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Enzymatic Synthesis of Deoxyribonucleic Acid

XV. PURIFICATION AND PROPERTIES OF A POLYMERASE FROM *BACILLUS SUBTILIS**

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Nuclease activity persists in the most purified deoxyribonucleic acid polymerase preparations from normal and bacteriophage T2-infected *Escherichia coli* (1, 2). It has therefore been difficult to determine whether such a nucleolytic activity is an invariable component of bacterial polymerase. An opportunity to explore a source of polymerase which was promising in this respect was suggested by a survey of a variety of microorganisms for nuclease activity.[1] *Bacillus subtilis* was identified in a group of organisms which have low levels of nuclease activity; since extracts of this organism have substantial polymerase levels, the polymerase to nuclease ratio compared to that in *E. coli* is relatively high. Purification of DNA polymerase from *B. subtilis* therefore might yield a nuclease-free enzyme and would also provide a polymerase to compare with the *E. coli* enzyme for specificity of primers, for relative effectiveness of various base analogues of the deoxynucleoside triphosphates, for synthesis of deoxyadenylate-deoxythymidylate copolymer *de novo*, and for the ability to incorporate a ribonucleotide into a DNA polymer.

This report describes the preparation of a polymerase from *B. subtilis* which has little or no nuclease activity. Studies of the properties of the enzyme reveal fundamental similarities to the *E. coli* polymerase. Quantitative comparisons of base analogue incorporation support the view that base pairing rather than enzyme specificity determines the incorporation of bases into DNA.

EXPERIMENTAL PROCEDURE

Materials

Unlabeled deoxyribonucleoside triphosphates were purchased from the California Corporation for Biochemical Research. ^{32}P-Labeled deoxyribonucleotides, labeled in the ester phosphate, were prepared as described previously (3). Deoxythymidine-2-^{14}C, purchased from the New England Nuclear Corporation, was enzymatically phosphorylated to dTTP (^{14}C-dTTP) (4). ^{14}C-Labeled dCTP was prepared from DNA obtained from a *Chromatium* species grown on $^{14}CO_2$ as the sole carbon source (5). Cytidine triphosphate labeled with ^{32}P in the ester phosphate was prepared by enzymatic phosphorylation of ^{32}P-labeled cytidine 5′-phosphate, prepared according to Hurwitz (6). The procedure for preparation of the base analogues of deoxynucleoside triphosphates was previously described (7).

^{32}P-Labeled DNA (10 μc per μmole of phosphate) was isolated from *E. coli* as described by Lehman (8). Calf thymus DNA was isolated according to Kay, Simmons, and Dounce (9), and *B. subtilis* (SB 19) DNA by the method of Marmur (10). The dAT copolymer[2] was prepared by synthesis *de novo* with the hydroxylapatite fraction of the *E. coli* DNA polymerase (11). ^{32}P-dGdC (-*pC-) and ^{14}C-dAT (-p*T-) polymers were synthesized in a primed reaction with *E. coli* polymerase (11, 12). Heat-denatured DNA was obtained by heating DNA in 0.005 M KCl at 100° for 10 minutes and cooling quickly in an ice bath. DNA terminally labeled at its 3′-hydroxyl end with ^{14}C-deoxythymidylate was prepared with *E. coli* DNA polymerase in a "limited reaction" (5).

Concentrations of the polynucleotides are expressed as equivalents of nucleotide phosphorus.

The hydroxylapatite fraction of *E. coli* DNA polymerase was prepared as described elsewhere (1). *E. coli* endonuclease had a specific activity of 6,360 units per mg of protein (carboxymethyl cellulose fraction) (13); DNA phosphatase, purified from *E. coli*, had a specific activity of 76,500 units per mg of protein (phosphocellulose pervaporate fraction) (14). Crystalline pancreatic DNase was obtained from Worthington Biochemical Corporation. Micrococcal DNase purified from *Micrococcus pyogenes* was a gift from Dr. C. A. Dekker.

DEAE-cellulose was purchased from Brown Company; Whatman phosphocellulose (P-70) from W. and R. Ralson, Ltd.; Hypatite C (hydroxylapatite) from Clarkson Chemical Company, Inc.; Dextran 500 from Pharmacia; polyethylene glycol (Carbowax 6000) from Union Carbide Chemical Company; antibiotic medium 3 from Difco Laboratories; and Superbrite glass beads from Minnesota Mining and Manufacturing Company.

Methods

Assay of B. subtilis DNA Polymerase—The assay measures the conversion of a ^{14}C- or ^{32}P-labeled deoxynucleoside triphosphate into an acid-insoluble product. The standard incubation mixture (0.3 ml) contained 20 μmoles of Tris-maleate-KOH buffer, pH 8.2, 2 μmoles of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, 6 mμmoles of dAT copolymer, 10 mμmoles each of dATP and ^{14}C-dTTP (2×10^6 c.p.m. per μmole), and 0.01 to 0.1 unit of enzyme. In some cases, 40 mμmoles of calf thymus DNA replaced the dAT polymer with 10 mμmoles each of dGTP and dCTP in addition to dATP and ^{14}C-dTTP. Enzyme dilu-

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[1] E. A. Pratt and I. R. Lehman, unpublished observations.

[2] The abbreviations used are: dAT copolymer, copolymer of deoxyadenylate and deoxythymidylate; dGdC, polymer consisting of homopolymers of polydeoxyguanylate-polydeoxycytidylate; the prefix "r" denotes "ribo."

TABLE I

Purification of enzyme

Fraction and step	Protein	Polymerase activity[a]		
		dAT primer		Thymus DNA primer, specific activity
		Total activity	Specific activity	
	mg/ml	*units* $\times 10^{-3}$	*units/mg*	
I. Cell extract	25.8	42.7	1.4	0.15
II. Phase separation	9.24	33	3.1	
III. Ammonium sulfate	30.6	19.8	5.4	0.41
IV. DEAE-cellulose	2.53	14.6	24.3	2.45
V. Phosphocellulose[b]	0.44	5.8	209	20.8
VI. Hydroxylapatite (VI-2)	0.094	—[c]	569 (1060)[d]	19 (100)[d]

[a] The unit of activity here, as noted in "Methods," is based on the incorporation of the labeled nucleotide only. Therefore, in terms of total nucleotide incorporated, the values with dAT primer are actually twice this amount and those with DNA primer approximately 4 times that amount.

[b] This step was performed many times on a smaller scale, and these values are calculated for the large scale procedure.

[c] See the text.

[d] Assays in the presence of hydroxylapatite Peak *a* (0.082 μg of protein).

tions were made in 0.05 M Tris buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol, bovine serum albumin (1 mg per ml), and 0.1 M ammonium sulfate.

After 30 minutes of incubation at 37°, the reaction was stopped by the addition of cold 7% perchloric acid, and the acid-insoluble fraction was isolated either by centrifugation (15) or by filtration (16) as described before. Radioactivity measurements were made in a windowless gas flow counter (Nuclear-Chicago) or in a Packard Tri-Carb scintillation counter.

One unit of enzyme is defined as the amount catalyzing the incorporation of 10 mμmoles of labeled deoxynucleotide into the acid-insoluble product. Specific activity is expressed as units per mg of protein.

