DNA-Dependent RNA Polymerase from *Pseudomonas* BAL-31. I. Purification and Properties of the Enzyme†

Stephen G. Zimmer*,‡ and Robert L. Millette§

ABSTRACT: The DNA-dependent RNA polymerase from *Pseudomonas* BAL-31, the host for bacteriophage PM2, has been purified 154-fold using differential centrifugation, chromatography on DEAE-cellulose, ammonium sulfate precipitation, and sucrose gradient centrifugations at low and high ionic strength. The resulting enzyme is free of enzyme activities which could interfere with transcription studies and is >85% pure as judged by polyacrylamide gel electrophoresis. Like other bacterial RNA polymerases, its subunit structure is $\beta'\beta\sigma\alpha_2$. From gel electrophoresis the β', β, and α subunits have approximately the same molecular weights as those from *Escherichia coli*, whereas the σ subunit is 5% larger (89,000 *vs.* 85,000). A summation of the subunits yields a molecular weight of 485,000 for the holoenzyme. Like other bacterial RNA polymerases, it sediments as a monomer (15 S) at low ionic strength (0.065) and as a dimer (22 S) at high ionic strength (0.75). Its activity is stimulated three-fold by monovalent cations (K^+, NH_4^+, Na^+) with additional stimulation provided by divalent cations (Mg^{2+}, Mn^{2+}). The transcription of phage PM2 form I (supercoiled) DNA has an ionic strength optimum of 0.26 for continuous long-term synthesis, and over an ionic strength range of 0.09–0.46 "plateau-type" kinetics are not observed. The σ subunit is required for optimal PM2 transcription. The enzyme is sensitive to the same inhibitors of transcription as the RNA polymerase from *E. coli*, it has a temperature optimum of 28°, and it is 50% inactivated by heating 10 min at 41°. It has template preference similar to *E. coli* polymerase and shows little preference for homologous templates. With various DNAs the order of template activities is T7 > PM2 I $\simeq$ T4 > PM2 II (relaxed circular form) > λ_c > calf thymus > BAL-31 DNA. Phage PM2 form I DNA is transcribed at a twofold greater rate than PM2 form II DNA by this enzyme.

Pseudomonas BAL-31, a marine bacterium isolated by Espejo and Canelo (1968a), serves as a host for the small, lipid-containing bacteriophage PM2 (Espejo and Canelo, 1968b). This phage is of particular interest for transcription studies in that its genome is a closed circular duplex DNA molecule of 6×10^6 daltons (Espejo *et al.*, 1969). Such a genome provides an ideal template for studying *in vitro* the effects of the DNA circularity, supercoiling, and single-strand breaks on the transcription process. From the purified phage relatively large quantities of the native supercoiled DNA (form I) can be easily purified, and these in turn can be modified by controlled "nicking" into the relaxed, circular DNA molecule (form II). A DNA molecule of such small size would be expected to code for only a few discrete mRNA species. This simplifies the problem of characterizing the individual transcription products, and facilitates analyzing the initiation and termination sites and events which generate these molecules. In contrast to the smaller single-stranded DNA phages, there is evidence for temporal controls in the gene expression of phage PM2 (Braunstein *et al.*, 1971) and thus it may provide us with a very simple system for studying the regulation of gene transcription.

Since there is evidence that RNA polymerases from different microorganisms show differences in their transcription specificity (von der Helm and Zillig, 1969), we considered it essential to utilize the DNA-dependent RNA polymerase from the bacteriophage's natural host, *Pseudomonas* BAL-31, in order to avoid the possibility of erroneous transcription by a nonhomologous enzyme. Therefore, as a prerequisite to study PM2 transcription, it was necessary to isolate the RNA polymerase from the host organism.

In this paper we describe a procedure for the purification of the DNA-dependent RNA polymerase from *Pseudomonas* BAL-31. We have characterized the enzyme with respect to its physical and enzymatic purity, molecular structure, stability, and template preferences; in particular we have studied its transcription requirements and the effect of inhibitors with PM2 DNA as template. A detailed study of its function in the transcription of the allomorphic forms of phage PM2 DNA is presented in the following publication (Zimmer and Millette, 1975).

Materials and Methods

Buffers and media used included: AMS buffer (Espejo and Canelo, 1968a); BAL-31 growth medium (2.4 g of Tris base, 5 g of glucose, and 8 g of nutrient broth per liter of AMS buffer, adjusted to pH 7.5 with HCl); BE buffer (Espejo and Canelo, 1968b); NT buffer and NTC buffer (Franklin *et al.*, 1969); buffer A_1 (0.02 M Tris-HCl (pH 8.1 at 4°)–0.01 M $MgCl_2$–0.1 M NaCl–0.5 mM EDTA–5 mM β-mercaptoethanol–10% glycerol, v/v); buffer A_2 (0.02 M Tris-HCl (pH 8.1 at 4°)–0.01 M $MgCl_2$–0.6 M NaCl–0.5 mM EDTA–2 mM β-mercaptoethanol–5% glycerol, v/v);

† From the Department of Pathology, University of Colorado Medical Center, Denver, Colorado 80220. *Received August 22, 1974.* This work was supported in part by Public Health Service Research Career Development Award 5-KO4 GM 19333 (R.M.), by Grant GB-23356 from the National Science Foundation (R.M.), and by National Institutes of Health Training Grants CA-5164 and GM-977 (S.Z.). This work was taken in part from a thesis submitted by S.Z., Department of Pathology, University of Colorado Medical Center, in partial fulfillment of requirements for the degree of Doctor of Philosophy. A preliminary report of this work was presented at the 1972 Annual Meeting of the American Society for Microbiology.

‡ Present address: Department of Pathology, Washington University School of Medicine, St. Louis, Mo. 63110.

§ Present address: Committee on Virology, The University of Chicago, Chicago, Ill. 60637.

buffer B (0.05 M Tris-HCl (pH 8.1 at 4°)–0.02 M $MgCl_2$–0.5 mM EDTA–5 mM β-mercaptoethanol–10% glycerol, v/v); buffer C (0.01 M Tris-HCl (pH 8.1 at 4°)–5 mM $MgCl_2$–0.05 M NaCl–0.5 mM EDTA–2 mM β-mercaptoethanol–5% glycerol, v/v).

Growth of Bacteria. Pseudomonas BAL-31 (generously supplied by R. Espejo) was grown in 20-l. batches in the following manner. Ten liters of BAL-31 growth medium was prewarmed to 28° in a New Brunswick M-114 fermenter, 500 ml of an overnight culture was added, and the cells were grown with vigorous aeration at 28° to a density of 2×10^9/ml (late log phase). Three liters of culture was removed and the cells were collected by centrifugation (7000 rpm for 10 min at 0° in a Sorvall GS-3 rotor) and held on ice. Concomitantly, 2 l. of fresh media was added to the culture. When the cell density again reached 2×10^9/ml, 3 l. was removed, and the above process was repeated until all of the bacteria were harvested.

The packed bacteria were washed twice by resuspension in 250 ml of cold AMS buffer, and collected by centrifugation at 20,000 rpm for 15 min at 0° in a Sorvall SS-34 rotor. The average yield was 6–7 g (packed, wet weight) of bacteria per liter of culture. The packed cells were frozen and stored at −20° until use (usually 2 days after harvesting). Under these growth conditions the bacteria have a doubling time of approximately 60 min.

Growth of Bacteriophage PM2. An 8-l. batch of *Pseudomonas* BAL-31 was grown, under the conditions described above, to a density of 3×10^8 cells/ml. $CaCl_2$ (8.8 g) was dissolved in the culture and purified bacteriophage PM2 (a gift of R. Espejo) was added at a multiplicity of 0.1–0.5. After lysis was completed, usually in 3 hr, the bacteriophage was harvested and purified according to the method described by Salditt *et al.* (1972) up to the first CsCl gradient. The phage band was collected and dialyzed overnight against two changes of 800 vol of BE buffer. If the DNA was not going to be extracted within 1 day, the dialysis was done against NTC buffer and the phage were stored at 4°.

