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Deoxyribonucleic Acid Polymerase from the Extreme Thermophile *Thermus aquaticus*

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A stable deoxyribonucleic acid (DNA) polymerase (EC 2.7.7.7) with a temperature optimum of 80°C has been purified from the extreme thermophile *Thermus aquaticus*. The enzyme is free from phosphomonoesterase, phosphodiesterase, and single-stranded exonuclease activities. Maximal activity of the enzyme requires all four deoxyribonucleotides and activated calf thymus DNA. An absolute requirement for divalent cation cofactor was satisfied by Mg^{2+} or to a lesser extent by Mn^{2+}. Monovalent cations at concentrations as high as 0.1 M did not show a significant inhibitory effect. The pH optimum was 8.0 in tris(hydroxymethyl)aminomethane-hydrochloride buffer. The molecular weight of the enzyme was estimated by sucrose gradient centrifugation and gel filtrations on Sephadex G-100 to be approximately 63,000 to 68,000. The elevated temperature requirement, small size, and lack of nuclease activity distinguish this polymerase from the DNA polymerases of *Escherichia coli*.

Studies on the biosynthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* during the last 20 years have contributed substantially to our knowledge of this fundamental cellular process in bacteria. The original enzyme DNA polymerase I, which was thought to be involved in DNA synthesis, has been purified and charcterized in a series of studies reported by Arthur Kornberg and his colleagues (2, 5, 8-10, 16). These studies included such aspects as the physical and chemical properties of the purified enzyme, the primer-template DNA required for the reaction, the product DNA, and the reaction mechanisms of the polymerization of deoxyribonucleotides into DNA.

In 1969 DeLucia and Cairns (7) discovered an amber mutant, PolA, of *E. coli* W3110 that appeared to lack DNA polymerase activity but synthesized DNA normally. The isolation of this mutant not only casts serious doubt on the role of DNA polymerase I in in vivo DNA replication, but also pointed out the complexity of this cellular process. As a result, the discovery of this mutant led to the search for other replication enzymes, and two new polymerases have since been isolated and characterized (14, 15, 19, 20, 31).

In contrast to the extensively researched replication phenomena in mesophiles, few attempts have been made to isolate DNA polymerases from thermophiles (27). Thermophiles are ubiquitous in nature, and many prokaryotic species thrive at temperatures above 45°C. Attempts to explain the proliferation of life at temperatures that destroy most mesophilic cellular components have resulted in widespread investigation in the last 25 years, with many of these studies focusing on protein structure. In this communication the purification and characterization of a thermophilic polymerase will be discussed in relation to what is known about DNA polymerases from mesophilic microorganisms.

MATERIALS AND METHODS

Strain. *Thermus aquaticus* YT-1 used in these experiments was supplied by Paul Ray, Burroughs Wellcome.

Culture medium. Cells were grown in a defined mineral salts medium containing 0.3% glutamic acid (which served as both a carbon and nitrogen source), which was supplemented with biotin and thiamin (0.1 mg/liter each) and nicotinic acid (0.05 mg/liter). The salts included in 1 liter of medium were: nitrilotriacetic acid, 100 mg; $CaSO_4 \cdot 2H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; NaCl, 8 mg; KNO_3, 103 mg; $NaNO_3$, 689 mg; $ZnSO_4$, 5 mg; H_3BO_3, 5 mg; $CuSO_4$, 0.16 mg; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; $CoCl_2$, 0.4 mg; $FeCl_3$, 0.28 mg; $MnSO_4 \cdot H_2O$, 22 mg; and Na_2HPO_4, 110 mg. The pH of the medium was adjusted to 8.0 with NaOH.

Growth conditions. Cells were grown initially in 500-ml Erlenmeyer flasks at 75°C in a New Brunswick water bath shaker. When the cultures reached a density of approximately 170 Klett units, 1 liter of these cells was transferred to 16-liter carboys, which were placed in hot-air incubators. In place of shaking, sterile air was bubbled through the cultures, and the temperature was maintained at 75°C. The cells were allowed to grow for 20 h before they were collected with a Sharples continuous-flow centrifuge.