DNase or dATase activities were determined by measurement of the conversion of ^{32}P-DNA or ^{14}C-dAT copolymer (2-^{14}C-thymine) to acid-soluble products as described by Lehman (8). The 0.3-ml incubation mixture contained 25 mμmoles of ^{32}P-DNA or 6 mμmoles of ^{14}C-dAT polymer, 20 μmoles of Tris-maleate-KOH buffer, pH 8.2, 2 μmoles of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, and 0.01 to 0.2 unit of enzyme. DNA phosphatase activity was assayed by the method described elsewhere (14). The 0.3-ml incubation mixture contained 50 mμmoles of 3′-phosphoryl-terminated ^{32}P-DNA (micrococcal endonuclease-treated ^{32}P-DNA), 20 μmoles of potassium phosphate buffer, pH 7.0, 3 μmoles of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, and 0.01 to 0.3 unit of enzyme. After 30 minutes of incubation at 37°, the acid-soluble fraction was isolated and treated with Norit, and the resulting acid-soluble, Norit-nonadsorbable ^{32}P was counted.

Treatment of Primers with Nucleases—E. coli DNA phosphatase treatment: The incubation mixture (1.0 ml) consisted of 240 mμmoles of dAT or 500 mμmoles of calf thymus DNA, 50 μmoles of Tris-maleate-KOH buffer, pH 7.0, 10 μmoles of $MgCl_2$, and 13 units of enzyme. After a 30-minute incubation at 37°, aliquots were used in the polymerase reaction without inactivating the enzyme. *E. coli* endonuclease treatment: The reaction mixture (1.0 ml) contained 240 mμmoles of dAT or 500 mμmoles of calf thymus DNA, 50 μmoles of Tris-HCl buffer, pH 7.8, 10 μmoles of $MgCl_2$, and 0.2 unit of *E. coli* endonuclease. After a 75-minute incubation at 37°, 20 mμmoles of soluble RNA were added to the reaction mixture to inhibit the endonuclease. Micrococcal nuclease treatment: The incubation mixture (1.0 ml) contained 240 mμmoles of dAT or 500 mμmoles of calf thymus DNA, 50 μmoles of Tris-HCl buffer, pH 8.6, 10 μmoles of $CaCl_2$, and 0.4 unit of micrococcal nuclease. After incubation for 60 minutes at 37°, the reaction mixture was chilled and dialyzed against 3 liters of 0.05 M KCl for 15 hours to remove $CaCl_2$. Further treatment of the micrococcal digest with *E. coli* DNA phosphatase was carried out on the dialyzed sample.

Determination of nucleotide sequence in the synthetic polymer was carried out by chemical hydrolysis in the diphenylamine-formic acid system described by Burton and Petersen (17).

Protein was determined by the method of Lowry *et al.* (18). Samples with interfering materials were first precipitated with cold 5% trichloroacetic acid. Optical measurements were made with the Zeiss PMQ II spectrophotometer.

RESULTS

Purification of Enzyme

All procedures were carried out at 0–5° unless otherwise indicated.

Growth of Bacteria—Wild-type *B. subtilis* (SB 19) was grown in 90 liters of broth (Difco antibiotic medium 3) with vigorous aeration at 37° in a fermentor and was harvested at the end of logarithmic phase. The yield of packed wet cells was approximately 3.3 g per liter of culture; the cell paste was frozen and stored at −20° until used.

Preparation of Cell Extract—In a 5-liter Waring Blendor equipped with a cooling jacket were mixed 300 g of cell paste, 900 g of glass beads (Superbrite, average diameter 200 μ), and 130 ml of 0.05 M glycylglycine buffer, pH 7.0, containing 0.002 M EDTA and 0.002 M glutathione, for 35 minutes at a rheostat setting of 80 volts. The speed was then reduced by two-thirds, 270 ml of the same buffer were added, and mixing was continued for 10 minutes. The glass beads were allowed to settle for 10 minutes, the supernatant fluid was decanted, and the beads were washed with 800 ml of the same buffer. The supernatant fluids were combined and centrifuged for 30 minutes at 10,000 × *g*. The resulting supernatant fluid (1,150 ml) was collected (Fraction I, Table I). The temperature was maintained at or below 7° throughout the procedure.

Removal of Nucleic Acids by Phase Partition[3] (*19*)—To Frac-

[3] A simpler purification procedure based on a protamine precipitation of polymerase followed by chromatography on DEAE-cellulose was abandoned for three reasons. The yields with the protamine step were variable, the capacity of DEAE-cellulose for the protamine fraction was low, and, most significant, the polymerase activity was distributed in two separate peaks on the DEAE-cellulose chromatogram. One of the peaks was eluted with 0.2 M phosphate buffer, pH 7.4 (20 to 50%), and the other with 0.32 M buffer (50 to 80%). When the latter peak was rechromatographed on DEAE-cellulose, over 70% of the activity was then eluted with 0.2 M buffer. We suspect that nucleic acid persisting in the protamine fraction was responsible for the heterogeneous character of polymerase activity on the DEAE-cellulose chromatogram. The phase separation method appeared to give a more complete and reproducible removal of nucleic acid from the polymerase.

tion I were added 132 ml of 20% (w/w) Dextran 500 solution and 370 ml of 30% (w/w) polyethylene glycol to give final concentrations (by weight) of 1.6% and 6.4% for dextran and polyethylene glycol, respectively (20).[4] NaCl (387 g) was added gradually to the solution (final concentration, about 4 M), and the mixture was stirred for 2 hours at 0° and then centrifuged at 500 × *g* for 10 minutes. The resulting 1480 ml of clear top phase (polyethylene glycol + protein) were recovered to give Fraction II, and the turbid bottom phase (280 ml, dextran + nucleic acids) was discarded.

Ammonium Sulfate—To remove the NaCl, Fraction II was dialyzed against 40 liters of 0.3 M potassium phosphate buffer, pH 7.4, containing 0.002 M EDTA and 0.01 M 2-mercaptoethanol, for 20 hours; the volume increased to 2,400 ml and 94% of the activity was recovered. Solid ammonium sulfate (480 g) was added gradually to the dialyzed fraction with continuous stirring, and the solution was transferred to a 4-liter separatory funnel. After 7 hours at 4°, a lower phase containing the enzyme separated from the upper polyethylene glycol phase. To 2,100 ml of the lower phase were added 192 g of ammonium sulfate, and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 10 minutes. Another 264 g of ammonium sulfate were added to the supernatant fluid, and the precipitate was recovered by centrifugation and dissolved in 120 ml of 0.3 M potassium phosphate buffer, pH 7.4, containing 0.002 M EDTA and 0.002 M glutathione (Fraction III). Fraction III was stored at −15° until used.

