RNA Synthesis in *Streptomyces antibioticus:* In Vitro Effects of Actinomycin and Transcriptional Inhibitors from 48-h Cells†

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ABSTRACT: Two forms of DNA-dependent RNA polymerase have been partially purified (about 100-fold relative to the crude extract) from 48-h old cells of *Streptomyces antibioticus*. The two forms show different Mg^{2+} optima for the incorporation of [^{3}H]UMP into RNA. Substances inhibiting transcription have been isolated by ammonium sulfate precipitation from one of the fractions produced during the polymerase purification. Actinomycin can be shown to inhibit RNA synthesis catalyzed by the *S. antibioticus* polymerases to a similar extent regardless of the template used. When *S. antibioticus* DNA is the template, actinomycin inhibits transcription by *S. antibioticus* polymerase to a degree that is significantly less than the observed actinomycin inhibition of synthesis catalyzed by *Escherichia coli* polymerase or by either *S. antibioticus* or *E. coli* polymerase with calf thymus DNA as the template. Using an assay previously developed, it was shown that the association constant for the binding of actinomycin to *S. antibioticus* DNA was increased by the presence of RNA polymerase in the binding mixture, while the association constant for the binding to calf thymus DNA was decreased by RNA polymerase. RNA synthesis in crude, cell-free extracts of 12-h old *S. antibioticus* cells (not producing actinomycin) is less refractory to actinomycin inhibition than synthesis catalyzed by extracts of 48-h old (actinomycin producing) cells, and both extracts catalyze appreciable RNA synthesis at actinomycin concentrations that completely inhibit RNA synthesis catalyzed by *E. coli* extracts.

Streptomyces antibioticus is one member of the group of organisms that produces the antibiotic actinomycin (Waksman, 1968). Although many of the details of the biosynthesis of actinomycin remain to be worked out, much attention has been given to the biochemical events that precede and accompany the production of actinomycin by *S. antibioticus* cultures. For example, during the growth period preceding the onset of actinomycin production, some significant changes occur in the capacity of the cultures to synthesize macromolecules. Net protein synthesis declines sharply, beginning about 9 h after innoculation, and continues to decline during the period in which the cells are preparing to make actinomycin (Katz and Weissbach, 1963; Collett and Jones, 1974). Net RNA synthesis also drops drastically during this period (Jones and Weissbach, 1970). In neither case, however, does macromolecular synthesis fall completely to zero. Basal levels of both protein and RNA synthesis may be maintained for several days after innoculation even though the cells are actively synthesizing actinomycin at this time (Jones and Weissbach, 1970; Collett and Jones, 1974).

The studies to be described below were performed to examine the changes in macromolecular synthesis that occur in aging *S. antibioticus* cultures in greater detail. In particular, it was of interest to determine the factors responsible for the decreased ability of aging cultures to synthesize RNA. It seemed unlikely that the production and accumulation of actinomycin could explain this observation, since the decrease in ability to incorporate precursors into RNA occurs long before cultures commence actinomycin synthesis (see below). The persistence of some RNA synthesis in an organism producing a potent inhibitor of DNA-dependent RNA synthesis also poses an intriguing regulatory problem. It has been previously shown that *S. antibioticus* cultures become less permeable to the antibiotic with increasing age (Marshall et al., 1968) but it is not clear that these observations can fully explain the continued RNA synthesis in cultures producing actinomycin. Nor do these studies account for actinomycin that might be present inside the cells. An alternative explanation for the partial sensitivity of RNA synthesis in *S. antibioticus* to actinomycin would involve the presence in those cells of a RNA synthesizing system that can function in the presence of the antibiotic. It seemed possible that such an activity might exist in an organism that produces actinomycin as a secondary metabolite. To examine this possibility, and to obtain information bearing generally on the regulation of RNA synthesis in *S. antibioticus,* the transcriptional machinery of *S. antibioticus* has been studied. In this report, the partial purification and some properties of two forms of DNA-dependent RNA polymerase from 48 h old *S. antibioticus* cells are described, and the ability of these enzymes to transcribe in the presence of actinomycin and transcriptional inhibitors from *S. antibioticus* is reported. In addition, evidence is presented for the existence of actinomycin binding proteins in *S. antibioticus* cells.

Materials and Methods

Materials. Calf thymus DNA (type V) and actinomycin D were obtained from Sigma. Deoxyribonuclease I was from Worthington, and pancreatic ribonuclease and Pronase (nuclease free) were from Calbiochem. [^{3}H]UTP (19.8 Ci/mmol), [^{3}H]GTP (9.35 Ci/mmol), [^{3}H]CTP (26.2 Ci/mmol), and [^{14}C]leucine (313 mCi/mmol) were supplied by New England Nuclear, while [^{14}C]ATP (58 mCi/mmol) and [^{3}H]uridine (49 Ci/mmol) were from Amersham/Searle. Frozen cells of *Escherichia coli* K12 were from Grain Processing Laboratories. [^{3}H]Actinomycin (95.23 mCi/mmol) was prepared as

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[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; ATP, adenosine 5′-triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate.

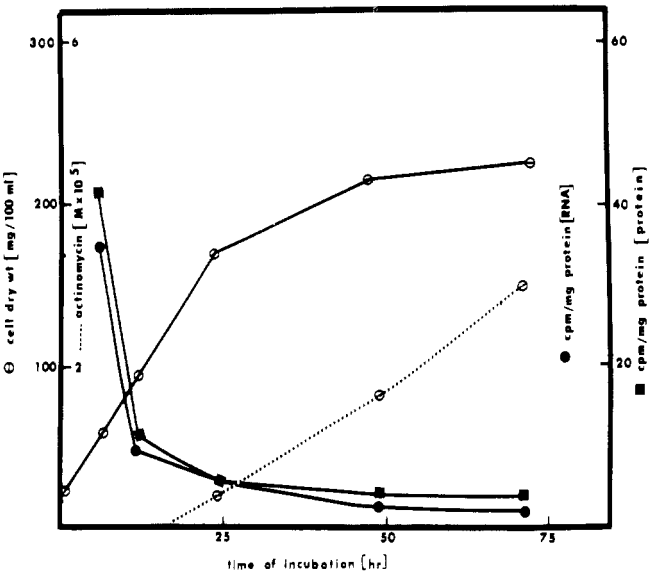

FIGURE 1: Growth characteristics of *S. antibioticus*. Growth conditions and techniques for labeling protein and RNA and for determining cell dry weight and actinomycin concentration are described under Materials and Methods.

previously described (Katz et al., 1965). *E. coli* RNA polymerase (specific activity 673 units/mg; Burgess, 1969) was from Sigma.

Buffer. Buffer 1 contained the following components: 0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM K-EDTA, 5% glycerol. Buffer 2 contained: 0.01 M Tris-HCl, pH 7.8, 0.01 M $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM potassium EDTA.

Growth of S. antibioticus and Assay for Actinomycin. S. antibioticus, strain 3720, was grown in NZ-amine medium, as previously described (Katz et al., 1958), in order to obtain an innoculum of sufficient size to allow subsequent actinomycin synthesis. After 48 h in this medium, the cells were washed with 0.9% NaCl and transferred to galactose-glutamic acid medium containing 0.1% glucose (Gallo and Katz, 1972). Cells were generally grown at 30 °C as 200-ml cultures in 500-ml Erlenmeyer flasks or as 1-l. cultures in 2-l. flasks containing galactose-glutamic acid-glucose medium. It is in this medium that the cells synthesize actinomycin. Cells were grown for 48 h with shaking (180 rpm) in a Labline hot air incubator, and are referred to below as "48-h cells." At the time of harvest, the actinomycin concentration in the growth medium was generally about 2×10^{-5} M as determined by the method previously described (Katz and Weissbach, 1963).

Analysis of the Growth Characteristics of S. antibioticus Cultures. A 200-ml *S. antibioticus* culture was grown in galactose-glutamic acid-glucose medium as described above. At various times after innoculation, 10-ml samples were removed from the culture and incubated for 30 min with 5 μCi of [^{3}H]uridine and 2 μCi of [^{14}C]leucine. At the end of the incubation the sample was brought to 10% with trichloroacetic acid. After standing at least 10–15 min at 0 °C, the precipitated cellular matter was washed three times with Cl_3CCOOH, resuspended in 5 ml of 10% Cl_3CCOOH, and heated for 10 min at 90 °C. The precipitates were sedimented in a clinical centrifuge, and aliquots of the supernatants were examined by liquid scintillation counting. The hot Cl_3CCOOH precipitates were washed three times with ethanol–ether (1:1, v/v) and dissolved in 5 ml of 0.5 M NaOH by heating for 30 min at 90 °C. Separate aliquots of the alkaline digests were used for protein determination (Lowry et al., 1951) and for determination of [^{14}C]leucine incorporation. Results of these experiments are expressed as cpm [^{3}H]uridine or [^{14}C]leucine incorporated per mg of protein (Figure 1). Actinomycin concentrations were determined using aliquots of the culture medium from the 200-ml culture described above, and separate 50-ml cultures were used to assess the increase in cell dry weight with time (Jones and Weissbach, 1970).