Unlabeled DNA. Unlabeled DNA from the purified phage was prepared by a modification of the procedure described by Espejo *et al.* (1969). Sarkosyl (Sigma Chemical Co.) was added to the purified phage to a final concentration of 0.2% (w/v) and the mixture was heated at 45° for 5 min. The clarified solution was extracted twice with equal volumes of BE saturated phenol (pH 7.5). The phenol phase and interface were re-extracted with 0.5 vol of BE buffer and the combined aqueous phases were extracted once with an equal volume of chloroform–octanol (9:1, v/v). The aqueous DNA solution was dialyzed overnight against two changes of 1 mM Tris-HCl (pH 8.1)–0.1 mM EDTA buffer, and stored at −20°. T4, T7, and λ DNAs were prepared by phenol and chloroform–octanol (9:1, v/v) extractions as described by Fuchs *et al.* (1967). *Pseudomonas* BAL-31 DNA was prepared according to the method of Marmur (1961).

^{3}H-Labeled PM2 DNA. Pseudomonas BAL-31 was grown in 3 l. of NTC buffer supplemented with 0.5% glucose and 0.2% casamino acids to a density of 3×10^8/ml and infected with PM2 phage at a multiplicity of 5–10. At 20 min post-infection, 0.5 mCi of [5-^{3}H]thymidine (Schwarz BioResearch Co.) was added to the culture, followed by another 0.5 mCi of [5-^{3}H]thymidine at 30 min post-infection. After purification of the phage and extraction of the DNA by the method described above, the labeled DNA had a specific activity of 16,000 cpm/μg.

Separation of PM2 Forms I and II DNA. DNA, CsCl (Harshaw), and ethidium bromide (Calbiochem) were mixed together in final concentrations of 20–30 μg/ml, 1.55 g/cm^3 (5.5 *m*), and 150 μg/ml, respectively. The DNA was separated into forms I (superhelical) and II (relaxed circular) by centrifugation for 24 hr at 43,000 rpm in a Spinco 50 rotor at 10°. The lower band (form I) and the upper band (form II) were removed separately by puncture of the nitrocellulose tube from the side with a 22 gauge hypodermic needle.

The ethidium bromide was removed by passing the DNA through a 0.9 × 6 cm Dowex-50 (Bio-Rad, AG 50W-X4, 200–400 mesh) cation exchange column which had been previously washed with 20 vol of 1 M Tris-HCl (pH 8.1), and equilibrated with 1 M NaCl–0.02 M Tris-HCl (pH 8.1). (It is important that the resin be rigorously washed with a cycle of 1 N HCl, H_2O, 1 N NaOH, and H_2O prior to this equilibration step.) The DNAs were dialyzed overnight against two changes of 600 vol of 1 mM Tris (pH 8.1)–0.1 mM EDTA, and stored at −20°.

Homogenization and Differential Centrifugation. One hundred grams of packed, frozen *Pseudomonas* BAL-31 bacterial pellets was chopped into small pieces, suspended in 100 ml of buffer A_1 to thaw, and homogeneously mixed with 300 ml of 0.1-mm glass beads (Edmund Bühler, Tübigen). The cells were disrupted by shaking for 6 or 10 min at 0°, in 125- or 200-ml stainless steel beakers, respectively, at 400 vibrations/sec in a Vibrogen cell mill (Edmund Bühler, Tübigen). The glass beads were removed by suction filtration on a sintered glass funnel and washed with approximately 300 ml of buffer A_1. The resulting homogenate was centrifuged at 20,000 rpm for 20 min at 0° in a Sorvall SS-34 rotor. The clear supernatant was carefully removed and the pellets were twice resuspended in 40 ml of buffer A_1 and centrifuged as above. The supernatants were combined to give the "crude extract." This was centrifuged in the Spinco 42 rotor for 1.5 hr at 40,000 rpm at 2° to yield the "high-speed supernatant."

DEAE-Cellulose Column Chromatography. The high-speed supernatant was treated with 5 μg/ml of DNase I (Worthington) for 30 min at 4°, and loaded onto a 5 × 25 cm column of DEAE-cellulose (0.98 mequiv/g, Type 20, Schleicher & Schuell) which had been previously equilibrated with buffer B + 0.1 M NaCl. The unadsorbed protein was washed through the column with 800 ml of buffer B + 0.1 M NaCl at a flow rate of 10 ml/min. RNA polymerase was eluted from the column with 600 ml of buffer B + 0.17 M NaCl at a flow rate of 5 ml/min. After elution of the enzyme the column was washed with 600 ml of buffer B + 0.27 M NaCl to ensure that no significant amounts of enzyme remained bound to the column. Polymerase-containing fractions having at least one-half the specific activity of the peak fraction were pooled from the 0.17 M NaCl eluent ("DEAE fraction").

Ammonium Sulfate Precipitation. To the DEAE fraction an equal volume of ammonium sulfate solution (Schwarz/Mann, enzyme grade), saturated at 0° in buffer A_1 lacking NaCl, pH adjusted to 8.1, was added slowly, and with stirring, to give a 50% saturated solution. After standing at 0° for 1 hr, the precipitate was removed by centrifugation for 30 min at 20,000 rpm at 0° (Sorvall SS-34 rotor). The supernatant was brought to 60% saturated ammonium sulfate by adding a volume of saturated ammonium sulfate solution equal to one-half the original volume

of the DEAE fraction, allowed to stand at 0° for 1 hr, and centrifuged as before. The precipitate, containing RNA polymerase, was dissolved in approximately 5 ml of buffer C ("60% ammonium sulfate fraction").

Low Salt Sucrose Density Gradient Centriguation. The 60% ammonium sulfate fraction was dialyzed twice against 600 vol of buffer C for 1.5 hr each time. The dialyzed enzyme was then diluted to a concentration of 20 mg/ml or less with buffer C and 3-ml aliquots were layered onto 55 ml of 7–25% (w/v) sucrose gradients in buffer C. The gradients were centrifuged in a Spinco SW25.2 rotor for 24 hr at 25,000 rpm at 2°, collected into 1.0-ml fractions, and assayed for enzyme activity. The enzyme-containing fractions having at least one-half the peak specific activity were pooled and precipitated with 70% saturated ammonium sulfate. The precipitate was dissolved in buffer A_2 to give the "low salt fraction."

High Salt Sucrose Density Gradient Centrifugation. The low salt fraction was adjusted to 20 mg/ml of protein or less with buffer A_2 and 1-ml aliquots were layered onto three 32-ml 7–25% (w/v) sucrose gradients in buffer A_2. The gradients were centrifuged at 25,000 rpm for 24 hr in the SW25.1 rotor at 2°, collected into 0.75-ml fractions, and assayed for enzyme activity. Only the constant specific activity peaks were pooled and precipitated with 70% saturated (at 0°) ammonium sulfate. The precipitate was dissolved in approximately 1 ml of buffer A containing 50% glycerol (w/v) and stored at −20°. This is the "high salt fraction."

Standard Polymerase Assay. For enzyme assays during the purification procedure, each 0.1 ml of mixture contained 30 mM Tris-HCl (pH 8.2 at 25°), 10 mM $MgCl_2$, 150 mM KCl, 0.5 mM P_i, (to inhibit polynucleotide phosphorylase), 0.5 mM each of GTP, CTP, UTP (P-L Biochemicals), and [^{14}C]ATP (0.5 Ci/mol, Schwarz), 10 μg of calf thymus DNA (Worthington), and enzyme as indicated. The reaction mixtures were incubated for 20 min at 28°, chilled on ice, and diluted with 1 ml of ice cold distilled water. Nucleic acids were precipitated by the addition of 1.1 ml of ice cold 10% trichloroacetic acid, and collected by suction filtration on glass fiber filters. The filters were washed three times with ~10 ml of cold 5% Cl_3CCOOH, one time with cold 95% ethanol, and dried, and the radioactivity measured in a liquid scintillation spectrometer. One enzyme unit equals the amount of enzyme which will incorporate 1 nmol of [^{14}C]AMP with calf thymus DNA as template in 20 min at 28°.