DEAE-cellulose Chromatography—A column of DEAE-cellulose (8.3 cm² × 12 cm) was prepared and equilibrated with 0.02 M K_2HPO_4 containing 0.01 M 2-mercaptoethanol. Fraction III (70 ml) was dialyzed against 3 liters of 0.3 M potassium phosphate buffer, pH 7.4, containing 0.002 M EDTA and 0.01 M 2-mercaptoethanol, for 4 hours, and the dialysate (75 ml) was diluted to 450 ml with 0.01 M 2-mercaptoethanol before being adsorbed to the column at the rate of 4.5 ml per minute. The column was then washed with 200 ml of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.002 M 2-mercaptoethanol and 0.002 M EDTA. A linear gradient of elution was applied with 0.05 M and 0.5 M potassium phosphate, pH 7.4, as limiting concentrations. The total volume of the gradient was 2000 ml, and 0.01 M 2-mercaptoethanol and 0.002 M EDTA were present throughout the gradient. The flow rate was 4.5 ml per minute, and 40-ml fractions were collected. Of the activity applied to the column, 85% was eluted between 6.4 and 12 resin bed volumes of effluent. Peak fractions having specific activities of 15 to 41 were combined (360 ml) and concentrated to one-third this volume by dialysis against 30% polyethylene glycol in 0.3 M potassium phosphate buffer, pH 7.4, containing 0.002 M EDTA and 0.002 M glutathione. The DEAE-cellulose step was carried out with the remaining 50 ml of Fraction III on a smaller column (8.3 cm² × 10 cm). The peak fractions recovered from the two columns were combined to yield 240 ml of Fraction IV (74% recovery from Fraction III). Fraction IV was stored at −15° until used.

Phosphocellulose Chromatography—Fraction IV (80 ml), dialyzed against 8 liters of 0.05 M potassium phosphate buffer, pH 6.8 (containing 0.002 M EDTA and 0.01 M 2-mercaptoethanol), for 12 hours and diluted to 200 ml with 0.01 M 2-mercaptoethanol, was passed through a phosphocellulose column (15 cm² × 10 cm) previously equilibrated with 0.02 M potassium phosphate buffer, pH 6.5, containing 0.002 M EDTA and 0.01 M 2-mercaptoethanol. The column was washed with 300 ml of 0.05 M potassium phosphate buffer, pH 6.8, containing 0.002 M EDTA and 0.01 M 2-mercaptoethanol. The column was eluted with a linear gradient of potassium phosphate buffer, pH 6.8, from 0.05 M to 0.40 M. The buffer contained 0.01 M 2-mercaptoethanol and 0.002 M EDTA, and 500 ml of each buffer were used. Fractions of 10 ml were collected at 3-minute intervals. The enzyme was eluted between 2.2 to 3.7 resin bed volumes of effluent in a zone containing 15% of the initial protein. Fractions having specific activities of 172 to 302 were combined (55 ml, 51% recovery), concentrated by dialysis for 4 hours against 1 liter of 30% polyethylene glycol solution in the buffer used for washing the column, and then dialyzed against 2 liters of this buffer for 12 hours (Fraction V, 21 ml, 40% recovery from Fraction IV). It was used immediately for the next step of purification. In dilute solution at 0°, Fraction V lost half of its activity in 24 hours (see footnote *b*, Table I).

Hydroxylapatite Chromatography—A column of hydroxylapatite (0.9 cm² × 10 cm) was prepared and washed with 0.02 M potassium phosphate buffer, pH 6.8, containing 0.01 M 2-mercaptoethanol. Fraction V (19 ml) was applied to the column at a rate of 0.4 ml per minute, and the adsorbent was washed with 10 ml of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.01 M 2-mercaptoethanol. The enzyme was then eluted with a linear gradient of potassium phosphate buffer at pH 7.4, the limits of which were 0.05 M and 0.25 M. The buffers contained 0.01 M 2-mercaptoethanol and 80 ml of each buffer were used; fractions of 2 ml were collected every 5 minutes. The chromatogram is shown in Fig. 1.

A peak of nuclease activity was detected between 2.2 to 6.6 resin bed volumes of effluent (Peak *a*) whereas polymerase activity occurred between 6.6 to 13.3 resin bed volumes (Peak *b*) with only a trace of nuclease activity. The polymerase peak was divided into four portions as shown in Fig. 1. Each portion was concentrated as described previously in 0.2 M potassium phosphate buffer, pH 7.4, containing 0.002 M glutathione and 0.002 M EDTA, to give a protein concentration of about 0.1 mg per ml (Fractions VI-1 to VI-4).

Fraction VI failed to show enzyme proportionality to the amount of protein added (Fig. 2), and the recovery at this step was only 10 to 25%. Addition of Peak *a* material to the polymerase reaction mixture stimulated the activity of Fraction VI several fold, as shown in Fig. 2, improved enzyme proportionality, and increased the recovery of activity (about 50% from Fraction V).

Nuclease Activities in Enzyme Preparations

The nuclease and polymerase activities of various fractions are compared in Table II. Values for the most purified *E. coli* DNA polymerase are also provided for reference. The polymerase to nuclease activity ratio in the crude extract (Fraction I) of *B. subtilis* was higher to begin with than that of the extensively purified *E. coli* polymerase. In Fraction V, the nuclease activity was relatively greater on native DNA than on heat-denatured DNA or dAT polymer. However, chromatography on the hydroxylapatite column separated this nuclease activity (Peak *a*, Fig. 1) from the polymerase fraction, and only a trace of nuclease activity could be detected in Fraction VI.[5]

[4] We gratefully acknowledge the advice and help of Dr. Per-Åke Albertsson with this procedure.

