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Purification and Properties of DNA-Dependent RNA Polymerase from *Bacillus subtilis* Vegetative Cells

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A purification procedure to prepare highly purified DNA-dependent RNA polymerase from *Bacillus subtilis* vegetative cells is described. The enzyme consists of four different subunits, β', β, σ and α (molecular weights 154000 for β' and β, 56000 for σ and 43000 for α) in a molar ratio 1:1:1:2. By phosphocellulose chromatography RNA polymerase has been disscociated in σ subunit and core enzyme, containing β', β and α subunits in a molar ratio 1:1:2; the σ subunit stimulates the activity of core polymerase with φ 29 DNA but not with poly[d(A—T)]. The general properties of the enzyme are also described.

We have described recently the subunit composition of highly purified DNA-dependent RNA polymerase from *Bacillus subtilis* vegetative cells [1]. The enzyme consists of at least three different subunits, β, σ and α, with molecular weight values of approximately 150000, 55000 and 43000, respectively [1]. However, a detailed description of the purification procedure as well as a systematic study of the properties of the enzyme is lacking.

We describe here the purification procedure we have developed to obtain highly purified RNA polymerase from *B. subtilis*, the dissociation of the enzyme in σ subunit and core polymerase and the general properties of the enzyme.

MATERIALS AND METHODS

Chemicals

Alumina, polyethyleneglycol 6000, coomassie brilliant blue and DEAE-cellulose (0.76 mequiv./g) were obtained from Serva Feinbiochemica; dextran 500, bovine serum albumin, ovalbumin and immunoglobulin G from Sigma; ammonium sulfate (enzyme grade) from Mann; cellulose Munktell was purchased from BioRad Laboratories; phosphocellulose P11 (7.4 mequiv./g) was from Whatman; unlabelled ribonucleoside triphosphates were from P-L Biochemicals and [4-^{14}C]UTP from the Radiochemical Center (Amersham); sodium dodecylsulfate, obtained from Sigma, was recrystallized from 95% ethanol; acrylamide and *N*,*N*′-methylenebisacrylamide, obtained from Serva, were recrystallized as described by Loening [2]; β-galactosidase and calf thymus DNA were from Worthington; poly[d(A-T)] from Miles Laboratories and 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole (butyl-PBD) from CIBA. All other chemicals were reagent grade.

φ29 DNA and T4 DNA were prepared from purified φ29 (supplied by V. Rubio) and T4 phages by treatment with pronase (1 mg/ml) in the presence of 0.5% sodium dodecylsulfate and phenol extraction. Their sedimentation coefficients were 24 and 27 S, respectively. ^{14}C-labelled φ29 DNA was supplied by J. Ortín. *B. subtilis* DNA was prepared as described by Okamoto *et al.* [3]; its sedimentation coefficient was 32 S. ^{14}C-labelled R17 RNA was obtained by phenol extraction of purified ^{14}C-labelled R17 phage [4].

Bacteria

Bacillus subtilis 168 (leu$^-$ met$^-$ thr$^-$ su$^-$) was obtained from Dr. C. P. Georgopoulos. The cells were grown to the mid log phase in a medium containing the salts indicated by Anagnostopoulos and Spizizen [5] supplemented with 0.1 M NaCl, 0.01 mM $MnCl_2$, 20 mM D-glucose, 0.02% casein acid hydrolysate, 0.2 mM L-tryptophan, 0.05% yeast extract and 0.05% tryptone. The cells were kept at −20 °C till used.

Buffers

All the buffers were prepared with deionized and distilled water. Buffer I contained 10 mM Tris-HCl pH 8.4, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 1 mM EDTA. Buffer II contained 10 mM Tris-HCl pH 8.4, 5 mM 2-mercaptoethanol, 1 mM EDTA, 25 mM KCl and 15% (v/v) glycerol.

Unusual Abbreviation. Butyl-PBD, 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole.

Enzymes. DNA-dependent RNA polymerase or ribonucleoside triphosphate : RNA-nucleotidyl transferase (EC 2.7.7.6); pancreatic ribonuclease (EC 2.7.7.17); pancreatic deoxyribonuclease (EC 3.1.4.5); polynucleotide phosphorylase (EC 2.7.7.8).

Column Chromatography

DEAE-cellulose and phosphocellulose were treated as described by Burgess [6]. φ29 DNA-cellulose was prepared essentially as described by Alberts *et* al. [7] using native φ29 DNA at a concentration of about 2 mg/ml; 0.6 mg of φ29 DNA were adsorbed per ml of packed cellulose.

Protein Determination

Protein was determined by the procedure of Lowry *et al.* [8] using bovine serum albumin as standard. When the ratio of absorbance at 280 to 260 nm was greater than one, protein was also determined spectrophotometrically measuring the absorbance at 280 and 260 nm [9].

RNA Polymerase Assay

The assay mixture for the determination of RNA polymerase activity contained, in a total volume of 0.1 ml, the following components: 60 mM Tris-HCl pH 7.8, 12 mM $MgCl_2$, 50 mM NH_4Cl, 10 mM 2-mercaptoethanol, 0.1 mM ATP, GTP and CTP, 0.1 mM [^{14}C]UTP (specific activity 2.5), 10 μg of φ29 DNA and enzyme as indicated in each case. Where indicated, 50 μg of bovine serum albumin were added to the reaction mixture. The enzyme was diluted in buffer II containing 500 μg of bovine serum albumin per ml. After 10 min at 37 °C the incubation mixture was chilled, precipitated with cold 5% (w/v) trichloroacetic acid and filtered through discs of glass fiber paper (Whatman GF/C, 2.4 cm diameter). After drying, radioactivity in the samples was estimated either in a Nuclear Chicago gas-flow counter or in a Packard Tri-Carb scintillation spectrometer using as scintillation liquid a solution containing 4 g of butyl-PBD per liter of toluene.