Preparation of S. antibioticus DNA. S. antibioticus DNA was isolated from extracts of 48-h cells. Cells (5 g) were disrupted by grinding with an equal weight of alumina in the cold, and the grindate was extracted with 20 ml of buffer 1. The extract was centrifuged for 10 min at 20 000*g* and the supernatant was extracted with an equal volume of chloroform–isoamyl alcohol (24:1, v/v). Additional extractions were performed with water-saturated phenol until the phenol–water interface was free of observable protein. Three more chloroform extractions were performed to insure complete deproteinization. The DNA was obtained by carefully layering an equal volume of 2-propanol over the final aqueous phase and spooling onto a glass rod by gentle stirring. The DNA was dissolved in 2 ml of 0.1 × saline–citrate (0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0) and isolated again by spooling as above. The DNA was then dissolved in 2 ml of 0.1 × saline–citrate and its concentration determined by the indole method using calf thymus DNA as a standard (Ceriotti, 1952).

RNA Polymerase Assay. The assay for RNA synthesis under cell-free conditions was performed essentially as described by Burgess (1969) for *E. coli* RNA polymerase. Reaction mixtures (generally 0.1 ml) contained per ml: Tris-HCl, pH 7.8, 40 μmol; $MgCl_2$, 10 μmol; K-EDTA, 0.1 μmol; dithiothreitol, 0.1 μmol; KCl, 150 μmol; potassium phosphate, pH 7.5, 0.5 μmol; bovine serum albumin, 0.5 mg; three unlabeled nucleoside triphosphates, 0.15 μmol each; [^{3}H]UTP, 1.25 nmol; calf thymus DNA (in some experiments), 0.15 mg; and enzyme. Duplicate assay tubes were incubated for 5 min at 30 °C when the reaction was stopped by the addition of cold 7.5% Cl_3CCOOH containing 2% sodium pyrophosphate. Cl_3CCOOH precipitates were collected on glass fiber filters, dried, and analyzed by liquid scintillation counting. Under the conditions described, [^{3}H]UMP incorporation was linear for at least 10 min, and when purified polymerases were used, the level of incorporation in the absence of DNA was essentially identical with the zero time control (generally equivalent to about 0.04 pmol of [^{3}H]UMP). All assays carried out on fractions obtained during the enzyme purification were supplemented with 0.1 mg of total yeast RNA prior to filtration of Cl_3CCOOH precipitates. This procedure was observed to increase the recovery of labeled RNA synthesized during the assay, particularly when more purified enzyme fractions were used. Assay duplicates generally varied no more than ±10%. One unit of enzyme activity represents the incorporation of 1 pmol of [^{3}H]UMP (about 11 350 cpm) in 5 min under the assay conditions described.

Partial Purification of S. antibioticus RNA Polymerase. Step 1, crude extract: this and all other purification steps were carried out at 0–4 °C unless otherwise noted. The crude extract was prepared from 15 g of 48-h old galactose–glutamic acid–glucose grown *S. antibioticus* cells as described previously (Jones and Weissbach, 1970). The cells were suspended in 30 ml of buffer 1 containing 0.3 M KCl for disruption in the Omnimixer homogenizer. The extracts were brought to 5 μg/ml with DNase and freed of debris by centrifugation for 10 min at 17 300*g*. The supernatant from this centrifugation was then incubated for 5 min at 37 °C. This incubation was

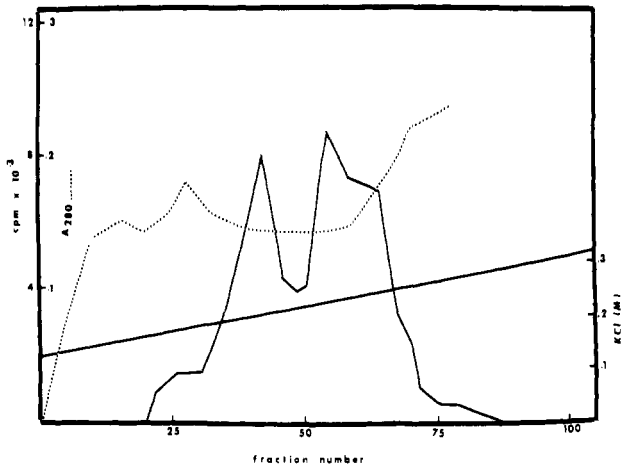

FIGURE 2: DEAE-cellulose chromatography of *S. antibioticus* RNA polymerase. Gradient conditions were as described in the text and fractions 30–46 and 50–70 were pooled. The shape of the KCl gradient is also depicted in the figure.

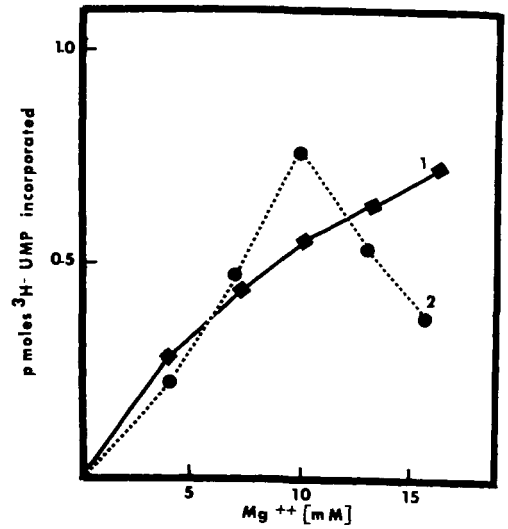

FIGURE 3: Dependence of *S. antibioticus* RNA polymerase activity on added Mg^{2+}. Aliquots of tubes 42 and 54 of Figure 2 were assayed as described under Materials and Methods in the presence of varying concentrations of Mg^{2+}. Calf thymus DNA was the template used. The Mg^{2+} contributed by the enzyme solutions was about 4 mM and has been added to the Mg^{2+} concentrations with which the assays were supplemented. The concentrations of polymerase used were about 40 μg/ml. Blank (zero time) incorporation was equivalent to about 0.04 pmol of [^{3}H]UMP.

critical to the subsequent purification of the enzyme. Step 2, high-speed supernatant: the incubated supernatant from the previous step was centrifuged at 200 000*g* for 60 min to sediment ribosomes. Step 3, ammonium sulfate precipitation: the 200 000*g* supernatant was diluted to a protein concentration of 3.9 mg/ml and brought to 55% saturation with solid ammonium sulfate, added gradually, and the resulting suspension was stirred at 0 °C for 30 min. The pH of the solution was maintained by the addition of small amounts of a 10% NH_4HCO_3 solution as needed. The precipitated protein was collected by centrifugation for 10 min at 20 000*g* and was redissolved in buffer 2. Step 4, glycerol gradient centrifugation: the protein solution from the previous step was diluted to 12–21 ml (depending on the recovery of protein from the previous step) with buffer 2 and equal aliquots were applied to each of four to six 35-ml glycerol gradients (10–30%) prepared in buffer 2. Gradients were centrifuged in the Spinco SW 27 rotor for 30 h at 69 000*g*. At the end of the centrifugation, gradients were collected from the bottom of the centrifuge tubes and assayed for polymerase. Step 5, batchwise DEAE-cellulose chromatography: the polymerase containing fractions from the glycerol gradient were pooled and applied to a 1 × 10 cm column of DEAE-cellulose equilibrated with buffer 2 containing 25% glycerol. The column was washed with this buffer containing 0.12 M KCl until the effluent was free of protein (A_{280}) and the polymerase was eluted by washing the column with 0.28 M KCl in the same buffer. Step 6, rechromatography on DEAE-cellulose: the RNA polymerase peak from the batchwise DEAE-cellulose column was diluted to 20 ml with buffer 2 containing 25% glycerol and applied to a second column of DEAE-cellulose (1 × 10 cm) equilibrated with the same buffer. This column was developed with a linear gradient in 240 ml of 0.12–0.30 M KCl in the indicated buffer. The column was washed at a flow rate of 40 ml/h and 2.0-ml fractions were collected. Two peaks of RNA polymerase activity were eluted by this procedure as is shown in Figure 2. Because the two peaks obtained were eluted at salt concentrations that were rather close (about 0.20 and 0.23 M KCl, respectively), the peak fraction from each polymerase peak was retained for examination of the Mg^{2+} dependency of RNA synthesis catalyzed by each form of the polymerase. The differences in Mg^{2+} dependency depicted in Figure 3 were obscured when the pooled, concentrated RNA polymerase peaks were assayed for Mg^{2+} dependency. The fractions employed were numbers 42 and 54 of Figure 2.

