DNA-Dependent RNA Polymerase from the Thermophilic Bacterium *Caldariella acidophila*. Purification and Basic Properties of the Enzyme†

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ABSTRACT: A DNA-dependent RNA polymerase has been isolated from *Caldariella acidophila*, a thermophilic bacterium living in acidic hot springs at temperatures ranging from 63 to 89 °C. The enzyme was purified 180-fold and is composed of five different subunits having the following molecular weights: a = 127 000, b = 120 000, c = 72 000, d = 65 000, and e = 38 000. The enzyme is activated by Mn^{2+} and Mg^{2+} and exhibits optimal activity in the presence of 0.5 mM Mn^{2+}. The activity depends on ionic strength, with a maximum at 0.25 M KCl, and exhibits a pH optimum at 7.8 in the presence of Tris-HCl buffer. The enzyme shows a high degree of thermophilicity, its temperature optimum being 80 °C in the in vitro assay. The thermophilicity of *C. acidophila* RNA polymerase allows studies on enzyme–template interactions to be performed in a temperature range where many templates are close to their T_m.

RNA polymerase from bacterial sources has been widely employed in the study of the mechanism of reaction concerning the various steps leading to RNA synthesis. The enzyme from *Escherichia coli* has been used also in investigating the kinetic properties of some mammalian chromatin templates (Cedar and Felsenfeld, 1973; Meilhac and Chambon, 1973; Cox, 1973). With the aim of further elucidating some characteristics of RNA synthesis, we have isolated and purified a highly thermophilic RNA polymerase from the bacterium *C. acidophila*. This microorganism, isolated from hot springs, grows at pH 3.5 in the temperature range 63–89 °C and has an optimal growth temperature at 87 °C. The temperature range at which the enzyme exhibits optimal activity is 70–90 °C and allows studies on enzyme–template interactions to be performed in proximity to the melting temperature of many DNA templates. Some properties of the *C. acidophila* enzyme, such as pH and metal ion requirements, resemble those of mesophilic RNA polymerases.

Two other thermophilic enzymes, isolated from *Thermus aquaticus* (Air and Harris, 1974) and *Bacillus stearothermophilus* (Remold-O'Donnell and Zillig, 1969), have a lower thermophilicity and different optimal ionic strength requirements and Mn^{2+}/Mg^{2+} activity ratios.

The basic properties of this enzyme and the purification procedure are presented here. The second part of this work, to be reported, is a general study of transcription at temperatures close to the template T_m.

Materials and Methods

Chemicals. The sources of essential chemicals were the following: yeast extract and casamino acids (Difco); dithiothreitol and bovine serum albumin (Calbiochem); double-distilled glycerol and ammonium sulfate (Merck); unlabeled ribonucleotide triphosphates (Sigma); [U-^{14}C]UTP (350 mCi/mmol) and Liquifluor (New England Nuclear); yeast RNA (Boehringer); glass fiber filters and DEAE-cellulose DE-52 (Whatman). Acrylamide, *N,N'*-methylenebis(acrylamide) and *N,N,N',N'*-tetramethyleneethylenediamine were from Kodak; sodium dodecyl sulfate, specially pure, was from BDH. DNase I (RNase free) was from Worthington, and glass beads (200 mesh) were from Fisher. All other chemicals, from various suppliers, were reagent grade. All solutions were made with quartz-distilled water.

Buffers. Buffer I contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5% v/v glycerol. Buffer II contained 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% v/v glycerol.