Standard Transcription Mixture. For studies with purified polymerase, the "standard polymerase assay" mixture was used with the following modifications. Inorganic phosphate was deleted, the pH of the Tris-HCl solution was 8.3, and the $MgCl_2$ concentration was 30 mM. Reaction volumes and DNA and enzyme concentrations were varied as indicated in the appropriate figure or table legend.

Assay of Enzymatic Impurities. DNase. Exonuclease and large amounts of endonuclease activities in RNA polymerase were assayed by the appearance of Cl_3CCOOH soluble radioactivity from ^{3}H-labeled T4 DNA. Twenty micrograms of ^{3}H-labeled T4 DNA (3600 cpm/μg) was incubated with 20 μg of enzyme in 100 μl of standard polymerase reaction mixture, lacking ribonucleoside triphosphates and calf thymus DNA, for 6 hr at 28°. The mixtures were chilled, diluted with 100 μl of 10% Cl_3CCOOH, and centrifuged, and 100 μl of the supernatant was assayed for radioactivity in Kinard's scintillation liquid (Kinard, 1957).

The conversion of form I to form II DNA was used as a more sensitive endonuclease assay. Five micrograms of ^{3}H-labeled PM2 DNA composed of approximately 33% form I (16,000 cpm/μg) was incubated in 100 μl of standard polymerase assay mixture, lacking ribonucleoside triphosphates and calf thymus DNA, for 2 hr at 28°. The reactions were then terminated by adding 10 μl of 1 N NaOH. The mixture was layered on a linear 5–20% (w/v) sucrose gradient in 0.1 M NaCl–0.1 N NaOH–0.001 M EDTA and centrifuged in the Spinco SW50 rotor for 1.5 hr at 45,000 rpm at 4°. Fractions were collected and assayed for radioactivity in Kinard's scintillation fluid. The presence of DNase was indicated by a decrease in the ratio of form I to form II DNA.

RNase. Exonuclease and large amounts of endonuclease were assayed in the same way as for exo-DNase activity except that 20 μg of *E. coli* ^{14}C-labeled rRNA (4700 cpm/μg) was substituted for the T4 DNA. A more sensitive endonuclease assay was the cleavage of phage R17 RNA. Assay tubes containing 2.5 μg of ^{3}H-labeled R17 RNA (38,000 cpm/μg) and 7 μg of enzyme were incubated for 1 hr at 28° in 25 μl of 10^{-2} M Tris-HCl (pH 7.5)–10^{-3} M EDTA. The RNA was analyzed by electrophoresis on 2.4% polyacrylamide–0.1% sodium dodecyl sulfate gels (Bishop *et al.*, 1967). The 0.5 × 9 cm gels were run for 90 min at 90 V, removed, frozen on Dry Ice, and cut into 1.5-mm sections. The RNA was solubilized by overnight incubation at 37° in 0.3 ml of NCS (Amersham/Searle) diluted 1:5 in toluene. Radioactivity was determined by counting in Kinard's scintillation liquid.

POLYNUCLEOTIDE PHOSPHORYLASE. This was assayed by the exchange of $^{32}P_i$ into ADP according to the method of Ochoa *et al.* (1963). Each assay tube contained 3×10^5 cpm of $^{32}P_i$ and 70 μg of RNA polymerase. Incubation was for 30 min at 28°.

POLYRIBOADENYLATE SYNTHETASE. RNA polymerase (22–35 μg) was added to 100 μl of standard reaction mixture containing 0.5 mM [^{14}C]ATP (2 Ci/mol) as the only ribonucleoside triphosphate. After incubation for 30 min at 28° the reaction was terminated by chilling on ice and Cl_3CCOOH insoluble radioactivity was determined as described above.

POLYPHOSPHATE KINASE. RNA polymerase (7–36 μg) was added to 100 μl of standard reaction mixture (minus calf thymus DNA) containing [γ-^{32}P]CTP (525 cpm/pmol) as the sole NTP. Incubation was for 1 hr after which Cl_3CCOOH precipitable radioactivity was determined.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. Proteins were analyzed on 0.5 × 9 cm gels, containing 5% acrylamide and 0.1% sodium dodecyl sulfate (SDS), prepared and run according to the method of Shapiro *et al.* (1967). Ten to twenty microgram samples of protein in 50 μl of a buffer containing 0.1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol, 0.01 M sodium phosphate (pH 7.1), 10% (v/v) glycerol, and 0.002% (w/v) Bromophenol Blue were heated at 65° for 10 min, cooled to room temperature, and layered onto the gels. Electrophoresis was for 3 hr at 60 V in 0.1 M sodium phosphate (pH 7.1) and 0.1% SDS. The gels were stained for 3 hr in a 0.025% solution of Coomassie Brilliant Blue in methanol–acetic acid–water (5:1:5), and destained in a diffusion destainer (Hoeffer Scientific Instruments) in 10% Cl_3CCOOH. Destaining was stopped by a 7.5% acetic acid–5% methanol solution. The gels were scanned at 550 nm on a recording spectrophotometer (Gilford Instrument Lab) at

Table I: Purification of DNA-Dependent RNA Polymerase from *Pseudomonas* BAL-31.[a]

Fraction	Total Protein (mg)	Total Units	Sp Act. (units/mg)	% Yield	Stability,[b] $t_{1/2}$ (Days)
Crude extract	5330	16,300	3.1	100	11
High-speed supernatant	4460	15,700	3.5	96	16
DEAE-cellulose	1150	15,100	13.1	92	6.0
60% $(NH_4)_2SO_4$	172	12,842	74.8	79	2.2
Low salt gradient	45.6	6,175	135	38	3
High salt gradient	7.2	3,425	479	21	5

[a] The figures are averages of seven enzyme preparations each from 100 g of packed cells (wet weight). [b] An aliquot of each fraction was left at 0° and assayed in the standard polymerase assay at 2-day intervals over a period of 10 days. The 60% $(NH_4)_2SO_4$ fraction was dialyzed into buffer C before assay since the enzyme is stable in its precipitated form.

two different chart speeds and the areas under the peaks were measured. The $\beta + \beta'$ areas were determined together since these peaks are poorly resolved.

Molecular Weight Determination. The molecular weights of the RNA polymerase subunits were determined by comparison of their electrophoretic mobility with those of proteins of known molecular weight. The standards were run in parallel gels in the same experiment. The BAL-31 polymerase was electrophoresed in duplicate, one alone and one with either bovine serum albumin or DNase I as internal standard. The molecular weights of the polymerase subunits were determined from the standard plot of mobility *vs.* log molecular weight.

Determination of Sedimentation Constants. For high ionic strength values, purified *E. coli* and *Pseudomonas* BAL-31 polymerases were dialyzed against buffer A_2 for 2 hr with one buffer change. Each enzyme (0.2 ml; 0.5 mg) was layered onto separate 12.5-ml, 7–25% (w/v) linear sucrose gradients in buffer A_2. Centrifugation was in the SW41 rotor at 37,000 rpm for 20 hr at 2°. Fractions were collected from the bottom of the tubes and assayed for enzyme activity by the "standard polymerase assay." Low ionic strength runs were carried out in the same manner but using buffer C for the dialysis and gradients and centrifuging for 24 hr. The *s* values were calculated from the position of *E. coli* polymerase, using values of 15.0 S for the monomer and 24 S for the dimer of this enzyme (Berg and Chamberlin, 1970), assuming constant velocity throughout the gradients.

E. coli DNA-dependent RNA polymerase was prepared as previously described (Millette and Trotter, 1970).

Results

Purification of Pseudomonas BAL-31 RNA Polymerase. Table I summarizes the purification of the enzyme according to the procedure described under Materials and Methods. The protein recovered in the final fraction is composed of >85% RNA polymerase as determined from SDS–polyacrylamide gel electrophoresis. This represents a 154-fold purification over the crude extract and a 21% yield of the original enzyme activity. Varying with the individual preparation and the strictures used when selecting the enzyme containing fractions, the purification and the yield obtained by this procedure have ranged from 117- to 187-fold and from 12 to 23%, respectively, and the purity of the final preparation has ranged from 75 to 97% RNA polymerase. The BAL-31 polymerase exhibits lowest stability at all stages of purification subsequent to DEAE-cellulose chromatography (Table I). However, the final enzyme preparation stored at −20° in buffer A_1 containing 50% glycerol has a half-life of approximately 60 days.