For most of the studies described below, forms 1 and 2 (designation based on the order of elution from the DEAE-cellulose column) were used in concentrated form. For these studies, fractions from the DEAE-cellulose column were pooled as indicated in the legend to Figure 2, diluted with an equal volume of buffer 2–25% glycerol, reapplied to 1 × 10 cm columns of DEAE-cellulose, and eluted batchwise with 0.28 M KCl in the indicated buffer.

Assay for Enzymatic Impurities. Solubilization of total [^{3}H]RNA (from *S. antibioticus*, Jones, 1975a) and [^{3}H]DNA (from virus SV-40, a gift of Dr. W. R. Folk) was used to assay for RNase and DNase activities, respectively. Samples of labeled polynucleotides were incubated for 60 min at 37 °C with 40–70 μg/ml of purified polymerase under the polymerase assay conditions described above, but in the absence of nucleoside triphosphates and unlabeled DNA. Polynucleotide phosphorylase was assayed by the method of Thang et al. (1971) using *E. coli* tRNA.

Preparation of S. antibioticus and E. coli Cell-Free Extracts. S. antibioticus cells were grown in galactose–glutamic acid–glucose medium as described above, harvested 12 or 48 h after innoculation, washed with 0.9% NaCl, and frozen until use. Generally, 1.6 g of cells (*S. antibioticus* or *E. coli*) were suspended in 5 ml of buffer 1 and sonicated for 2 min in 40-s bursts. The cells were maintained at a temperature of 0–10 °C during this treatment by immersing the container in an ice–salt bath. Debris was removed by centrifugation at 20 000*g* for 10 min and the supernatants from this centrifugation were filtered through a 2 × 30 cm column of Sephadex G-25 equilibrated with buffer 1. The protein eluting in the void volume of this column was collected. Gel filtered supernatants are referred to below as "crude extracts". RNA synthesis catalyzed by crude extracts was assayed as described above for RNA polymerase except that no DNA was added. Assay mixtures generally contained about 2.5 mg/ml of extract protein. Some

TABLE I: Partial Purification of *S. antibioticus* RNA Polymerase.[a]

Step	Volume (ml)	Protein (mg)	Units[b]	Spec Act.[c]	Purification
(1) Crude extract	35.4	287	1619	5.6	
(2) 200 000*g* supernatant	50	182	2146	11.8	2.1
(3) $(NH_4)_2SO_4$ precipitate	12	77	2066	26.8	4.8
(4) Glycerol gradient	52	10.7	1374	128	22.9
(5) 1st DEAE-cellulose	25.5	6.4	1236	193	34.5
(6) 2nd DEAE-cellulose					
Peak 1 pool[d]	4.5	1.0	483	469	83.8
Peak 2 pool[d]	4.0	1.4	1004	717	128

[a] Starting with 15 g of 48 h old *S. antibioticus* cells. [b] One unit of enzyme activity was defined and measured as described under Materials and Methods. [c] Units/mg of protein. [d] Data from all the relevant fractions of Figure 2 were used in computing these values.

assays contained, per ml: [^{14}C]ATP (43.1 nmol), or [^{3}H]GTP (5.35 nmol) or [^{3}H]CTP (1.25 nmol).

Miscellaneous Methods. Liquid scintillation counting was performed with a Beckman LS-250 liquid scintillation spectrometer. Thermal denaturation of DNA was performed essentially as described by Marmur and Doty (1962) and *S. antibioticus* DNA was found to contain about 25% G-C pairs. The binding of actinomycin to DNA was measured as previously described via ethanol precipitation of the actinomycin–DNA complex onto glass fiber filters (Jones, 1976). In some experiments the binding data were analyzed by the method of Scatchard (1949).

Results

Growth Characteristics of S. antibioticus Cultures. As a first step in this study, it was deemed necessary to examine the growth characteristics of the *S. antibioticus* cells and their abilities to synthesize actinomycin, RNA, and protein under the growth conditions described above. Results of an experiment in which these factors were examined are shown in Figure 1. It can be seen that the cell dry weight increases throughout the 3-day incubation, with the culture reaching stationary phase between 24 and 48 h after innoculation. Actinomycin does not become detectable in the growth medium until about 24-h post-innoculation. The ability of the culture to incorporate labeled macromolecular precursors declines drastically between 6- and 12-h post-innoculation, but detectable levels of incorporation are observed as late as 72-h post-innoculation in this experiment. In experiments not shown here it has been observed that: (1) both protein and RNA synthesis can continue for many days (albeit at low levels compared to vegetatively growing cultures) after innoculation (Collett and Jones, 1974, and unpublished results); (2) both 12 and 48 h old cells synthesize RNA species with the sedimentation properties of ribosomal RNA (Jones, 1975a); and (3) the [^{3}H]uridine incorporated by 12-, 48-, and 72-h cells can be completely solubilized with pancreatic RNase.

Partial Purification of S. antibioticus RNA Polymerase. As can be seen in Table I and Figure 2, two forms of RNA polymerase from 48 h *S. antibioticus* cells can be separated by DEAE-cellulose chromatography. Form 1 (eluting first from the DEAE-cellulose column of Figure 2) was purified about 80-fold with respect to the crude extract and form 2 about 120-fold. It should be mentioned that, under the assay conditions described under Materials and Methods, the specific activity of the *S. antibioticus* polymerase is very much lower than that commonly observed for microbial RNA polymerases. This low specific activity is totally the result of the conditions chosen for the assay of the polymerase during the purification. In preliminary experiments it was found to be extremely difficult to detect the polymerase activity in crude extracts of *S. antibioticus* cells when all four nucleoside triphosphates were present at 0.15 mM and [^{14}C]ATP was the labeled precursor. Some incorporation could be detected, but this incorporation generally required the utilization of such large amounts of extract protein that a linear relationship between [^{14}C]AMP incorporation and protein concentration could not be observed (in the experiments of Figure 7, for example, a protein concentration of about 2.5 mg/ml was required to produce readily detectable levels of [^{14}C]AMP incorporation). To alleviate this problem, high specific activity (about 20 Ci/mmol) [^{3}H]UTP was utilized to assay for RNA synthesis at a low concentration (about 1.25 μM). Under these conditions, RNA synthesis could be detected at a crude extract protein concentration of 0.2 mg/ml. When partially purified *S. antibioticus* RNA polymerase, rather than crude extracts, was assayed with all four nucleoside triphosphates (including [^{14}C]ATP) at 0.15 mM, the specific activities observed were 27 and 83.7 n*mol* of [^{14}C]AMP incorporated per mg of protein for forms 1 and 2, respectively, or an average specific activity of 59.3 nmol/mg. This value is only about tenfold less than the specific activity reported by Burgess (1969) for purified *E. coli* polymerase, and compares favorably with a value of 82 nmol/mg reported for 50-fold purified enzyme in that study.

Both forms 1 and 2 of the *S. antibioticus* polymerase were heterogeneous as regards protein content when examined by acrylamide gel electrophoresis (data not shown), but no contamination of either polymerase with RNase, DNase, or polynucleotide phosphorylase could be observed under the conditions described under Materials and Methods (data not shown).

As can be seen in Figure 3, when the peak fractions from the DEAE-cellulose column of Figure 2 were assayed in the presence of varying Mg^{2+} concentrations, differing responses were observed. The Mg^{2+} optimum for RNA synthesis catalyzed by the enzyme from tube 54 (form 2) was about 10 mM, whereas the enzyme from tube 42 (form 1) was still increasing in activity at 16 mM. Since the *S. antibioticus* polymerase forms are contaminated with other proteins, it is possible that the difference in Mg^{2+} optima is due to the presence of Mg^{2+} binding proteins associated with form 1 and is not due to distinctive characteristics of the polymerase itself. It seems possible, therefore, that the two polymerase forms separated by DEAE-cellulose chromatography represent a single gene product.

Forms 1 and 2 of the polymerase were identical in their responses to added KCl and to DNA from calf thymus or *S. antibioticus*, although maximal RNA synthesis by either

TABLE II: Examination of Various Protein Fractions for Ribonuclease Activity.[a]

Enzyme Fraction Added	cpm Recovered
None	6895
Crude extract[b]	6484
55–90% proteins[c]	6991

[a] Incubations were conducted using the conditions described for the RNA polymerase assay except that nucleoside triphosphates and DNA were omitted. Assay tubes contained about 7000 cpm of total, [^{3}H]uridine labeled RNA and were incubated 5 min at 32 °C after which the contents of each tube were collected by precipitation with Cl_3CCOOH and subjected to liquid scintillation counting. The specific activity of the added RNA was 10 500 cpm/A_{260}. [b] 250 μg of protein added. [c] 60 μg of protein added.