Determination of Contaminating Enzymic Activities

DNAase. Exonuclease activity was assayed by determining the ethanol-soluble radioactivity of labelled DNA. ^{14}C-labelled φ29 DNA (0.05 μg, 65000 counts $\times$ min^{-1} $\times$ μg^{-1}) was incubated in the presence of 10 μg of purified RNA polymerase in the reaction mixture described for the RNA polymerase assay except for the absence of nucleoside triphosphates and DNA. After 1 h at 37 °C, the mixture was chilled in ice and the DNA was precipitated by addition of two volumes of absolute ethanol in the presence of cold φ29 DNA (25 μg) and 0.5 M NaCl. After 10 min in ice the mixture was centrifuged and the supernatant transferred to small glass vials (4.5 $\times$ 1.2 cm) containing a disc of glass fiber paper (Whatman GF/A, 2.4 cm diameter) touching the bottom of the vial. The vials were heated in an oven at 90 °C till dryness, and the radioactivity was determined in a scintillation spectrometer.

Endonuclease activity was determined by incubating 10 μg of RNA polymerase in the presence of native ^{14}C-labelled φ29 DNA as indicated before for the assay of exonucleolytic activity. After 1 h at 37 °C the DNA was denatured by addition of 0.1 M NaOH and the mixture was centrifuged in a 5—20% (w/v) sucrose gradient in 0.1 M NaOH—0.9 M NaCl—0.01 M EDTA for 2 h at 48000 rev./min in a SW50L rotor of the Spinco L250 ultracentrifuge at 0 °C.

RNAase. Exonuclease activity was determined by incubation of ^{14}C-labelled polyuridylic acid (10 μg, 2000 counts/min) with 25 μg of purified RNA polymerase for 1 h at 37 °C as described above for the DNAase assay. The mixture was chilled in ice and the RNA was precipitated by addition of two volumes of absolute ethanol in the presence of yeast RNA (0.5 mg) and 0.1 M NaCl. After 15 min at 0 °C the precipitate was removed by centrifugation and the supernatant counted as before.

Endonuclease activity was determined by incubation of ^{14}C-labelled R17 RNA (1 μg, 1400 counts/min) with 25 μg of purified RNA polymerase for 1 h at 37 °C. Sodium dodecylsulfate at a final concentration of 0.1% was added and the mixture was incubated for 2 min at 37 °C and then centrifuged in a 5—20% (w/v) sucrose gradient in 0.1 M Tris-HCl pH 7.8, 0.01 M EDTA for 3 h at 49000 rev./min in a SW50L rotor of the Spinco L250 ultracentrifuge at 0 °C.

Polynucleotide Phosphorylase. The exchange of [^{32}P]phosphate into UDP was determined as indicated by Reiner [10]. The incubation mixture for the exchange reaction was that described by Burgess [6] using 10 μg of purified RNA polymerase per assay.

Polyacrylamide Gel Electrophoresis

Gels (12 cm long) containing 5% (w/v) acrylamide and 0.15% (w/v) *N,N'*-methylenebisacrylamide were prepared as described by Viñuela *et al.* [11].

The sample for electrophoresis was dialyzed against distilled water, dried out under a nitrogen stream and the residue dissolved in 0.2—0.3 ml of a solution containing 0.01 M sodium phosphate pH 7.2, 1% (w/v) sodium dodecylsulfate, 1% (v/v) 2-mercaptoethanol and 2 M urea and heated for 5 min in a bath of boiling water. 10 μl of a 0.2% (w/v) bromophenol blue solution were used as indicator. The electrode compartments were filled with a buffer containing 0.1 M sodium phosphate, pH 7.1 and 0.1% (w/v) sodium dodecylsulfate. Electrophoresis was carried out at room temperature at a constant voltage of 2.5 volt/cm for about 15 h. After electrophoresis the gels were stained wth a 0.25% (w/v) solution of coomassie brilliant blue in methanol—acetic acid—water (5:1:5, v/v/v) for at least 6 h. The gels were destained either by shaking in 7.5% acetic acid or electrophoretically in the same solution. Markers were

run in parallel gels in order to calculate the molecular weight values of the polypeptide chains of RNA polymerase as described by Shapiro *et al.* [12].

For the separation of the β' and β subunits the system of electrophoresis described by Laemmli [13] was used. The separation gel (10 cm long) contained 7.5% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylenebisacrylamide; the stacking gel (3 cm long) contained 3% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylenebisacrylamide. The rest of the components were used as described by Laemmli. Electrophoresis was carried out at a constant voltage of 100 volts until the bromophenol blue marker reached the bottom of the gel (about 6 h). The proteins were stained as indicated before.

Purification of RNA Polymerase

All operations were carried out at 0—4 °C.

Preparation of Extracts. 100 g of cells were ground with 200 g of alumina during 60 min and extracted with 400 ml of buffer I. The crude extract was centrifuged for 30 min at 10000 × *g* in the SS34 rotor of a Sorvall centrifuge and the pellet discarded.

Phase Partition and Ammonium Sulfate Precipitation. The supernatant (350 ml) was treated with 123 ml of 30% (w/w) polyethyleneglycol 6000 and 45 ml of 20% (w/w) dextran 500, both dissolved in water, according to the procedure described by Babinet [14]. After stirring for 30 min the mixture was centrifuged 10 min at 14500 × *g*. Two phases were obtained; the upper phase, containing the polyethyleneglycol, was discarded. To the dextran phase (50 ml), 123 ml buffer I, 50 ml 30% polyethyleneglycol and 26.2 g NaCl were added. The mixture was stirred for 30 min and centrifuged as before; the polyethyleneglycol phase was again discarded. To the dextran phase (50 ml), 123 ml buffer I, 50 ml 30% polyethyleneglycol and 47 g NaCl were added. The mixture was stirred for 2 h and centrifuged as before. The dextran phase was discarded. The polyethyleneglycol phase, containing the RNA polymerase activity, was dialyzed for 2 h against 6 liters of buffer I, changing the buffer every 30 min. After dialysis, ammonium sulfate (16.3 g/100 ml) was added; the mixture was stirred for 30 min and centrifuged 5 min at 10000 × *g*. Two phases were obtained; the upper phase, containing the polyethyleneglycol, was discarded. To the lower phase, ammonium sulfate (7 g/100 ml) was added; the mixture was stirred for 30 min, centrifuged 45 min at 23500 × *g* and the precipitate discarded. Finally, ammonium sulfate (10 g/100 ml) was added to the supernatant. After stirring and centrifuging as before the precipitate was dissolved in 35 ml of buffer II (fraction AS I).