HOMOGENIZATION AND DIFFERENTIAL CENTRIFUGATION. Disruption of the bacteria produces a large amount of pink-orange flocculent material which may be bacterial membrane components. Failure to free the cell extract of this material hinders the fractionation of the enzyme on DEAE-cellulose and lowers the recovery in subsequent purification steps. Therefore, after the low-speed centrifugations of the crude extract it is best to draw off the clear supernatant fluid with a pipet, so as not to include any of the underlying pinkish debris. If this material is carried over to the high-speed centrifugation stage, it will not pack well on the ribosome pellet, and will contaminate the high-speed supernatant fraction.

DEAE-CELLULOSE COLUMN CHROMATOGRAPHY. When a linear salt gradient was used to elute the enzyme, only about 20–30% of the enzyme activity was recovered and up to 70% of this activity was lost upon storage of the enzyme overnight at 0°. This may have been caused by inactivation due to low protein concentration or to partial dissociation of the enzyme subunits. These difficulties were overcome when the enzyme was eluted with a NaCl step gradient as illustrated in Figure 1. A small amount of enzyme activity is lost in the flow-through fraction (0.1 M NaCl) whereas the bulk of the enzyme (>90%) elutes in the 0.17 M NaCl peak. A variable amount of polymerase activity remains bound to the column and is eluted in the 0.27 M fraction. The presence of the flocculent material at this

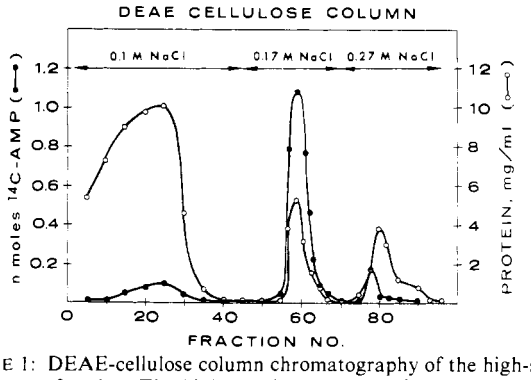

FIGURE 1: DEAE-cellulose column chromatography of the high-speed supernatant fraction. The high-speed supernatant fraction, containing 4.9 g of protein, was adsorbed to a 5 × 25 cm DEAE-cellulose column which had been previously equilibrated with buffer B + 0.1 M NaCl. The column was washed and the enzyme eluted as described under Materials and Methods. Fractions of 20 ml were collected.

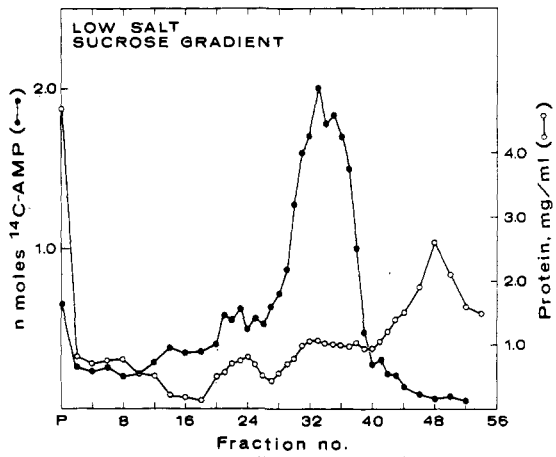

FIGURE 2: Low salt sucrose gradient sedimentation. A total of 53 mg of protein in 3.3 ml was applied to each of three 55-ml 7–25% sucrose gradients in buffer C. The gradients were centrifuged at 25,000 rpm in a Spinco SW25.2 rotor for 24 hr at 2°. Fractions of 1.0 ml were collected by pumping from the bottom of the tube. Fractions 29–38 were pooled along with similar fractions from the other two gradients.

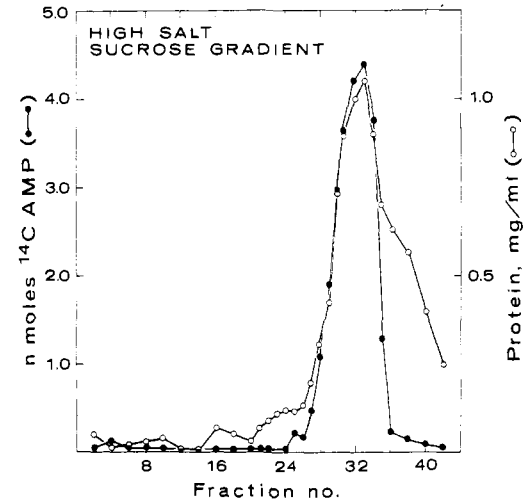

FIGURE 3: High salt sucrose gradient sedimentation. A total of 6 mg of protein from the low salt sucrose gradient sedimentation step in 1.0 ml was layered on each of three 32-ml 7–25% sucrose gradients in buffer A_2. The gradients were centrifuged at 25,000 rpm in the Spinco 25.1 rotor for 24 hr at 2°. Fractions of 0.75 ml were collected by pumping from the bottom (fraction 1) of the tube, and assayed for enzyme activity. Fractions 29–34 were pooled.

stage causes a slight increase in the amount of enzyme eluting in the 0.27 M NaCl wash, a significant increase in the amount of enzyme appearing in the flow-through fraction, and an overall reduction in yield. The DEAE-cellulose step usually results in a four- to sixfold purification and an 85–100% recovery of the enzyme. Since the stability of the enzyme decreases markedly at this point (see Table I), the purification procedure should not be interrupted until the enzyme is precipitated in 60% ammonium sulfate after the DEAE step.

AMMONIUM SULFATE PRECIPITATION. Precipitation of the enzyme is best accomplished with a pH adjusted, saturated (at 0°) ammonium sulfate solution. The use of solid, finely ground ammonium sulfate resulted in consistently lower enzyme recoveries. Since the enzyme is inactivated by brief exposure to low pH, the loss may be due to localized regions of low pH produced during the dissolution of the solid ammonium sulfate. The vigorous stirring required when solid ammonium sulfate is used may also result in losses due to surface denaturation. The enzyme is quite stable if left in the precipitated form at this stage.

LOW SALT SUCROSE GRADIENT SEDIMENTATION. The sedimentation profile for the enzyme in a 7–25% (w/v) sucrose gradient in buffer C is shown in Figure 2. Under these ionic conditions (μ = 0.065) the enzyme sediments with a peak at 22 S (see below), ahead of the bulk of the contaminating proteins. Although this step removes most of the contaminating proteins, it results in a relatively low-fold (1.8×) purification. This can be accounted for by loss of activity due to the inherent instability of the enzyme at this point. The reason for the instability is unknown. However, it does not seem to be due to the buffer conditions or to protease contamination, as the enzyme remains unstable over a wide range of salt concentrations (0.05–1.0 M NaCl) and pH (6.5–9.5), and it is not stabilized by the addition of 1 mg/ml of bovine serum albumin. Also, substrate protection of the enzyme (with 0.05 mM ATP and 0.05 mM GTP present in the sucrose gradients) did not increase the recovery of enzyme activity.

HIGH SALT SUCROSE GRADIENT SEDIMENTATION. This procedure, first used by Zillig *et al.* (1970b), takes advantage of the fact that this enzyme, like *E. coli* RNA polymerase (Richardson, 1966; Zillig *et al.*, 1966; Berg and Chamberlin, 1970), sediments as a monomer at 15 S under conditions of high ionic strength (μ = 0.65). Thus, most of the contaminating proteins which cosediment with the 22S form in the low salt gradient are removed in this step. Figure 3 depicts the sedimentation profile obtained by this method. Only those fractions having essentially the same maximum specific activity are pooled. The average purification achieved is a 3.5-fold increase in specific activity over the previous fraction. The sedimentation profile (Figure 3) is that obtained when the enzyme was selected for maximum specific activity in the previous steps. When the enzyme is selected for high yields at each step, more contaminating protein is found in the fractions preceding the enzyme peak.