Bacteria. C. acidophila, strain MT-4, was isolated from an acidic hot spring in Agnano, Naples (De Rosa et al., 1975). The bacteria were grown at 87 °C in 25-l. batches in a Terzano (Italy) fermentor with slow mechanical agitation and an aeration flux of 2.5–3 l./min. The culture medium contained 1 g/l. of yeast extract, 1 g/l. of casamino acids, 3.1 g/l. of KH_2PO_4, 2.5 g/l. of $(NH_4)_2SO_4$, 0.20 g/l. of $MgSO_4 \cdot 7H_2O$, 0.25 g/l. of $CaCl_2 \cdot 2H_2O$. The pH was adjusted to 3.0 with 0.1 N H_2SO_4. The cells were harvested in the logarithmic phase of growth at a concentration of 0.5 g of dry cells per liter of culture medium by continuous flow centrifugation in an Alfa-Laval Model LAB 102B-20 separator. The pellet was washed twice in 10 mM Tris-HCl, pH 7.5, and collected by centrifugation at 10 000 rpm for 30 min. Bacteria could be stored at −20 °C for months without any loss of enzymic activity. The base composition of DNA from *C. acidophila,* strain MT-4, was 38–39% in (G + C).

Protein Determination. Protein concentration was determined by the procedure of Lowry et al. (1951). Bovine serum albumin was used for standardization. The ratio of the absorbances at 260 and 280 nm was also used as an esti-

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mate of both protein and DNA content at different stages of purification (Warburg and Christian, 1942).

RNA Polymerase Assay. The standard mixture for the polymerase activity assay contained in a total volume of 0.1 ml: 0.1 mM each GTP, CTP, and ATP, 14.8 μM unlabeled UTP, 5.2 μM [U-^{14}C]UTP (367 mCi/mmol), 0.2 mg/ml of native calf thymus DNA, 1 mM $MnCl_2$, 0.18 M KCl, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 30 mM borate buffer, pH 8.0 at 20 °C, and enzyme. The mixture was incubated either in stoppered test tubes or in glass sealed ampoules at 80 °C for 10 min. The reaction was quenched in ice and stopped by the addition of 0.1 ml of a solution containing 0.1 M $Na_4P_2O_7$ adjusted to pH 7 with HCl, 2 mg/ml of yeast RNA, 2 mg/ml of bovine serum albumin, 5 mM UTP, followed by the addition of 5% sodium dodecyl sulfate. Assay mixtures were precipitated with 2 ml of 10% trichloroacetic acid in 40 mM $Na_4P_2O_7$. After 10 min the precipitates were collected on Whatman GF/C glass fiber filters and washed five times with 3-ml aliquots of 5% trichloracetic acid, 40 mM $Na_4P_2O_7$, and twice with 2 ml of ice-cold water. Filters were dried for 20 min under an infrared lamp and counted in a Nuclear Chicago Mark IV scintillation counter using 4% Liquifluor in toluene. One activity unit is defined as the amount of enzyme which incorporates 1 pmol of UMP into the insoluble acid product in 10 min at the conditions used for the assay.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (7.5 and 12.5%) were prepared and run according to the procedure of Laemmli (1970). The following proteins were used as molecular weight markers: myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, lactate dehydrogenase, and chymotrypsinogen.

Purification of the Enzyme. Enzyme Extraction and DNase Treatment. All purification steps were performed at room temperature except for centrifugations, which were carried out at 4–5 °C. Twenty grams of frozen bacteria was washed with 25 ml of 0.01 M Tris-HCl buffer, pH 7.5, and collected by centrifugation. The pellet was ground with 35 g of glass beads and 35 ml of buffer I in the stainless steel chamber of a Sorvall Omni-Mixer for 5 min at half speed and 5 min at full speed. The homogenate was transferred into a beaker, adjusted with 0.5 ml of a freshly prepared 1 mg/ml solution of DNase I in buffer I, and incubated at 30 °C for 30 min with occasional stirring. The beads were removed by centrifugation at 200 rpm for 10 min in the 872 rotor of the IEC B20A centrifuge, washed with 35 ml of buffer I, and centrifuged again at 500 rpm for 10 min. The two supernatants were combined and centrifuged at 45 000 rpm for 45 min in a Spinco Ti 60 rotor.