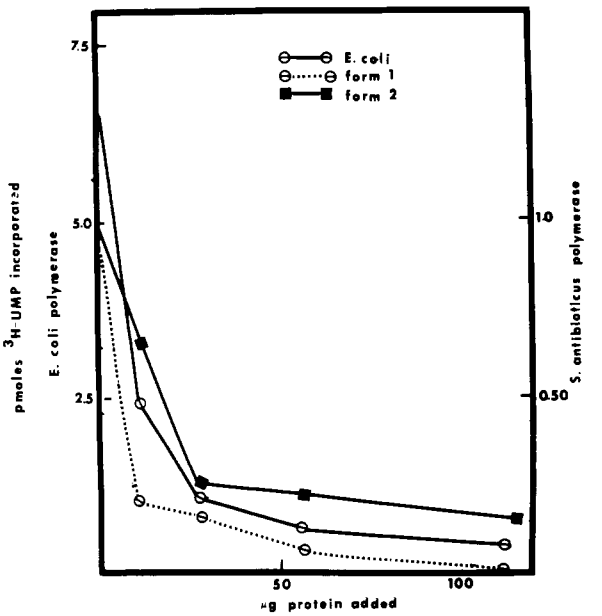

FIGURE 4: Effects of the 55–90% fraction on the activity of RNA polymerase. Assays for [^{3}H]UMP incorporation were performed as described under Materials and Methods. Reaction mixtures contained 35 μg/ml of *S. antibioticus* polymerase form 1, 53 μg/ml of form 2, or 74 μg/ml of *E. coli* polymerase. Increasing amounts (expressed as protein concentration) of the material recovered by ammonium sulfate precipitation of the supernatant from step 3 of the polymerase purification were added to assay tubes as indicated in the figure. All values have been corrected for zero time incorporation.

polymerase required higher levels of *S. antibioticus* than calf thymus DNA. In addition, the level of incorporation of [^{3}H]UMP was higher with *S. antibioticus* DNA than with calf thymus DNA at comparable DNA concentrations (see, for example, Table III below). Sucrose gradient analysis of RNA's synthesized by forms 1 and 2 revealed a number of discrete RNA species including species with the sedimentation coefficients of *S. antibioticus* ribosomal RNA (data not shown). It should also be noted that the total number of enzyme activity units recovered increased during the early stages of the purification (Table I). This increase was often as high as twofold between steps 1 and 3. The possible significance of this increase will be discussed in greater detail below. The complete purification of the *S. antibioticus* polymerase and an examination of the differences between forms 1 and 2 will be described in detail in a subsequent publication.

Evidence for the Existence of Proteins Inhibiting RNA Synthesis in S. antibioticus. The observed increase in the number of enzyme units with continued purification of the *S. antibioticus* RNA polymerase (Table I) suggested that substances inhibitory to the polymerase were removed during the purification procedure. It was reasoned that these substances might be responsible, at least in part, for the decrease in RNA synthesis observed in aging *S. antibioticus* cells "in vivo" (Figure 1). Three types of experiments were performed to examine this possibility more fully. In the first type of experiment, advantage was taken of the observation that the specific activity of crude cell extracts of 12-h *S. antibioticus* cells (which have not yet commenced actinomycin production) in directing [^{3}H]UMP incorporation was always several times higher than the specific activity of extracts of actinomycin producing 48-h cells (see Table VI below). When aliquots of 12- and 48-h cell extracts were mixed and assayed together, it was observed that the level of [^{3}H]UMP incorporation was only 50% of that predicted from the activities of the two extracts assayed separately (data not shown).

Further experiments have shown that, although the specific activity of 48-h cell extracts is consistently lower than that of 12-h cell extracts in RNA synthesis, the total RNA polymerase activity present in 48-h cell extracts increases significantly after batchwise DEAE-cellulose chromatography of the extract, whereas the total activity of 12-h cell extracts is essentially unchanged by this treatment. Thus, in the second type of experiment designed to examine the polymerase inhibitors, 5 mg of 48-h cell extract protein was applied to a 2.5-ml column of DEAE-cellulose equilibrated with buffer 1. The column was washed with buffer 1, then with buffer 1 plus 0.3 M KCl. It was observed that, although a total activity equivalent to 1.86 pmol of [^{3}H]UMP incorporated was applied to the column, an activity equivalent to 5.68 pmol was recovered. In a comparable experiment, 5.63 mg of 12-h cell extract protein, yielding 7.09 pmol of [^{3}H]UMP incorporated, was applied to a similar DEAE-cellulose column, and activity equivalent to 7.25 pmol was recovered. These data also suggest the presence of inhibitors of RNA polymerase in extracts of 48-h *S. antibioticus* cells, but that these inhibitors are absent, or present in lower amounts, in extracts of 12-h cells.

Finally, the direct isolation of some inhibitory substances has been achieved. The supernatant obtained after the ammonium sulfate polymerase purification step (step 3 of Table I) was used as the source of inhibitors. This supernatant was brought to 90% saturation with ammonium sulfate and the precipitate was collected by centrifugation and dissolved in 0.1 original volume of Buffer 1. Aliquots of the dissolved precipitates (referred to below as the "55–90% fraction") were then added to RNA polymerase assay mixtures containing concentrated polymerase forms 1 or 2 or *E. coli* RNA polymerase as the enzyme source. The results of these experiments are shown in Figure 4. It can be seen that the 55–90% fraction is extremely effective in inhibiting RNA synthesis catalyzed by both polymerase forms 1 and 2 and *E. coli* polymerase. Transcription was not completely inhibited by the 55–90% fraction in the experiments of Figure 4, but complete inhibition could be achieved by further increasing the amount of the 55–90% fraction added. Table II presents evidence against the presence of RNase as a major component of the 55–90% fraction. In these experiments, total [^{3}H]uridine labeled RNA from *S. antibioticus* was incubated with an aliquot of the 55–90% fraction for 5 min at 32 °C under the conditions used for RNA polymerase assay, but in the absence of polymerase. It can be seen that the recovery of acid insoluble RNA was

TABLE III: Effects of the 55–90% Ammonium Sulfate Fraction from *S. antibioticus* on Transcription.

System	pmol of [^{3}H]UMP Incorporated by Polymerase		
	E. coli	Form 1[a]	Form 2[a]
Complete	6.64	0.27	1.02
Complete plus 55–90% fraction	1.37	0.10	0.27
Complete plus 55–90% fraction (RNase or DNase treated)	–	–	0.33
Complete plus 55–90% fraction (trypsin treated)	–	–	0.98
Complete plus 55–90% fraction (pronase treated)	–	–	0.58
Complete plus 55–90% fraction (heated)	–	–	0.44
Complete minus *S. antibioticus* DNA + calf thymus DNA	11.65	–	0.71
Complete minus *S. antibioticus* DNA + calf thymus DNA + 55–90% fraction	9.39	–	0.68
Complete plus 300 μg/ml of DNA	–	–	0.40
Complete plus 106 μg/ml of polymerase	–	–	0.48
Complete plus actinomycin (4×10^{-5} M)	–	–	0.41
Complete plus actinomycin + 55–90% fraction	–	–	0.18

[a] From *S. antibioticus*. In the complete system, reaction mixtures contained 35 μg/ml of form 1 polymerase, 53 μg/ml of form 2 polymerase or 74 μg/ml of *E. coli* polymerase. Tubes were incubated for 10 min at 32 °C in the presence or absence of 55–90% fraction equivalent to 28 μg of protein, which had been dialyzed overnight against buffer 1. RNase and DNase treated 55–90% fractions were digested with 10 μg/ml of pancreatic RNase or DNase 1 for 30 min at 37 °C. Trypsin and pronase treated 55–90% fractions were digested at enzyme-substrate ratios of 1:15. All enzyme digests were subsequently dialyzed for 5–6 h vs. buffer 1, and a tenfold excess of soybean trypsin inhibitor was added to the trypsin digest after dialysis. Heated 55–90% fraction was boiled for 5 min and the precipitated protein was removed by brief centrifugation. In all relevant experiments, the amount of treated 55–90% fraction added was equivalent to a volume of the original, dialyzed 55–90% fraction containing 28 μg of protein. Calf thymus or *S. antibioticus* DNA's were present at concentrations of 150 μg/ml unless otherwise noted.

essentially unaffected by the presence of the 55–90% fraction in the assay tubes.