Enzymatic Purity. RNA polymerase prepared by this method is generally free of contaminating enzyme activities which can interfere with studies of RNA synthesis (see Materials and Methods). No RNase or DNase activities could be detected as assayed by the cleavage of ^{3}H-labeled R17 RNA and the nicking of PM2 form I DNA, respectively. Polynucleotide phosphorylase activity in the final enzyme fraction is negligible; with 70 μg of polymerase, <0.03 nmol of $^{32}P_i$ was exchanged with ATP. Traces of polyphosphate kinase have been found in some polymerase preparations. For example, with 36 μg of RNA polymerase ≤0.2 pmol of ^{32}P was incorporated from γ-[^{32}P]CTP. Therefore, each preparation should be carefully monitored for this contaminant, and any residual polyphosphate kinase activity can be inhibited by including 0.1 mM ADP in the reaction mixture.

The *Pseudomonas* BAL-31 polymerase is capable of synthesizing poly(A) from ATP in the absence of the other three triphosphates (data not shown). This enzyme activity has been found in all of our polymerase preparations. It increases linearly with increasing polymerase concentration, and is equal to approximately $^{1}/_{12}$ of the DNA-dependent RNA polymerase activity. Although this activity is independent of DNA, it is stimulated 1.5-fold by PM2 II DNA (50 μg/ml). The polyriboadenylate synthetase activity is only slightly inhibited by CTP, but when both CTP and GTP are present, the synthetase activity is inhibited >80%.

Subunit Composition and Molecular Weights. If we

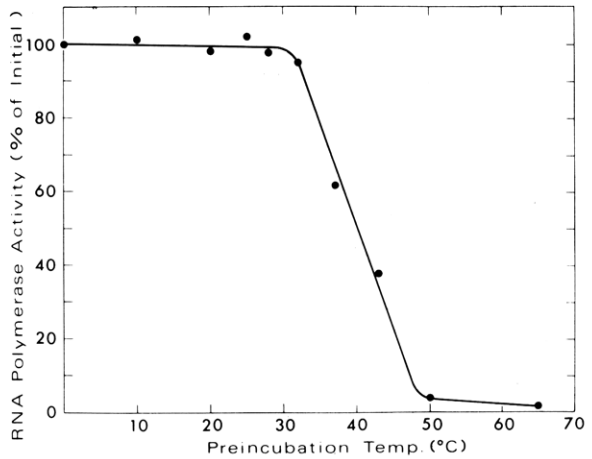

FIGURE 4: Thermal inactivation of *Pseudomonas* BAL-31 polymerase. Purified RNA polymerase (4.2 μg) in 100 μl of the standard polymerase assay mixture lacking DNA was incubated for 10 min at the indicated temperatures. PM2 DNA (10 μg) was then added, followed by a 20-min incubation at 28°. The reaction was stopped by chilling on ice and the samples were assayed as described under Materials and Methods. One hundred per cent activity represents the incorporation of 6.03 nmol of [^{14}C]AMP in 20 min.

adopt the nomenclature proposed for the protein subunits of *E. coli* polymerase (Burgess *et al.*, 1969; Burgess, 1969b), the *Pseudomonas* BAL-31 polymerase is also seen to consist of β', β, σ, and α subunits (Figure 4). The β', β, and α subunits have essentially the same electrophoretic mobility as those of *E. coli* polymerase (Herzfeld and Zillig, 1971), while the σ subunit of *Pseudomonas* BAL-31 polymerase is about 5% larger than that of *E. coli*.

As with RNA polymerase from *E. coli*, the BAL-31 σ subunit can be separated from the core enzyme by phosphocellulose column chromatography (Burgess, 1969a). However, the BAL-31 polymerase is not as strongly retained on the column as is the *E. coli* enzyme, and thus appreciable amounts of holoenzyme, in addition to σ, appear in the phosphocellulose flow-through fraction. This necessitates further purification of σ such as by glycerol gradient centrifugation (Burgess *et al.*, 1969). The minimal enzyme, which by polyacrylamide–SDS gel electrophoresis is seen to consist of only the β', β, and α subunits, can transcribe calf thymus DNA as well as the holoenzyme, but its ability to read PM2 DNA is greatly reduced (Figure 5). Readdition of the σ containing glycerol gradient fraction to the core enzyme results in a fourfold stimulation of transcription of PM2 DNA (data not shown). The BAL-31 σ subunit can also be separated from the core enzyme by chromatography on hydroxylapatite. Thus, in contrast to the *E. coli* K12 polymerase, the *Pseudomonas* BAL-31 σ seems to be less tightly bound to the core. This has also been observed for the *E. coli* B RNA polymerase (King and Nicholson, 1971).

The molecular weights of the enzyme subunits have been determined by the method of Shapiro *et al.* (1967) by electrophoresis with known standards on 5% polyacrylamide–0.1% SDS gels (Figure 6). The standards fall on a straight line within the 30,000–160,000 molecular weight range. From these data the molecular weights determined for the *Pseudomonas* BAL-31 polymerase subunits are: β' = 165,000; β = 155,000; σ = 89,000; α = 38,000.

The molar ratios of the enzyme subunits were determined from densitometer tracings of Coomassie blue stained polyacrylamide gels as described under Materials and Methods.

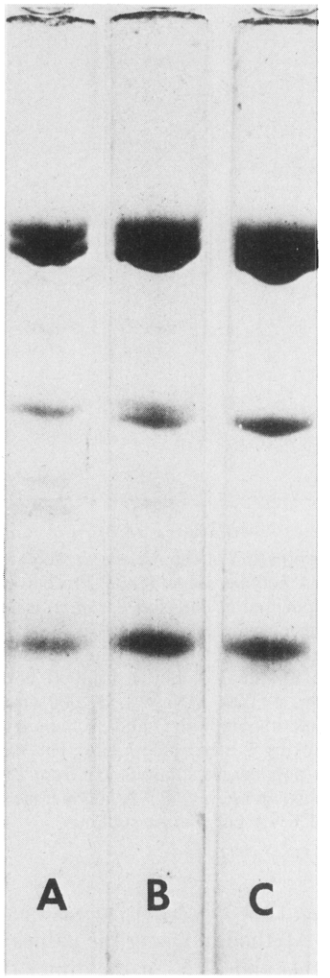

FIGURE 5: Subunit structure of *Pseudomonas* Bal-31 and *E. coli* polymerases. Polyacrylamide gels containing 5% acrylamide and 0.1% SDS were prepared and stained as described under Materials and Methods. Electrophoresis was for 3 hr at 60 V: (A) 12 μg of *Pseudomonas* BAL-31 polymerase; (B) 6 μg of *Pseudomonas* BAL-31 polymerase + 7 μg of *E. coli* polymerase; (C) 14 μg of *E. coli* polymerase.

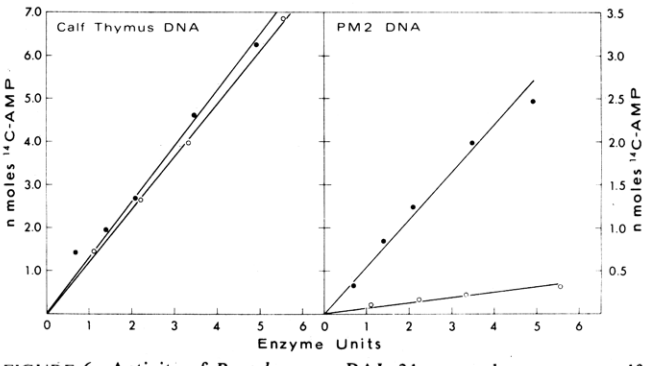

FIGURE 6: Activity of *Pseudomonas* BAL-31 core polymerase on calf thymus and PM2 DNA. Equal units of core polymerase (373 U/mg) or holoenzyme (528 U/mg) were assayed in 100 μl of standard polymerase assay mixture containing either 10 μg of calf thymus DNA or 4 μg of PM2 I DNA. Incubation was for 20 min at 28°: (●) holoenzyme; (○) core polymerase.

