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Enzymatic Synthesis of Deoxyribonucleic Acid

XXV. PURIFICATION AND PROPERTIES OF DEOXYRIBONUCLEIC ACID POLYMERASE INDUCED BY INFECTION WITH PHAGE T4+ *

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MEHRAN GOULIAN,‡ ZOLTAN J. LUCAS,§ AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California 94304

SUMMARY

DNA polymerase induced in *Escherichia coli* by phage T4 *am* N82 has been prepared in a highly purified state. The molecular weight of about 112,000 for this enzyme compares with a value of 109,000 for the host (*E. coli*) DNA polymerase. The amino acid compositions of the two enzymes, however, are distinctive, especially the half-cystine value of 15 residues for the phage enzyme and 3 for the bacterial enzyme. The purified phage enzyme contains an exonuclease activity which is physically inseparable from the polymerase activity but is suppressed or obscured by conditions favorable for replication. The polymerase requires a single stranded template, with a free 3′-hydroxyl terminus, which is replicated to an extent that approaches but never exceeds the DNA input. The product is a helical structure in which the newly synthesized strand is covalently linked from its 5′-terminus to the 3′-terminus of the template. Various features of the replication process and the product suggest a mechanism in which the 3′-terminus of the template first loops back upon itself and then serves as the priming end for replication of the remainder of the template. The evidence indicates that the phage polymerase, unlike the *E. coli* enzyme, is unable to initiate new strands or utilize a fully helical DNA as template.

The increase in rate of deoxyribonucleic acid synthesis associated with infection by virulent coliphages (1) was shown, in the case of phages T2, T4, and T5, to be accompanied by the appearance of a new DNA polymerase (2–4). Recent work with conditional lethal mutants has justified the earlier presumptions that this enzyme, unique to phage infection, is the product of a phage gene and is essential for synthesis of phage DNA (5, 6).

In the present study, we have examined the properties which distinguish the phage-induced polymerase from the host enzyme: the requirement for a single stranded template and the limited extent of replication. Such studies, we anticipate, will extend our understanding of DNA synthesis in general and phage DNA replication in particular.

A procedure is presented for preparation of highly purified phage T4 polymerase, followed by a description of the physical and enzymatic properties of the enzyme. Studies of the nature of the DNA synthesized suggest a mechanism for the action of phage T4 polymerase *in vitro*.

EXPERIMENTAL PROCEDURE

Materials

Unlabeled deoxyribonucleoside triphosphates were purchased from Calbiochem or Pabst and purified by chromatography on DEAE-Sephadex (A-25) with a triethylammonium bicarbonate gradient. α-^{32}P-Labeled deoxyribonucleoside triphosphates were prepared as described previously (7).

^{3}H-DNA from M13 phage was isolated as described by Mitra *et al.* (8). The linear form of M13 DNA was prepared by limited exposure to *Neurospora* endonuclease (0.05 unit per μmole[1] of DNA for 20 min). Under conditions described by Linn and Lehman (9), approximately two-thirds of the DNA circles were cleaved, and the linear forms were obtained by fractionation in an alkaline sucrose gradient (8). ^{3}H-labeled T7 DNA and T4 DNA were prepared by the procedure of Richardson, Inman, and Kornberg (10); ^{3}H-labeled T7 DNA was digested with micrococcal nuclease by the method of Richardson, Schildkraut, and Kornberg (11). A single stranded form of T7 DNA was prepared by digestion with λ-exonuclease to the limit of 50% acid solubility as described by Little (12), followed by alkaline denaturation at 50°. ^{3}H-Labeled λ DNA was a gift from Dr. Walter Doerfler.

[1] Molarity of DNA refers to concentration of nucleotide residues.

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‡ Special Fellow, National Institutes of Health. Present address, Department of Medicine, University of Chicago, Chicago, Illinois 60637.

§ American Cancer Society Fellow. Present address, Department of Surgery, Stanford University School of Medicine, Palo Alto, California 94304.

Salmon sperm and calf thymus DNAs were isolated by the method of Kay, Simmons, and Dounce (13); partial pancreatic digestion of this DNA was carried out by the procedure of Oleson and Koerner (14). Preparation of *Bacillus subtilis* DNA was based on the method of Massie and Zimm (15). ^{3}H-dAT copolymer[2] was made in a primed incubation with ^{3}H-dATP by the method of Schachman *et al.* (16). Poly dC was isolated by Cs_2SO_4 density gradient sedimentation (17) from dI:dC that had been prepared in a primed incubation (17). The poly dC was heated at pH 3 for 5 min at 95° to reduce contamination with small fragments of dI. Poly dA, made with calf thymus terminal deoxynucleotidyltransferase (18), was a gift from Dr. F. J. Bollum. Unlabeled and ^{32}P-labeled *Escherichia coli* DNAs were isolated according to the method of Lehman (19).

The following enzymes were used: exonuclease III, prepared by the method of Richardson and Kornberg (20); exonuclease I (hydroxylapatite fraction), Lehman and Nussbaum (21); λ-exonuclease, Little, Lehman, and Kaiser (22); *Neurospora* endonuclease, Linn and Lehman (23); *E. coli* alkaline phosphatase, Worthington Biochemical Corporation; 5′-nucleotidase, Lehman, Roussos, and Pratt (24); and micrococcal nuclease, Cunningham, Catlin, and Privat de Garilhe (25).

DEAE-cellulose and phosphocellulose were products of Bio-Rad. Hydroxylapatite and Sephadex G-25 were purchased from Clarkson and Pharmacia, respectively. Streptomycin sulfate was the gift of Merck, Sharp and Dohme.

Methods

Assay of Polymerase—The assay measures the conversion of ^{32}P-labeled deoxyribonucleoside triphosphates to an acid-insoluble product. The incubation mixture (0.3 ml) contained 67 mM Tris buffer (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 16.6 mM ammonium sulfate, 6.7 μM EDTA, 0.33 mM sodium fluoride, 167 μg of bovine plasma albumin per ml, 0.2 mM alkali-denatured salmon sperm DNA, 0.033 mM concentration each of dCTP, dATP, dTTP, and dGTP (one of which was labeled in the α position with ^{32}P, 1 to 5 × 10^6 cpm per μmole), and 0.04 to 0.40 unit of enzyme. Enzyme dilutions were made in 0.05 M Tris (pH 7.5), 0.1 M ammonium sulfate, bovine plasma albumin (1 mg per ml), and 0.01 M 2-mercaptoethanol. After a 30-min incubation at 37°,[3] the reaction was stopped by addition of 0.1 ml of a carrier solution containing 0.1 M sodium pyrophosphate, 0.1 M EDTA, and 100 μg of calf thymus DNA per ml. Acid-insoluble material was collected and washed on glass filters (26), and radioactivity was measured in a liquid scintillation counter. Sodium fluoride, which served to inhibit deoxycytidine triphosphatase activity, was omitted in assays of fractions beyond the phosphocellulose step in the purification procedure. A unit of enzyme activity[4] is defined as the amount catalyzing incorporation of 10 mμmoles of total nucleotide into an acid-insoluble product during the period of incubation. The assay was linear in the range of 0.04 to 0.40 unit of enzyme.

[2] The abbreviations used are: dAT copolymer, copolymer of deoxyadenylate and deoxythymidylate; poly dC, polydeoxycytidylate; poly dA, polydeoxyadenylate; dI:dC, homopolymers of the deoxyribonucleotides of hypoxanthine and cytosine, hydrogen-bonded together.

[3] All enzyme incubations described in this paper were conducted at 37° unless stated otherwise.

[4] This definition of a polymerase unit differs from that used previously (2) in that it is based on total rather than labeled nucleotide.

DNA Synthesis—Incubations for extensive synthesis were the same as for assay except that the concentration of each deoxyribonucleoside triphosphate was increased to 0.3 mM, and the amounts of enzyme and template[5] were varied. Aliquots for assay of the extent of reaction were added to 0.1 ml of carrier solution, and acid-insoluble radioactivity was determined as in the assay procedure.

Nuclease Assays—Assay of the nuclease associated with T4 polymerase was ordinarily carried out by the method described by Oleson and Koerner (14); the substrate was salmon sperm DNA partially digested by pancreatic DNase. Appearance of acid-soluble material was followed spectrophotometrically. One unit refers to that amount of enzyme releasing 10 mμmoles of acid-soluble nucleotide in 20 min at 30°. In those assays in which nuclease activity toward ^{32}P-labeled *E. coli* DNA was determined, the conditions were the same except that incubation was conducted at 37° instead of 30°, and the appearance of acid-soluble radioactivity was measured.