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Preparation of enzyme extract. Subsequent to collection, the cells were suspended in standard KP buffer (0.02 M potassium phosphate [pH 7.5], 8% [vol/vol] glycerol, 0.005 M ethylenediaminetetraacetate [EDTA], 0.01 M NaN_3, 0.01 M KCl). The cells were ruptured in a Branson 20-kc Magnetostrictive ultrasonic oscillator, operated at 3.5 A for 45 s. The extracts were then spun in a Sorvall RC-2B centrifuge at 17,300 × *g* for 20 min. The supernant fluid served as the crude extract.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (23).

Enzyme assays. (i) Duplicate DNA polymerase assays were carried out in disposable glass test tubes. The reaction mixture (125 μl) contained: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), 25 mM; 2-mercaptoethanol, 1 mM; $MgCl_2$, 10 mM; KCl, 25 mM; deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP), and ^{3}H-labeled thymidine 5'-triphosphate ([^{3}H]dTTP; specific activity, 38.8 mCi/mmol), each 0.15 mM; and activated calf thymus DNA (prepared by the method of Loeb et al. [22]), 12.5 μg. After 30 min of incubation at 80°C in sealed tubes, the assay was stopped by chilling the tubes in an ice bath for a few minutes. Samples of 100 μl were then pipetted onto 25-mm Schleicher and Schuell filter paper disks and immediately dropped into ice-cold 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate for at least 1 h. This was followed by two changes of 5% trichloroacetic acid. The first change contained 0.1 M sodium pyrophosphate. Finally, the disks were run through 30-min washes in ether-ethanol (1:1, vol/vol), ether-ethanol (3:1, vol/vol), and ether. The disks were air dried, and the amount of [^{3}H]dTTP incorporated into an acid-insoluble product was measured in a Packard scintillation spectrometer with a 5% counting efficiency for ^{3}H under these experimental conditions. One unit of enzyme is defined as the amount of enzyme that will incorporate 10 nmol of [^{3}H]dTTP into acid-insoluble material at 80°C in 30 min.

(ii) The exonuclease assays were conducted in a reaction mixture containing 25 mM Tris-hydrochloride (pH 8.0); 1 mM 2-mercaptoethanol; 10 mM $MgCl_2$; 12 μg of ^{3}H-labeled DNA prepared by the method of Reuben et al. (25), and approximately 0.02 U of polymerase in a final volume of 125 μl. After 30 min of incubation at 80°C, the tubes were chilled and the acid-insoluble material was measured by the filter paper disk method as described previously for the polymerase. The amount of acid-soluble counts released was used as a measure of exonuclease activity.

(iii) Alkaline phosphomonoesterase activity was assayed in a reaction mixture containing 1 mM *p*-nitrophenyl phosphate, 0.1 M Tris-hydrochloride buffer (pH 8.0), and approximately 0.02 U of polymerase in a final volume of 0.5 ml. After 20 min at 80°C, the reactions were stopped by chilling the tubes and then adding 0.5 ml of 0.1 N NaOH. The formation of *p*-nitrophenol was determined spectrophotometrically at 410 nm. *E. coli* alkaline phosphatase was used as a control. In addition, the phosphomonoesterase activity was assayed in the standard DNA polymerase assay mixture.

(iv) Alkaline phosphodiesterase I was assayed in a manner similar to that for alkaline phosphatase, except that 1 mM thymidine 5-monophospho-*p*-nitrophenyl ester was used as a substrate. Phosphodiesterase II activity was measured with 1 mM thymidine 3-monophospho-*p*-nitrophenyl ester as substrate and 0.1 M sodium succinate buffer, pH 6.5. In addition, both substrates were assayed in the standard DNA polymerase assay mixture.

Polyacrylamide gel electrophoresis. Disc-gels were prepared by the method of Davis (6). The separating gel (8.0 by 0.5 cm) contained 7% acrylamide, whereas the spacer gel (1.5 by 0.5 cm) contained 2% acrylamide. Electrophoresis in a Canalco model 66 electrophoresis bath was carried out at room temperature in 2.5 mM Tris-1.9 mM glycine buffer (pH 9.5) for 2.5 h at 2 mA. The gels were stained with 0.1% Coomassie brillant blue in 7% acetic acid and subsequently destained by diffusion in 7% acetic acid. For the recovery of the DNA polymerase activity from the gel, the gels were prepared in exactly the same manner, except that there was a 1-h electrical prerun to remove ammonium persulfate, which inhibits the enzymatic activity of the polymerase. Samples were then applied and electrophoresis was continued. The gels were removed from the tubes, sliced into 2-mm disks, and placed directly into tubes containing the polymerase assay mixture. No additional process for the elution of the enzyme off gel was required since the incubation temperature of the polymerase was 80°C.