[5] Assays of this fraction for nuclease based on the destruction

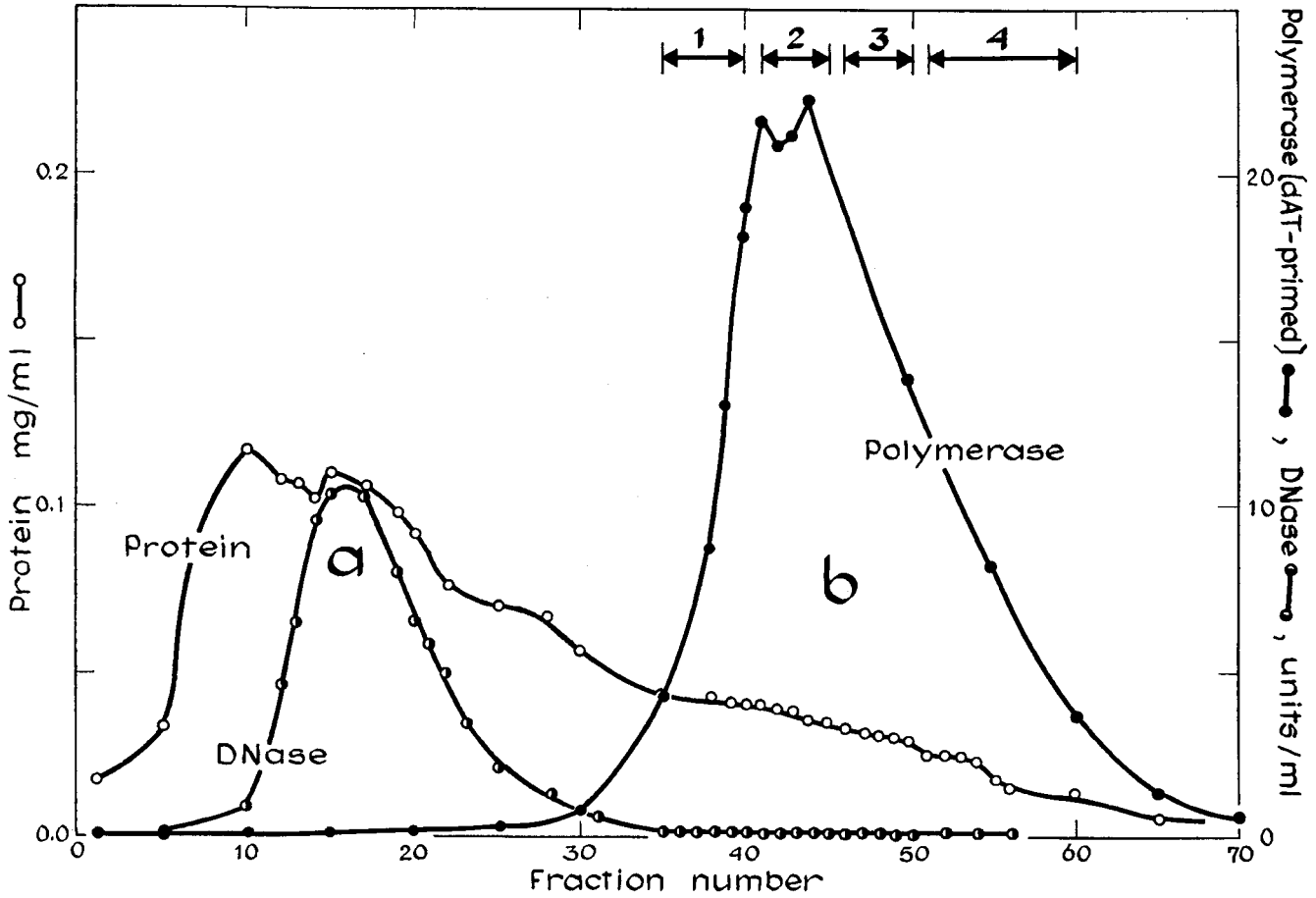

FIG. 1. Chromatography of Fraction V on hydroxylapatite. Elution was achieved with a linear gradient of potassium phosphate buffer ranging from 0.05 to 0.25 M at pH 7.4. The eluate fractions were assayed for protein, DNase, and polymerase (dAT-primed) as described in "Methods." Nuclease fractions are labeled as *a*, and polymerase fractions as *b*. The contents of tubes 35 to 40, 41 to 45, 46 to 50, and 51 to 60 were pooled and concentrated separately (Fractions VI-1, -2, -3, and -4, respectively).

The pH optimum of the DNase activity in Fractions V and VI and Peak *a* was 8.2 in Tris-maleate-KOH buffer, and $MnCl_2$ (0.2 μmole) could be used to replace $MgCl_2$ (2 μmoles). All three fractions showed DNA phosphatase activity (cleavage of inorganic phosphate from 3′-phosphoryl ends of DNA molecules) (14) in constant ratio to DNase activity; the ratios for Fractions V and VI and Peak *a* were 0.26, 0.22, and 0.19, respectively. The DNase in Peak *a* can apparently initiate its attack at the 3′-hydroxyl ends of DNA. This was shown with DNA terminally labeled at its 3′-hydroxyl terminus with ^{14}C-deoxythymidylate as a substrate (5). When 85% of the radioactivity was rendered acid-soluble, only 3% of the nucleotides in the DNA had been released, as judged by ultraviolet absorption of the acid-soluble fraction. This is a result similar to that obtained with venom diesterase (5), an exonuclease known to initiate its stepwise hydrolysis from the 3′-hydroxyl end of DNA (21).

The stimulatory effect of Peak *a* on polymerase activity is most probably due, as in the *E. coli* studies (22), to removal of inhibitory 3′-phosphoryl ends from DNA chains and to the production of new 3′-hydroxyl ends in their place. The same stimulatory effect was obtained when the purified *E. coli* DNA phosphatase (14) was used in place of Peak *a*. Since the *E. coli* DNA phosphatase-exonuclease has the dual capacity to act as a DNA phosphatase and exonuclease (cleaves the 3′-phosphoryl linkage of mono- or diesterified phosphate residues of a DNA chain), it seems likely that the Peak *a* enzyme of *B. subtilis* is also a single protein with both functions.

Properties of Enzyme

pH Optimum—With Fraction V, the maximal rate of synthesis in the dAT-primed reaction occurred around pH 8.2 in Tris-maleate-KOH buffer (Fig. 3). The same results were obtained with Fraction VI and Tris-maleate-KOH or glycine buffer.

Requirements for Reaction—A DNA primer, a divalent metal, and the appropriate deoxynucleoside triphosphates were essential for the reaction (Table III). Under the assay conditions and at the level of dAT copolymer used, $MnCl_2$ was almost as effective as $MgCl_2$ at low concentrations (2×10^{-4} M) but was very inhibitory at a higher concentration (1×10^{-3} M) (Fig. 4).

Incorporation of Base Analogues—With the *E. coli* and T2 polymerases, it has been shown that analogues of the naturally occurring bases could serve as substitutes in a manner governed by the base pairing of adenine to thymine and of guanine to cytosine, as proposed by Watson and Crick for the double helical

of *B. subtilis* transforming factor activity were generously performed by Dr. Walter Bodmer. With 1.88 μg of this fraction incubated with 6.2 mμmoles of DNA for 60 minutes at 37°, 90% of the transforming factor activity was retained.

structure of DNA (23). The same replacements, in the form of deoxynucleoside triphosphates, were found with Fraction V of *B. subtilis* polymerase. Thus, uracil and its 5-bromo and 5-fluoro derivatives could replace thymine; hydroxymethyl-, 5-methyl-, 5-bromo-, and 5-fluorocytosine could replace cytosine; and hypoxanthine could replace guanine. Xanthine was not incorporated, and *N*-methyl-5-fluorocytosine gave very little incorporation when used in place of dCTP (Table IV). The values obtained are virtually indistinguishable from those obtained with the *E. coli* polymerase (hydroxylapatite fraction) tested in parallel experiments.

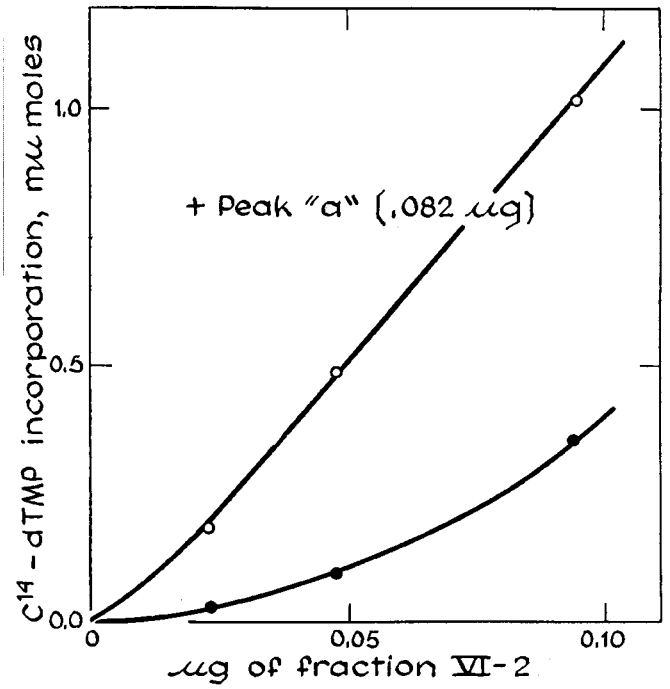

FIG. 2. Proportionality of the polymerase activity of Fraction VI-2. The standard dAT-primed assay was used with and without addition of 0.082 μg of Peak *a* of the hydroxylapatite chromatogram.