From an average of five separate enzyme preparations, setting $\beta + \beta' = 2$, these values are: $(\beta + \beta'):\sigma:\alpha$ = 2.0:0.78:2.3.

The sedimentation constants for *Pseudomonas* BAL-31 RNA polymerase were determined at low (μ = 0.065) and high (μ = 0.75) ionic strengths by sucrose gradient centrif-

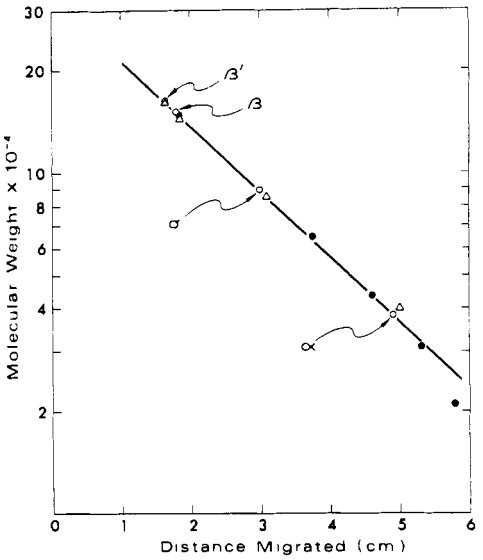

FIGURE 7: Determination of the molecular weights of the *Pseudomonas* BAL-31 RNA polymerase subunits. Proteins of known molecular weight as well as purified *E. coli* and *Pseudomonas* BAL-31 RNA polymerases were electrophoresed on 5% polyacrylamide gels as described under Materials and Methods. The marker proteins and molecular weights were: immunoglobulin G, 150,000; bovine serum albumin, 65,000; ovalbumin, 44,000; DNase I, 31,000 (monomer); α-chymotrypsin, 21,600. Immunoglobulin G and α-chymotrypsin were prepared in SDS buffer lacking 2-mercaptoethanol. The molecular weights of the *E. coli* RNA polymerase subunits are from Zillig *et al.* (1970a): (●) marker proteins; (△) *E. coli* RNA polymerase subunits; (○) *Pseudomonas* BAL-31 RNA polymerase subunits.

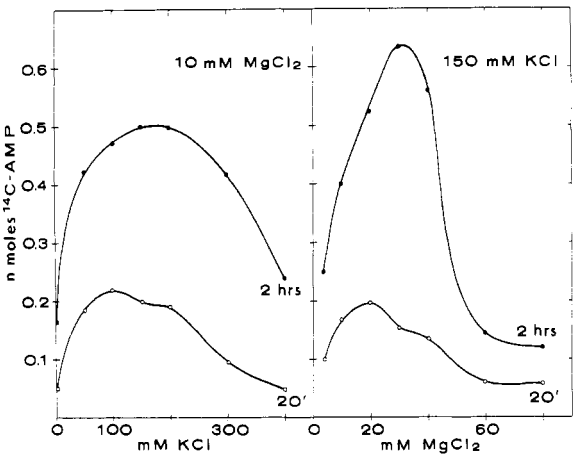

FIGURE 8: Effect of KCl and $MgCl_2$ on *Pseudomonas* BAL-31 RNA polymerase activity. Each polymerase assay mixture (100 μl) contained 7 μg of *Pseudomonas* BAL-31 RNA polymerase and 10 μg of PM2 I DNA. The other components were as described under Materials and Methods for the standard transcription mixture. Ten-microliter samples were taken at different times of synthesis, and the 20-min and 2-hr values are plotted. Each point is the average of three separate determinations.

ugation with *E. coli* RNA polymerase as a standard (see Materials and Methods). Using the values of 15.0 S for the monomer (low μ) and 24 S for the dimer (high μ) of the *E. coli* enzyme (Berg and Chamberlin, 1970), we have calculated sedimentation ceofficients of 14.8 S, at low ionic strength, and 22 S, at high ionic strength, for the *Pseudomonas* BAL-31 RNA polymerase. Thus, like the *E. coli* polymerase, this enzyme appears to sediment as a dimer at low ionic strength and as a monomer at high ionic strength. The similarity of the sedimentation coefficients for the two polymerases indicates that the *Pseudomonas* BAL-31 polymerase has a molecular weight very close to that of the *E. coli* enzyme (4.7×10^5 daltons, Berg and Chamberlin, 1970). This observation, along with the molecular weights and molar ratios of the subunits, specifies a subunit composition of $\beta'\beta\alpha_2\sigma$ for the holoenzyme and $\beta'\beta\alpha_2$ for the core enzyme. A summation of the subunit molecular weights yields molecular weights of 396,000 daltons for *Pseudomonas* BAL-31 core polymerase and 485,000 daltons for the holoenzyme.

Requirements for Optimal Enzyme Activity. Pseudomonas BAL-31 polymerase, like other DNA-dependent RNA polymerases, has an absolute requirement for DNA, a divalent cation, and all four ribonucleoside triphosphates. Its activity is further stimulated by increasing the ionic strength with both monovalent and divalent cations (see below). Although potassium is the cation most frequently used in transcription studies, sodium or ammonium ions work almost as well giving at least 90% of the incorporation seen with K^+. Manganese can be substituted for Mg^{2+} at an optimal concentration of 3.5 mM whereas higher Mn^{2+} concentrations cause the DNA to precipitate. The enzyme does not require bovine serum albumin or mercaptoethanol for optimal enzyme activity. Bovine serum albumin has been shown to stimulate *Bacillus subtilis* RNA polymerase activity (Avila *et al.*, 1971) and to be required for T7 polymerase activity (Chamberlin and Ring, 1973).

The enzyme has a broad pH optimum between 8.0 and 9.5 with both PM2 and calf thymus DNA templates. Even at pH values of 7.5 and 10 the activity is still 70% of maximum.

Thermal Inactivation and Temperature Optimum. The thermal inactivation curve for the BAL-31 RNA polymerase is depicted in Figure 7. No loss of enzyme activity is detected until the preincubation temperature exceeds 30°. A 10-min preincubation at 40° destroys 50% of the enzyme activity, while temperatures of 50° or greater completely inactivate the enzyme.

The temperature optimum for *Pseudomonas* BAL-31 polymerase activity is 28–32°, with PM2 I DNA as template in the standard transcription mixture. Therefore, for transcription studies, we have used an incubation temperature of 28° to avoid possible heat inactivation of the enzyme (see Figure 4). This optimal temperature for BAL-31 RNA polymerase activity *in vitro* coincides with the optimal temperature for growth of the bacterium (Espejo and Canelo, 1968a).

Effect of Salt Concentration on Enzyme Activity. As with the DNA-dependent RNA polymerase from *E. coli* (Fuchs *et al.*, 1967), the synthesis of RNA by *Pseudomonas* BAL-31 polymerase is markedly dependent on the salt concentration. Figure 8 shows the effect of varying both the KCl and $MgCl_2$ concentrations on short (20 min) and long (2 hr) term synthesis using PM2 I DNA as template. At a constant $MgCl_2$ concentration of 10 mM, there is a three- to fourfold stimulation of transcription by KCl with optima at 100 mM for short and 150 mM for long term synthesis. At a constant KCl concentration of 150 mM, the enzyme shows a $MgCl_2$ optimum at 20 mM for short term and a sharp optimum at 30 mM for long term synthesis. Thus, Mg^{2+} provides further stimulation of synthesis at the optimum monovalent ion concentration and promotes more linear synthesis kinetics. This was previously observed for the *E. coli* enzyme (Fuchs *et al.*, 1967). The most linear and continuous rate of RNA synthesis is thus achieved at 150 mM KCl and 30 mM $MgCl_2$. However, at all salt concentrations shown

Table II: Inhibitors of *Pseudomonas* BAL-31 RNA Polymerase Activity.