Sucrose gradient centrifugation. Purified DNA polymerase (fraction IV, 50 μl) and bovine serum albumin (BSA) (2 mg) were layered on a linear sucrose gradient of 6 to 20%. The gradient also included 0.01 M Tris (pH 7.5), 0.001 M EDTA, 8% glycerol, 0.01 M NaN_3, and 0.5 M NaCl. Centrifugations were preformed in a Spinco SW50L rotor in a preparative Beckman ultracentrifuge at 38,000 rpm for 14 h at 4°C. Fractions (0.2 ml) were collected from the top of the gradient with an ISCO density gradient fractionator. BSA was assayed for absorbance at 280 nm, and the DNA polymerase was assayed by enzymatic activity. Approximate molecular weight for the DNA polymerase was determined by the method of Martin and Ames (24).

Molecular weight estimation by gel filtration. A column (Pharmacia K 16/70) was packed with Sephadex G-100 that had been preequilibrated in standard KP buffer containing 0.5 M NaCl and 0.01 M NaN_3. The void volume was determined by using blue dextran, and three proteins of known molecular weight were used as standards: BSA, 68,000; ovalbumin, 43,000; and lysozyme, 14,300. The approximate molecular weight of the DNA polymerase was determined in reference to a linear plot of the three standard proteins by the method of Andrews (1).

Chromatographic procedures. All chromatography was conducted at room temperature. Pharmacia chromatographic columns (K9, 15, 16, 50) and accessories were used. Flow rates of 40 ml/h for diethylaminoethyl (DEAE)-Sephadex and phosphocellulose

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and 6 ml/h for DNA cellulose were maintained through the use of a Harvard multispeed peristaltic pump. Fractions were collected with a Büchner automatic fraction collector. Phosphocellulose (Whatman grade P-1) was prepared by the method of Loeb (21), and the DNA cellulose was prepared by the method of Litman (18).

Chemicals and reagents. Calf thymus DNA type V, the four deoxyribonucleotides, pancreatic deoxyribonuclease (DNase), and BSA were purchased from Sigma Chemical Co. Ribonuclease (RNase) A and T_1 were purchased from Worthington Biochemicals Corp. The lysozyme and ovalbumin came from Schwarz/Mann. The radioactive isotopes [^{3}H]dTTP and [^{3}H]thymine were obtained from Schwarz Bio-Research, and [^{3}H]dATP was from New England Nuclear Corp. Chemicals and reagents for general use were of analytical grade and were purchased from Fisher Scientific Co.

RESULTS

Purification of DNA polymerase from *T. aquaticus*. For the purification, crude extract was prepared from 16 liters of cells as described above. Fraction I, 176 ml of sonically treated crude extract, was placed on a DEAE-Sephadex A-50 column that had been equilibrated previously with standard KP buffer. The column used was a Pharmacia K50/60 with flow adaptors and a set bed volume of 800 ml. A linear 2-liter gradient of 0.01 to 0.4 M KCl was used in standard KP buffer. The DNA polymerase eluted at a salt concentration of approximately 0.15 M. The pooled fractions were dialyzed against standard KP buffer (fraction II) and placed on a phosphocellulose column with a set bed volume of 40 ml packed in a K15/30 Pharmacia column. The peak of activity eluted at a salt concentration of 0.15 M KCl in a 400-ml linear gradient (0.01 to 0.4 M KCl in standard KP buffer). The pooled fractions were then dialyzed against 1 mM Tris-hydrochloride (pH 8.0) containing 1 mM β-mercaptoethanol, 0.1 M NaCl, 8% (vol/vol) glycerol, and 1 mM EDTA (fraction III). After dialysis, 500 g of BSA per ml was added to the enzyme preparation. The inclusion of BSA in this step of purification is necessary since its omission results in complete loss of polymerase activity. The enzyme preparation was then loaded on a preequilibrated DNA cellulose column and washed with 4 bed volumes of Tris buffer containing 1 mM EDTA, 8% (vol/vol) glycerol, 1 mM β-mercaptoethanol, and 500 μg of BSA per ml. The major peak of polymerase activity was eluted with 0.6 M NaCl. The peak fractions were pooled and dialyzed against KP buffer (fraction IV). A summary of the purification is shown in Table 1. The specific activity of fraction IV could not be determined due to the presence of BSA. Attempts were made to remove the BSA from the enzyme preparation on phosphocellulose and were partially successful. The major problem was the loss of over 95% of the activity during the process; therefore, we used fraction IV for the characterization of the enzyme since it was stable at -4°C for at least 3 months.