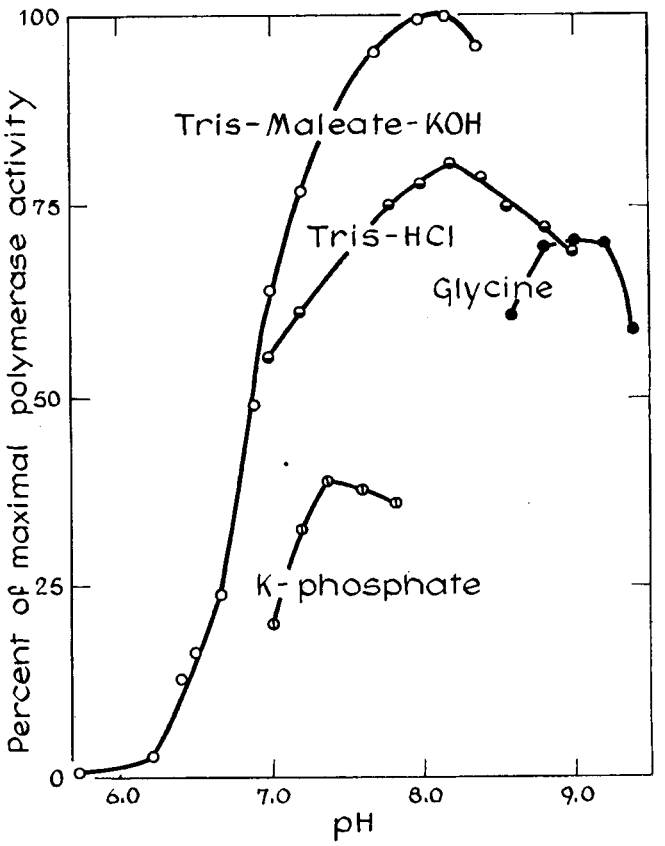

FIG. 3. Effect of pH and buffer on polymerase activity. Activity was measured in the routine dAT-primed assay except that the buffer indicated replaced the usual buffer. The pH of each buffer (0.05 M) was determined at room temperature. To each assay was added 0.88 μg of Fraction V, and the value in Tris-maleate-KOH at pH 8.2 was set at 100%.

Attempt to Incorporate a Ribonucleoside Triphosphate—As described by Berg, Fancher, and Chamberlin (24), *E. coli* DNA polymerase, in the presence of Mn^{++}, incorporates ribonucleoside triphosphates in place of their deoxy counterparts. However,

TABLE II

Comparison of nuclease and polymerase activities in various enzyme fractions

Assay conditions for the *B. subtilis* enzymes are described in "Methods." *E. coli* polymerase assay conditions were like those used for *B. subtilis*, except that potassium phosphate buffer (pH 7.4, 0.06 M) was used. Nuclease activities in *E. coli* polymerase fraction were assayed at pH 9.2 in glycine buffer.

Primer or substrate	Polymerase, Fraction			Nuclease, Fraction				Polymerase to nuclease ratio, Fraction			
	I	V	VI-2	I	V	VI-2	Peak *a*	I	V	VI-2	*E. coli* hydroxylapatite
	mμmoles nucleotide incorporated/mg protein			*mμmoles nucleotide or phosphate released/mg protein*							
Heated DNA				13.7[a]	43.7						
Native DNA	6.3	685	850 (4,250)[b]	3.1	234	28.7	827	2.03	2.94	29.6 (148)[b]	0.58
dAT	31.9	4,400	10,650 (21,300)[b]	6.2	119	3.2	346	5.14	37	3,328 (6,680)[b]	1.47
3′-Phosphoryl-terminated DNA					62	6.4	153				

[a] When $MgCl_2$ was replaced by $CaCl_2$ (0.2 μmole per 0.3 ml of assay mixture), this value was 1,780 and less than 1.9 with Fraction V.

[b] Values assayed in the presence of 0.082 μg of hydroxylapatite Peak *a*; this amount of Peak *a* had no detectable nuclease activity by itself.

TABLE III

Requirements for reaction

The complete system for the reactions is described in "Methods." For Columns 1 and 2, respectively, 0.19 and 2.84 μg of Fraction VI-1 were used.

System	^{14}C-dTMP incorporated	
	dAT primer	Thymus DNA primer
	mμmoles	
Complete	0.435	0.461
Minus primer	<0.005[a]	<0.006
Minus $MgCl_2$	<0.005	<0.005
Minus $MgCl_2$, plus $MnCl_2$[b]	0.394	0.395
Minus dATP	0.012	0.012
Minus dATP, plus dAMP	0.013	0.010
Minus dATP, plus dGTP	0.007	
Minus dGTP		0.013
Minus dCTP		0.020
Minus dATP, dGTP, dCTP		0.019

[a] Synthesis of dAT *de novo* occurs after a lag period of 2 to 5 hours.

[b] $MnCl_2$, 0.05 μmole.

B. subtilis DNA polymerase fails to utilize a ribonucleoside triphosphate when examined under the conditions described in Table V. With rCT^{32}P in place of dCT^{32}P, no incorporation was observed either with Mg^{++} or with Mn^{++}. rATP and rGTP also failed to replace dATP or dGTP, respectively, in supporting the incorporation of ^{14}CdTTP. The value for incorporation with the four deoxynucleoside triphosphates was 0.31 mμmole; in the absence of dATP or dGTP or upon replacement of dATP by rATP or dGTP by rGTP, there was no detectable synthesis, *i.e.* less than 0.01 mμmole.

Effects of Various Treatments of Primers on Priming Activities—In order to study the influence of end groups of primer molecules on polymerase reactions, dAT polymer and calf thymus DNA were treated with the following nucleases: (*a*) *E. coli* DNA phosphatase-exonuclease, an enzyme known to specifically remove 3′-phosphoryl end groups from native DNA; (*b*) *E. coli* endonuclease and pancreatic DNase, which cleave phosphodiester bonds of DNA to produce 3′-hydroxyl and 5′-phosphoryl termini throughout the molecule; (*c*) micrococcal endonuclease, which cleaves phosphodiester bonds of DNA, producing 3′-phosphoryl and 5′-hydroxyl termini; and (*d*) subsequent treatment of the micrococcal endonuclease digestion product with *E. coli* DNA phosphatase to remove 3′-phosphoryl end groups. Priming activities of these DNAs for Fraction VI-2 and highly purified *E. coli* polymerase were determined (Table VI). With both dAT polymer and thymus DNA, removal of the 3′-phosphate by DNA phosphatase resulted in an increase of priming activities for *B. subtilis* as well as for *E. coli* polymerase. The treatment with the endonucleases which produce 3′-hydroxyl and 5′-phosphoryl ends also stimulated priming activities of both primers, whereas treatment with micrococcal nuclease resulted in marked reduction of priming activities. It should be noted that although the