Ammonium Sulfate Precipitation and DEAE-Cellulose Chromatography. The high-speed supernatant from the previous step was precipitated with 0.33 g/ml of solid $(NH_4)_2SO_4$, corresponding to 50% saturation at 25 °C. After stirring for 20 min, the precipitate was collected by centrifugation for 40 min at 60 000 rpm in a Spinco Ti 60 rotor, resuspended in buffer II, and dialyzed overnight against 100 volumes of the same buffer at 4 °C. Subsequent centrifugation in a Ti 50 rotor at 35 000 rpm for 40 min removed any residual turbidity and left a clear amber supernatant. The last supernatant (13 ml) was applied to a 13 × 2.5 cm DEAE-cellulose column equilibrated with buffer II and washed with 70 ml of the same buffer. The column was then eluted with a linear gradient from 0 to 0.5 M KCl in 300 ml of buffer II. Elution rate was 1 ml/min. Fractions

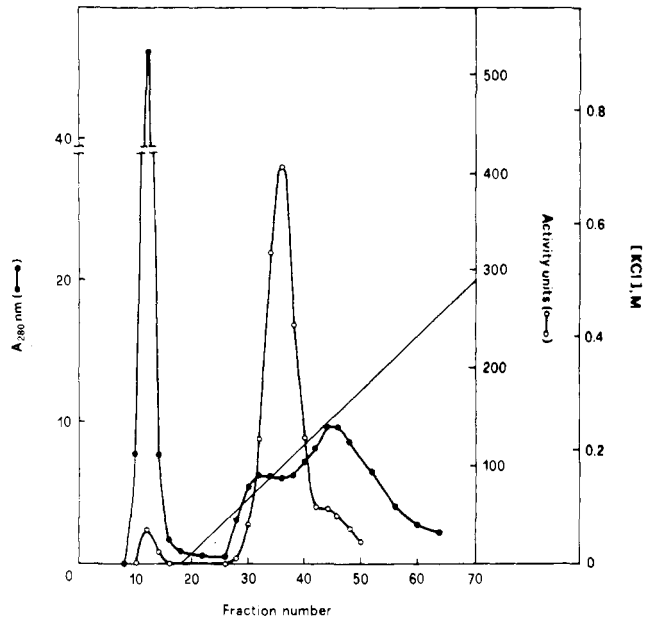

FIGURE 1: DEAE-cellulose column chromatography. Dialyzed ammonium sulfate fraction (13 ml) was applied to a 13 × 2.5 cm column which had been equilibrated with buffer II. The column was washed and the enzyme eluted as described in Materials and Methods. Fractions of 5 ml were collected. Aliquots of 20 μl were used to assay the enzymatic activity. (●) Absorbance at 280 μm; (○) RNA polymerase activity.

containing RNA polymerase activity were pooled and stored at 5 °C.

Glycerol Gradient Centrifugation. Twenty-seven milliliters of pooled DEAE fraction were brought to 1 M KCl by the addition of solid salt and layered over a 10–30% v/v glycerol isovolumetric (van der Zeijst and Bult, 1972) gradient in buffer II, 1 M KCl in a Ti 14 zonal rotor. The appropriate gradient shape was obtained with a Beckman Model 141 gradient pump. The gradient was centrifuged at 40 000 rpm, corresponding to a maximal 140 000*g*, for 22 h. After deceleration to 3000 rpm, the gradient was pumped out from the rotor. The active fractions were pooled and dialyzed against buffer II. After dialysis the enzyme was concentrated by adsorption on a 0.8 × 2.5 (inner diameter) cm DEAE-cellulose column equilibrated with buffer II at a 0.5 ml/min flow. The enzyme was eluted with 0.3 M KCl in buffer II in 3-ml fractions; the activity was usually recovered in three to four fractions. The pooled fractions were diluted to a final volume of 40 ml and subjected to gradient centrifugation at conditions similar to those relative to the high-salt glycerol gradient, except for KCl concentration which was 50 mM.