Polyacrylamide gel electrophoresis. Since BSA had been added to the enzyme preparation, we had no means of estimating the purity of the final enzyme preparation based on specific activity. Therefore, to get an estimate of purity, polyacrylamide gel electrophoresis was carried out. The enzyme was first passed through hydroxylapatite, to remove contaminating DNA eluted from the DNA cellulose, before it was loaded onto the gels. The results can be seen in Fig. 1, where BSA was run as a control (gel A). The enzyme preparation (gel B) was run in duplicate, since one gel was stained with Coomassie brilliant blue and the other was sliced and assayed for polymerase activity (plotted below gel B). The peak of activity corresponds to one of four major bands. Two of the four major bands appear to represent BSA, but it also should be mentioned that protein contaminants in the final preparation could migrate along with them and thus be hidden from detection.

Contaminating enzyme activities. Fraction IV was tested for a number of possible contaminating activities that might interfere with the DNA polymerase reaction. DNA polymerase (0.02 U per reaction tube) was assayed as described above for alkaline phosphomonoesterase and acid phosphodiesterases I and II. All of these activities were at background levels.

Exonuclease assays were conducted as described previously, using single-stranded (heat-denatured) ^{3}H-labeled λ DNA as a substrate. Experimental conditions and the amount of radioactive counts solubilized after 30 min of incubation are listed in Table 2. Only the pancreatic DNase I control (assayed at 37°C) was shown to be effective in solubilizing the ^{3}H-labeled λ DNA. Under these experimental conditions, the final enzyme preparation appears to be free from exonuclease activity in the presence and absence of ATP.

Temperature optimum. The effect of temperature on the catalytic activity of the enzyme can be seen in Fig. 2. The temperature optimum for the incorporation of [^{3}H]dTTP into acid-insoluble material was 80°C. At this temperature, the amount of incorporation was 10 to 15 times greater than that which was measured at 37°C. This optimum profile may reflect melt-

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TABLE 1. *Summary of the purification procedure*[a]

Fraction	Vol (ml)	Total polymerase activity (U)	Total protein (mg)	Yield of activity (%)	Sp act (U/mg)	Purification (fold)
Crude	176	2,080	975.0		2.13	
DEAE-Sephadex	210	4,613	197.5	221	23.3	10.9
Phosphocellulose	132	1,900	16.0	91	118.7	55.7
DNA-cellulose	63	685		33		

[a] One unit of polymerase equals the incorporation of 10 nmol of [^{3}H]dTTP into acid-soluble material at 80°C in 30 min.