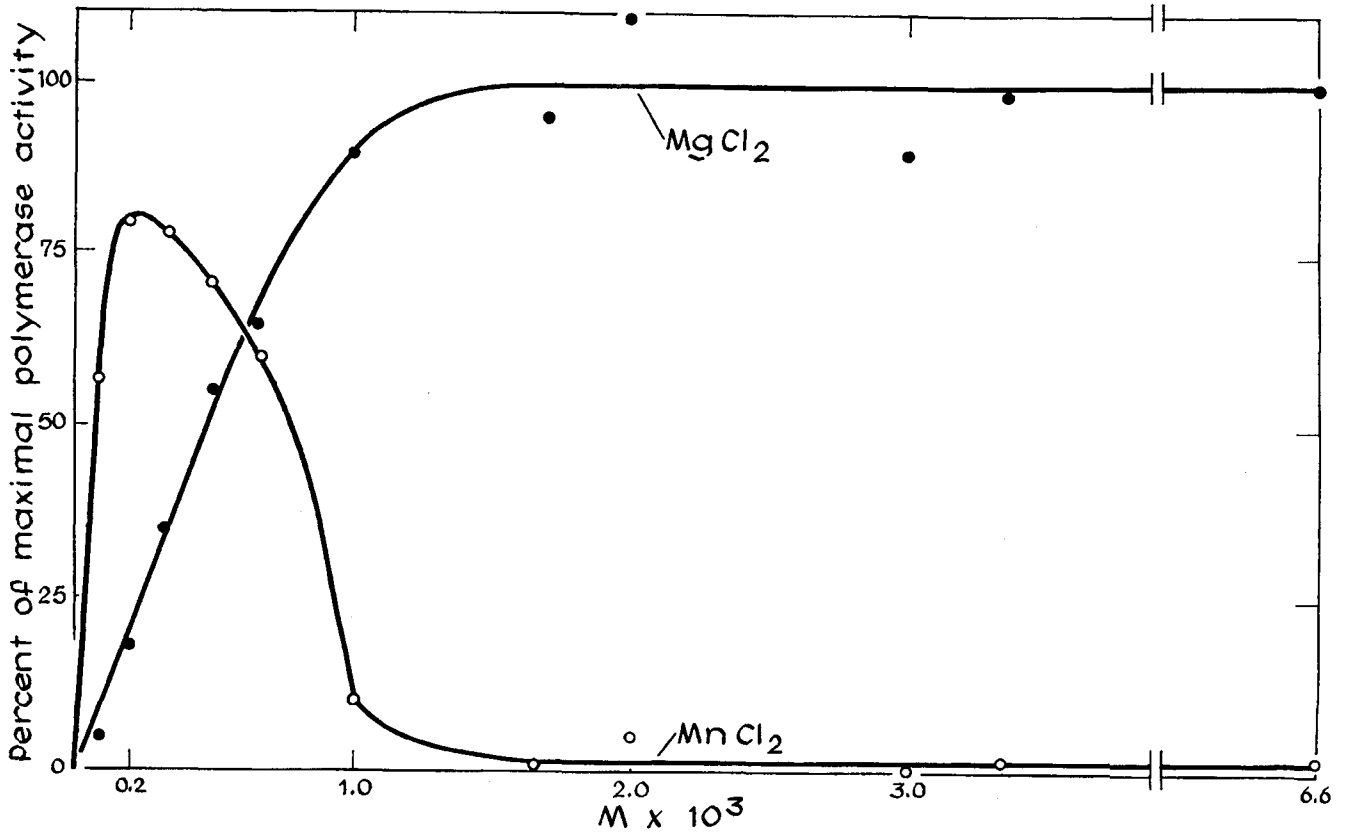

FIG. 4. The influence of metal ion concentration on the rate of polymerase activity. The standard dAT-primed assay was used, except that the metal ion concentration was varied, as shown. To each assay was added 0.88 μg of Fraction V enzyme, and the value with 2 μmoles of $MgCl_2$ per assay mixture (0.66×10^{-2} M) was set at 100%.

latter treatment does not change the total number of 3′-hydroxyl and 5′-phosphoryl end groups, there was a marked decrease in priming activity. This priming activity could be restored by the enzymatic removal of 3′-phosphoryl end groups from the primer molecule by the DNA phosphatase.

These results indicate that the 3′-hydroxyl terminus is necessary for the reaction of both polymerases whereas a 3′-phosphoryl end is inhibitory.

Heat denaturation of thymus DNA resulted in little change of priming activity compared with native DNA under the conditions used in this experiment.

TABLE IV

Replacement of deoxynucleoside triphosphates by various base analogues in polymerase reaction

Reaction mixtures were those of the standard assay with 40 mμmoles of calf thymus DNA activated with *E. coli* DNA phosphatase (see "Treatment of Primers with Nucleases") and 1.3 μg of Fraction V. Incubations were carried out for 30 minutes at 37°. Control values were measured as rates of radioactive deoxynucleotide incorporation into DNA in the presence of the four usual nucleoside triphosphates but in the absence of the analogue. Values were 0.34 mμmole of ^{14}C-dTMP for Columns 2, 3, and 4, and 0.19 mμmole of ^{14}C-dCMP for Column 1. Omission of one of the deoxynucleoside triphosphates gave 2 to 6% of the control values. Values in parentheses were obtained with *E. coli* polymerase (0.28 μg of hydroxylapatite fraction) in potassium phosphate buffer, pH 7.4.

Analogue used in form of deoxynucleoside triphosphates	Deoxynucleoside triphosphate replaced by analogue			
	dTTP	dATP	dCTP	dGTP
	% *control value*			
Uracil	69 (61)	3	6	3
5-Bromouracil	91 (93)	3	3	2
5-Fluorouracil	28 (27)	6	6	4
5-Hydroxymethylcytosine	6	6	107 (61)	6
5-Methylcytosine	0	2	118 (127)	5
N-Methyl-5-fluorocytosine	5	5	12 (10)	3
5-Fluorocytosine	3	3	82 (55)	6
5-Bromocytosine	3	4	98 (129)	6
Hypoxanthine	5	3	7	69 (50)
Xanthine	7	5	7	4

TABLE V

Attempt to incorporate a ribonucleoside triphosphate

As in "Methods," assay mixtures contained 20 μmoles of Tris-maleate-KOH, pH 8.2, 10 mμmoles each of dATP, dTTP, and dGTP, 40 mμmoles of calf thymus DNA, 10 mμmoles of dCT^{32}P (2×10^6 c.p.m. per μmole) or 100 mμmoles of rCT^{32}P (2×10^6 c.p.m. per μmole), 2 μmoles of $MgCl_2$ or 0.05 μmole of $MnCl_2$, and 2.84 μg of Fraction VI-1 enzyme. Incubation was performed for 30 minutes at 37°.

Metal	Substrate	Incorporation of dCM^{32}P or rCM^{32}P
		mμmole
$MgCl_2$	dCT^{32}P	0.317
$MnCl_2$	dCT^{32}P	0.314
$MgCl_2$	rCT^{32}P	<0.01[a]
$MnCl_2$	rCT^{32}P	<0.01[a]

[a] Prolonged incubation (120 minutes) or the use of 0.5 μmole of $MnCl_2$ and 350 mμmoles of calf thymus DNA did not produce any detectable increase of incorporation of rCM^{32}P.

TABLE VI

Comparison of activities of various primers for B. subtilis and E. coli polymerases

Pretreatment of dAT copolymer and calf thymus DNA was carried out as described in "Methods." The pretreated dAT, 6 mμmoles, was used as primer in the standard incubation mixture, with 0.024 μg of *B. subtilus* polymerase (Fraction VI-2) or 0.007 μg of *E. coli* polymerase (hydroxylapatite fraction). The pretreated calf thymus DNA, 40 mμmoles, was used as primer in the standard incubation mixture, with 0.47 μg of *B. subtilis* polymerase or 0.28 μg of *E. coli* polymerase. The rates of incorporation of nucleoside monophosphates for nontreated dAT primer were 11.4 and 53.4 mμmoles per μg of Fraction VI-2 and *E. coli* polymerase, respectively, and were set at 100%.