Results

Enzyme Purification. The elution pattern from the DEAE-cellulose column is shown in Figure 1. A single peak of enzyme activity was eluted at 175 mM KCl. The activity and OD_{280} profiles relative to the high-salt glycerol gradient are shown in Figure 2. The fractions from the low-salt glycerol gradient had no detectable absorbance and were concentrated on the same DEAE-cellulose column used after the high-salt glycerol gradient step. Purification of the enzyme is summarized in Table I.

Enzyme Characterization. The subunit composition of *C. acidophila* RNA polymerase, studied by sodium dodecyl sulfate gel electrophoresis (Figure 3), had a complexity

Table I: Purification of DNA-Dependent RNA Polymerase from *Caldariella acidophila*.[a]

	Total Protein (mg)	Total Enzyme Act. (U)	Yield (%)	Specific Act. (U/mg)	Purification (fold)
High-speed supernatant	1421	274 960	100	193	1.0
DEAE-cellulose	226	470 000	171	2 083	11.0
High salt glycerol gradient	18.3	204 624	74	11 200	58.3
Low salt glycerol gradient	7.7	104 676	38	49 550[b]	186.0[b]

[a] The data given refer to the purification of 20 g of cells. [b] Purest fraction.

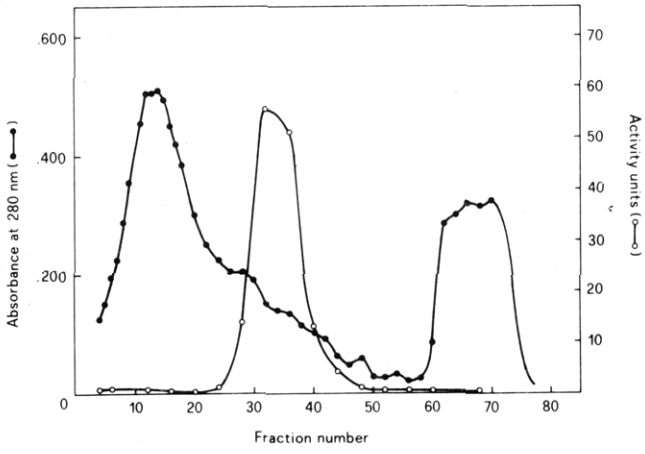

FIGURE 2: High salt glycerol gradient. Twenty-seven milliliters of pooled DEAE-cellulose fraction containing 226 mg of protein were layered over a 10–30% v/v isovolumetric gradient in buffer II, 1 M KCl in a Ti-14 zonal rotor. The gradient was centrifuged at 40 000 rpm for 22 h. Ten-milliliter fractions were collected from the rotor after deceleration to 3000 rpm. Aliquots of 20 μl were used for enzymatic activity assay. (●) Absorbance at 280 nm; (○) RNA polymerase activity.

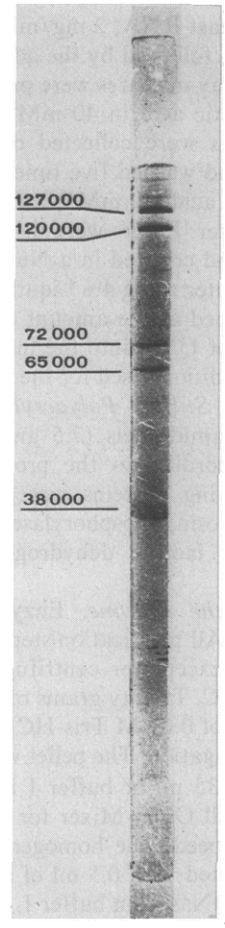

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *C. acidophila* RNA polymerase. Discontinuous gels were prepared and run as described in Methods. One hundred microliters of the highest specific activity fraction from the low-salt glycerol gradient was electrophoresed at 4 mA per gel.

comparable to that of other bacterial polymerases (Darnall and Klotz, 1975). Following the electrophoretic pattern of the fractions from the low-salt glycerol gradient, it was possible to give a tentative assignment to the bands indicated in the figure. The molecular weights of the subunits were determined in separate 7.5% polyacrylamide gels. The estimated values are as follows: a = 127 000, b = 120 000, c = 72 000, d = 65 000, and e = 38 000. These values are subjected to an error of ±5%, except for the largest two where the error is ±10%.