DEAE-Cellulose Chromatography. Fraction AS I was diluted to 210 ml with buffer II and passed through a column of DEAE-cellulose, previously equilibrated with the same buffer, at a flow rate of 1.5 ml/min. Then, a linear gradient was started with 200 ml of buffer II and 200 ml of buffer II containing 0.5 M KCl and finally the column was washed with 130 ml of the last buffer. The fractions containing the activity (Fig. 1) were pooled and kept at 0—4 °C overnight.

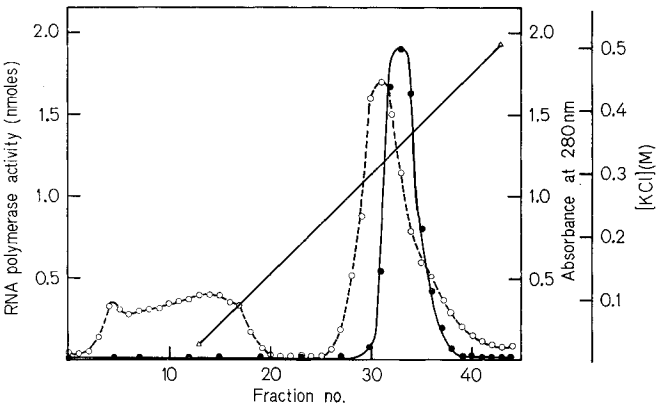

Fig. 1. *Chromatography of RNA polymerase on DEAE-cellulose.* Fraction AS I (280 mg of protein) was passed through a column (20 × 3 cm) of DEAE-cellulose and eluted as described in the text. Fractions of 13 ml were collected. Fractions 0 (90 ml) to 12 contained the flow-through material. The gradient was started in fraction 13 and the final washing of the column with 0.5 M KCl was started in fraction 44. Aliquots of 10 µl were used to assay the enzymatic activity. O----O, absorbance at 280 nm; ●——●, RNA polymerase activity, measured as the amount of [^{14}C]UMP incorporated in 10 min; △——△, KCl concentration

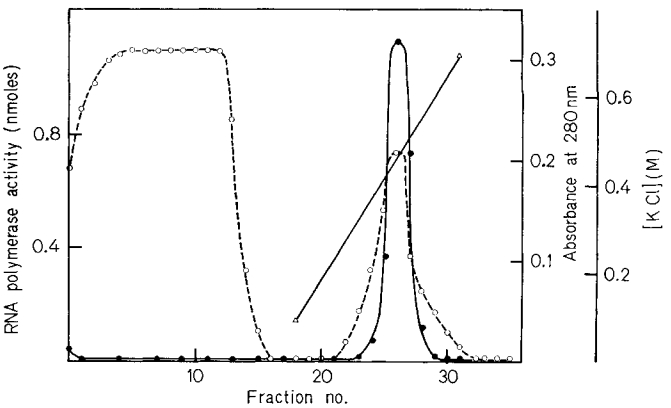

Fig. 2. *Chromatography of RNA polymerase on φ29 DNA-cellulose.* The pooled fractions from the DEAE-cellulose column (175 mg of protein) were passed through a column (9 × 3 cm) of φ29 DNA-cellulose and eluted as described in the text. Fractions of 11 ml were collected. Fractions 0 (100 ml) to 17 contained the flow-through material. The gradient was started in fraction 32. Aliquots of 10 µl were used to assay the enzymatic activity. O----O, absorbance at 280 nm; ●——●, RNA polymerase activity, measured as the amount of [^{14}C]UMP incorporated in 10 min; △——△, KCl concentration

Table 1. *Summary of* B. subtilis *RNA polymerase purification*
The data given refer to the purification of 100 g of cells. Activity units are expressed as nmoles of UMP incorporated in 10 min under the assay conditions described in Materials and Methods

Fraction	A_{280}/A_{260}	Total protein	Total activity	Specific activity	Yield
		mg	units	units/mg	%
1. Extract	0.53	5950	24900	4	100
2. AS I	0.89	280	27100	97	109
3. DEAE-cellulose peak	1.39	175	18800	107	79
4. DNA-cellulose, AS II	1.55	10	9850	970	40

DNA-Cellulose Chromatography. The pooled fractions from the DEAE-cellulose column were diluted three-fold with buffer II lacking KCl and passed through a column of φ29 DNA cellulose, equilibrated with buffer II containing 0.1 M KCl, at a flow rate of 0.45 ml/min. The column was washed with 70 ml of the same buffer; then a linear gradient was started with 70 ml of buffer II containing 0.1 M KCl and 70 ml of buffer II containing 0.7 M KCl and the flow rate was increased to 1.25 ml/min. Finally, the column was washed with 30 ml of buffer II containing 1.0 M KCl. The active fractions (Fig. 2) were pooled and precipitated by addition of solid ammonium sulfate to 70% saturation, keeping the pH between 8 and 8.4 by addition of 0.1 M NH_4OH. After 30 min, the precipitate was centrifuged 30 min at $23500 \times g$ and dissolved in 3.5 ml of buffer II (Fraction AS II). Small aliquots of the enzyme were kept frozen at -20 °C. Table 1 shows a summary of the results of the purification.

Stability of RNA Polymerase

In order to keep the enzyme active a pH between 8 and 8.4 is required. Addition of 15% (v/v) glycerol and KCl (between 25 and 100 mM) is essential to maintain the stability of RNA polymerase. When stored at -20 °C under the conditions described before, the enzyme is stable for several months.

RESULTS AND DISCUSSION

Purity of RNA Polymerase

As shown previously [1] *B. subtilis* RNA polymerase is highly purified after chromatography on φ29 DNA-cellulose; by sedimentation on a glycerol gradient there is a single protein peak which overlaps with the peak of enzyme activity [1]. However, sometimes, a small amount of protein with a molecular weight value of approximately 110000 is present in the enzyme preparation. This protein can be removed by centrifugation in a 10–30% (v/v) glycerol gradient as described previously [1].