Primer and pretreatment	Relative priming activity	
	B. subtilis Fraction VI-2	*E. coli* hydroxylapatite
dAT copolymer		
None	100	100
E. coli DNA phosphatase	312	329
E. coli endonuclease	344	250
Pancreatic DNase	366	
Micrococcal endonuclease	35	16
Micrococcal endonuclease followed by *E. coli* DNA phosphatase	268	180
Thymus DNA		
None	6.7	2.8
Heated at 100° and quickly cooled	8.6	3.5
E. coli DNA phosphatase	31	37
E. coli endonuclease	70	63
Heat denaturation of *E. coli* endonuclease-treated DNA	74	73
Pancreatic DNase	28	
Micrococcal endonuclease	2.5	1.4
Micrococcal endonuclease followed by *E. coli* DNA phosphatase	49	45

Net Synthesis of DNA—With Fraction VI-2 and *B. subtilis* native DNA as primer, 2.5 replications (2.5 parts synthesized DNA per unit of primer) were obtained in 25 hours. With heat-denatured DNA as primer, the initial rate of synthesis was similar to that with native primer; however, the rate decreased after 0.15 replication and in 25 hours of incubation only 0.64 replication was observed (Fig. 5). The thymine to cytosine ratio of the replication product was checked at intervals with two identical reaction mixtures, one containing ^{14}C-dTTP and the other ^{32}P-dCTP. The ratio was 1.57 at 0.08 replication and 1.35 between 0.15 and 1.0 replication; the thymine to cytosine ratio in *B. subtilis* is 1.3.

dAT Synthesis, de Novo and Primed—*B. subtilis* polymerase carries out extensive replication of dAT polymer when primed with dAT made by *E. coli* polymerase. As shown in Fig. 6, 70% of the added substrate was made acid-precipitable in 3 hours as determined by ^{14}C-dTMP incorporation (6.2 replications). Unlike the case with even the most purified *E. coli* polymerase fractions, this product was not degraded after prolonged incubation (more than 10 hours). Without added primer, synthesis of dAT *de novo* was observed after a 3- to 5-hour lag

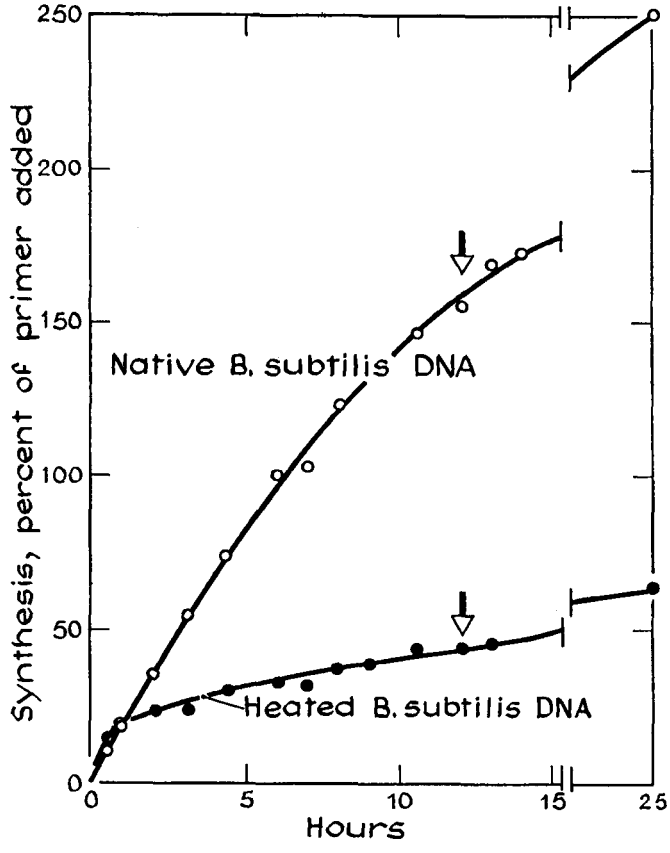

FIG. 5. The course of replication of native and heated DNA. The reaction mixture contained (in 1.5 ml) 100 μmoles of Tris-maleate-KOH, pH 8.2, 10 μmoles of $MgCl_2$, 1.5 μmoles of 2-mercaptoethanol, 0.125 μmole each of dATP, dCTP, dGTP, and ^{14}C-dTTP (2×10^6 c.p.m. per μmole), 62 mμmoles of *B. subtilis* DNA, 50 μl of enzyme diluent, and 18.8 μg of Fraction VI-2. The reaction was carried out at 37°. At intervals, 50-μl aliquots were taken for measurement of acid-precipitable radioactivity. At the time shown by the *arrow*, an additional 2 μg of enzyme were added.

period. The copolymeric nature of the product of synthesis *de novo* (with dATP and ^{14}C-dTTP) was determined by hydrolysis with diphenylamine-formic acid (12, 17). Acidic depurination with subsequent scission of the polynucleotide chain at the depurinated sites results in acid solubilization of the residues of the hydrolyzed polynucleotide. ^{32}P-DNA isolated from *E. coli* and dGdC and dAT polymers made by *E. coli* polymerase were also subjected to the same treatment to serve as standards. Under the conditions which result in complete acid solubilization of *E. coli* DNA or dAT copolymer, there is no significant acid solubilization of pyrimidine-labeled dGdC polymer. The dAT polymer synthesized by *B. subtilis* polymerase was rendered acid-soluble to the extent of 98% (Table VII), suggesting that it is a copolymer of deoxythymidylate and deoxyadenylate units like that formed *de novo* by *E. coli* DNA polymerase.

DISCUSSION

The finding that the DNA polymerase from *B. subtilis* may be freed of virtually all nuclease activity contrasts with the persistence of such activity in the purified *E. coli* and T2 phage polymerase preparations. It is clear, therefore, that even if an exonucleolytic activity should prove to be an intrinsic capacity of a pure DNA polymerase, this is not an essential property of all DNA-synthesizing enzymes. On the other hand, it should not be assumed that any nuclease activity associated with polymerase is necessarily unrelated to DNA replication. For example, the DNA phosphatase-exonuclease is found in both the *B. subtilis* and *E. coli* polymerase preparations and separated by hydroxylapatite chromatography only in the last step of the purification procedure. This enzyme, by removing phosphoryl groups attached to the 3'-hydroxyl termini of DNA chains, converts inhibitory DNA to an active primer. Such 3'-phosphoryl groups may occur as a result of endonuclease action or be produced by shearing forces; for some reason, such groups do occur in native DNA isolated by usual methods, as may be inferred from the many fold increase in priming activity of the DNA after treatment with the DNA phosphatase-exonuclease.

The DNA primer requirements of the *B. subtilis* polymerase are remarkably similar to those of the *E. coli* polymerase. The reaction rate with dAT is about 20 times faster than with native DNA, and the rates with native and single stranded DNA are about equal. The inhibitory effect of 3'-phosphoryl ends and the activating effect of 3'-hydroxyl ends are also seen with both enzymes. By contrast, the T2 phage-induced polymerase (2) and the enzyme from calf thymus (25) require single stranded DNA; with the T2 enzyme, dAT is a relatively poor primer and the production of additional 3'-hydroxyl ends in native DNA does not activate its priming capacity. It should be emphasized that these comparisons are based on rather gross characteristics of the DNA preparations and that the finer details of the DNA may reveal important distinctions between the *B. subtilis* and *E. coli* polymerases.