Evidence pertaining to the nature of the inhibitors is provided in Table III. The inhibitors were not dialyzable, and their inhibitory activity was completely reversed by prior digestion with trypsin, and partially reversed by digestion with pronase, followed by dialysis. Soybean trypsin inhibitor was used to inactivate any residual trypsin present in the tryptic digest. When the trypsin inhibitor was omitted, trypsin digestion reduced the inhibitory activity of the 55–90% fraction by only 60%. These results suggest the possibility that residual tryptic activity caused partial inactivation of the polymerase when soybean trypsin inhibitor was omitted. Polymerase inactivation may also explain the observation that pronase digestion of the 55–90% fraction only produced a 60% decrease in inhibitor activity. The inhibitory activity was not affected by digestion with DNase or RNase, and control experiments revealed that the inhibition by RNase treated 55–90% fraction was not due to the presence of the RNase itself. Approximately 20% of the inhibitory activity was abolished by heating for 5 min at 100 °C. Thus, at least some of the inhibition seems to be due to heat stable proteins present in the 55–90% fraction. The proteins show some specificity for *S. antibioticus* transcription complexes, since transcription of *S. antibioticus* DNA by homologous or heterologous polymerase was inhibited by the protein fraction, while transcription of calf thymus DNA (or salmon sperm or bacteriophage T4 DNA's, not shown) was essentially insensitive to inhibition by this fraction.

The data of Table III further show that the inhibitory effects of the 55–90% fraction could be partially overcome by increasing the polymerase or DNA concentrations in the reaction mixture. The 55–90% proteins do not, however, appear to counteract the inhibition of RNA synthesis by actinomycin. It can be seen in Table III that in the presence of 4×10^{-5} M actinomycin, actinomycin synthesis catalyzed by polymerase form 2 was inhibited by about 60%. When the 55–90% fraction was added along with actinomycin, the inhibition increased to 82%.

Effects of Actinomycin on Transcription by RNA Polymerase from S. antibioticus and E. coli. In this introduction to this report it was suggested that the persistence of RNA synthesis in actinomycin producing cultures of *S. antibioticus* could be partially due to some peculiar features of the cellular DNA, the RNA polymerase, or both. To further examine these possibilities, the effects of actinomycin on RNA synthesis catalyzed by the purified polymerases has been examined. Figure 5 shows the effects of varying concentrations of actinomycin on the activities of the purified *S. antibioticus* polymerase and *E. coli* polymerase in the presence of calf thymus or *S. antibioticus* DNA. The two DNA preparations were present in approximately equal amounts (by weight) in all the experiments of Figure 5.

The data of Figure 5 reveal the following: (1) actinomycin inhibits transcription by *S. antibioticus* polymerase forms 1 and 2 to somewhat similar extents regardless of the DNA template used (compare data for polymerase forms 1 and 2 in Figure 5a,b); (2) RNA synthesis catalyzed by both polymerase forms 1 and 2 is more resistant to actinomycin inhibition when homologous DNA is used as template than when heterologous DNA is the template (compare data for calf thymus and *S. antibioticus* DNA's in Figure 5a, b); (3) transcription with *E. coli* polymerase using *S. antibioticus* DNA as template is only slightly less sensitive to actinomycin than the corresponding synthesis of RNA with calf thymus DNA as template (Figure 5c); (4) RNA synthesis catalyzed by the *S. antibioticus* RNA polymerase with homologous DNA as template is more resistant to actinomycin inhibition than synthesis catalyzed by *E. coli* polymerase with the same template (compare dashed lines of Figure 5a,b,c). These data strongly suggest that some components of the transcriptional machinery of *S. antibioticus* are refractory to actinomycin inhibition of RNA synthesis, as compared to systems containing heterologous DNA and RNA polymerase. Subsequent studies were designed to examine the possible mechanisms responsible for the observations of Figure 5.

Possible Mechanisms for Actinomycin Resistant Tran-

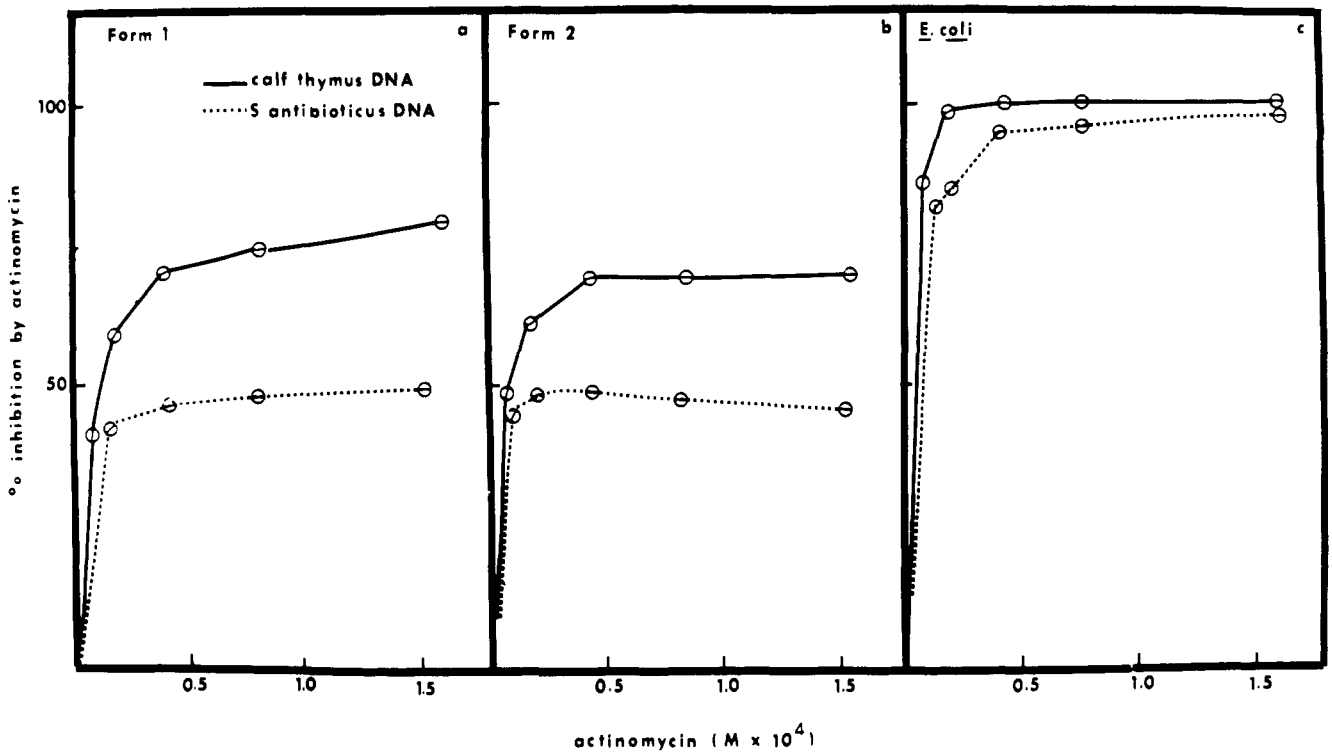

FIGURE 5: Effects of actinomycin on transcription by *S. antibioticus* and *E. coli* RNA polymerases. Each polymerase preparation was assayed at 10 mM Mg^{2+} (final concentration), with 150 μg/ml of calf thymus or *S. antibioticus* DNA as template. Assays contained 46 μg/ml of *S. antibioticus* polymerase form 1, or 70 μg/ml of form 2, or 70 μg/ml of *E. coli* polymerase. Maximal (100%) incorporation of [^{3}H]UMP in the absence of added actinomycin utilizing *S. antibioticus* DNA as a template was 0.37 pmol (form 1), 1.33 pmol (form 2), and 6.88 pmol (*E. coli*). Levels of incorporation were slightly lower with *S. antibioticus* polymerase and calf thymus DNA as template and slightly higher with *E. coli* polymerase and calf thymus DNA as template. Zero time controls were equivalent to 0.04 pmol. The indicated concentrations of actinomycin were added to some assay tubes, and the results are expressed as percentage inhibition of control incorporation by actinomycin.

scription in S. antibioticus. Considering the suspected mode of action of actinomycin in inhibiting DNA-dependent RNA synthesis (Reich and Goldberg, 1964), there appear to be three possible mechanisms directly involving RNA polymerase that might account for the relative insensitivity of transcription by *S. antibioticus* polymerase to actinomycin: (1) the attachment of *S. antibioticus* polymerase to homologous DNA might prevent actinomycin binding to the DNA and bring about the irreversible displacement of any previously bound actinomycin; (2) *S. antibioticus* polymerase might displace actinomycin from DNA sequences that are being actively transcribed, but the antibiotic might be rebound when the enzyme traverses those sequences; (3) *S. antibioticus* polymerase may not displace actinomycin from the DNA at all, but may simply be capable of transcribing sequences to which the drug remains bound. A fourth mechanism would not involve insensitivity of the transcriptional apparatus to actinomycin, but rather postulates the existence of actinomycin binding proteins (or other substances) in cells producing the antibiotic. These substances might sequester actinomycin and prevent its interaction with DNA. Evidence relating to this possibility will be presented below.