As will be shown later (Fig. 7A), the RNA polymerase obtained by φ29 DNA-cellulose chromatography contains three bands, β, σ and α. From the densitometry tracings the enzyme is calculated to be 95–99% pure, depending on the preparation.

Contaminating Activities

The DNAase, RNAase and polynucleotide phosphorylase activities of RNA polymerase purified by DNA-cellulose chromatography were assayed as indicated in Materials and Methods.

Exonuclease. No solubilization of radioactivity was detected by incubation of labelled φ29 DNA or polyuridylic acid with RNA polymerase.

Endonuclease. There was no change in the distribution of radioactivity in a sucrose gradient of φ29 DNA or R17 RNA after incubation with RNA polymerase.

Polynucleotide phosphorylase. No detectable exchange of ^{32}P into UDP was observed after incubation with RNA polymerase.

Number of Subunits of B. subtilis *RNA Polymerase*

By electrophoresis on polyacrylamide gels containing 5% acrylamide in the presence of 1% sodium dodecylsulfate, three protein bands (β, σ and α) are obtained with purified *B. subtilis* RNA polymerase (Fig. 7A). In order to separate the β subunit into β' and β (see Fig. 3) we used the electrophoresis system described by Laemmli [13]. Losick *et al.* [15] have also characterized four subunits in *B. subtilis* RNA polymerase.

In our purified preparation we have not detected the ω subunit described as a component of *Escherichia coli* RNA polymerase [6]. Preliminary experiments suggest that enzyme with the ω subunit elutes from the DEAE-cellulose column after the main peak of activity.

Molecular Weights of the Subunits of B. subtilis *RNA Polymerase*

The molecular weights of the subunits of RNA polymerase were determined according to Shapiro *et al.* [12] by comparing their electrophoretic mobilities with those of marker proteins of known molecular weights (Fig. 4). The estimated molecular weight values of each subunit are shown on Table 2. Similar values have been obtained by Losick *et al.* [15].

Fig. 3. *Separation of the β' and β subunits by gel electrophoresis.* Gels containing 7.5% and 3% acrylamide were prepared and stained as described in Materials and Methods. Protein migration was towards the anode, shown at the bottom. Electrophoresis was carried out at a constant voltage of 100 volts for 6 h. The sample for electrophoresis contained 22 μg of purified RNA polymerase

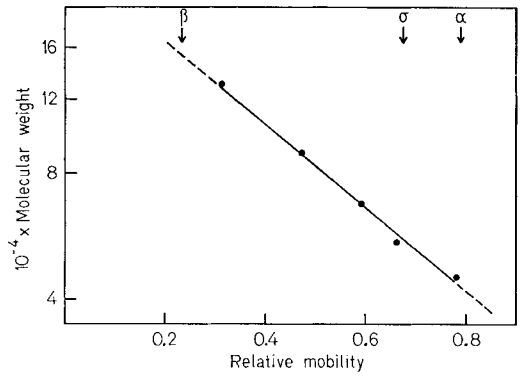

Fig. 4. *Estimation of the molecular weights of the subunits of* B. subtilis *RNA polymerase.* Proteins of known molecular weight as well as purified RNA polymerase were dissociated and subjected to electrophoresis on 5% acrylamide gels as described in Materials and Methods and their mobilities relative to the tracking dye determined. The marker proteins used were: β-galactosidase (100 μg), molecular weight 130000; bovine serum albumin (20 μg), molecular weight 67500; ovalbumin (100 μg), molecular weight 45000 (monomer) and 90000 (dimer); immunoglobulin G (40 μg), molecular weight 55000 (heavy chain). The mobilities of the subunits of RNA polymerase are indicated by arrows

Table 2. *Molecular weights of the subunits of* B. subtilis *RNA polymerase*
Molecular weights were estimated as described in Materials and Methods and in Fig. 4. The given values are the average of 30 determinations

Subunit	Molecular weight
$\beta'\beta$	154000 ± 6000
σ	56000 ± 2000
α	43000 ± 2000

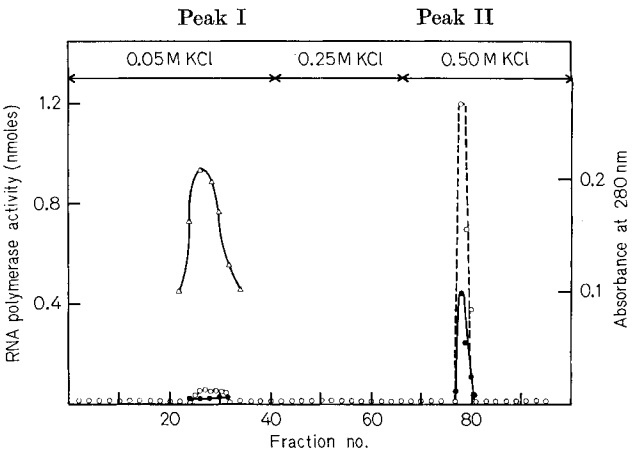

Fig. 5. *Chromatography of RNA polymerase (DNA-cellulose fraction) on a phosphocellulose column.* RNA polymerase (3.6 mg) purified by DNA-cellulose chromatography was diluted to 30 ml with buffer II containing 0.05 M KCl and passed through a column (12 × 1.5 cm) of phosphocellulose, equilibrated with the same buffer, at a flow rate of 0.1 ml/min. The column was washed with 20 ml of the same buffer at a flow rate of 1 ml/min, then with 35 ml of buffer II containing 0.25 M KCl and finally with 50 ml of buffer II containing 0.50 M KCl. Fractions of 1.4 ml were collected. Activity was assayed in aliquots of 10 μl using φ29 DNA as template. O----O, absorbance at 280 nm; ●——●, RNA polymerase activity, measured as the amount of [^{14}C]UMP incorporated in 10 min; △——△, RNA polymerase activity in the presence of 10 μl of fraction 78 (peak II)

Molar Ratio of the RNA Polymerase Subunits

The molar ratio of the subunits of RNA polymerase, calculated from the densitometry tracings, was 0.86:0.48:1 which agrees with the structure $\beta'\beta\sigma\alpha_2$. A molecular weight of 450000 can be calculated for this structure; this value is close to that obtained by sedimentation in glycerol gradient [1,15,16]. In some preparations, however, the molar ratio of the σ subunit is lower than 1; this probably means that some enzyme molecules have lost their σ subunit and contain only the β', β and α subunits (core enzyme).