Other Methods—Amino acids were analyzed by the methods of Moore and Stein (27), modified for greater sensitivity with a long path flow cell and range card (28). Analyses were carried out in a Spinco model 120B amino acid analyzer with Spinco AA15 and PA35 resins in the 58- and 10-cm columns, respectively.

Equilibrium centrifugation experiments were carried out in a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics.

Sucrose gradient sedimentation was run in a 5 to 20% gradient according to the procedure of Martin and Ames (29). The material was collected directly on Whatman No. 3MM paper discs, which were then washed with 5% trichloracetic acid and ethanol before radioactivity measurements. Sedimentation coefficients were determined from an internal DNA reference marker.

Polyacrylamide gel electrophoresis employed 7.5% gels (0.5 × 5 cm) and the conditions for electrophoresis and elution described by Falaschi and Kornberg (30).

Alkaline denaturation of DNA was ordinarily carried out for 5 min in 0.15 N NaOH at room temperature, followed by chilling and neutralization with a predetermined amount of HCl.

Protein was determined by the method of Lowry *et al.* (31) after precipitation with cold trichloracetic acid. The standard was bovine plasma albumin (Armour).

RESULTS

Purification of Enzyme

Except as noted, all steps were carried out at 0–4°. Centrifugations were done at 15,000 × *g* for 15 min. The purification procedure is summarized in Table I.

Preparation of Extract—E. coli B cells were grown from 1% inocula in 100-liter quantities, at 37°, with aeration. The medium contained the following, per liter: K_2HPO_4, 13.2 g; KH_2PO_4, 3.26 g; Difco Casamino acids, 10 g; DL-tryptophan, 0.1 g; cysteine HCl, 0.13 g; $(NH_4)_2SO_4$, 2.6 g; $MgSO_4$, 0.25 g; glucose, 10 g; $Fe(NH_4)_2(SO_4)_2$, 0.4 mg. At an optical density (590 mμ) of 1.0, the culture was infected with 3 × 10^{12} T4 *am* N82 per liter (multiplicity, 4).[6] After 75 min of further incuba-

[5] The DNA required for activity of the enzyme will be referred to as template, although it serves a primer function as well.

[6] The T4 mutant was kindly provided by Dr. Robert Edgar. This amber mutant was used, with the nonpermissive host *E.*

tion, the culture was chilled and harvested by centrifugation, and the cell paste was stored at −20° until the next step.

Pooled cell paste from two 100-liter cultures, totaling 552 g, was blended with glass beads in glycylglycine buffer (0.05 M, pH 7.0)-2 mM EDTA-2 mM reduced glutathione as described previously (26), and after centrifugation yielded 2300 ml of extract (Fraction I, Table I).

Streptomycin Precipitation—To Fraction I were added, with stirring, 5100 ml of 2 mM reduced glutathione-2 mM EDTA, followed by 2200 ml of 5% streptomycin sulfate during a 30-min interval. After additional stirring for 20 min, the precipitate was collected by centrifugation and suspended in 1290 ml of potassium phosphate buffer, 0.05 M, pH 7.0, containing 2 mM reduced glutathione (Fraction II, Table I).

Autolysis—Fraction II was made 3 mM in $MgCl_2$ and incubated at 37° until 95% of the ultraviolet-absorbing material at 260 mμ had become acid-soluble (26) (approximately 90 min). The autolysate was then rapidly chilled to 0°, and the supernatant (Fraction III, Table I) was separated by centrifugation.

Ammonium Sulfate Fractionation—To each liter of Fraction III were added 218 g of ammonium sulfate with stirring during a 30-min interval. After an additional 30-min stirring period, the precipitate was removed by centrifugation. Additional ammonium sulfate, 78 g per liter of supernatant, was added with the same procedure. The precipitate was collected and dissolved in 120 ml of potassium phosphate buffer, 0.05 M, pH 7.4, containing 2 mM EDTA and 2 mM reduced glutathione. This solution was passed through a column of Sephadex G-25 (20 cm^2 × 50 cm) equilibrated with potassium phosphate buffer, 0.05 M, pH 6.5, and 0.01 M 2-mercaptoethanol, and followed by the same buffer. Active fractions were pooled (Fraction IV, Table I).

Phosphocellulose Chromatography—Fraction IV was applied to a column of phosphocellulose (10 cm^2 × 20 cm) that had been equilibrated with potassium phosphate buffer, 0.05 M, pH 6.5, and 0.01 M 2-mercaptoethanol. This was followed by a linear gradient of 3 liters of potassium phosphate buffer, pH 6.5, with limits of 0.05 M and 0.4 M, containing 0.01 M 2-mercaptoethanol. The flow rate was 2.5 ml per min, and 40-ml fractions were collected. The peak of enzyme activity emerged after passage of approximately 2 liters of the gradient solution. Fractions with specific activity of greater than 12,000 were pooled (Fraction V, Table I).

DEAE-cellulose Chromatography—The volume of Fraction V was reduced from 220 ml to approximately 20 ml by dialysis against solid sucrose; fresh changes of dry sucrose were applied as necessary to achieve the volume reduction. The sample was then dialyzed against potassium phosphate buffer, 0.02 M, pH 7.4, and 0.01 M 2-mercaptoethanol and applied to a column of DEAE-cellulose (1.6 cm^2 × 25 cm) that had been equilibrated against the same buffer. A linear gradient of 800 ml of potassium phosphate buffer, pH 6.3, was applied, with limits of 0.02 M and 0.15 M, containing 0.01 M 2-mercaptoethanol. The flow rate was 0.6 ml per min, and 5-ml fractions were collected. The peak of protein and polymerase activity appeared after passage of approximately 300 ml of gradient solution. The active fractions were pooled, concentrated by vacuum ultrafiltration (20), and dialyzed against 0.1 M Tris-HCl buffer, pH 7.5, and 1 mM reduced glutathione (Fraction VI, Table I). Fraction VI, stored in liquid nitrogen, retained 84% of the original activity after 10 months.

coli B, because specific activity of crude extracts is approximately 5 to 10 times the level obtained with wild type phage T4 or T2 (2, 3).

TABLE I

Purification of enzyme

Fraction	Units	Protein	Specific activity	Over-all yield
	× 10^{-5}	*mg/ml*	*units/mg*	%
I. Extract	30	16.2	78	100
II. Streptomycin	38	14.8	200	127
III. Autolysis	26	3.1	370	87
IV. Ammonium sulfate	20	29.5	530	67
V. Phosphocellulose	11	0.33	15,300	37
VI. DEAE-cellulose	7.3	0.67	33,000	24
VII. Hydroxylapatite	1.2[a]	0.86	31,000	8

[a] Only 10.6 mg of the 22 mg of Fraction VI were purified on hydroxylapatite; the yield for this step is corrected to apply to the total amount of Fraction VI, but includes the 16% loss in activity of the latter after storage in liquid nitrogen.

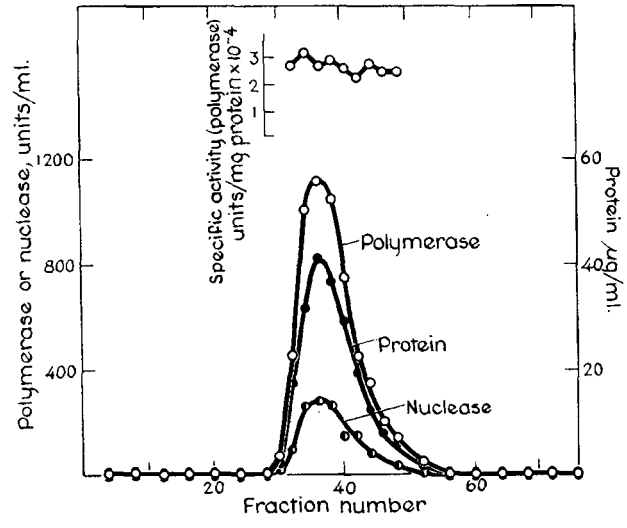

FIG. 1. Hydroxylapatite chromatography of Fraction VI. The DEAE-cellulose fraction (Fraction VI) (10.6 mg) was eluted from hydroxylapatite as described in the text. Each fraction contained 20 ml. Nuclease assays were performed by the method of Oleson and Koerner (14).