Evidence against the first of the four possibilities listed above is presented in Table IV. In these experiments, the binding of actinomycin to DNA was measured utilizing the binding assay previously described (Jones, 1976), under conditions in which active transcription could take place. It can be seen that the amount of actinomycin bound to the input DNA does not change appreciably during the incubation period, even though data from parallel incubations indicated that the RNA polymerase was actively transcribing (in the presence of unlabeled actinomycin at the same concentration as in the experiments of Table IV) during the entire period. The limits of detection of the assay in question are probably within 10% of the observed binding of actinomycin to DNA, so that the decrease in actinomycin bound observed with *S. antibioticus* polymerase (Table IV) is probably within the limits of experimental error. Thus, it can be stated that at least 90% of the actinomycin initially bound to DNA remains bound during the entire course of the experiments of Table IV, during which active transcription of the added DNA was possible.

Direct experiments to examine possible mechanisms 2 and

TABLE IV: Time Course of Actinomycin Binding to *S. antibioticus* DNA in the Presence of RNA Polymerase.

Time of Incubation (min)	cpm of [^{3}H]actinomycin *E. coli* Polymerase	Bound Using Form 2[a]
4	4526	5018
6	4860	4986
8	4836	4645
10	4860	4652

[a] From *S. antibioticus*. [^{3}H]Actinomycin (10 000 cpm, 4.36 mCi/mmol, 5.5×10^{-5} M, final concentration) was added to incubation mixtures prepared as described in the legend to Figure 6. The binding of actinomycin to DNA was measured as described in the legend to Figure 6 and the data represent cpm of [^{3}H]actinomycin bound to *S. antibioticus* DNA at each time point in the presence of RNA polymerase. Reaction mixtures were identical with those normally used for RNA synthesis except that unlabeled UTP was present.

TABLE V: Parameters for the Binding of Actinomycin to *S. antibioticus* and Calf Thymus DNAs in the Presence and Absence of RNA Polymerase.[a]

Polymerase Added	K_{app} (M^{-1})		B_{app}	
	Calf Thymus	*S. antibioticus*	Calf Thymus	*S. antibioticus*
None	2.8×10^6	2.1×10^5	8.2	16
E. coli	2.4×10^6	2.4×10^5	10.2	20
S. antibioticus	1.6×10^6	2.9×10^5	8.2	16

[a] The binding of [^{3}H]actinomycin to *S. antibioticus* and calf thymus DNA's was measured via ethanol precipitation of the complexes as described in the legend to Figure 6. In the experiments with calf thymus DNA, the DNA concentration was 50 μg/ml.

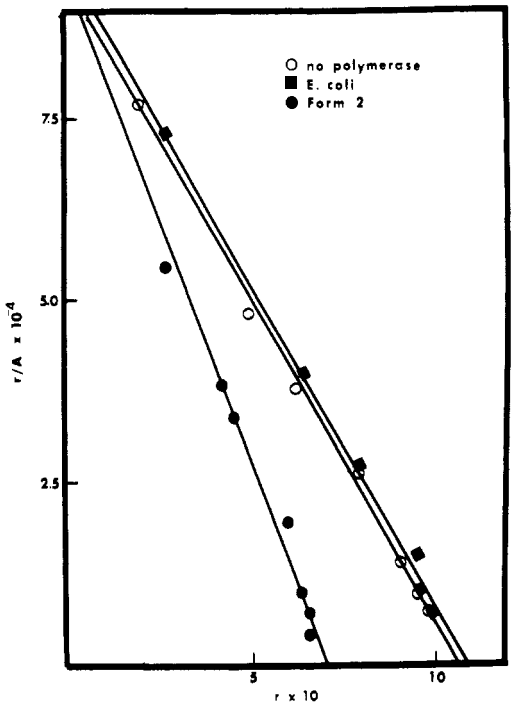

FIGURE 6: Binding isotherms for the formation of complexes between actinomycin and *S. antibioticus* DNA. Assays were conducted in 0.1 ml reaction mixtures containing all components normally used for RNA synthesis except that unlabeled UTP was substituted for [^{3}H]UTP. Reaction tubes contained 100 μg/ml of *S. antibioticus* DNA, and were incubated for 3 min at 25 °C in the presence of RNA polymerase (53 μg/ml of *S. antibioticus* form 2 or 74 μg/ml of *E. coli* polymerase), or in its absence, then [^{3}H]actinomycin (95.23 mCi/mmol) was added to give final concentrations of 7×10^{-6} to 1.4×10^{-4} M. After 5 min of additional incubation, the actinomycin–DNA complexes were collected via ethanol precipitation onto glass fiber filters. The filters were dried and examined by liquid scintillation counting. Control experiments showed that RNA synthesis did indeed occur in identical reaction mixtures incubated without actinomycin; r = moles actinomycin bound/mol of total DNA base pairs; A = concentration of free actinomycin.

3 above have not been devised. However, it was reasoned that the transient displacement of actinomycin from DNA by *S. antibioticus* polymerase (mechanism 2) might be practically manifested as an apparent decrease in the affinity of the antibiotic for DNA, since this displacement would shift the binding equilibrium toward the regeneration of reactants. It thus seemed possible that the association constant and/or the number of binding sites per nucleotide pair for the binding of actinomycin to DNA might be decreased if mechanism 2 (but not mechanism 3) were operative. To examine this possibility, the binding of low concentrations of [^{3}H]actinomycin to *S. antibioticus* DNA was measured in the presence and absence of *E. coli* or *S. antibioticus* RNA polymerase, under conditions in which active transcription could take place. Actinomycin binding was measured as described (Jones, 1976) and details of the experiment are given in the legend to Figure 6. It was shown in the previous study (Jones, 1976) that the assay employed gives association constants (K_{app}) that are reasonably close to those measured by equilibrium dialysis. For example, in the experiments described below, K_{app} for the binding of actinomycin to calf thymus DNA was calculated to be 2.8×10^6 M^{-1} using the ethanol precipitation assay, as compared with a value of 2.3×10^6 M^{-1} determined by equilibrium dialysis (Muller and Crothers, 1968).

In Figure 6, typical Scatchard plots (Scatchard, 1949) are presented for the binding of actinomycin to *S. antibioticus* DNA in the presence and absence of RNA polymerase, and in Table V, the apparent binding constants and nucleotide pairs per binding site are presented. The values of Table V represent the average of two experiments including the one described in Figure 6. Binding data for calf thymus DNA are also included in Table V. It can be seen that the binding isotherms in question were linear over the actinomycin concentration range tested. The lines were drawn by least-squares analysis of the binding data, and their correlation coefficients were 0.99, 0.93, and 0.99 for the binding in the absence of polymerase, in the presence of *E. coli* polymerase and in the presence of *S. antibioticus* polymerase, respectively. Table V indicates that the binding constant in the absence of polymerase was 2.1×10^5 M^{-1}. In the presence of *E. coli* polymerase, the binding constant increased slightly to 2.4×10^5 M^{-1} and in the presence of *S. antibioticus* polymerase, the binding constant increased to 2.9×10^5 M^{-1}. There was essentially no change in the number of nucleotides involved in the actinomycin binding sites in the presence of *S. antibioticus* RNA polymerase, while a slight increase in the number of nucleotide pairs per binding site was observed with *E. coli* polymerase (Table V). A different type of result was observed when calf thymus DNA was the substrate used. In these experiments, the binding constant decreased in the presence of polymerase, again with no appreciable change in the estimated size of the binding site (Table V). In several experiments, the constant for actinomycin binding to *S. antibioticus* DNA has been observed to increase in the presence of RNA polymerase. No decrease in K_{app} for the binding of actinomycin to *S. antibioticus* has ever been observed in the presence of *E. coli* or *S. antibioticus* enzyme. Thus, actinomycin seems to be bound to the appropriate nucleotide sequences of the *S. antibioticus* DNA in the presence of homologous polymerase as well as in its absence, but the binding is somewhat more efficient when the enzyme is present. These results, of course, also argue against an irreversible displacement of actinomycin by polymerase (mechanism 1 above), as well as against a transient displacement mechanism

TABLE VI: Comparison of RNA Synthesizing Activities of Crude Extracts of 12- and 48-h *S. antibioticus* Cells.