Inhibitor	μg of Enzyme	Inhibitor Concn (M) 50% Inhibition	Inhibitor Concn (M) Complete Inhibition
Rifampicin	7.0	3.5×10^{-7}	1.4×10^{-6}
Streptolydigin	5.6	7.5×10^{-6}	9×10^{-5}
Ethidium bromide	7.2	8.6×10^{-6}	2.8×10^{-5}
Mercaptoethanol	9.0	7×10^{-3}	1.4×10^{-2}
Actinomycin D[a]	7.0		10 μg/ml
Heparin[a]	7.2		10 μg/ml

[a] Inhibition curves not run. In each standard transcription mixture of 100 μl, described under Materials and Methods, 5 μg of PM2 I DNA was used. Except for actinomycin D and heparin, varying amounts of each inhibitor were added to each reaction. Synthesis was for 20 min at 28°.

Table III: Activity of RNA Polymerase on Various DNA Templates.[a]

DNA	nmol of [^{14}C]AMP Incorpd *P.* BAL-31 Polymerase	nmol of [^{14}C]AMP Incorpd *E. coli* Polymerase
Calf thymus	0.525	0.368
PM2 I	1.69	1.18
PM2 II	0.802	0.524
T4	1.47	2.16
T7	3.64	4.22
BAL-31	0.381	
λ_c	0.856	

[a] Each tube contained the standard transcription mixture with 3 μg of the indicated DNA and either 2.4 μg of *Pseudomonas* BAL-31 or 1.2 μg of *E. coli* RNA polymerase in a volume of 50 μl. Incubation was for 20 min at 28°.

(Figure 8) "plateau-type" kinetics, such as seen with T4 DNA transcription at low ionic strength (Fuchs *et al.*, 1967), are not observed. At higher salt concentrations ($\mu \geq 0.35$) enzyme activity is strongly inhibited. The residual activity observed at the highest salt concentrations may be due to the fact that salts were added last to the reaction mixtures. This would allow some enzyme molecules to initiate at 0°, thereby escaping salt inhibition (Fuchs *et al.*, 1967).

These data show that the ionic strength optima differ for short and long term synthesis. For 20 min of synthesis the ionic strength optima are 0.16 when KCl is varied and 0.24 when $MgCl_2$ is varied. For 2 hr synthesis the optima are 0.24 when KCl is varied and 0.27 when $MgCl_2$ is varied. This shift in ionic strength optima with longer incubation time was reported earlier for T4 DNA transcription by *E. coli* polymerase (Fuchs *et al.*, 1967).

Inhibitors of Pseudomonas BAL-31 RNA Polymerase Activity. The same compounds which inhibit the *E. coli* RNA polymerase also inhibit the *Pseudomonas* BAL-31 enzyme. Table II lists the various inhibitors tested and the concentrations required to give 50 and 100% inhibition. The concentrations of streptolydigin and ethidium bromide required to give 50% inhibition are similar to those reported for *E. coli* polymerase (Cassani *et al.*, 1970; Richardson, 1973). However, the concentration of rifampicin needed to inhibit this enzyme appears to be about tenfold higher than for the *E. coli* enzyme (Hartmann *et al.*, 1967; Cassani *et al.*, 1970). With 7 μg of *Pseudomonas* BAL-31 polymerase no RNA synthesis can be detected in the presence of 10 μg/ml of actinomycin D or heparin.

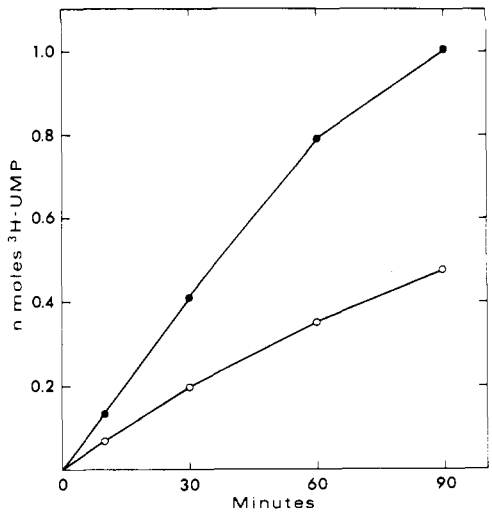

FIGURE 9: Time course of RNA synthesis on PM2 I and PM2 II DNA. A 60-μl standard transcription mixture contained [^{3}H]UTP (2840 cpm/nmol) as the labeled substrate, 4.5 μg of either PM2 I or PM2 II DNA, and 3.2 μg of *Pseudomonas* BAL-31 RNA polymerase. Incubation was at 28°. Aliquots of 10 μl were taken at the times indicated, diluted into 0.5 ml of H_2O (0°), and assayed for incorporated radioactivity by Cl_3CCOOH precipitation (Materials and Methods): (●) PM2 I directed synthesis; (○) PM2 II directed synthesis.

Template Preferences. The activity of *Pseudomonas* BAL-31 polymerase with nonlimiting amounts of various DNA templates is compared with that of *E. coli* polymerase in Table III. In general, both enzymes show a similar pattern of template preferences with the DNAs tested. Maximal activity for both polymerases is on T7 DNA, and both show a twofold greater activity on PM2 I than on PM2 II. *Pseudomonas* BAL-31 polymerase shows similar activities on PM2 I (homologous template) and T4 DNA (heterologous template), while *E. coli* polymerase shows a definite preference for T4 over PM2 I DNA.

Transcription of PM2 DNA. We have shown above that PM2 I DNA supports continuous transcription *in vitro* over a wide range of salt concentrations and that the form I DNA is a more active template than form II. To further elucidate these differences in template activity we have analyzed the kinetics of transcription of the two circular forms of phage PM2 DNA (Figure 9). Both templates show initially linear synthesis which continues for at least 90 min. However, throughout the time course the rate of transcription of the supercoiled DNA (form I) is 2.2-fold greater than that of the relaxed DNA (form II).

Discussion

We have described a method for obtaining the DNA-dependent RNA polymerase from *Pseudomonas* BAL-31 in highly purified form. The procedure was designed to overcome enzyme losses due to the presence of large amounts of flocculent material, presumably membrane components, in

the cell homogenate. It utilizes a DEAE-cellulose batch elution procedure (Burgess, 1969a) in order to avoid inactivation of this enzyme which occurs with a linear salt gradient elution, plus low and high salt sucrose gradient centrifugations (Zillig *et al.*, 1970b). The latter technique utilizes the reversible aggregation behavior of the polymerase (Richardson, 1966; Zillig *et al.*, 1966) to free it of contaminating proteins which do not exhibit this property. Thus, at high ionic strength (μ = 0.75) the *Pseudomonas* BAL-31 polymerase sediments as a monomer at 15 S, while at low ionic strength (μ = 0.065) it behaves as a 22S dimer. This seems to be a general feature of RNA polymerases from prokaryotes (Zillig *et al.*, 1966; Lee-Huang and Warner, 1969; Herzfeld and Zillig, 1971). Further purification by hydroxylapatite chromatography, although an excellent purification step for *E. coli* RNA polymerase (Richardson, 1966), is unsuitable for the BAL-31 enzyme in that it causes dissociation of the σ subunit. In the final stage, the enzyme is purified 154-fold over the crude extract and is free of contaminating enzyme activities which could interfere with transcription studies.

Like all bacterial DNA-dependent RNA polymerases studied thus far, this enzyme consists of four subunits assembled in the stoichiometry of $\beta'\beta\alpha_2\sigma$. The core subunits ($\beta'$, β, and α) have essentially the same molecular weights as those of *E. coli* polymerase while the σ subunit is somewhat larger (89,000 *vs.* 85,000). Differences in the size of σ factors have been observed with other bacterial RNA polymerases (Johnson *et al.*, 1971; Avila *et al.*, 1971; Hermoso *et al.*, 1972).

The enzymatic properties of *Pseudomonas* BAL-31 RNA polymerase are very much the same as those of other bacterial DNA-dependent RNA polymerases. However, several differences are worth mentioning. We have shown that *Pseudomonas* BAL-31 RNA polymerase is able to synthesize poly(A) in the absence of DNA. This is like the RNA polymerases from *Micrococcus lysodeikticus*, *Pseudomonas indigofera*, and *Azotobacter vinlandii* (Fox *et al.*, 1963; Tani *et al.*, 1968; Krakow, 1968) but in contrast to the *E. coli* polymerase which requires DNA for poly(A) synthetase activity (Chamberlin and Berg, 1962). Poly(A) synthesis by *Pseudomonas* BAL-31 polymerase is inhibited by GTP, but not by CTP, while with the other bacterial polymerases this reaction is inhibited strongly by either GTP or CTP (Fox *et al.*, 1963; Tani *et al.*, 1968; Krakow, 1968).