Hydroxylapatite Chromatography[7]—Fraction VI (15.8 ml), from liquid nitrogen storage, was diluted with an equal volume of 0.02 M 2-mercaptoethanol and adjusted to pH 6.5 with 0.05 M KH_2PO_4 containing 0.01 M 2-mercaptoethanol. The diluted fraction was applied to a column of hydroxylapatite (4.5 cm^2 × 15 cm) that had been washed with 2500 ml of 0.05 M potassium phosphate buffer, pH 6.5, and 0.01 M 2-mercaptoethanol. The column was washed with 100 ml of the same buffer, and then a linear gradient of 1500 ml of potassium phosphate, pH 6.5, with limits of 0.05 M and 0.3 M, containing 0.01 M 2-mercaptoethanol, was applied. The flow rate was 0.8 ml per min. Protein and enzyme activity appeared in a single peak midway in the gradient (Fig. 1). Tubes with greater than 300 units per ml were pooled,

[7] Although the hydroxylapatite step resulted in a large loss of activity without increase in specific activity, it was included because it removed traces of residual endonuclease activity still detectable in Fraction VI.

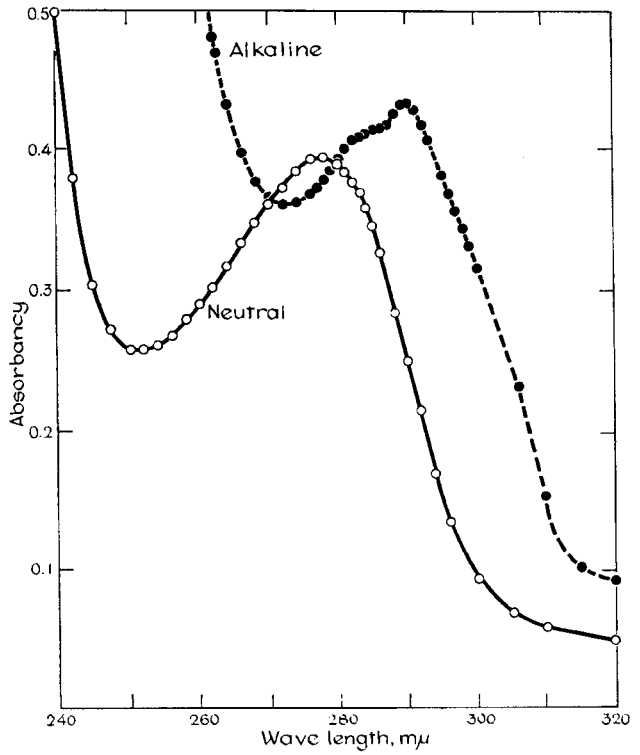

FIG. 2. Ultraviolet absorption spectrum of the enzyme. The spectrum of Fraction VII was determined in a Zeiss PMQ II spectrophotometer. Light path was 1 cm; protein concentration was 0.23 mg per ml. The solvent for the neutral spectrum was 9 mM NH_4HCO_3, pH 8.0, and for the alkaline spectrum it was 9 mM NH_4HCO_3-0.1 N NaOH.

concentrated by dialysis against solid sucrose, and dialyzed exhaustively against 50% glycerol-0.2 M potassium phosphate, pH 6.5, containing 0.01 M 2-mercaptoethanol. This material (Fraction VII) had a protein concentration of 0.86 mg per ml and when stored at −20° retained full activity for 10 months. Except where stated otherwise, this fraction was used for the experiments described below.

Physical Properties

Ultraviolet Spectrum—The hydroxylapatite fraction (Fraction VII) exhibited a characteristic protein absorption spectrum at neutral and alkaline pH (Fig. 2). The absorption at 280 mμ of a solution containing 1 mg per ml (in 0.01 M NH_4HCO_3, pH 8; 1-cm light path) was 1.40 when using a protein concentration calculated from the amino acid analysis and corrected for light scattering (32). The ratio of absorbance at 280 mμ to that at 260 mμ was 1.71 after a similar correction.

Amino Acid Composition—The amino acid composition, summarized in Table II, shows several differences from that of the *E. coli* polymerase. The T4 enzyme has 15 half-cystine residues, whereas the *E. coli* enzyme has 3,[8] a point of particular interest in view of the great sensitivity of the phage-induced enzyme to sulfhydryl inactivation, to be cited below. Other notable differences are higher serine and isoleucine contents in the phage enzyme, and lower values for alanine, leucine, and phenylalanine.

Molecular Weight—Equilibrium centrifugation was carried out by the high speed procedure of Yphantis (35). The molecular weight obtained by this method emphasizes low molecular weight components in a multicomponent system and does not represent an average molecular weight. However, the plot of log of concentration with respect to the square of distance from the center of rotation gave a straight line and thus showed no evidence for more than one component in the region of the cell that could be analyzed (Fig. 3). The slopes derived from two separate experiments gave molecular weights of 110,000 and 114,000, based on a partial specific volume of 0.734 calculated from the amino acid composition (36). This molecular weight may be compared with a value of 109,000 for the *E. coli* polymerase (32).

Homogeneity—A high degree of purity of the hydroxylapatite fraction is indicated by three findings: the relatively constant specific activity determined across the protein peak in the hydroxylapatite chromatogram (Fig. 1), a single protein band obtained on electrophoresis of 20 μg of Fraction VII in polyacrylamide gel (analysis of 15 μg of Fraction VI under the same conditions revealed several minor components), and the linear function (of log of concentration with respect to distance squared) in the Yphantis equilibrium sedimentations (Fig. 3).

Associated Nuclease Activity

The presence of endonuclease activity was tested for by incubating ^{3}H-labeled M13 DNA or ^{3}H-labeled T7 DNA with 5

TABLE II

Amino acid composition

Duplicate samples were hydrolyzed in 6 N HCl with a crystal of phenol (32), after exhaustion of air, for 24 or 78 hours at 110°. Half-cystine was determined as cysteic acid after oxidation with performic acid (33).

Amino acid	Content
	moles/112,000 g
Lysine	85
Histidine	16
Arginine	46
Half-cystine	15
Aspartic acid[a]	113
Threonine[b]	29
Serine[b]	65
Glutamic acid[a]	113
Proline	40
Glycine	61
Alanine	55
Valine[c]	55
Methionine	35
Isoleucine[c]	86
Leucine	59
Tyrosine	47
Phenylalanine	12
Tryptophan[d]	12

[a] Includes amide.
[b] Extrapolated to zero time of hydrolysis.
[c] Value after 78 hours of hydrolysis.
[d] Calculated from spectral analysis (34).

[8] T. M. Jovin, P. T. Englund, and L. L. Bertsch, personal communication.

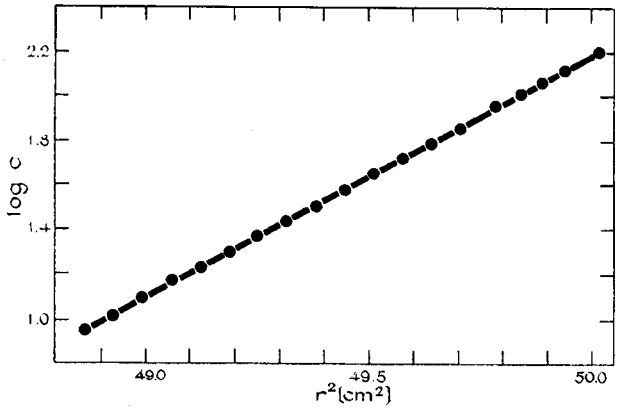

FIG. 3. Sedimentation equilibrium analysis of the enzyme. A 2.3-mm column was analyzed in a double sector cell with sapphire windows and with Rayleigh interference optics. Rotor speed was 19,160 rpm; protein concentration, 0.50 mg per ml; temperature, 6°; buffer, 0.1 M KCl-0.05 M potassium phosphate (pH 6.8)-0.01 M 2-mercaptoethanol. *Abscissa*, square of distance from center of rotation (r); *ordinate*, logarithm of vertical fringe displacement (c) (proportional to protein concentration). The positions of the meniscus and bottom of sample were at 47.77 cm² and 50.25 cm², respectively.

TABLE III

Production of 5′-mononucleotides as result of nuclease activity in purified enzyme fractions

Heat-denatured ^{32}P-labeled *E. coli* DNA (120 mμmoles) was incubated with 11 units of T4 polymerase until 6% and 18% of the DNA was made acid-soluble. $^{32}P_i$ released by treatment of the acid-soluble fraction of the digested DNA with either bacterial alkaline phosphatase or 5′-nucleotidase was measured as Norit-nonadsorbable material.

Enzyme	Release of $^{32}P_i$	
	6% acid-soluble DNA	18% acid-soluble DNA
	%	%
Bacterial alkaline phosphatase	103	99
5′-Nucleotidase	103	101

units of enzyme per mμmole of DNA for 6 hours under conditions for synthesis, except that one deoxyribonucleoside triphosphate was omitted; the DNA was then analyzed by sucrose gradient sedimentation at alkaline pH. In studies with both DNA samples, the sedimentation profiles were unchanged as a result of incubation with the enzyme, showing the absence of single strand scissions and indicating the absence of endonuclease activity.