From the above resulted it seems that the structure of *B. subtilis* RNA polymerase is similar to that of the *E. coli* enzyme [17—19], the main difference being the molecular weight of the σ subunit which is smaller in *B. subtilis* than in *E. coli* RNA polymerase.

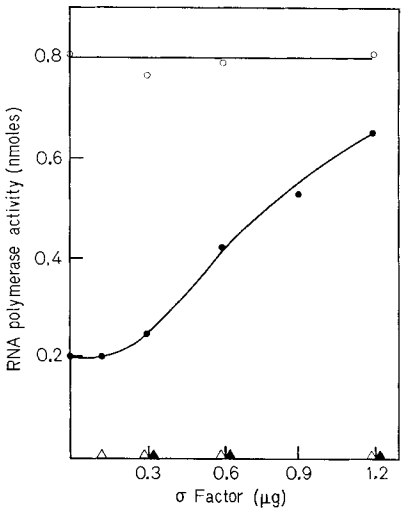

Fig. 6. *Stimulation of core RNA polymerase by the sigma factor.* Peak I (sigma factor) obtained by phosphocellulose chromatography of pure RNA polymerase was added to 2 μg of peak II (core enzyme) from the same column and the activity was assayed with φ29 DNA and poly[d(A-T)], respectively, using 2.5 μg of poly[d(A-T)] per assay. ●, activity of core enzyme with φ29 DNA in the presence of σ factor; ○, activity of core enzyme with poly[d(A-T)] in the presence of σ factor; ▲, activity of σ factor with φ29 DNA; △, activity of σ factor with poly[d(A-T)]. Activity was measured as the amount of [^{14}C]UMP incorporated in 10 min

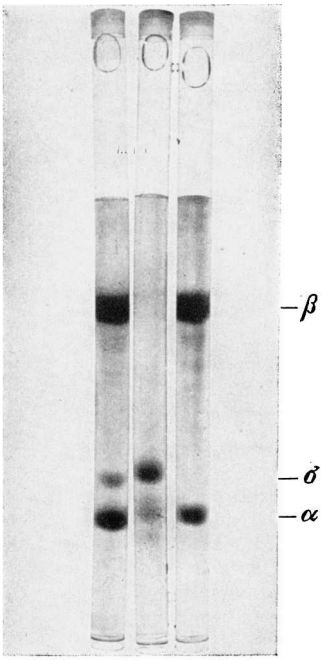

Fig. 7. *Electrophoresis on polyacrylamide gels of RNA polymerase after φ29 DNA-cellulose chromatography and of peaks I and II obtained by phosphocellulose chromatography.* 12 cm gels containing 5% acrylamide and 1% sodium dodecylsulfate were prepared and stained as indicated in Materials and Methods. Protein migration was towards the anode, shown at the bottom. Electrophoresis was carried out at a constant voltage of 2.5 volt/cm for 14 h. (A) 60 μg of RNA polymerase after DNA-cellulose chromatography; (B) 30 μg of peak I from the phosphocellulose column (Fig. 5); (C) 60 μg of peak II from the same column

Isolation of Core RNA Polymerase and Sigma Subunit by Phosphocellulose Chromatography

When RNA polymerase purified after the DNA-cellulose chromatography step was passed through a column of phosphocellulose according to the procedure described by Burgess *et al.* [17], two protein peaks, I and II, were obtained (Fig. 5). Peak I had no activity either with φ29 DNA or with poly[d(A-T)] (Fig. 6). Peak II had some activity with φ29 DNA; this activity was increased by addition of peak I (Figs 5 and 6) indicating that peak II lacked some component present in peak I. However, addition of peak I to peak II had no effect when poly[d(A-T)] was used as template (Fig. 6); peak II by itself was about three times more active with poly[d(A-T)] than with φ29 DNA. At the concentration of templates used, the RNA polymerase holoenzyme is about twice as active with φ29 DNA than with poly-[d(A-T)].

The electrophoresis on polyacrylamide gels of peaks I and II as well as that of RNA polymerase before chromatography on phosphocellulose are shown in Fig. 7. Peak I contained σ subunit and a small amount of α in a molar ratio 2.7:1. Peak II contained the β and α subunits in a molar ratio 1:1 and the σ subunit was lacking. The RNA polymerase before chromatography contained β, σ and α subunits in a molar ratio 0.83:0.42:1. When peak II was centri-

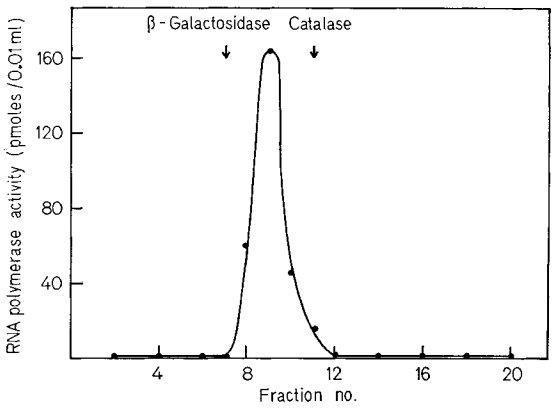

Fig. 8. *Centrifugation of peak II from phosphocellulose in a glycerol gradient.* Peak II (125 μg) from the phosphocellulose column (Fig. 5) was mixed with 1 mg of β-galactosidase and 1.5 mg of catalase, in a final volume of 0.15 ml, and layered on top of a 15—30% (v/v) glycerol gradient in 0.01 M Tris-HCl pH 8.4, 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 0.05 M KCl. The centrifugation was carried out in the SW50L rotor in a Spinco L250 ultracentrifuge for 18 h at 35000 rev./min at 0 °C. RNA polymerase activity, measured as the amount of [^{14}C]UMP incorporated in 10 min, was assayed in the fractions in the presence of added σ factor using φ29 DNA as template

