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, \( \beta' \), \( \beta \), \( \sigma \), and \( \alpha \) (molecular weights 154,000 for \( \beta' \) and \( \beta \), 56,000 for \( \sigma \) and 43,000 for \( \alpha \)) in a molar ratio 1:1:1:2. By phosphocellulose chromatography RNA polymerase has been dissociated in \( \sigma \) subunit and core enzyme, containing \( \beta' \), \( \beta \), and \( \alpha \) subunits in a molar ratio 1:1:2; the \( \sigma \) subunit stimulates the activity of core polymerase with \( \varphi 29 \) 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 *B. subtilis* vegetative cells [1]. The enzyme consists of at least three different subunits, \( \beta' \), \( \beta \), and \( \sigma \), with molecular weight values of approximately 150,000, 55,000 and 43,000, 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 \( \sigma \) subunit and core polymerase and the general properties of the enzyme.

MATERIALS AND METHODS

**Chemicals**

Alumina, polyethylene glycol 6000, coomassie brilliant blue and DEAE-cellulose (0.76 mequiv./g) were obtained from Serva; 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]; \( \beta \)-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.

\( \varphi 29 \) DNA and T4 DNA were prepared from purified \( \varphi 29 \) (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 \( \varphi 29 \) 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. Georgopoulous. 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.
Column Chromatography

DEAE-cellulose and phosphocellulose were treated as described by Burgess [6]. p29 DNA-cellulose was prepared essentially as described by Alberts et al. [7] using native p29 DNA at a concentration of about 2 mg/ml; 0.6 mg of p29 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 MgCl₂, 50 mM NH₄Cl, 10 mM 2-mercaptoethanol, 0.1 mM ATP, GTP and CTP, 0.1 mM [³⁴C]UTP (specific activity 2.5), 10 µg of p29 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. ³¹C-labelled p29 DNA (0.05 µg, 63000 counts × min⁻¹ × 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 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 ¹⁴C-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 ¹⁴C-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 [³²P]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 under 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 with 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 10,000 × 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) polyethylene glycol 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 at 14,500 × g. Two phases were obtained; the upper phase, containing the polyethylene glycol, was discarded. The dextran phase (50 ml), 123 ml buffer I, 50 ml 30% (w/w) polyethylene glycol and 26.2 g NaCl were added. The mixture was stirred for 30 min and centrifuged as before; the polyethylene glycol phase was again discarded. To the dextran phase (50 ml), 123 ml buffer I, 50 ml 30% (w/w) polyethylene glycol and 47 g NaCl were added. The mixture was stirred for 2 h and centrifuged as before. The dextran phase was discarded. The polyethylene glycol 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 10,000 × g. Two phases were obtained; the upper phase, containing the polyethylene glycol, 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 28,500 × 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.
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 q29 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 NH₄OH. After 30 min, the precipitate was centrifuged 30 min at 23 500 x 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 q29 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 q29 DNA-cellulose chromatography contains three bands, \( \beta, \sigma \) and \( \alpha \). From the densitometry tracings the enzyme is calculated to be 85–99\% 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 q29 DNA or polyuridylic acid with RNA polymerase.

Endonuclease. There was no change in the distribution of radioactivity in a sucrose gradient of q29 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 (\( \beta, \sigma \) and \( \alpha \)) are obtained with purified B. subtilis RNA polymerase (Fig. 7A). In order to separate the \( \beta \) subunit into \( \beta' \) and \( \beta \) (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 \( \omega \) subunit described as a component of Escherichia coli RNA polymerase [6]. Preliminary experiments suggest that enzyme with the \( \omega \) 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].