The *Pseudomonas* BAL-31 RNA polymerase exhibits a temperature optimum and thermal stability consistent with its marine origin. It has optimal activity at 28° and is 50% inactivated by a 10-min exposure to 41°. The bacterium itself has an optimal growth temperature at 28° and will not grow above 35°. It is of interest to compare this with other RNA polymerases of bacteria from different thermal environments. The RNA polymerase from the enteric bacterium, *E. coli*, has an optimal temperature of 37° and is 50% inactivated by heating at 55° for 10 min (Remold-O'Donnell and Zillig, 1969). The polymerase from *Bacillus stearothermophilus*, an organism indigenous to hot springs and having an optimal growth temperature of 45°, is 50% inactivated by a 10-min exposure to 65° (Remold-O'Donnell and Zillig, 1969). Thus it appears that RNA polymerases of bacteria from different thermal environments reflect their origin in terms of their temperature optima and thermal lability.

Most bacterial RNA polymerases usually show highest activity on homologous phage DNA templates. For instance the RNA polymerase from *P. putida* prefers the homologous bacteriophage gh-1 DNA (Johnson *et al.*, 1971), *B. subtilis* polymerase prefers ϕ29 DNA (Avila *et al.*, 1971), and the *E. coli* enzyme prefers T4 and T7 DNA (Table III) over DNA templates from nonhomologous sources. *Pseudomonas* BAL-31 polymerase differs in this respect in that it shows no strong preference for its homologous bacteriophage templates (Table III) and, in fact, exhibits template preferences similar to that of the *E. coli* enzyme.

One of the main purposes in isolating the *Pseudomonas* Bal-31 RNA polymerase was to utilize it in transcription studies with bacteriophage PM2 DNA. We have shown here that PM2 DNA is an efficient template for *in vitro* transcription by *Psuedomonas* BAL-31 polymerase. Furthermore, this transcription is sensitive to the usual inhibitors of RNA polymerase that block initiation (rifampicin and heparin) and chain elongation (streptolydigin). As in the transcription of homologous templates by other bacterial RNA polymerases, σ subunit is required for efficient transcription of PM2 DNA by this enzyme. Removal of σ by phosphocellulose or hydroxylapatite chromatography results in a ninefold reduction in enzyme activity with PM2 form I DNA.

We have been particularly interested in the effects of DNA supercoiling circularity and single-strand breaks on the various phases of the transcription process. PM2 DNA is particularly amenable to such studies since the supercoiled form I DNA of relatively low complexity (6×10^6 daltons) can be extracted in high yields from the virus. The relaxed circular molecules (form II) which may arise either spontaneously (through handling or endogenous DNases) or by controlled "nicking" with DNase I, may be easily separated by dye-buoyant density centrifugation. In the studies reported here, we have shown that both PM2 form I and form II DNA support initially linear and continuous RNA synthesis for at least 90 min. The significant difference between the two templates, however, is that the supercoiled molecule (form I) provides a twofold higher rate of synthesis. Such higher template activity of form I DNA has been observed for other circular duplex DNAs—ϕX174RF (Hayashi and Hayashi, 1971), SV40 (Westphal, 1971), and λ circles (Botchan *et al.*, 1973). The basis for the difference in transcription efficiencies of the two DNA forms and the effect of supercoiling on the initiation and termination of PM2 DNA transcription will be the subject of the following paper (Zimmer and Millette, 1975).

Acknowledgments

Thanks are due to Ann Nicholas for constructive criticism of the manuscript.

References

Avila, J., Hermoso, M., Vinñuela, E., and Salas, M. (1971), *Eur. J. Biochem. 21*, 526.

Berg, D., and Chamberlin, M. (1970), *Biochemistry 9*, 5052.

Bishop, D. H. L., Claybrook, J. R., and Spigelman, S. (1967), *J. Mol. Biol. 26*, 373.

Botchan, P., Wang, J. C., and Echols, H. (1973), *Proc. Nat. Acad. Sci. U.S. 70*, 3077.

Braunstein, S. N., Datta, A., and Franklin, R. M. (1971), *Virology 46*, 161.

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

Burgess, R. R. (1969b), *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. R., Burgess, R., and Goodman, H. M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 59.
Chamberlin, M., and Berg, P. (1962), *J. Mol. Biol. 8,* 708.
Chamberlin, M., and Ring, J. (1973), *J. Biol. Chem. 248,* 2235.
Espejo, R. T., and Canelo, E. S. (1968a), *J. Bacteriol. 95,* 1887.
Espejo, R. T., and Canelo, E. S. (1968b), *Virology 34,* 738.
Espejo, R. T., Canelo, E. S., and Sinsheimer, R. L. (1969), *Proc. Nat. Acad. Sci. U.S. 63,* 1164.
Fox, C. F., Robinson, W. S., and Weiss, S. B. (1963), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 22,* 463.
Franklin, R. M., Salditt, M., and Silbert, J. A. (1969), *Virology 38,* 627.
Fuchs, E., Millette, R. L., Zillig, W., and Walter, G. (1967), *Eur. J. Biochem. 3,* 183.
Hartmann, G., Honikel, K. O., Knüsel, F., and Nüesch, J. (1967), *Biochim. Biophys. Acta 145,* 843.
Hayashi, Y., and Hayashi, M. (1971), *Biochemistry 10,* 4212.
Hermoso, J. M., Avila, J., Jimenez, F., and Salas, M. (1972), *Biochim. Biophys. Acta 277,* 280.
Herzfeld, F., and Zillig, W. (1971), *Eur. J. Biochem. 24,* 242.
Johnson, J. C., De Backer, M., and Boezi, J. A. (1971), *J. Biol. Chem. 246,* 1222.
Kinard, F E. (1957), *Rev. Sci. Instrum. 28,* 293.
King, A. M. Q., and Nicholson, B. H. (1971), *J. Mol. Biol. 62,* 303.
Krakow, J. S. (1968), *Biochim. Biophys. Acta 166,* 459.
Lee-Huang, S., and Warner, R. C. (1969), *J. Biol. Chem. 244,* 3793.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Marmur, J. (1961), *J. Mol. Biol. 3,* 208.
Millette, R. L., and Trotter, C. D. (1970), *Proc. Nat. Acad. Sci. U.S. 66,* 701.
Ochoa, S., Krakow, J. S., and Basilico, C. (1963), *Methods Enzymol. 6,* 3.
Remold-O'Donnell, E., and Zillig, W. (1969), *Eur. J. Biochem. 7,* 318.
Richardson, J. P. (1966), *Proc. Nat. Acad. Sci. U.S. 55,* 1616.
Richardson, J. P. (1973), *J. Mol. Biol. 78,* 703.
Salditt, M., Braunstein, S. N., Camerini-Otero, R. D., and Franklin, R. M. (1972), *Virology 48,* 259.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.
Tani, T., McFadden, B., Homann, H. R., and Shishiyama, J. (1968), *Biochim. Biophys. Acta 161,* 309.
von der Helm, K., and Zillig, W. (1969), *FEBS Lett. 3,* 76.
Westphal, H. (1971), *in* Lepetit Colloquium on RNA Polymerase and Transcription, Silvestri, L., Ed., Amsterdam, North-Holland Publishing Co., p 77.
Zillig, W., Fuchs, E., and Millette, R. (1966), *Procedures Nucleic Acid Res.,* 323.
Zillig, W., Fuchs, E., Palm, P., Rabussay, D., and Zechel, E. (1970a), *in* Lepetit Colloquium on RNA Polymerase and Transcription, Silvestri, L., Ed., Amsterdam, North-Holland Publishing Co., p 151.
Zillig, W., Zechel, K., and Halbwachs, H.-J. (1970b), *Z. Physiol. Chem. 351,* 221.
Zimmer, S. G., and Millette, R. L., (1975), *Biochemistry,* following paper.