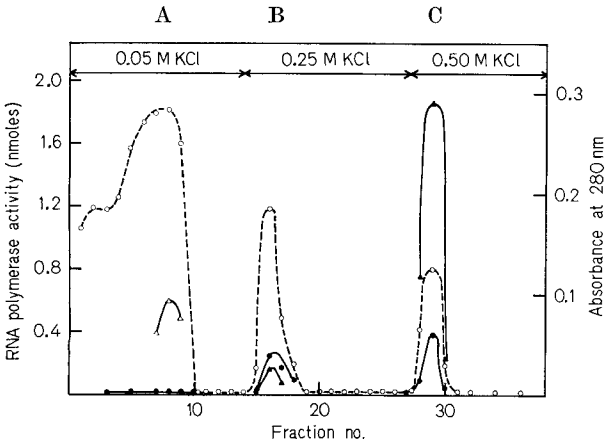

Fig. 9. *Chromatography of RNA polymerase (DEAE-cellulose fraction) on a phosphocellulose column.* RNA polymerase (5.7 mg) purified by DEAE-cellulose chromatography was diluted to 21 ml with buffer II containing 0.05 M KCl and passed through a column (12 × 1.5 cm) of phosphocellulose, equilibrated with the same buffer, at a flow rate of approx. 0.3 ml/min. Fractions of about 3 ml were collected. The column was washed with 20 ml of the above buffer, then with 40 ml of buffer II containing 0.25 M KCl and finally with 40 ml of buffer II containing 0.50 M KCl. Activity, measured as the amount of [^{14}C]UMP incorporated in 10 min, was assayed in aliquots of 10 µl with φ29 DNA and poly-[d(A-T)], respectively, using 2.5 µg of poly[d(A-T)] per assay. O----O, absorbance at 280 nm; ●——●, activity with φ29 DNA; ▲——▲, activity with poly[d(A-T)]; △——△, activity with φ29 DNA in the presence of 10 µl fraction 29

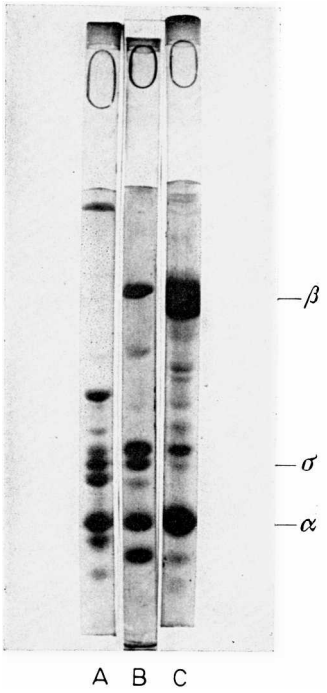

Fig. 10. *Electrophoresis on polyacrylamide gels of peaks A, B and C obtained by phosphocellulose chromatography of partially purified RNA polymerase (DEAE-cellulose fraction).* 12 cm gels containing 5% acrylamide and 1% sodium dodecyl-sulfate were prepared and stained as indicated in Materials and Methods. Protein migration was towards the anode, shown at the bottom. Electrophoresis was carried out at a constant voltage of 2.5 volt/cm for 15 h. (A) 125 µg of peak A from the phosphocellulose column (Fig. 9); (B) 100 µg of peak B; (C) 90 µg of peak C from the same column

fuged in a glycerol gradient in the presence of β-galactosidase and catalase as markers, a molecular weight value of 370000 was obtained (Fig. 8). This and the results of electrophoresis indicate that peak II is the core RNA polymerase containing β', β and two α subunits. The above results show that the stimulation of peak II (core enzyme) by peak I when φ29 DNA is used as template is due to the σ subunit.

Our results confirm and extend those of Losick *et al.* [15] and Kerjan and Szulmajster [20]. They have reported the stimulation of the core RNA polymerase by the protein peak which is not retained by phosphocellulose at 0.05 M KCl using φe DNA and T4 DNA, respectively, as templates. In addition, we have shown that, in the case of purified RNA polymerase, this protein peak consists mainly of σ subunit. However, there was a discrepancy between our results and those of Kerjan and Szulmajster using partially purified preparations of RNA polymerase; they found three protein peaks eluting from the phosphocellulose column at 0.05, 0.23 and 0.40 M KCl, respectively, and we only obtained two peaks. When we subjected a less purified preparation of RNA polymerase (after DEAE-cellulose chromatography) to phosphocellulose chromatography as described before, three protein peaks, A, B and C, eluting at 0.05, 0.25 and 0.50 M KCl, respectively, were obtained (Fig. 9). Peak A had no activity. Peak B had some activity with φ29 DNA and poly[d(A-T)]. Peak C was about six times more active with poly[d(A-T)] than with φ29 DNA. Addition of peak A to peak C produced a stimulation when assayed with φ29 DNA and had no effect when poly[d(A-T)] was used as template. The electrophoresis in polyacrylamide gels of peaks A, B and C are shown in Fig. 10. Besides some impurities present in the three peaks, peak A contained σ and α subunits in a molar ratio 1:2.5, peak C contained β and α subunits in a molar ratio approximately 0.8:1 and peak B showed the presence of β, σ and α subunits in a molar ratio 1:1:3, indicating the this peak has a decreased amount of β and σ subunits. Bautz *et al.* [21] have reported that, in the case of the *E. coli* RNA polymerase, peak B obtained by phosphocellulose chromatography does not have the β' subunit; however, in that case, the α subunit is also proportionally reduced.