Exonuclease activity was detectable by the action of the enzyme on ^{32}P-labeled *E. coli* DNA. Approximately 3.0 μμmoles of ^{32}P-labeled *E. coli* DNA per unit of Fraction VII were made acid-soluble in 30 min. This rate was increased 3-fold by prior heat denaturation of the DNA. With a DNA substrate partially digested with pancreatic deoxyribonuclease, the nuclease activity was about 50 times that observed with native ^{32}P-labeled *E. coli* DNA. Digestion of dAT copolymer by the enzyme proceeded at about the same rate as for partially digested DNA. The exonucleolytic nature of the nuclease activity was indicated by the appearance of 5′-mononucleotides as the exclusive product following partial digestion (Table III). 5′-Mononucleotides were identified by susceptibility to the action of 5′-nucleotidase.

Nuclease activity as reflected by loss of template was not evident under the conditions of synthesis of DNA (Fig. 4*A*) but was detectable to a moderate extent with dAT template (Fig. 4*B*). In the absence of deoxyribonucleoside triphosphates, the nuclease activity of the enzyme was greatly enhanced (Fig. 4, *A* and *B*). The maximum inhibitory effect of triphosphates on the nuclease activity required the complete complement necessary for replication of the template, but smaller effects were obtained with less than a full complement (Table IV). Triphosphates not involved in replication, for example, dGTP and dCTP with dAT as template, produced no effect (Table IV).

The close association of nuclease and polymerase activities is evident from the constant ratio of these activities in fractions

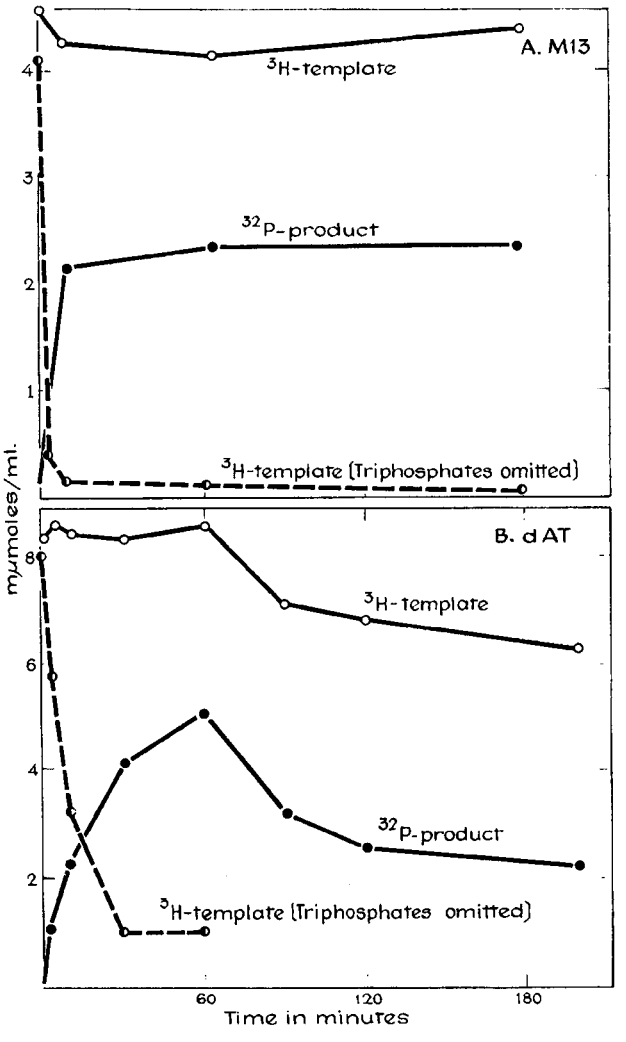

FIG. 4. Effect of the enzyme on M13 DNA and dAT, in the presence and absence of deoxyribonucleoside triphosphates. In *A*, incubations contained 2 mμmoles of linear ^{3}H-labeled M13 DNA and 7 units of Fraction VII in 0.4 ml; in *B*, incubations contained 8 mμmoles of ^{3}H-dAT and 30 units of Fraction VII in 1.0 ml. Conditions for DNA synthesis were those described in "Methods," except that deoxyribonucleoside triphosphates were omitted where indicated. *Abscissa*, time of incubation; *ordinate*, acid-precipitable DNA per ml of incubation mixture.

from hydroxylapatite chromatography (Fig. 1) and in material eluted from polyacrylamide gel (Fig. 5). The ratios of polymerase to nuclease activity (measured as in Fig. 1) in pooled fractions from phosphocellulose chromatography (Fraction V), DEAE-cellulose chromatography (Fraction VI), hydroxylapatite chromatography (Fraction VII), and polyacrylamide gel electrophoresis (Fig. 5) were 1.84, 1.80, 1.80, and 1.92 (units per unit), respectively. An additional indication of the inseparability of the nuclease and polymerase activities was the similarity of their rates of heat inactivation (Fig. 6).

TABLE IV

Effect of deoxyribonucleoside triphosphates on nuclease activity of enzyme

The incubation conditions were those used for assay of polymerase activity as in "Methods," except for omission or addition of deoxyribonucleoside triphosphates as indicated. The experiment with ^{3}H-labeled T7 DNA used 9 mμmoles of DNA and 24 units of enzyme in a 0.10-ml volume; for the experiment with ^{3}H-dAT, 1.7 mμmoles of dAT and 4 units of enzyme were contained in 0.021 ml. Each deoxyribonucleoside triphosphate was present at a concentration of 0.2 mM for the experiment with ^{3}H-labeled T7 DNA, and 0.1 mM with ^{3}H-dAT. The activities refer to relative amount of acid-soluble nucleotide after 15 min at 37°; 100% activity represents the digestion of 0.9 mμmole of ^{3}H-labeled T7 DNA and 1.2 mμmoles of ^{3}H-dAT.

Substrate and additions	Nuclease activity
	%
^{3}H-T7 DNA	
No additions	100
dGTP	51
dGTP, dATP	19
dGTP, dATP, dCTP	4
dGTP, dATP, dCTP, dTTP	<1
^{3}H-dAT	
No additions	100
dGTP, dCTP	89
dATP, dTTP	9

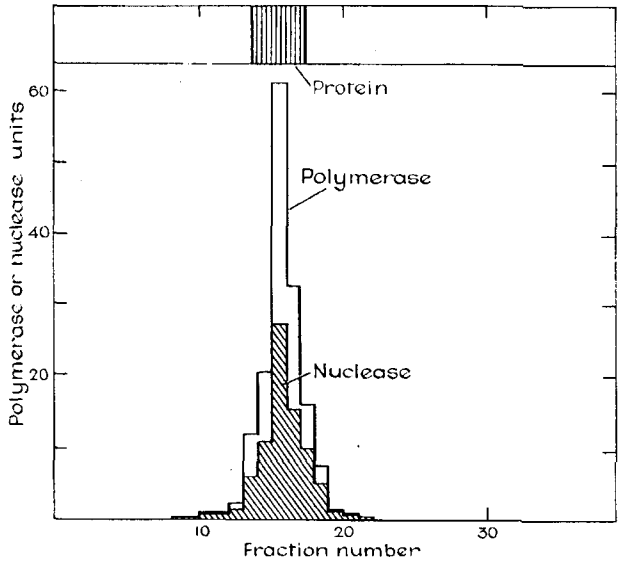

FIG. 5. Polymerase and nuclease activities in fractions obtained by polyacrylamide gel electrophoresis. Electrophoresis was carried out on 17 μg of Fraction VII; 1.25-mm segments of the gel were subsequently eluted. Recovery of polymerase activity was 30%. Values for polymerase and nuclease refer to the amount per segment and are plotted according to location in the gel; the area under the nuclease plot is indicated by *hatching*. The protein band in an identical gel, run in parallel and stained for protein, is shown at the *top* in alignment with the *abscissa*.

Requirements for Activity

pH Optimum—Maximum activity was obtained over the pH range of about 8 to 9 (Fig. 7).

Divalent Metal—There was no detectable polymerase activity in the absence of divalent cation. Maximum activity was observed at 6 mM Mg^{++}. Mn^{++} at an optimal concentration of 0.1 mM sustained a rate about one-fourth of that obtained with 6 mM Mg^{++}.