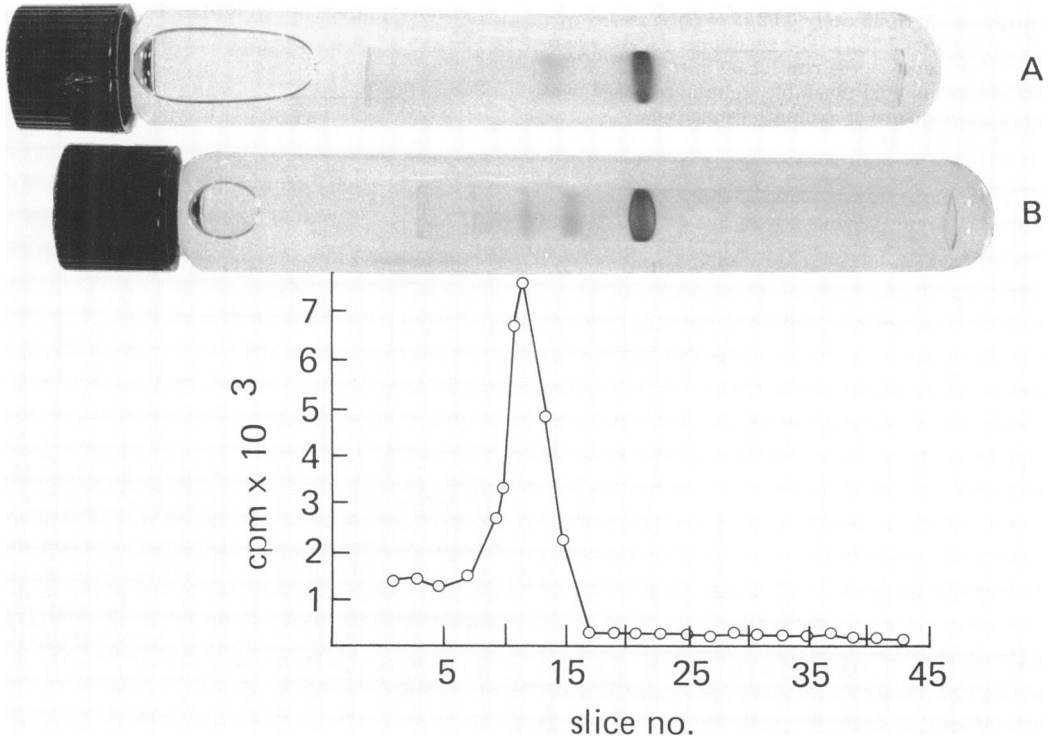

FIG. 1. *Gel electrophoresis of purified enzyme. Gel A, Electrophoresis of 25 μg of BSA at room temperature in 2.5 mM Tris-1.9 mM glycine buffer (pH 9.5). Gel B, Electrophoresis of 50 μl of fraction IV, which was stained for protein. The graph below gel B represents a gel of a size identical to that of B, which was subjected to electrophoresis at the same time but was sliced and assayed for activity as described in the text.*

ing of the template since the melting temperature of the DNA under these experimental conditions is 76°C. Thus, the decrease in catalytic activity above 80°C may be due to the denaturation of the template rather than the DNA polymerase since the enzyme has reduced activity with single-stranded DNA.

pH optimum. The effect of pH on the activity of the enzyme with three different buffer systems can be seen in Fig. 3. The pH optimum for the enzyme is in the range of 7.0 to 8.0 but varies with the buffer used. The highest activity was found with Tris-hydrochloride buffer, pH 7.8. The lowest activity occurred in potassium phosphate buffer, a situation which has been reported for mesophilic polymerases (16, 29).

Effect of divalent cations. This thermophilic DNA polymerase, like all other known polymerases, has an absolute requirement for divalent cations. Optimal activity is obtained with 10 mM Mg^{2+} (Fig. 4). Manganese was only partially effective and showed an optimum of 2 mM. Calcium ion was completely ineffective (data not shown).

Effect of monovalent cations. The addition of relatively low levels of KCl and NaCl stimulates the catalytic activity of the polymerase.

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TABLE 2. *Absence of single-stranded exonuclease specific activity*

Experimental conditions	% of cpm solubilized
Control (−DNA polymerase)[a]	0
DNA polymerase	0
DNA polymerase + 2 mM ATP	0
RNase A[b]	0
Pancreatic DNase I[b]	67.3

[a] The reaction mixture contained 25 mM Tris-hydrochloride (pH 8.0), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 12 μg of single-stranded ^{3}H-labeled λ DNA, and approximately 0.02 U of polymerase in a final volume of 125 μl. The reactions, except where otherwise stated, were run for 30 min at 80°C. The percentage of acid-insoluble counts released was used as a measure of exonuclease activity.

[b] Assayed at 37°C.

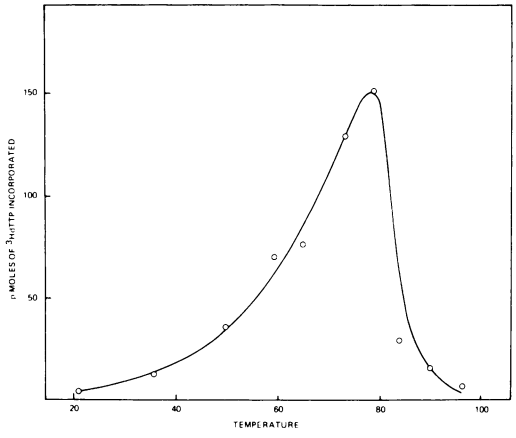

FIG. 2. *Effect of temperature on the activity of the DNA polymerase. DNA polymerase (0.02 U per assay) was incubated for 30 min in the standard reaction mixture at different temperatures. Fractions were then taken, and the conversion of [^{3}H]dTTP into acid-insoluble material was measured.*

The optimal concentrations of NaCl is 40 mM, whereas that of KCl is 60 mM. Above 100 mM, both salts inhibit the activity of the polymerase. These data are in contrast to polymerase I of *E. coli*, which is relatively insensitive to a high salt concentration but shows effects similar to those seen with polymerase II (16).