Table 3. *Requirements for RNA polymerase activity*
The activity is expressed as pmoles of [^{14}C]UMP incorporated into cold 5% trichloroacetic acid precipitable material in 10 min. All the values are the average of duplicate assays. The complete system is that described in Materials and Methods. 2 μg of purified RNA polymerase were used per assay. The effect of the omission of 2-mercaptoethanol on the enzymatic activity was not studied due to its presence in buffer II, in which the RNA polymerase was stored

System	Activity
	pmoles/10 min
Complete	855
+ bovine serum albumin, 50 μg	1704
− NH_4Cl	509
− $MgCl_2$	0
− ATP	0
− DNA	0

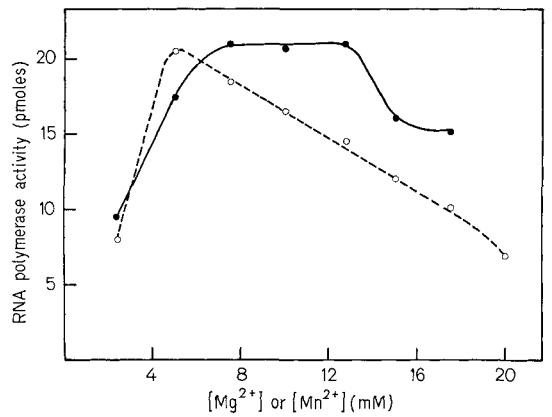

Fig. 11. *Effect of Mg^{2+} and Mn^{2+} concentration on the RNA polymerase activity.* The assay conditions were those described in Materials and Methods. 4 μg of purified RNA polymerase were used per assay. The values are the average of duplicate assays. ●——●, Mg^{2+}; O----O, Mn^{2+}. Activity was measured as the amount of [^{14}C]UMP incorporated in 10 min

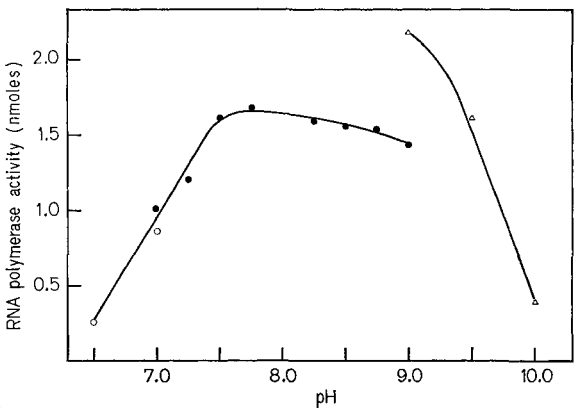

Fig. 12. *Dependence of RNA polymerase activity on pH.* The assay conditions were those described in Materials and Methods. 4 μg of purified RNA polymerase were used per assay. The values are the average of duplicate assays. The buffers used were the following: pH 6.5 and 7.0, Tris-acetate (O); pH 7.0 to 9.0, Tris-HCl (●); pH 9.0 to 10.0, glycine-NaOH (△). Activity was measured as the amount of [^{14}C]UMP incorporated in 10 min

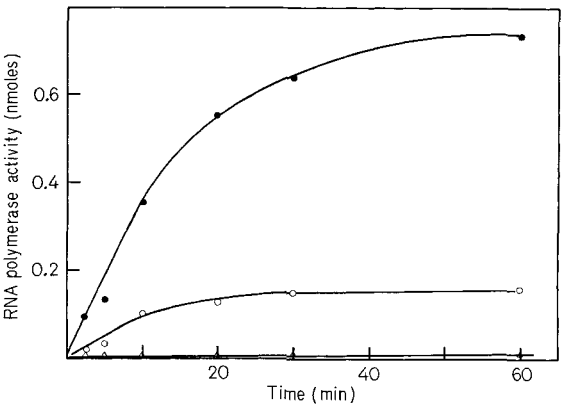

Fig. 13. *Time course of the RNA polymerase reaction in the absence and presence of KCl.* The assay conditions were those described in Materials and Methods. 8 μg of RNA polymerase were used per 0.2 ml of incubation mixture. At the indicated times aliquots of 10 μl were removed from the incubation mixture and the radioactivity determined as described in Materials and Methods. ●, without KCl; O, 0.1 M KCl; △, 0.2 M KCl. Activity was measured as the amount of [^{14}C]UMP incorporated in10 min

Properties of the Enzymatic Reaction

Some of the requirements for the RNA polymerase activity are shown on Table 3. The addition of bovine serum albumin stimulates RNA synthesis about two-fold. The activity is completely dependent on Mg^{2+}, nucleoside triphosphates and DNA. As shown in Fig. 11, Mn^{2+} can replace Mg^{2+} without loss of activity; the maximum rate of synthesis is observed at 5 mM Mn^{2+} and between 8 and 12 mM Mg^{2+}. In the case of the RNA polymerase purified from sporulating cells, Mn^{2+} can also replace Mg^{2+} although not as efficiently [16]. Fig. 12 shows the activity of RNA polymerase as a function of pH; the maximal activity is between pH 7.5 and 8.5 and it drops to about 20% at pH 6.5 and 10.

The kinetics of the RNA polymerase reaction in the absence and presence of KCl is shown in Fig. 13. In contrast to the effect on *E. coli* RNA polymerase [22,23], KCl inhibits strongly the activity of the *B. subtilis* enzyme, the inhibition being about 90% at 0.2 M KCl (see Fig. 14). Similar results have been recently reported by Whiteley and Hemphill [24]. The effect of rifamycin on RNA synthesis is shown on Fig. 15; 0.01 μg of rifamycin completely inhibit the activity of RNA polymerase (5 μg).

B. subtilis RNA polymerase was completely inactivated by heating for 5 min at 60 °C in buffer II (Fig. 16).

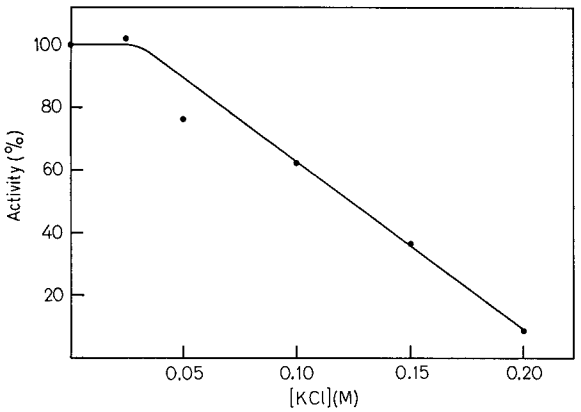

Fig. 14. *Effect of KCl on the RNA polymerase activity.* The assay conditions were those described in Materials and Methods, adding 50 μg of bovine serum albumin per 0.1 ml of incubation mixture. 4 μg of purified RNA polymerase were used per assay. The values are the average of duplicate assays. An activity of 100% represents the incorporation of 1.99 nmoles [^{14}C]UMP in 10 min

