyacrylamide gel electrophoresis (see below), indicat-

ing that the subunit of RNA polymerase had been released during gh-1 DNA-directed RNA synthesis. All of the RNA polymerase molecules, however, had not released σ. Based on the amount of free ^{35}S-labeled σ 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 σ.

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.*, 1 equiv of σ/equiv 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.

Effect of Binding ^{35}S-Labeled RNA Polymerase to gh-1 DNA on the Interaction between Core Polymerase and the σ Subunit. After centrifugation, all of the ^{35}S-labeled RNA ploymerase molecules which had sedimented as free enzyme contained σ (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). σ 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 σ occurred during centrifugation as the enzyme–gh-1 DNA complex moved through the gradient. Accordingly, σ 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 σ 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 σ 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 σ trailed behind the enzyme–gh-1 DNA complex throughout gradient. When 0, 50, 100, or 200 mM KCl was present in the gradients, σ did not dissociate during centrifugation fromRNA polymerase molecules which had not been reacted with gh-1 DNA.

Release of the σ 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 σ found near the top of the sucrose gradient which contained 50 mM KCl (lower diagram of Figure 3) and the amount of σ 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 σ. The other 40% of the enzyme molecules in the complex did not release σ. Presumably, the enzyme molecules which released σ were engaged in RNA synthesis and those which did not release σ 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,b).

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 min 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 to gh-1 DNA prior to centrifugation sedimented as free 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 σ 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 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 σ, *i.e.*, 1 equiv of sigma/equiv 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 σ found in free enzyme (inset A, lower diagram). Free ^{35}S-labeled enzyme at the 13S position of the sucrose gradient contained a full compliment of σ (inset B, lower diagram). The ^{35}S-labeled protein near the top of the gradient was identified as σ (inset C, lower diagram). The amount of the free ^{35}S-labeled σ near the top of the gradient was equivalent to the amount of σ 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

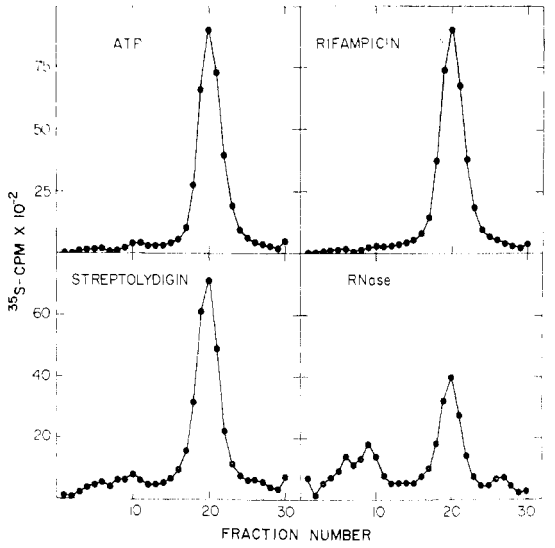


FIGURE 5: The effect of ATP, rifampicin, streptolydigin, and RNase on the formation of a 200 mM KCl stable complex. Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, 25 μg/ml of ^{35}S-labeled RNA polymerase, 100 μg/ml of 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 of rifampicin (upper right diagram), plus 0.33 mM streptolydigin (lower left diagram), or plus 6 μg/ml of 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 [^{3}H]-CMP into RNA was decreased by 98% over the control rate in the presence of either 1 μg/ml of rifampicin or 0.33 mM streptolydigin in the reaction mixture.

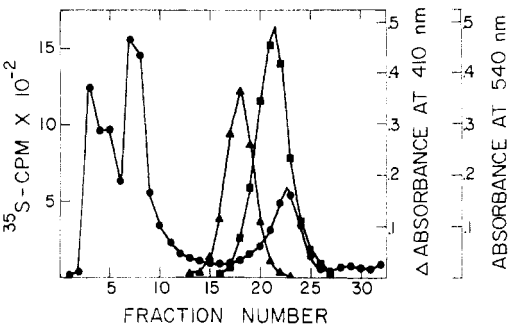


FIGURE 6: Determination of the sedimentation coefficient of the σ subunit of RNA polymerase released during gh-1 DNA-directed RNA synthesis. Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 20 mM KCl, 250 μg/ml of bovine serum albumin, and either 0.4 mM each of ATP, GTP, UTP, and CTP, 100 μg/ml of gh-1 DNA, and 10 μg/ml of ^{35}S-labeled RNA polymerase, or 420 μg/ml of *E. coli* alkaline phosphatase, or 25 A_{540} units/ml of rabbit hemoglobin were incubated for 3 min 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 5–20% 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 of 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 (■).