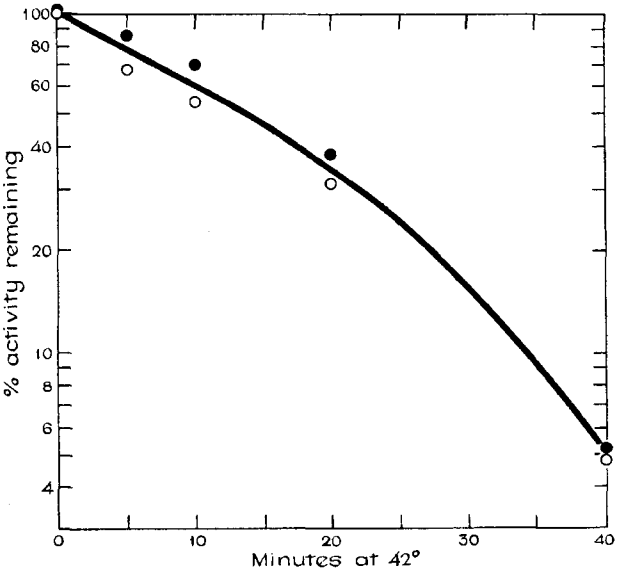

FIG. 6. Heat inactivation of polymerase and nuclease. Fraction VII, 8 μg per ml, was incubated at 42° in 0.05 M Tris-HCl (pH 7.5), 0.1 M $(NH_4)_2SO_4$, and 1 mg of bovine plasma albumin per ml. At the times indicated, aliquots were chilled and made 0.01 M in 2-mercaptoethanol. Assays for nuclease (14) and polymerase were conducted at 30°.

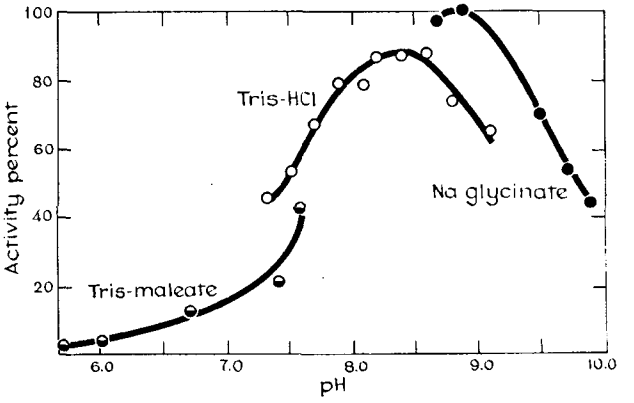

FIG. 7. pH-activity curve for the enzyme. The standard assay was used, with substitution of the indicated buffers at 0.05 M; 0.056 unit of enzyme was added per tube. pH was measured at room temperature. The activity with sodium glycinate, pH 9.0, was arbitrarily taken as 100%.

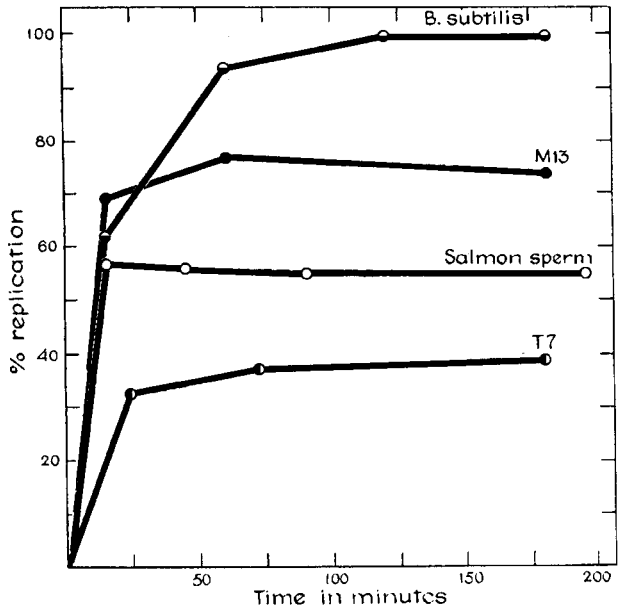

FIG. 8. Extent of replication of various DNA templates by the enzyme. Incubation conditions for synthesis were those described in "Methods." The incubations contained the following: 2.9 mμmoles of alkali-denatured ^{3}H-labeled M13 DNA (linear form) and 8 units of enzyme in 0.23 ml; 1.8 mμmoles of ^{3}H-labeled T7 DNA (treated with λ-nuclease and then denatured) and 6 units of enzyme in 0.1 ml; 0.9 mμmole of alkali-denatured salmon sperm DNA and 4 units of enzyme in 0.1 ml; or 5.8 mμmoles of alkali-denatured *B. subtilis* DNA and 150 units of enzyme in 0.4 ml.

Template—Like the T2 (2) and T5 polymerases (4), the T4 polymerase required DNA template in a single stranded form. In general, the highest rates were seen with denaturated salmon sperm DNA, but rates varied widely depending upon source and specific preparation. For example, the rates with denatured DNA from *E. coli*, calf thymus, and T4 phage were 4, 1.5, and 1%, respectively, of the rate with denatured salmon sperm DNA. It is assumed that these differences are primarily a reflection of the content of ends available for initiation of synthesis. The extent of synthesis also varied widely; it never exceeded 1 full template equivalent, although in some cases this was closely approached (Fig. 8).

Native DNA had little or no template activity. For example, the amount of acid-insoluble material formed with native T7 DNA after 4 hours of incubation was less than 1% of the template, compared to 35% for the same preparation after denaturation with alkali. Unfractionated, circular, single stranded M13 phage DNA was replicated approximately 3% in 3 hours of incubation, with 95% of the observed synthesis occurring in the first 20 min of incubation. From the kinetics of replication and the augmentation that occurs with ring scission (see experiments below with linear M13, Figs. 4, 8 and 12), it is assumed that this low level of synthesis with M13 DNA results from replication of the small amount of linear forms contaminating such preparations. It appears, therefore, that in addition to being single stranded, DNA must have a free end to serve as a template for T4 polymerase.

The synthetic copolymer dAT (16), which is a good template for the *E. coli* polymerase although double stranded, also serves as template for the T4 enzyme. Rates with two different preparations of dAT were 5 and 9% of that with denatured salmon sperm DNA; a value of 11% was reported for the polymerase induced by T2 infection (4). The maximum extent of synthesis with dAT as template reached 58% of the input, after which there was progressive loss of the synthesized product (Fig. 4).

Two different preparations of poly dC were incubated with dGTP under synthetic conditions; the extent of replication of one sample was less that 5%, and of the other less than 1%. Replication of poly dA with dTTP was less than 0.5%.

Native DNA made partially single stranded by digestion with exonuclease III (37) is capable of supporting synthesis with the T4 polymerase. The extent of synthesis was equivalent to the extent of prior digestion by exonuclease III (Fig. 9). Inasmuch as the enzyme was unable to initiate synthesis from the 5′-end (see below), it is assumed that the mechanism is the same as the "repair" reaction described for *E. coli* DNA polymerase; namely, there is extension at the 3′-hydroxyl terminus to fill in the denuded portions of the molecule (10). Repair-type synthesis carried out by the phage enzyme stopped abruptly upon completion of repair, as would be expected from its inability to act on fully helical DNA.

Inhibition by 3′-Phosphoryl Termini—A micrococcal nuclease digest of DNA exerted an inhibitory effect upon the activity of the T4 polymerase. For example, addition of T7 DNA treated with micrococcal nuclease to untreated, denatured T7 DNA, in a ratio of 1:10, reduced the rate of synthesis to 42% of that obtained with the same amount of untreated, denatured T7 DNA alone. The corresponding value for a 1:1 mixture was 5%. This is interpreted, as with *E. coli* polymerase, as inhibition of the enzyme by 3′-phosphoryl termini (11). The inhibition was completely reversed by removal of the 3′-phosphates with exonuclease III (DNA phosphatase-exonuclease) (11).

Other Requirements—In common with the other purified DNA polymerases, the T4 polymerase requires all four deoxyribonu-

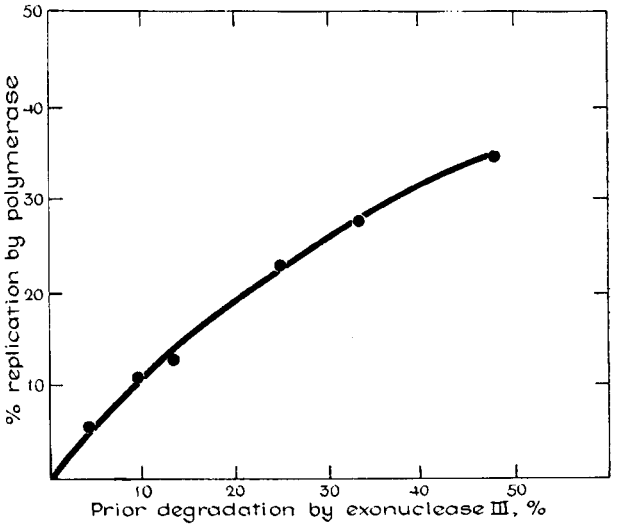

FIG. 9. Repair of partially single stranded DNA. *E. coli* DNA (59 mμmoles) that had been subjected to graded extents of digestion with exonuclease III (10) was incubated for 150 min with 10 units of the enzyme in a volume of 0.3 ml and under standard conditions for synthesis. Extent of replication is plotted against the extent of prior digestion. The figures in both cases are based on the amount of DNA present before digestion with exonuclease III.