Kinetic Properties. The rate of RNA synthesis depends on the ionic strength of the incubation mixture (Figure 4). In the presence of 0.230 M KCl the reaction proceeds with slightly decreasing slope in the time interval investigated, while at 50 mM KCl a plateau is reached after 50 min. The activation of RNA polymerase by different divalent cations is shown in Figure 5. Mn^{2+} is more effective than Mg^{2+} in stimulating enzyme activity, its optimal concentration being 0.5 mM. In contrast, Mg^{2+} does not exhibit a sharp activation peak and its effect levels off at 5 mM. No synergistic effect of the two ions was observed. The effect of ionic strength on the activity is shown in Figure 6. The optimal activity was obtained in the presence of 250 mM KCl. The effect of pH was studied using two different buffer systems (Figure 7). In the presence of Tris-HCl buffer, the pH optimum was 7.8.

Thermophilicity and Thermostability of the Enzyme. The enzyme shows a remarkable degree of thermophilicity, as illustrated in Figure 8. Optimal temperature for activity in vitro is 80 °C. The activity gradually decreases at higher tempeature and the enzyme is 50% active at 90 °C.

Discussion

DNA-dependent RNA polymerase from *C. acidophila* was purified following the general procedures common to purifications of the same enzyme from other bacterial sources. The extremely thermophilic enzyme behaves similarly to corresponding enzymes from mesophilic bacteria in the purification steps, especially with regard to salt concentration necessary for elution from DEAE-cellulose and ionic strength dependent association–dissociation behavior.

The isovolumetric gradient obtained according to van der

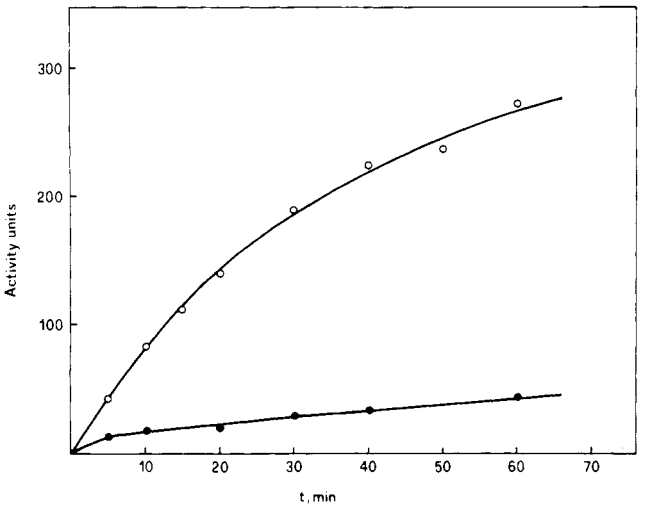

FIGURE 4: Time course of the RNA polymerase reaction at low- and high-salt concentration. Standard assays, except for salt concentration, were performed using 10 μl of concentrated low-salt glycerol gradient fraction. A 1-ml complete reaction mixture for each salt concentration was prepared and divided into 0.1-ml aliquots in sealed glass ampoules. At the time indicated, the reaction was stopped and the radioactivity determined as described in Materials and Methods. (●) With 50 mM KCl; (○) 230 mM KCl.