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-min 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 σ (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 σ.

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 σ 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 σ 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 min rather than the standard 3-min 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 σ found near the top of the sucrose gradient was detected with the longer times of incubation.

Determination of the Sedimentation Coefficient of the σ Subunit of RNA Polymerase Released during gh-1 DNA-Directed RNA Synthesis. The sedimentation coefficient of σ was determined using centrifugation through sucrose density gradients containing 50 mM KCl. *E. 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 σ sedimented as a nearly symmetrical peak with a sedimentation coefficient of 4.1–4.5 S.

Effect of Binding ^{35}S-Labeled RNA Polymerase to Polyribonucleotides on the Release of the σ Subunit. The effect of binding ^{35}S-labeled RNA polymerase to several polyribonucleotides on the release of the σ 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 σ was detected near the top of the gradient (upper diagrm 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 σ subunit. The binding of the enzyme to poly(U) in the absence of manganese chloride, however, resulted in the release of σ. If binding and centrifugation were carried out in the absence of manganese chloride, free σ was detected near the top of the sucrose gradient (lower

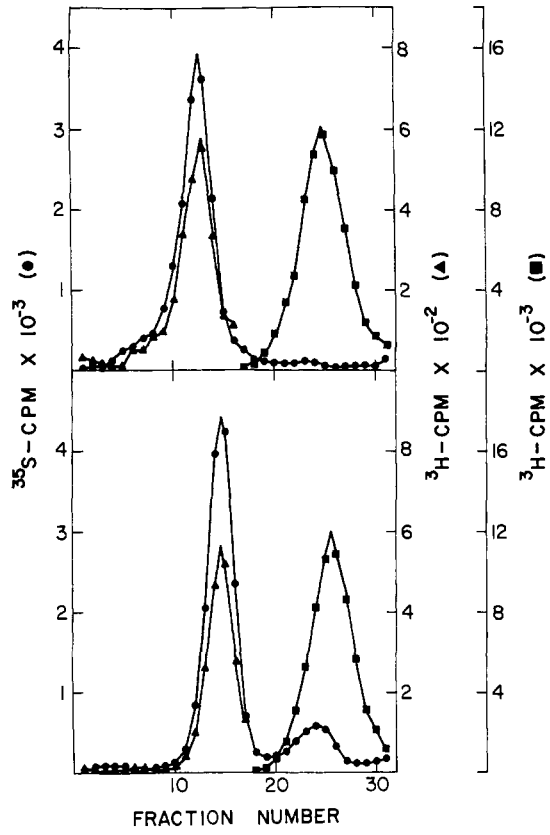


FIGURE 7: The effect of binding ^{35}S-labeled RNA polymerase to [^{3}H]poly(U) on the release of the σ subunit of RNA polymerase. Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 100 μM [^{3}H]poly(U) (5.8×10^5 cpm/μmole), 250 μg/ml of bovine serum albumin, 10 μg/ml of ^{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 min and cooled. A sample (0.10 ml) from each reaction mixture was layered on a sucrose gradient prepared by layering a 4.2-ml 5–20% 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 of 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).