Studies of the utilization of nucleoside triphosphates by the several bacterial polymerases reveal differences that reflect on the mechanism of replication. The relative rates of reaction with a number of base analogues of the deoxynucleoside triphosphates are remarkably similar with the *B. subtilis*, *E. coli*, and T2 phage-induced polymerases. From this it seems likely that the determination of which bases are selected in assembling the DNA chain is made by the template rather than by the enzyme. On the other hand, the capacity of the *E. coli* polymerase but not the *B. subtilis* enzyme to utilize ribonucleoside triphosphates must be a specific attribute of the enzyme. These results fortify the hypothesis that the sequential ordering of the bases is primarily due to directions by the DNA template.

One of the purposes in purifying the DNA polymerase from *B. subtilis* was to discover a source of the enzyme which might prove more amenable to purification than is *E. coli*. This was disappointing, in that the *B. subtilis* enzyme was far more difficult to obtain free of nucleic acids, was less stable, and was recovered in smaller yields. The specific activity of the most purified *B. subtilis* preparation was only about one-tenth that of the best *E. coli* fractions.

SUMMARY

1. A deoxyribonucleic acid polymerase has been purified from *Bacillus subtilis* and its properties compared to the polymerase from *Escherichia coli*.

2. Unlike the *E. coli* polymerase, the purified preparation has barely detectable exonucleolytic activity and, as with the *E. coli* enzyme, no measurable endonuclease activity. Hydroxylapatite chromatography, the last step in the purification procedure,

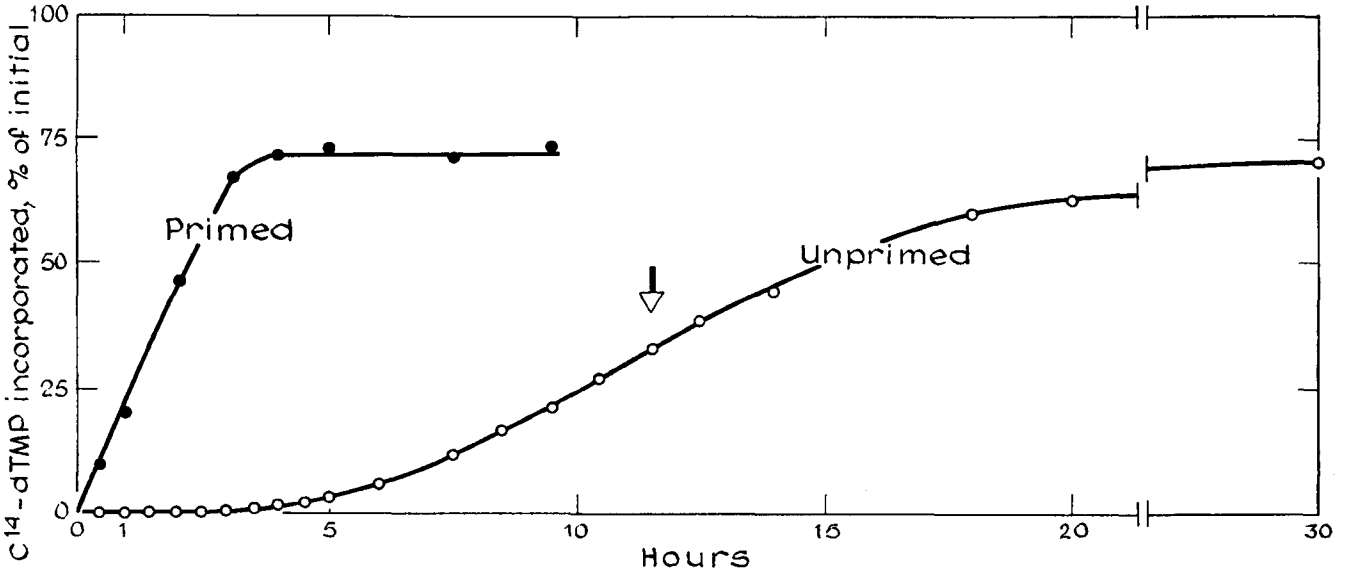

FIG. 6. Synthesis of dAT copolymer, *de novo* and primed. A 3-ml reaction mixture contained 0.5 μmole each of dATP and ^{14}C-dTTP (2×10^6 c.p.m. per μmole), 200 μmoles of Tris-maleate-KOH buffer, pH 8.2, 20 μmoles of $MgCl_2$, 3 μmoles of 2-mercaptoethanol, and 0.2 μl of enzyme diluent. For primed synthesis, 60 mμmoles of dAT polymer made by *E. coli* polymerase were added, and for both primed and unprimed reactions, 35 μg of Fraction VI-2 were present. Aliquots (30 μl) were taken at intervals, and acid-precipitable count was determined. After 20 hours, 1 ml of reaction mixture was taken from the unprimed reaction for chemical determination of nucleotide sequence.

TABLE VII

Demonstration that dAT is a copolymer

dAT polymer was synthesized in a nonprimed reaction from ^{14}C-dTTP and unlabeled dATP with *B. subtilis* polymerase (Fraction VI-2) and then hydrolyzed by the method of Burton and Petersen (17). Each polymer (0.5 ml, 0.2 μmole per ml, 10^6 c.p.m. per ml) was incubated with 1 ml of freshly prepared 3% diphenylamine in formic acid for 17 hours at 30°. A 0.3-ml aliquot was used for the assay of acid-insoluble radioactivity before and after hydrolysis as described previously (12). ^{32}P-Labeled preparations of *E. coli* DNA and of dGdC and ^{14}C-labeled dAT polymers made by *E. coli* polymerase were treated in the same manner to serve as controls.

Preparation	Distribution of label	Acid-precipitable radioactivity after hydrolysis		
		0 time	17 hrs	
		c.p.m.	c.p.m.	%
dAT made by *B. subtilis* polymerase	-p*T-	4,278	93	2.2
E. coli DNA	—[a]	12,050	144	1.2
dAT made by *E. coli* polymerase	-p*T-	2,733	29	1.1
dGdC made by *E. coli* polymerase	-*pC-	1,525	1,444	95

[a] Uniform.

identifies and separates a DNA phosphatase-exonuclease (14), just as in the case of *E. coli*.

3. The priming activities of deoxyadenylate-deoxythymidylate copolymer (dAT copolymer) and of native DNA, and the effect on priming of heating and deoxyribonuclease treatment of DNA are essentially the same with the purified *B. subtilis* and *E. coli* polymerases. Replication of native DNA and synthesis *de novo* of dAT copolymer proceed readily, and in the latter case to the nearly complete utilization of the deoxynucleoside triphosphate substrates provided. Because of the absence of nucleases, the synthesized dAT remains intact even after prolonged incubation.

4. Various base analogues of the naturally occurring deoxynucleoside triphosphates substitute in the *B. subtilis* polymerase system in a fashion quantitatively identical with that determined in the *E. coli* system. These and other data reaffirm that adenine to thymine and guanine to cytosine base pairing between template and substrate, rather than enzyme specificity, determines the sequential polymerization of nucleotides.

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