TABLE V

Effect of salt concentration on rate of synthesis

All incubations were conducted under the assay conditions as in "Methods," except for omission of ammonium sulfate.[a] NH_4Cl was added at the concentrations noted. All incubations contained 0.34 unit of Fraction VII. The highest activity, with 0.05 M NH_4Cl, represented 3.0 mμmole of acid-insoluble nucleotide in 30 min and was taken as 100%.

Concentration of NH_4Cl	Relative activity
M	%
0.00[a]	63
0.02	75
0.05	100
0.10	83
0.20	3

[a] In addition to 0.067 M Tris-HCl, the mixture contained 0.01 M NaCl which was included with the DNA.

cleoside triphosphates, dATP, dGTP, dTTP, dCTP, or their analogues, for synthesis of DNA. The concentration of each deoxyribonucleoside triphosphate ordinarily used for assays was 0.033 mM; however, enzyme activity was augmented 3-fold by increasing this to 0.33 mM. Omission of one, two, or three triphosphates reduced the activity to 0.63, 0.23, and 0.10%, respectively, of that obtained with all four present. Unlike the *E. coli* polymerase (38), replacement of Mg^{++} by Mn^{++} did not permit substitution of a ribonucleoside triphosphate for a deoxyribonucleoside triphosphate. Under the usual assay conditions with either 6 mM Mg^{++} or 0.2 mM Mn^{++}, replacement of dCTP by CTP or of dATP by ATP resulted in less than 1% of the normal activity.

A total salt concentration of approximately 0.10 M was necessary for optimal activity (Table V); however, if this was increased by the presence of 0.2 M NH_4Cl to a total salt concentration near 0.3 M, a level required for optimal activity with the phage T5 polymerase (4), there was 97% inhibition (Table V). The stimulatory and inhibitory effects were identical with NH_4Cl, NaCl, and KCl.

Sulfhydryl reagents, either reduced glutathione or 2-mercaptoethanol, were included throughout the purification procedure, during storage of enzyme, and in all incubations except where noted. When the level of 2-mercaptoethanol in the incubation mixture was reduced from 10 to 1 mM, the rate of synthesis dropped 63%, while with 2-mercaptoethanol omitted entirely it dropped by 81%. In the presence of *p*-hydroxymercuribenzoate at a concentration of 10^{-4} M, the rate was less than 2% of normal; the level of enzyme was 0.02 μg per ml, and bovine plasma albumin (0.15 mg per ml) was present.

Nature of Product

Hybrid Nature of Template-Product Complex—With a density label, bromouracil, in the product, the product-template complex exhibited a broad range of hybrid density values and product to template ratios (Fig. 10). Most of the material closely approached but did not exceed the density corresponding to 50% bromouracil and a product to template ratio of 1 (Fig. 10). The over-all ratio in this experiment, determined directly on the incubation mixture, was 0.71.

Sedimentation of the product-template hybrid was slower than of template alone in a neutral sucrose gradient containing 1 M sodium chloride, and the lower sedimentation rate could be ascribed to the conversion to a helical structure with increased molecular weight. Thus, a linear M13 DNA fraction of 23 S, corresponding to a molecular weight of 1.2×10^6 (40), was replicated to an extent of 92%, yielding a product-template complex of 15 S. The molecular weight calculated for a helical molecule of the latter sedimentation value is 2.6×10^6 (40).

Covalent Attachment of Product and Template—In an alkaline sucrose gradient, the template and product were found to be associated and to have a faster sedimentation rate than the template alone (Fig. 11). Inasmuch as no secondary structure remains at this alkaline pH, template and product are judged to be linked through a covalent bond.

Attachment of Product to 3′-Hydroxyl of Template—Digestion of the product-template complex with exonuclease III, an enzyme that attacks at the 3′-hydroxyl termini of double stranded DNA (37), quantitatively removed product material with minimal loss of template (Fig. 12*A*). In contrast, λ-exonuclease, an enzyme that attacks only at the 5′-phosphoryl termini of double stranded molecules (12), removed only template material (Fig. 12*B*).

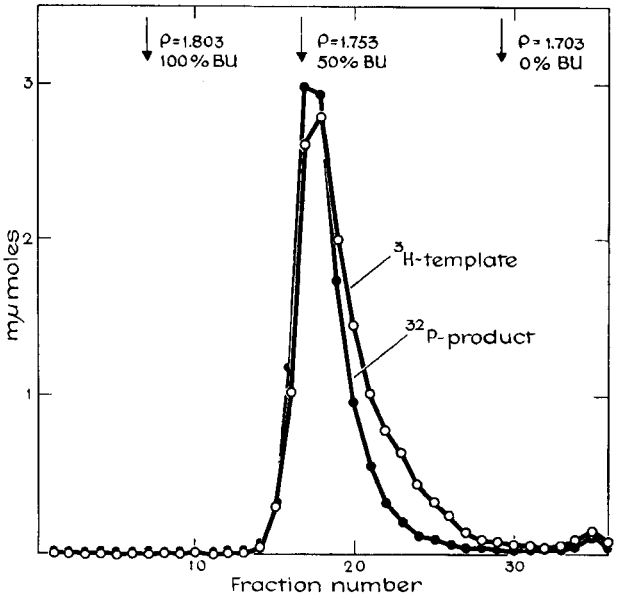

FIG. 10. Neutral CsCl density gradient centrifugation of the product-template complex. Alkali-denatured ^{3}H-labeled λ DNA was replicated under synthesis conditions except for the replacement of dTTP by bromodeoxyuridine triphosphate. Incubation volume was 1.0 ml, with 54 mμmoles of template and 300 units of enzyme. After 6 hours of incubation, the mixture was made 0.01 M in EDTA and heated at 60° for 10 min to inactivate the enzyme. Density gradient centrifugation was carried out in 8.5 ml of CsCl, ρ = 1.745, in 0.01 M Tris, pH 8.0, and 1 mM EDTA. The No. 50 rotor was run in the Spinco model L ultracentrifuge at 45,000 rpm for 80 hours at 25°. After measurement of specific gravity, the fractions were precipitated with acid and collected on glass filters as for the polymerase assay. Parallel centrifugations were carried out with a portion of the incubation mixture and an internal marker of ^{3}H-labeled λ DNA. Positions are indicated for the densities of native λ DNA containing 100, 50, and 0% bromouracil (*BU*). The value of ρ for λ DNA agrees with data of Baldwin and Shooter (39) for *E. coli* DNA, which has the same base composition as λ DNA. The ρ values for bromouracil-containing λ DNA were calculated by using a value of 0.10 for the density difference between 0 and 100% bromouracil estimated from the difference in density between dAT and the copolymer of deoxyadenylate and bromodeoxyuridylate (39).

This evidence for masking of the 3′-terminus of the template and the 5′-terminus of the product supports the tentative conclusion that a phosphodiester bridge between these termini links template and product.

Nondenaturability—The covalent linkage between product and template constitutes, in effect, a form of terminal cross-linkage between the two strands of the hybrid. Since cross-linked DNA renatures with great facility (41), it might be expected that the structure produced with T4 polymerase would have similar properties. This was tested by sedimenting the template-product hybrid in a neutral sucrose gradient after alkaline denaturation. Under the conditions used, denatured DNA, because of its collapsed structure, sediments much more rapidly than helical DNA of the same molecular weight (40). The "denatured" hybrid, sedimenting with a value of 15 S, was not distinguishable from the untreated product-template complex (Fig. 13), thus indicating a facile reassumption of secondary structure. This result was confirmed by the resistance of alkali-treated hybrid to exonuclease I, an enzyme active only on single stranded DNA (19). Less than 30% of the product and template were digested by the latter enzyme after the alkaline treatment in an experiment in which the control, alkali-denatured T7 DNA, underwent 97% digestion. These experiments also provide additional support for covalent attachment between product and template.

Proposed Model

Several aspects of the action of T4 polymerase, when considered together, suggest a "hairpin" or "loop" model for replication of single stranded DNA, as illustrated in Fig. 14*A*. These aspects are as follows: (*a*) requirement for a linear, single stranded template, or partially single stranded as in the case of DNA treated with exonuclease III; (*b*) inhibition by 3′-phosphoryl termini; (*c*) hybrid nature of the product-template complex; (*d*) covalent attachment between the 5′-end of the product and the 3′-end of the template; (*e*) facile renaturation of the product template hybrid; and (*f*) limitation to one or less than one replication of the template.