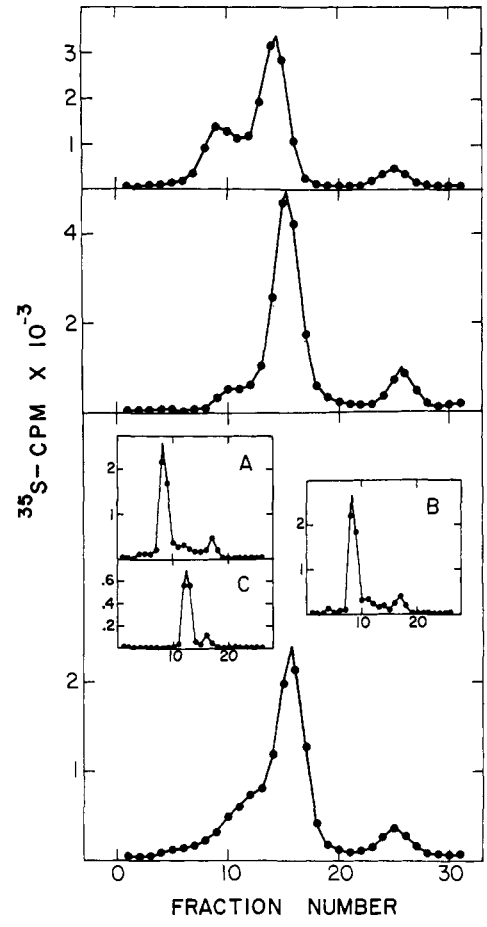


FIGURE 8: The effect of binding ^{35}S-labeled RNA polymerase to poly(C) and poly(A) on the release of the σ subunit of RNA polymerase. Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 10 μg/ml of ^{35}S-labeled RNA polymerase, and either 2 mM $MnCl_2$, 250 μg/ml of bovine serum albumin, and 100 μM poly(C) (upper diagram), or 250 μg/ml of 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 min 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 of bovine serum albumin (upper diagram), or 100 μg/ml of bovine serum albumin (middle diagram), or 2 mM $MnCl_2$ (lower diagram) as described in the legend to Figure 7. Fractions 11 (inset A), 16 (inset B), and 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 ordinate is expressed as [^{35}S]CPM $\times 10^{-2}$ and the abscissa as gel fraction number.

diagram of Figure 7). About 70% of the enzyme molecules released σ.

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 σ subunit. In these experiments, 40–60% of the enzyme molecules released σ. 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 σ and that the ^{35}S-labeled protein that was detected near the top of the sucrose gradient was σ (insets, lower diagram of Figure 8).

Effect of Binding ^{35}S-Labeled RNA Polymerase to Polydeoxyribonucleotides on the Release of the σ 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 σ subunit, *i.e.*, no peak of ^{35}S-labeled σ 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 σ 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 σ was observed, indicating that the binding of ^{35}S-labeled RNA polymerase to these polydeoxyribonucleotides did not result in the release of the σ subunit. In the case of denatured gh-1 DNA, SDS–polyacrylamide gel electrophoresis analysis

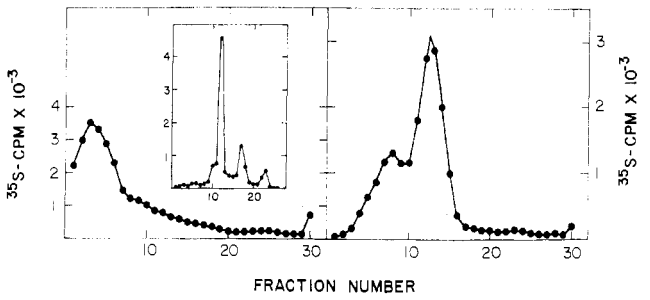


FIGURE 9: Effect of binding ^{35}S-labeled RNA polymerase to denatured gh-1 DNA and to poly[d(A-T)] on the release of the σ subunit of RNA polymerase. Reaction mixtures (0.12 ml) which contained 20 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol and either 4 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM KCl, 100 μg/ml of denatured gh-1 DNA, and 25 μg/ml of ^{35}S-labeled RNA polymerase (left diagram) or 2 mM $MnCl_2$, 100 μM poly[d(A-T)], and 17 μg/ml of ^{35}S-labeled RNA polymerase (right diagram) were incubated at 30° for 3 min 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 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 the first 15 min and then at 8 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 $\times$ 10^{-2} and the abscissa as gel fraction number.