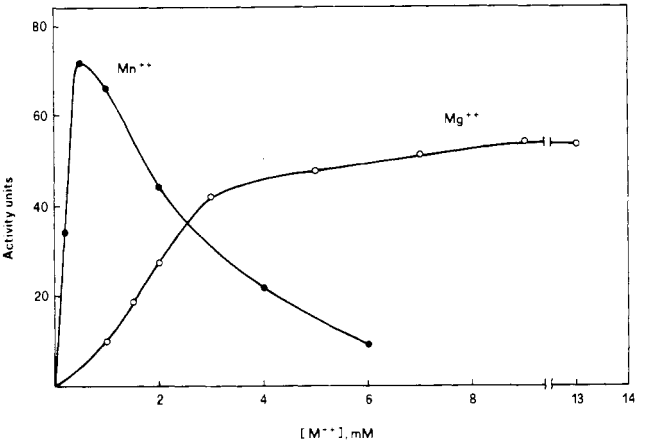

FIGURE 5: Effect of divalent cations on the RNA polymerase activity. Assays were standard except for metal ion concentration. Ten-microliter aliquots of concentrated low-salt glycerol gradient fraction were used for each assay. (●) Mn^{2+}; (○) Mg^{2+}.

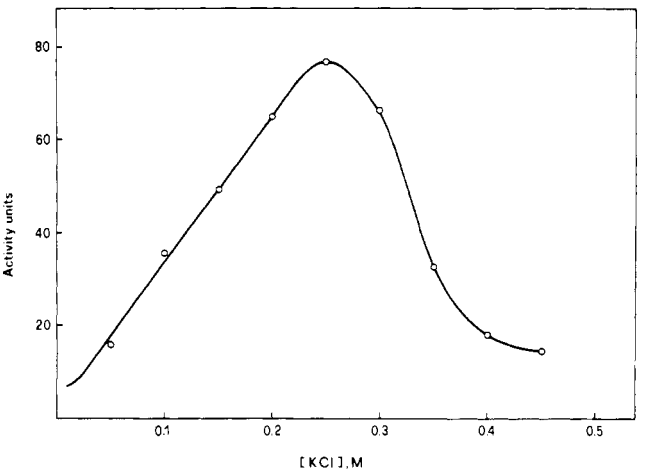

FIGURE 6: Effect of KCl on the RNA polymerase activity. Assays were standard except for KCl concentration. Ten microliters of concentrated low-salt glycerol gradient fraction was used.

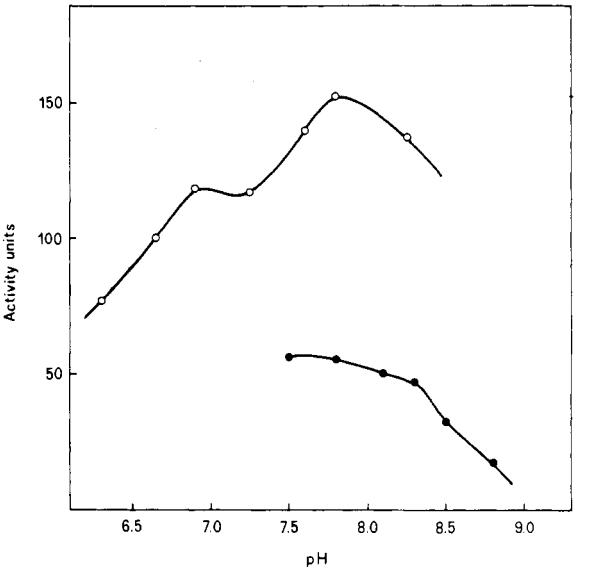

FIGURE 7: Dependence of the RNA polymerase activity on pH. Assays were standard except for the buffer employed. Ten microliters of concentrated low-salt glycerol gradient fraction was used. pH was measured experimentally at the assay temperature using model mixtures having the same composition. (○) Tris-HCl buffer, 80 mM final concentration; (●) borate buffer 60 mM final concentration.

Zeijst and Bult (1972) gave better resolution than did gradients linear with respect to volume in the same glycerol concentration range.