According to the model (Fig. 14*A*), initiation occurs at the 3′-hydroxyl terminus of the single stranded template, which has looped back, a looping which may be facilitated by fortuitous base pair homology between the terminal residues and regions

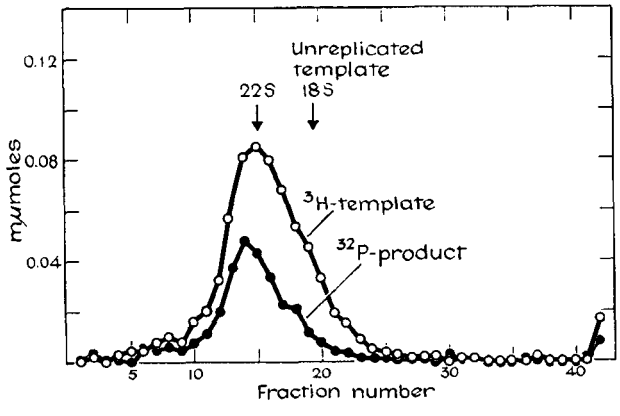

FIG. 11. Alkaline sucrose gradient sedimentation of the product-template complex. Linear ³H-labeled M13 DNA (0.7 mμmole) and 2.8 units of enzyme were incubated in 0.07 ml for 3 hours under conditions for synthesis. After addition of 3 μmoles of EDTA and 0.8 μmole of sodium pyrophosphate, the mixture was centrifuged in a sucrose gradient containing 0.2 M NaOH, 0.8 M NaCl, and 1 mM EDTA at 37,600 rpm (Spinco SW 39 rotor) for 8 hours at 10°. A duplicate experiment with an internal marker in the sedimentation analysis was used to determine the sedimentation constants.

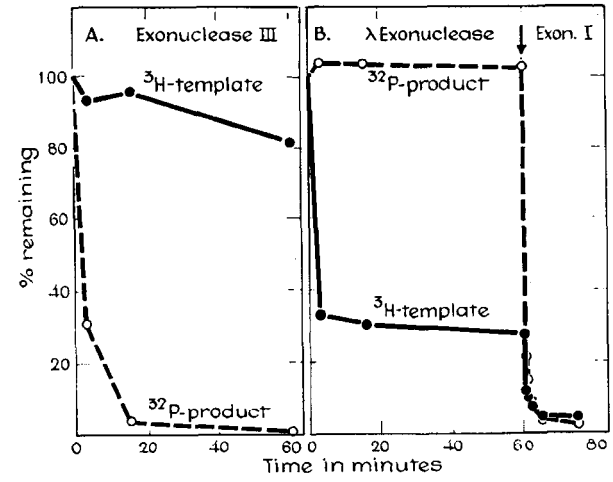

FIG. 12. Selective susceptibility of the product-template complex to action of specific exonucleases. Linear ³H-labeled M13 DNA (0.45 mμmole) was incubated in 0.10 ml with 2.0 units of enzyme for 3 hours under conditions for synthesis. Polymerase was inactivated by heating at 65° for 20 min. *A*, exonuclease III (20 units) was added and aliquots, taken at the times indicated, were assayed for acid-insoluble ³H-template and ³²P-product. *B*, λ-exonuclease (0.6 unit) was used in place of exonuclease III. In a second part of this experiment, exonuclease I (*Exon. I*) (5 units per mμmole of DNA) was added at 60 min as indicated by the *arrow*.

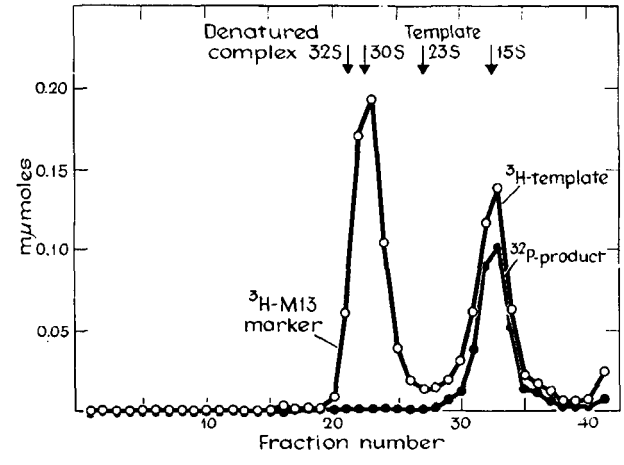

FIG. 13. Neutral sucrose gradient sedimentation of alkali-treated product-template complex. ³H-Labeled M13 DNA (0.4 mμmole) and 1.6 units of enzyme were incubated for 3 hours under conditions for synthesis. The product was denatured with alkali as described in "Methods," a marker of ³H-labeled M13 DNA was added, and the mixture was centrifuged in a sucrose gradient containing 1 M NaCl, 0.01 M Tris (pH 7.8), and 1 mM EDTA at 24,500 rpm for 9½ hours at 10°. The sedimentation constant of the template (23 S) was determined in a separate analysis with the same marker. The *s* value of a fully denatured product-template complex in which the template was replicated to an extent of 0.8 was calculated as 32 S by an empirical formula (40).

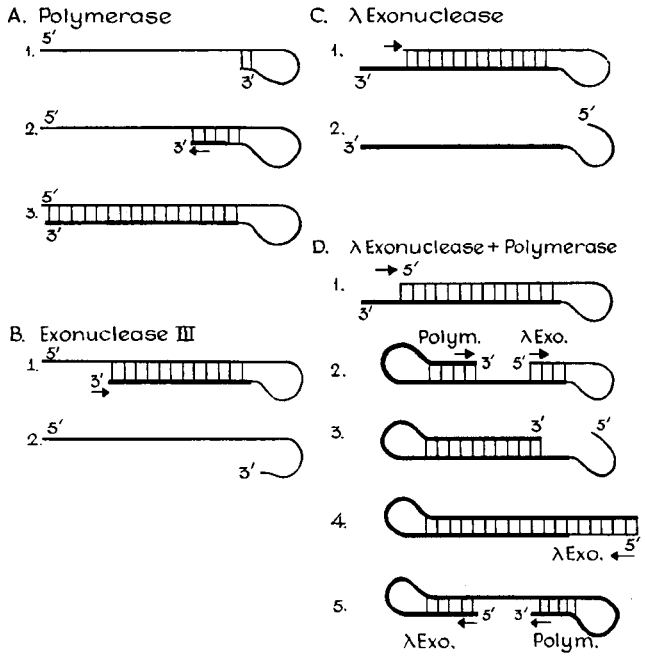

FIG. 14. Schematic representation of a proposed model for the action of T4 polymerase, and effects of nucleases on the product-template complex. *A*, replication of single stranded template by polymerase. *1* represents a single strand of template DNA (*light line*) in which the 3′-terminus loops back to permit initiation by the polymerase (*2*). With a small amount of synthesis (*2*) (*heavy line*), the loop is fixed in that position and synthesis ("repair") continues to the 5′-terminus (*3*). *B*, effect of exonuclease III on the isolated product-template complex shown in *A*, *3*. *C*, effect of λ-exonuclease action on the isolated product-template complex of *A*, *3*. *D*, action of λ-exonuclease (λ*Exo.*) on product-template complex in the continued presence of polymerase (*Polym.*). The sequence of events in the experiment of Fig. 15 is shown in terms of the model. Digestion of primer (*1*) is followed by formation of a new loop and a wave of new synthesis "chasing" the wave of digestion (*2*, *3*). When the loop region has been replicated (*4*), λ-exonuclease can resume action, with repetition of the process, eventually making product material available to this nuclease (*5*).

along the same strand. This configuration now resembles a DNA molecule partially digested by exonuclease III, which is known to be an effective template. Chain growth occurs by the same mechanism as in the case of the *E. coli* polymerase (42), with attack by the terminal 3′-hydroxyl of the template upon the incoming deoxynucleoside triphosphate, the selection of which is determined by base pairing with the corresponding nucleotide in the template. After replication has proceeded for a short distance the loop, otherwise unstable, is irreversibly fixed. Further replication, as in repair of DNA made partially single stranded with exonuclease III, continues to the 5′-terminus of the template, where it stops because the DNA is now fully helical at the 3′-growing end. The failure of homopolymers as templates is consistent with the absence of the postulated base pairing prerequisite for looping. The inhibition by 3′-phosphoryl termini is expected if initiation occurs only at the 3′-hydroxyl terminus. Facile renaturation of the product-template complex is readily explained by the extensive complementarity within the hybrid molecule.