Requirements for DNA polymerase reaction. Various components of the reaction mixture were removed to see what effect this would have on in vitro DNA synthesis. Low activity in the absence of template DNA, Mg^{2+}, or deoxyribonucleoside triphosphates indicates the need for these components (Table 3).

Molecular weight estimation. (i) Sucrose gradient centrifugation. The approximate molecular weight of the purified DNA polymerase was determined by the method of Martin and Ames (24). The DNA polymerase activity peak corresponds to the optical density of the marker BSA (Fig. 5); this would indicate that its approximate molecular weight is about 68,000.

(ii) Gel filtration on Sephadex G-100. A Sephadex G-100 column was standardized and used to determine the molecular weight of the DNA polymerase as described in Materials and Methods. It can be seen in Fig. 6, plotting log molecular weight versus V_e/V_o, that the approximate molecular weight is 63,000.

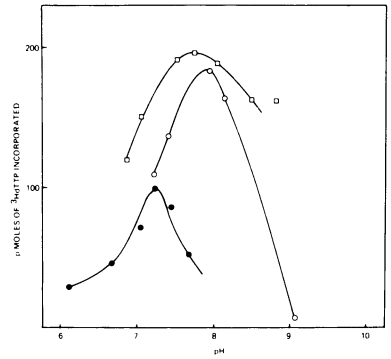

FIG. 3. *Effect of pH on the activity of the DNA polymerase from T. aquaticus. DNA polymerase (0.02 U per assay) was assayed in the standard reaction mixture with various buffers. The reactions were run at 80°C for 30 min, and corrections were made for the temperature coefficients of the various buffers used. Symbols: □, activity in 25 mM Tris-hydrochloride buffer; ○, activity in 25 mM glycine-sodium hydroxide buffer, ●, activity in 25 mM potassium phosphate buffer.*

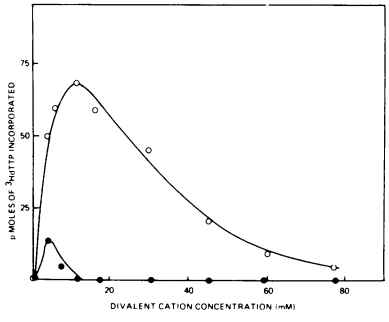

FIG. 4. *Effect of divalent cations on the DNA polymerase. DNA polymerase (0.02 U per assay) in the standard reaction mixture was used, except that the divalent cation concentration was varied as indicated. Symbols: ○, activity in $MgCl_2$, ●, activity in $MnCl_2$.*

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TABLE 3. *Requirements for DNA polymerase reaction*

Reaction components	[^{3}H]dTTP activity incorporated (pmol)	% Control
Complete reaction mixture[a]	102.9	100
$-Mg^{2+}$	6.0	6
-DNA	2.9	3
-dATP, dGTP, dCTP	18.6	18
-dATP	28.2	28
-dCTP	37.5	37
-dGTP	38.9	39
-dTTP[b]	20.7	21

[a] The complete reaction mixture contained in 125 μl: 25 mM Tris-hydrochloride (pH 8.0); 25 mM KCl; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol, 0.15 mM dATP, dCTP, dGTP, and [^{3}H]dTTP; 12.5 μg of calf thymus DNA; and 0.01 U of enzyme.

[b] [^{3}H]dATP was used instead of [^{3}H]dTTP.