The enzyme exhibited a certain degree of inactivation at concentrations lower than 10 μg/ml, especially at low ionic strength. A rapid system of concentration was afforded by the use of a small DEAE-cellulose column; upon elution with increasing ionic strengths, the enzyme could be concentrated up to 100-fold with full recovery of activity. The purification table (Table I) shows an increase in the yield at the DEAE-cellulose step, as has been reported for other purifications (Kedinger et al., 1972; Cacace and Nucci, 1973) and generally attributed to the removal of nucleic acids which interfere, to some extent, with the polymerase assay. The enzyme at the final stage of purification was purified 186-fold over the crude extract and was DNA dependent. The subunit composition of the enzyme resembles that of other bacterial RNA polymerases. On the other hand both the complexity of *C. acidophila* RNA polymerase and the reduced molecular weight of its larger subunits support the idea that thermophiles could develop an efficient transcriptional system probably by a selective reduction of the polypeptide chain length rather than a simplified quaternary structure. Even at the extremes of temperature of the assay, synthesis of RNA is activated by as high as 0.25 M KCl, while it is drastically reduced at low salt concentration. This result compares favorably with the behavior of the RNA polymerase isolated from *B. stearothermophilus,* which presents a similar but less pronounced activation by ionic strength at optimal temperature. The optimal salt concentration (0.25 M KCl) for RNA polymerase activity from *C. acidophila* is among the highest reported in the literature for bacterial RNA polymerases and reflects a high stability in the association of enzyme protomers and the enzyme–DNA complex. There is indication that DNA strongly stabilizes the enzyme against thermal inactivation. The activi-

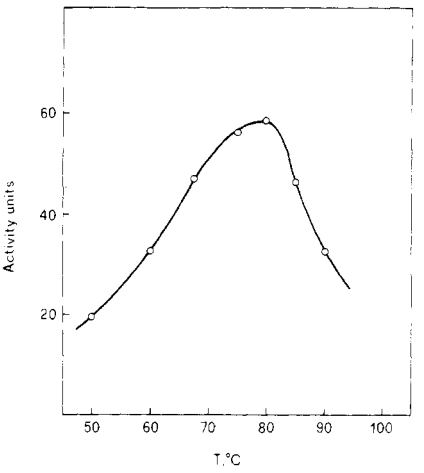

FIGURE 8: Dependence of RNA polymerase activity on temperature. Assays were standard except for the temperature of incubation. Ten microliters of concentrated low-salt glycerol gradient fraction was used.

ty in the presence of Mn^{2+} and Mg^{2+} is enhanced by the former ion while other bacterial RNA polymerases are optimally activated by Mg^{2+}. Remold-O'Donnell and Zillig (1969) have reported only 30% activity for *B. stearothermophilus* enzyme in the presence of Mn^{2+} when compared with Mg^{2+} at its optimal concentration. On the other hand, *C. acidophila* enzyme exhibits an optimal activity in the presence of Mg^{2+} that is only 70% of the optimal activity in the presence of 0.5 mM Mn^{2+}.

The effect of pH upon activity is similar to that found in mesophilic enzymes. In the presence of borate buffer the rate of RNA synthesis is 40% of that observed in Tris buffer at the same pH. However, no inhibition by higher borate concentrations was observed up to 100 mM borate. Due to the high temperature dependence of Tris ionization constants (0.022 pH unit/°C as experimentally determined in the conditions of the assay), borate buffer (0.004 pH unit/°C) was routinely employed for standard activity assay. The high temperature optimum of *C. acidophila* RNA polymerase reflects the physiological conditions for the growth of the bacterium. The assay optimum in vitro is only 7 °C below the normal temperature for growth and is closer to physiological conditions than that found for any other RNA polymerase isolated from thermophilic microorganisms (Remold-O'Donnell and Zillig, 1969; Air and Harris, 1974). Studies on the thermostability of the enzyme show that, at 90 °C in the presence of DNA at the concentration used for the assay, the $t_{1/2}$ of the inactivation process is 60 min (Cacace et al., 1976). This value is exceptionally high when compared with *B. stearothermophilus* polymerase which is 50% inactivated in 10 min at 65 °C or with *T. aquaticus* polymerase which is 35% inactivated after 60 min at 60 °C.

The peculiar thermophilicity and thermostability of *C. acidophila* RNA polymerase permit detailed studies on enzyme-template interactions in a temperature range at which many templates are close to their T_m value. Such studies are currently in progress in our laboratories.

Acknowledgments

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