System[a]	cpm of [^{3}H]UMP Incorporated by 12-h Extract	48-h Extract
Complete	4080 (12380)	1420 (6651)
Complete plus calf thymus DNA	6462	2508
Complete minus ATP	1482	560
Complete minus CTP	647	760
Complete minus GTP	128	325
Complete minus phosphate buffer	6655	2120
Complete plus DNase (25 μg)	238	126
Complete plus RNase (25 μg)	0	0

[a] Assays for RNA synthesis were performed as described under Materials and Methods except that no DNA was added to most incubations (complete system). Zero time control values (about 600 cpm) have been subtracted from all the data in the table. Figures in parentheses represent the specific activity (cpm/mg of protein) for each extract under the assay conditions used. All assays contained an equal amount of 12- or 48-h extract protein.

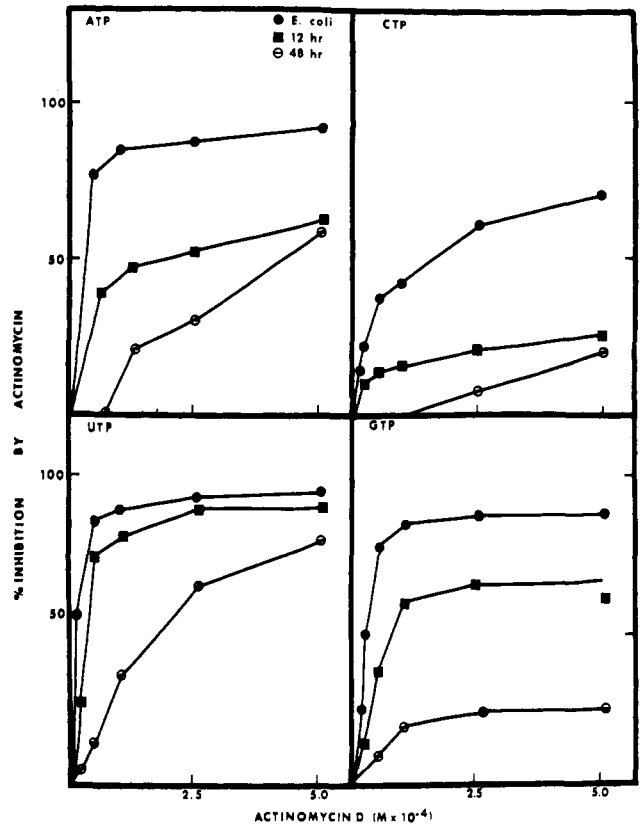

FIGURE 7: Effects of varying concentrations of actinomycin D on the RNA synthesizing activity in crude extracts of *E. coli* and 12- and 48-h *S. antibioticus* cells. Assays for RNA synthesis were performed as described under Materials and Methods in the presence or absence of varying concentrations of actinomycin. Reaction mixtures contained about 2.5 mg/ml of total protein in each case. Maximum incorporation in the absence of actinomycin was at least 0.15 pmol of [^{3}H]UMP, 11.1 pmol of [^{14}C]AMP, 0.32 pmol of [^{3}H]GMP, and 0.31 pmol of [^{3}H]CMP. All values were corrected for zero time incorporation (generally less than one-fifth of the maximum incorporation observed) and results are expressed as percentage inhibition of control incorporation by actinomycin.

(mechanism 2). Although the exact details of the mechanism of actinomycin resistant transcription will require further scrutiny, the binding studies suggest that *S. antibioticus* polymerase can transcribe DNA sequences to which actinomycin is bound without first displacing the antibiotic from those sequences.

Effects of Actinomycin on RNA Synthesis in Crude Extracts of S. antibioticus Cells. Since *S. antibioticus* produces actinomycin during the normal course of its development, and since it is capable of synthesizing RNA, while simultaneously producing the antibiotic, it seemed possible that resistance of the transcriptional machinery to actinomycin might be an acquired characteristic, with the degree of resistance increasing as the amount of actinomycin synthesized by the cells increases. Thus, it was reasoned that the transcriptional machinery of 48-h old cells might be less sensitive to actinomycin than that of 12-h old cells, since 12-h cells have not yet commenced actinomycin synthesis. These predictions were examined by using crude extracts of 12- and 48-h *S. antibioticus* cells for RNA synthesis, as described under Materials and Methods. Crude extracts of *E. coli* K12 were prepared and assayed in an identical fashion for comparison.

The general characteristics of RNA synthesis in crude *S. antibioticus* extracts are summarized in Table VI. It can be seen that the ability to catalyze [^{3}H]UMP incorporation into acid insoluble products was detectable in extracts of both 12- and 48-h cells. In terms of specific activity, extracts of 12-h cells were two to three times more active than extracts of 48-h cells (Table VI). This difference is presumably due to the presence of transcriptional inhibitors in the extracts of 48-h cells (see above). An increase in [^{3}H]UMP incorporation was observed when potassium phosphate buffer was deleted from the reaction mixtures. This compound was routinely included in all RNA polymerase assays as an inhibitor of polynucleotide phosphorylase, as suggested by Burgess (1969). The [^{3}H]UMP incorporating activity of the cell-free extracts was stimulated by the addition of 0.15 mg of calf thymus DNA to the incubation mixtures, and was completely abolished by incubation of the reaction mixtures with DNase or RNase. Further, maximal incorporation of [^{3}H]UMP directed by 12- or 48-h extracts was partially dependent on the presence of the other nucleoside triphosphates in the reaction mixtures. The reason for this partial dependency is not clear, and may indicate the presence of small amounts of unlabeled triphosphates in the cell extracts. It is clear from the data of Figure 7 that extracts of 12- and 48-h cells can incorporate label from ATP, CTP, and GTP into acid insoluble products. As has been previously reported, extracts of 12- and 48-h cells catalyze the synthesis of RNA's with the sedimentation properties of ribosomal RNA (Jones, 1975a).

The effects of actinomycin on RNA synthesis by extracts of 12- and 48-h *S. antibioticus* cells and by *E. coli* extracts were examined with the results presented in Figure 7. As can be seen, 12- and 48-h *S. antibioticus* extracts were able to synthesize RNA under circumstances in which the activity of *E. coli* extracts (prepared in an identical fashion) was severely inhibited. Further, RNA synthesis catalyzed by 48-h extracts was much less sensitive to the antibiotic than synthesis catalyzed by extracts of 12-h cells. For example, when [^{3}H]UTP was the labeled precursor, 5×10^{-5} M actinomycin decreased RNA synthesis catalyzed by *E. coli* extracts by nearly 90%, whereas synthesis catalyzed by the extracts of 12-h *S. antibioticus* cells dropped by 75% and synthesis catalyzed by extracts of 48-h cells, by only 15% (Figure 7). At 5×10^{-5} M actinomycin, using [^{14}C]ATP as the labeled precursor, equally dramatic results were obtained. At this concentration, the levels of RNA synthesis were decreased by 80%, 40%, and 0%, in

TABLE VII: Binding of [^{3}H]actinomycin by Crude Cell Extracts and Purified RNA Polymerase.

Fraction Analyzed	Total Protein Analyzed (mg)	cpm of [^{3}H] actinomycin Bound Expected	Found
Crude extract—*E. coli*	5.08	785	1108
Crude extract—12-h *S. antibioticus*	1.37	785	1010
Crude extract—48-h *S. antibioticus*	0.40	785	1529
S. antibioticus polymerase form 2	0.07	785	814

[a] Samples of each fraction were dialyzed against [^{3}H]actinomycin as described in the text. The expected cpm of actinomycin bound was determined by counting a 0.1-ml aliquot of the dialysis medium at equilibrium, and assuming that the concentration of actinomycin found inside the dialysis bag would equal that outside if no binding to extract or polymerase proteins took place. The cpm found was obtained by counting a 0.1-ml aliquot of the solutions inside the dialysis bags at equilibrium.

incubations containing extracts of *E. coli*, 12- and 48-h *S. antibioticus* cells, respectively. Similar results were obtained when [^{3}H]GTP and [^{3}H]CTP were the labeled precursors (Figure 7). It should be noted that the data presented may only reflect the minimum difference in sensitivity between 48-h extracts and 12-h or *E. coli* extracts, since no effort was made to determine the concentration of actinomycin actually present in the extract of 48-h cells. The actinomycin sensitivity of RNA synthesis in this extract may, thus, have been overestimated.

Sucrose gradient analysis of the RNA's synthesized by *S. antibioticus* extracts in the presence of actinomycin have revealed that RNA's with the sedimentation coefficients of ribosomal RNA are among the products of transcription (Jones, 1975a).