The effects of specific exonucleases on the structure proposed in this model are shown schematically in Fig. 14, *B* and *C*. Exonuclease III attacks at the 3′-terminus of the helical molecule (37) and removes only product material (Figs. 14*B* and 12*A*), whereas λ-exonuclease, which acts on the 5′-terminus (12), removes only the template (Figs. 14*C* and 12*B*). Since both enzymes are inactive with single stranded DNA, the complementary portion of the hybrid is in each case spared from attack. Conversion of the product to single stranded form, as in the experiment with λ-exonuclease, makes the product susceptible to exonuclease I action (Figs. 14*C* and 12*B*) and should also permit resumption of polymerase action (Fig. 14*D*). The latter prediction led to the experiment described in Fig. 15. Addition of λ-exonuclease to a replication mixture containing fully replicated DNA and active polymerase was followed by a burst of synthesis paralleling the digestion of template. Thus the digestion by λ-exonuclease at the 5′-terminus of template enables the 3′-terminus of the product to loop back and the polymerase to start synthesis again (Fig. 14*D*). The subsequent loss of template (Fig. 15) can be explained as the result of still another round of digestion, which becomes possible when the nuclease and polymerase reach the site of the original loop (Fig. 14*D*).

Heterogeneity in product to template ratios was observed in density gradients run with CsCl or with sucrose at alkaline pH (Fig. 11), indicating that the extent of replication was not the same for all template molecules. According to the mechanism proposed, this suggests that the loops are of varying size. The close approach to a full replication of all molecules of some DNAs (Fig. 8), or of a fraction of the molecules of other DNAs (as revealed by density studies in Fig. 10), indicates that the portion of

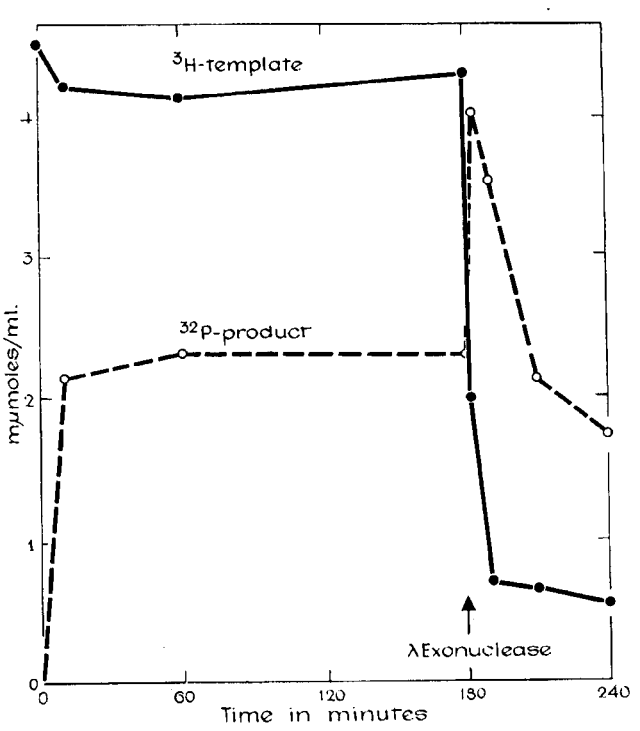

FIG. 15. Effect of λ-exonuclease on the product-template complex in the presence of T4 polymerase. The experiment was carried out as in Fig. 12, except that polymerase was not inactivated prior to addition of λ-exonuclease; no exonuclease I was added. Figures on the *ordinate* represent acid-precipitable ^{3}H-template and ^{32}P-product during the period of replication and after addition of λ-exonuclease.

template remaining in the form of a single stranded loop in the final product-template complex can be very small.

DISCUSSION

The replication by T4 polymerase of a single stranded DNA template leads to a product which is in a complex with the template. In this complex, the template strand is covalently attached by its 3′-terminus to the 5′-terminus of the product. According to our current notions, as presented in Fig. 14, the enzyme produces this structure by extension of the single stranded DNA at the 3′-terminus, which has looped back, thereby permitting the strand to act as template. Subsequent replication to the 5′-terminus of the template then resembles the "repair" reaction, as in the replacement of the denuded portions of a DNA treated with exonuclease III (10), a reaction carried out by both the T4 and *E. coli* polymerase enzymes. The loop mechanism as proposed here for the replication of single stranded DNA by phage enzyme may also apply to the replication of such DNA by the *E. coli* polymerase. The principal difference in replicative functions between the phage and bacterial enzymes then appears to be the ability of the bacterial enzyme, and inability of the phage enzyme, to initiate new strands and to utilize fully helical DNA as template (43).

The DNA polymerase activity purified from calf thymus resembles the T4 polymerase in its requirement for denatured DNA and limitation to one replication of template (44). The evidence for rapid renaturation of the template-product complex produced by the thymus polymerase, and for the covalent attachment between template and product (44, 45), suggests that a loop mechanism may also apply to the thymus enzyme. The DNA polymerase activities from a variety of other animal tissues have, for the most part, shown a similar preference for single stranded template and limited replication (46). It seems likely, therefore, that these enzymes may all have the same mechanism, but more experimental data will be required to establish this.

Two other purified phage polymerases, induced by infections with T2 (2) and T5 (4), also require single stranded DNA and achieve a replication limited to 1 template equivalent. The similarities in properties between the purified T2 and T4 polymerases, in addition to the extensive areas in which the T2 and T4 bacteriophages themselves resemble each other (47), make it likely that the mechanism described for the T4 polymerase also applies to the T2 enzyme. The comparison between the T4 and T5 enzymes reveals certain dissimilarities at this time. The product of T5 polymerase action is readily separated by denaturation from its template (48), and the T5 enzyme is able to utilize ΦX174 DNA (49). If further work firmly establishes these observations, then the T5 enzyme will be distinctive as compared with the T4 enzyme in its capacity to initiate new strands in the replication of a single stranded template.

Another point of comparison between the T4 and T5 polymerases is their response to the ionic strength of the replication medium. The T4 enzyme activity is inhibited 97% by 0.2 M NH_4Cl, a salt concentration required for optimal activity with the T5 enzyme (4). This fact suggests that, if the T5 polymerase were to act by the loop mechanism proposed for the T4 enzyme, the salt effects would be primarily upon the enzymes rather than on the DNA templates.

Recent studies of the enzyme activities of extracts of nonpermissive bacteria infected with mutants of T4 have identified the structural gene for DNA polymerase (5). The fact that mutants in the same gene are unable to induce synthesis of phage DNA (50) indicates that the enzyme does serve an essential function in DNA synthesis. The gene-enzyme-function relationship is strengthened by the demonstration of temperature-dependent activity for the polymerase purified from temperature-sensitive mutants in the same gene (5). Some mutants in the polymerase gene have the additional property of inducing a high mutation rate in other genes (51). Although it would seem reasonable to ascribe the latter to errors in replication resulting directly from structural alterations in the polymerase enzyme, this has not yet been firmly established.

The genetic evidence for T4 polymerase as an integral component of DNA replication *in vivo* draws sharper attention to the paradoxical failure of the polymerase to replicate helical DNA *in vitro*. Proper expression of the enzyme function may require a different form of the enzyme present *in vivo*, or a specific form of template DNA (52), or additional factors, which may or may not be enzymatic in character. Unsuccessful experiments not described in this report have attempted to correct the deficiency *in vitro* by using "replicative forms" of T4 DNA extracted from infected cells (52), and by supplementation with the purified host cell polymerase or with crude extracts of infected cells. It seems clear that complexities of replication of the T4 chromosome are responsible for the current lack of success in reconstituting the entire event *in vitro*.

The nuclease activity of the highly purified T4 enzyme remains closely associated with polymerase activity under all of the conditions used in these experiments, similar to the result obtained by Short and Koerner in studies on partially purified T2 polymerase (53). A marked inhibition of nuclease activity occurs in the presence of a full complement of deoxyribonucleoside triphosphates. The inhibitory effect of the triphosphates appears to be related to their ability to support polymerase activity; thus with DNA as template the full inhibitory effect required that all four triphosphates be present, whereas with dAT as template dGTP and dCTP had no effect. Apparently the nuclease activity of the enzyme remains suppressed or is more than compensated for as long as the requirements for polymerase activity are met. The association between nuclease and polymerase in the T4 enzyme is reminiscent of the close relationship between *E. coli* polymerase and exonuclease II (26). For neither polymerase are the functions of the nuclease known, but it does invite the speculation that the nucleases may function in correction of errors made during replication. Perhaps errors by polymerase in base selection result in structures which are relatively unsuitable for additional polymerase action and are relatively more susceptible to excision of the mismatched base by the nuclease.

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