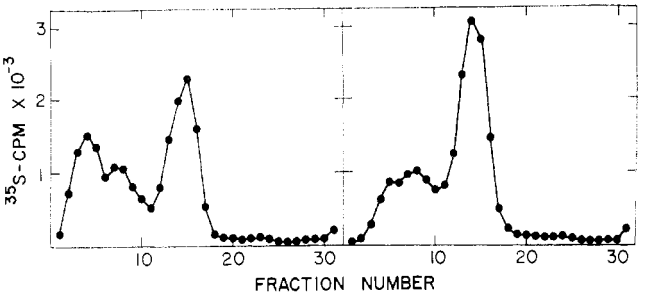


FIGURE 10: Effect of binding ^{35}S-labeled RNA polymerase to poly(dT) and poly(dC) on the release of the σ subunit of RNA polymerase. Reaction mixtures (0.12 ml) which contained 100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 2 mM $MnCl_2$, 250 μg/ml of bovine serum albumin, 10 μg/ml of ^{35}S-labeled RNA polymerase, and either 115 μM poly(dT) (left diagram) or 130 μM poly(dC) (right diagram) were incubated for 3 min 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 5–20% 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 of 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).

showed that RNA polymerase complexed to denatured gh-1 DNA and centrifuged through the sucrsoe gradient contained a full compliment of σ (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 σ was observed. For poly(dC) but not for poly(dT) release of σ 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 σ (data not shown).

In the studies of the release of the σ subunit during gh-1 DNA-directed RNA synthesis, the reaction mixture contained divalent metal ions, but the 50 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 σ found near the top of the sucrose gradients.

Discussion

^{35}S-Labeled *P. 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 3.6–3.6 to 2.2–1.0. Since the molar concentrations of β', β, and σ relative to α are 0.5–0.5 to 0.5–1.0 (Johnson *et al.*, 1970), 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 44,000*g* of β', of β, and of σ, there are twice as many sulfur-containing amino acids as in a gram-mole of α which is 44,000*g*. Burgess (1969) reported that for *E. coli* RNA polymerase, in each 39,000*g* of β' pulse β there are 1.3 times as many sulfur-containing amino acids as in a gram-mole of α which is 39,000*g*. The values for *E. coli* σ and for β' and β separately have not been reported.

σ was released from the enzyme–polynucleotide complex during gh-1 DNA-directed RNA synthesis. Within 3-min 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 σ. The other 40% of the enzyme molecules did not form a 200 mM KCl stable complex within 3 min 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 σ. 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 σ either because they have a direct impairment in the σ release process itself, or because some event in RNA synthesis which triggers σ release does not occur. Perhaps, σ release is triggered once the synthesis of nascent RNA of a certain nucleotide length is achieved (Krakow and Fronk, 1969). 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, σ 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 50–100 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 dam-

aged 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* σ released during gh-1 DNA-directed RNA synthesis was 4.1–4.5 S. The molecular weight of *P. putida* σ is 98,000 (Johnson *et al.*, 1971). Enzymes of globular conformation with molecular weights in the 100,000 range usually have sedimentation coefficients of 5.5–6.0 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 5.5–6.0 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* σ indicates that it does not have a globular conformation. The conformation of *E. coli* σ probably is also not globular. Its molecular weight and sedimentation coefficient are 95,000 and 4.5–5.0 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 σ. Binding of the enzyme to polyribonucleotides, on the other hand, with one exception, did result in the release of σ.

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 σ. In their studies, Krakow and von der Helm used electrophoresis in polyacrylamide gels to assay for the release of σ from the enzyme–polynucleotide complex. The effects that this method might have on the interaction between σ 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 σ 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 σ. 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 σ, in fact, usually only about 60% of the enzyme molecules released σ. Perhaps, the enzyme molecules which did not release σ on binding to the synthetic polyribonucleotides were the defective enzyme molecules which could not release σ during gh-1 DNA-directed RNA synthesis. Further experimentation is required before the mechanism of σ release following the binding of the enzyme to polynucleotides and during DNA-directed RNA synthesis is understood.

References

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

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

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

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

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

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

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

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

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

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

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

Hinkle, D. C., and Chamberlin, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 65.

Holleman, W. H. (1966), Ph.D. Thesis, Michigan State University, East Lansing, Mich.

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

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

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

Krakow, J. S., and von der Helm, K. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 73.

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

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

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

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

Ruet, A., Sentenac, A., and Fromageot, P. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 169.

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

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

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

Travers, A. (1971), *Nature (London), New Biol. 229*, 69.

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

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

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

Zillig, W., Zeckel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 47.