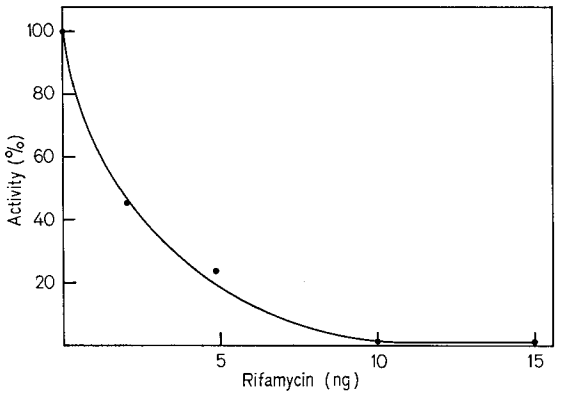

Fig. 15. *Effect of rifamycin on the activity of RNA polymerase.* The assay conditions were those described in Materials and Methods. 5 μg of RNA polymerase were used per assay. An activity of 100% represents the incorporation of 1.015 nmoles [^{14}C]UMP in 10 min

Table 4. *Activity of RNA polymerase with several templates*
The values for V and apparent K_m were calculated for each template using the assay conditions described in Materials and Methods in the presence of 50 μg of bovine serum albumin, at several concentrations of DNA. All the values are the average of duplicated assays. 3 μg of purified RNA polymerase were used per assay. The apparent K_m values are expressed as nmoles of nucleotide/ml

Template DNA	V	Apparent K_m
		μM
φ29	2360	54
B. subtilis	336	25
Poly[d(A-T)]	1120	23
T4	1120	151
Calf thymus	330	70

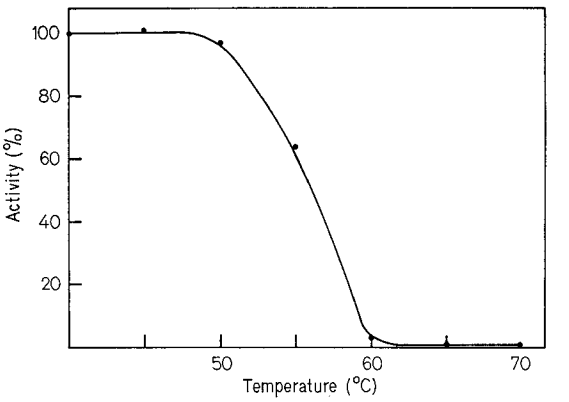

Fig. 16. *Heat inactivation of RNA polymerase.* Purified RNA polymerase, at a concentration of 0.4 mg/ml in buffer II, was heated for 5 min at the indicated temperatures and cooled in ice. The assay conditions for the determination of the residual activity were those described in Materials and Methods. 4 μg of enzyme were used per assay. The values are the average of duplicate assays. An activity of 100% represents the incorporation of 576 pmoles of [^{14}C]UMP in 10 min

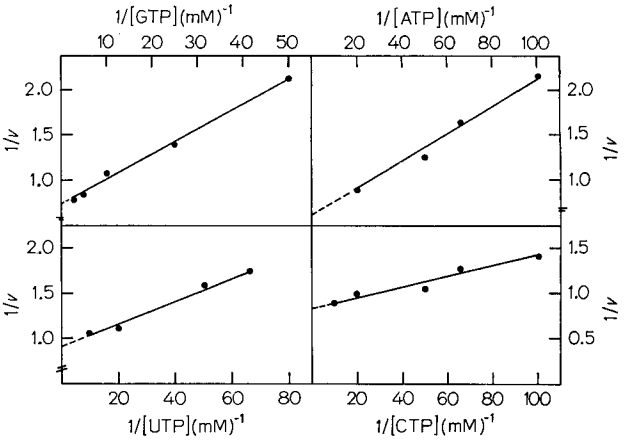

Fig. 17. *Effect of nucleoside triphosphate concentration on the RNA polymerase activity.* The assay conditions were those described in Materials and Methods in the presence of 50 μg of bovine serum albumin per 0.1 ml. A volume of 0.2 ml was used per assay. In each case three nucleotides were kept at high concentration (0.2 mM GTP and 0.1 mM CTP, UTP and ATP) and the fourth one was varied. The labelled nucleotide was always [^{3}H]UTP at a specific activity of 25 μCi/μmole. 1 μg of purified RNA polymerase was used per 0.2 ml of incubation mixture. Under these conditions the amount of nucleotide incorporated at the lowest concentration was about 20% of the total nucleotide present. Initial velocity, v, was measured in nmoles/min

The activity of the RNA polymerase with several native DNA templates is shown in Table 4. Denatured DNAs were completely inactive as templates for RNA synthesis. In contrast, RNA polymerase from sporulating cells is equally active on native or denatured DNA [16]. It can be seen in Table 4 that, at saturation, φ29 DNA is the best template, followed by poly-

[d(A-T)] and T4 DNA. *B. subtilis* and calf thymus DNAs are poor templates for RNA synthesis. However, RNA polymerase has a higher apparent affinity for *B. subtilis* DNA and poly[d(A-T)] than for the other templates.

The effect of the concentration of each nucleoside triphosphate at saturation of the other three nucleotides is shown in Fig. 17. As can be seen, CTP has the lowest K_m value (9 μM) and GTP the highest (36 μM); ATP and UTP have K_m values intermediate to those of CTP and GTP (20 μM, approximately). In the case of *E. coli* RNA polymerase Anthony *et al.* [25] have shown that the K_m value for the nucleotide which initiates the RNA chain is higher than that for the other three nucleotides. In our case, the highest K_m value corresponds to GTP; this could mean that the RNA chain is initiated with G when φ29 DNA is used as template. A direct study of the initial nucleotide in the RNA chains programmed with several DNA templates using *B. subtilis* RNA polymerase is presently being carried out.

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