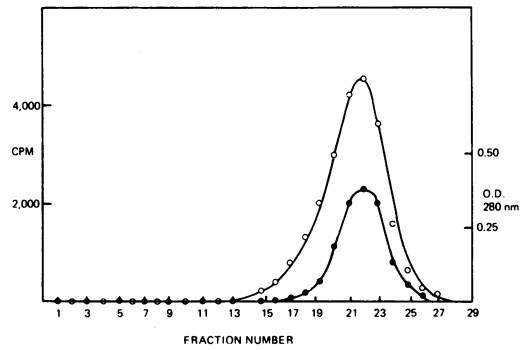

FIG. 5. *Molecular weight determination by sucrose gradient centrifugation. Fraction IV DNA polymerase (50 μl) and BSA (2.0 mg) were layered on a 5-ml linear sucrose gradient of 6 to 20% and run as described in the text. Fractions of 200 μl were collected and assayed for BSA by adsorbance at 280 nm (●) and DNA polymerase by catalytic activity (○).*

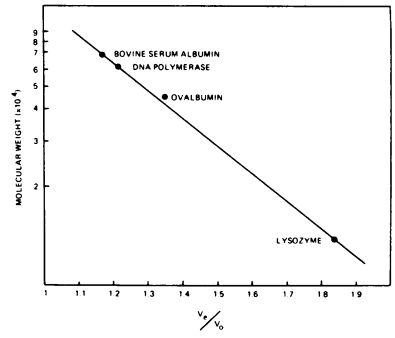

FIG. 6. *Estimation of the molecular weight of the DNA polymerase by gel filtration. A Sephadex G-100 column was run and assayed as described in the text. The molecular weight of the DNA polymerase was determined in reference to the three standard proteins by the method of Andrews (1).*

DISCUSSION

A stable thermophilic DNA polymerase has been isolated and purified. The gel data indicate that the final sample, although not homogenous, represents a relatively pure fraction containing BSA. Attempts to remove the BSA from the enzyme sample were only partially successful and resulted in extensive loss of the catalytic activity of the DNA polymerase, a situation which could have resulted from the low protein concentration of DNA polymerase after its separation from the BSA. The molecular weight of the enzyme has been estimated by sucrose gradient centrifugation to be 68,000, and by gel filtration it was estimated to be approximately 63,000, two sets of data which are relatively close in agreement. Its size, in relation to the three DNA polymerases from *E. coli*, is relatively small, although a DNA polymerase from *Bacillus subtilis* has been isolated which has a molecular weight of 46,000 (11). It should be pointed out that polymerase I from *E. coli* can be cleaved into two fragments by trypsin, one of molecular weight of 76,000 and the other of 34,000 (4). The large fragment retains the polymerase and 3′→5′ exonuclease activities, whereas the small fragment appears to be responsible for the 5′→3′ exonuclease activity. Thus, the question of whether the DNA polymerase isolated from *T. aquaticus* represents the native form of the enzyme in vivo or is a result of proteolytic cleavage during isolation cannot be answered at this time. The enzyme also appears different from the *E. coli* polymerases in that the final sample (fraction IV) is free from single-stranded exonuclease activity. The enzyme, like mesophilic DNA polymerases, requires all four deoxyribonucleoside triphosphates as well as DNA and Mg^{2+} for good catalytic activity. Omission of any one unlabeled deoxyribonucleoside triphosphate still gives approximately 35% maximal incorporation. This amount is higher than those reported for most bacterial polymerases but is similar to certain mammalian DNA polymerases (26). The incorporation probably represents the addition of two or three dTTP's to complementary ends.

The most unique feature of this enzyme is its temperature optimum of 80°C, which is 15°C higher than the optimal temperature reported for the DNA polymerase from *Bacillus stearothermophilus* (27). In addition to the scientific interest, this wide temperature range also provides the possibility of using this enzyme in

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gene synthesis in the form of a reverse transcriptase. Other DNA polymerases, such as polymerase I from *E. coli*, were shown under various experimental conditions to synthesize DNA by using ribonucleic acid (RNA) template (13). The DNA products of these DNA polymerases and the reverse transcriptases from tumor viruses appeared to have fallen short of completion of the entire gene (3, 28–30). One possible explanation for this could be interference by the secondary and tertiary structure of the RNA template in the completion of the DNA product. With an enzyme such as a thermophilic DNA polymerase, which has a wide temperature range, one might be able to overcome some of these difficulties, since it is possible to melt out the secondary and tertiary structure of the RNA template at elevated temperatures. We are currently investigating the possibility of using certain purified RNAs as template for this enzyme.

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