Another important characteristic of the purified *S. antibioticus* RNA polymerase is revealed by comparison of Figure 5a,b with Figure 7. It can be seen that RNA synthesis catalyzed by the purified polymerase is considerably more sensitive to actinomycin than synthesis catalyzed by crude extracts of *S. antibioticus* cells. For example, at 5×10^{-5} M actinomycin, [^{3}H]UMP incorporation by the purified polymerase forms 1 and 2 was inhibited by an average of 48% when homologous DNA was the template (Figure 5a,b). In contrast, 15% inhibition was observed when a crude extract of 48-h *S. antibioticus* cells was the polymerase source (Figure 7).

Finally, it was of interest to determine whether the data of Figure 7 could be partially explained by the existence of actinomycin binding proteins (or other substances) in the crude extracts of *S. antibioticus* cells that might be absent from purified polymerase preparations. To examine this possibility, aliquots of the crude extracts were digested for 30 min at 37 °C with 50 μg of DNase and then dialyzed for 48 h against a solution of [^{3}H]actinomycin (8×10^{-6} M containing about 2×10^6 cpm) in 0.05 M Tris-HCl, pH 7.6, 0.01 M KCl, 0.01 M $MgCl_2$. At the end of the dialysis, aliquots of the dialysis medium and the dialyzed extracts were examined by liquid scintillation counting with the results presented in Table VII. Results are expressed as cpm [^{3}H]actinomycin found outside or inside the dialysis bags. The expected cpm were calculated by counting a 0.1-ml aliquot of the medium and assuming that the concentration of actinomycin found inside the dialysis bag would be equal to that outside if no binding to extract proteins took place. It can be seen that all three crude extracts tested showed higher than expected [^{3}H]actinomycin concentrations inside the dialysis bags. For *E. coli* and 12-h *S. antibioticus* cell extracts, the increase in internal actinomycin concentration was about 30–40% relative to the dialysis medium, while the internal concentration was increased by twofold when the crude extract of 48-h *S. antibioticus* cells was used. Since the extracts had been extensively digested with DNase prior to dialysis, the binding factors must be substances other than DNA. Binding was abolished by trypsin digestion of the 48-h extract before dialysis, suggesting that the binding factors are proteins. The data of Table VII also show that the actinomycin binding factors were removed by partial purification of the *S. antibioticus* RNA polymerase. When polymerase form 2 was dialyzed against [^{3}H]actinomycin as described above, no increase in actinomycin concentration inside the dialysis bag (as compared with the external concentration at equilibrium) was observed.

Discussion

The data from the studies described above can be used to formulate plausible hypotheses to explain the decrease in RNA synthesis that precedes and accompanies actinomycin production in *S. antibioticus*, and the persistence of some RNA synthesis in cultures which are actively synthesizing the antibiotic. The decrease in RNA synthesis in actinomycin producing cultures seems to be due, at least in part, to the synthesis by these cultures of specific proteins that inhibit transcription. These proteins may bind to DNA and block transcription by DNA-dependent RNA polymerase. The data of Table III indicate that these proteins are rather specific for *S. antibioticus* DNA. The 55–90% ammonium sulfate fraction from 48-h *S. antibioticus* cells blocks transcription of *S. antibioticus* DNA by both homologous and heterologous RNA polymerases, but transcription of calf thymus, salmon sperm and bacteriophage T4 DNA's was essentially insensitive to these inhibitors at concentrations that produced almost 75% inhibition of transcription of *S. antibioticus* DNA (Table III). This mechanism can also explain the observation that the inhibition by the 55–90% fraction can be relieved by increasing the concentration of DNA or RNA polymerase in the reaction mixtures (Table III), if it is assumed that the affinity of the inhibitors for DNA is not very much greater than the affinity of the RNA polymerases for this template. If this is the case, one might expect that increased concentrations of polymerase would compete effectively with the inhibitors for binding sites on the DNA, and increased amounts of DNA would, of course, increase the concentration of nucleotide sequences to which no inhibitor is bound. The data of Table III suggest that the inhibitory substances are proteins, since the inhibitory activity of the 55–90% ammonium sulfate fraction can be destroyed by proteases. The activity was not affected by digestion with RNase or DNase. Experiments are in progress to purify specific proteins from the 55–90% ammonium sulfate fraction and to examine the ability of these proteins to bind to DNA.

The data presented above also suggest that the persistence of RNA synthesis in actinomycin producing cultures of *S. antibioticus* is due to a combination of two factors: (1) the presence of actinomycin binding proteins in cells producing the antibiotic; (2) some unique features of the transcription complexes formed between *S. antibioticus* DNA and RNA polymerase that allow transcription to procede in the presence of actinomycin. The data of Figure 7 indicate that RNA syn-

thesis in crude extracts of actinomycin producing *S. antibioticus* cells is less sensitive to actinomycin inhibition than RNA synthesis in crude extracts of younger cells, not producing the antibiotic, or *E. coli* cells. Table VII suggests that this difference may be due, not only to the formation of transcription complexes that are partially resistant to actinomycin (see further below), but also to the existence of actinomycin binding proteins in crude extracts of actinomycin producing cells. Actinomycin binding proteins were present in much lower amounts in 12-h old *S. antibioticus* cells and in *E. coli* cells.

In addition, transcription of *S. antibioticus* DNA by homologous RNA polymerase in vitro was shown to be refractory to actinomycin inhibition. This phenomenon was not observed with heterologous DNA or RNA polymerase. The data of Figure 6 and Table V argue against the displacement of actinomycin from the DNA by *S. antibioticus* RNA polymerase during transcription, since displacement might be expected to lead to a decrease in the association constant for the binding of actinomycin to DNA, or an increase in the apparent size of the actinomycin binding site. Such changes were not observed. In addition, no evidence for the existence of actinomycin binding proteins in the partially purified polymerase preparation was found when a sample of polymerase form 2 was dialyzed against radioactive actinomycin (Table VII). Thus, the *S. antibioticus* RNA polymerase appears to be capable of transcribing DNA sequences to which actinomycin remains bound.

It should be noted that the apparent constant for the binding of actinomycin to calf thymus DNA did decrease in the presence of RNA polymerase. This result is not completely surprising, since transcription of calf thymus DNA by *S. antibioticus* RNA polymerase was considerably more sensitive to actinomycin inhibition than transcription of *S. antibioticus* DNA (Figure 5). Thus, it seems possible that the conformation of transcription complexes formed between *S. antibioticus* DNA and RNA polymerase may differ from the conformation of complexes formed with calf thymus DNA and the same polymerase. In this regard, it is worthwhile to note that, at comparable concentrations of DNA and enzyme, *S. antibioticus* DNA was a more efficient template than calf thymus DNA for *S. antibioticus* RNA polymerase, while calf thymus DNA was a more efficient template for *E. coli* RNA polymerase (see, for example, Table III). It should also be mentioned that Beabealashvilly et al. (1973) reported a decrease in the association constant for the binding of actinomycin to calf thymus DNA (from 1.8×10^6 to 2.5×10^5) in the presence of *E. coli* polymerase. Although these workers studied actinomycin binding to a complex of DNA and polymerase that had been digested with DNase, and their studies were not performed under conditions in which transcription could take place, their results are consistent with the observations of Table V.

The studies presented in this report were performed to examine the regulation of transcription in an organism producing a transcriptional inhibitor as a secondary metabolite. The data presented provide evidence for the following hypotheses: (1) binding of transcriptional inhibitors (proteins) to *S. antibioticus* DNA is responsible for the decrease in RNA synthesis observed in aging cells in vivo; (2) complexes between *S. antibioticus* DNA and RNA polymerase are resistant to actinomycin inhibition of transcription and, thus, are capable of synthesizing RNA in vivo while actinomycin synthesis is occurring in the cells. The observed decrease in permeability to actinomycin observed in cells synthesizing the antibiotic (Marshall et al., 1968) would help to prevent the accumulation of high concentrations of the antibiotic in the cells, and the presence of actinomycin binding proteins in cells producing the antibiotic would lead to a decrease in the intracellular levels of the antibiotic available for complexing with DNA.

Previous studies have shown that protein synthesis declines concomitantly with RNA synthesis in *S. antibioticus* cells (Jones and Weissbach, 1970; Collett and Jones, 1974; Figure 1, above), and the molecular mechanism for this decline in translational capacity seems to involve modification of the cellular tRNA's and ribosomes (Jones, 1975b). Nevertheless, low levels of protein synthesis are maintained throughout actinomycin production (Collett and Jones, 1974), and the low levels of RNA synthesis alluded to above presumably provide RNA species to support this translation. It seems obvious that these low levels of RNA synthesis do not require the same number of active transcription complexes as might be present in vegetatively growing cells, and the RNA polymerase that is present at these later times must be capable of functioning in the presence of actinomycin. The hypotheses outlined above are totally consistent with this line of reasoning.

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