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Table 1. Summary of B. subtilis RNA polymerase purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( A_{260}/A_{280} )</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>0.83</td>
<td>3050</td>
<td>28000</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>2. AS I</td>
<td>0.89</td>
<td>280</td>
<td>27000</td>
<td>97</td>
<td>109</td>
</tr>
<tr>
<td>3. DEAE-cellulose peak</td>
<td>1.39</td>
<td>175</td>
<td>18800</td>
<td>107</td>
<td>79</td>
</tr>
<tr>
<td>4. DNA-cellulose, AS II</td>
<td>1.55</td>
<td>10</td>
<td>9850</td>
<td>970</td>
<td>40</td>
</tr>
</tbody>
</table>
**Bacillus subtilis RNA Polymerase**

Fig. 3. Separation of the $\beta'$ and $\beta$ subunits by gel electrophoresis. Gels containing 7.5%, 10%, and 30% 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: $\beta'$-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.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta'$</td>
<td>$154000 \pm 6000$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$56000 \pm 2000$</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$43000 \pm 2000$</td>
</tr>
</tbody>
</table>

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 x 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. $\odot$ absorbance at 280 nm; $\bullet$ RNA polymerase activity, measured as the amount of [14C]UMP incorporated in 10 min; $\triangle$ 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 $\beta'$βα2. 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 $\alpha$ subunit is lower than 1; this probably means that some enzyme molecules have lost their $\alpha$ subunit and contain only the $\beta'$, $\beta$ and $\alpha$ 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 $\alpha$ subunit which is smaller in B. subtilis than in E. coli RNA polymerase.
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 p29 DNA or with poly[d(A-T)] (Fig. 6). Peak II had some activity with p29 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 p29 DNA. At the concentration of templates used, the RNA polymerase holoenzyme is about twice as active with p29 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 $\sigma$ subunit and a small amount of $\alpha$ in a molar ratio 2.7:1. Peak II contained the $\beta$ and $\alpha$ subunits in a molar ratio 1:1 and the $\sigma$ subunit was lacking. The RNA polymerase before chromatography contained $\beta$, $\sigma$, and $\alpha$ subunits in a molar ratio 0.83:0.42:1. When peak II was centri-
fuged in a glycerol gradient in the presence of \(\beta\)-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 \(\beta', \beta\) and two \(\alpha\) subunits. The above results show that the stimulation of peak II (core enzyme) by peak I when \(\varphi 29\) DNA is used as template is due to the \(\sigma\) 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 \(\varphi 29\) 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 \(\sigma\) 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, 0.25 and 0.50 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 \(\varphi 29\) DNA and poly[d(A-T)], respectively, using 2.5 \(\mu\)g of poly[d(A-T)] per assay. Peak C was about six times more active with poly[d(A-T)] than with \(\varphi 29\) DNA. Addition of peak A to peak C produced a stimulation when assayed with \(\varphi 29\) 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 \(\sigma\) subunits in a molar ratio 1:2.5, peak C contained \(\beta\) and \(\alpha\) subunits in a molar ratio approximately 0.8:1 and peak B showed the presence of \(\beta, \sigma\) and \(\alpha\) subunits in a molar ratio 1:1:3, indicating the this peak has a decreased amount of \(\beta\) and \(\sigma\) 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 \(\beta'\) subunit; however, in that case, the \(\alpha\) subunit is also proportionally reduced.
Table 3. Requirements for RNA polymerase activity

The activity is expressed as pmoles of [14C]UMP incorporated into cold 50\%/ 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 \mu g 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.

<table>
<thead>
<tr>
<th>System</th>
<th>Activity pmoles/10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>855</td>
</tr>
<tr>
<td>+ bovine serum albumin, 50 \mu g</td>
<td>1704</td>
</tr>
<tr>
<td>- NH_4Cl</td>
<td>509</td>
</tr>
<tr>
<td>- MgCl_2</td>
<td>0</td>
</tr>
<tr>
<td>- ATP</td>
<td>0</td>
</tr>
<tr>
<td>- DNA</td>
<td>0</td>
</tr>
</tbody>
</table>

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 \mu g of rifamycin completely inhibit the activity of RNA polymerase (5 \mu g).

B. subtilis RNA polymerase was completely inactivated by heating for 5 min at 60 °C in buffer II (Fig. 16).
**Table 4. Activity of RNA polymerase with several templates**

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>( V )</th>
<th>Apparent ( K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \psi 29 )</td>
<td>2360</td>
<td>54</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>336</td>
<td>25</td>
</tr>
<tr>
<td>Poly[d(A-T)]</td>
<td>1120</td>
<td>23</td>
</tr>
<tr>
<td>T4</td>
<td>1120</td>
<td>151</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>330</td>
<td>70</td>
</tr>
</tbody>
</table>

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, \( \psi 29 \) 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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REFERENCES