

Enzymatic Synthesis of Deoxyribonucleic Acid

I. PREPARATION OF SUBSTRATES AND PARTIAL PURIFICATION OF AN ENZYME FROM *ESCHERICHIA COLI*

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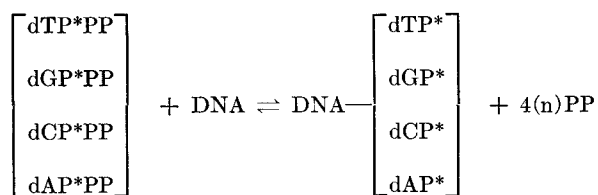
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(Received for publication, October 10, 1957)

In considering how a complex polynucleotide such as DNA¹ is assembled by a cell, the authors were guided by the known enzymatic mechanisms for the synthesis of the simplest of the nucleotide derivatives, the coenzymes. The latter, whether composed of an adenosine, uridine, guanosine, or cytidine nucleotide, are formed by a nucleotidyl transfer from a nucleoside triphosphate to the phosphate ester which provides the coenzymatically active portion of the molecule (1, 2). This condensation, which has been regarded as a nucleophilic attack (3) on the innermost or nucleotidyl phosphorus of the nucleoside triphosphate, results in the attachment of the nucleotidyl unit to the attacking group and in the elimination of inorganic pyrophosphate. By analogy, the development of a DNA chain might entail a similar condensation, in this case between a deoxynucleoside triphosphate with the hydroxyl group of the deoxyribose carbon-3 of another deoxynucleotide. Alternative possibilities involving other activated forms of the nucleotide (as, for example, nucleoside diphosphates which have proved reactive in the enzymatic synthesis of ribonucleic acid (4)) were not excluded.

Earlier reports (1, 2, 5-7) briefly described an enzyme system in extracts of *Escherichia coli* which catalyzes the incorporation of deoxyribonucleotides into DNA. Purification of this enzyme led to the demonstration that all four of the naturally occurring deoxynucleotides, in the form of triphosphates, are required. In addition, polymerized DNA and Mg⁺⁺ were found to be indispensable for the reaction. Deoxynucleoside diphosphates are inert; and as a further indication of the specificity of the enzyme for the triphosphates, the synthesis of DNA is accompanied by a release of inorganic pyrophosphate, and reversal of the reaction is specific for inorganic pyrophosphate.

These considerations have led to a provisional formulation of the reaction as follows:



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¹The following abbreviations are used: ATP, adenosine triphosphate; dATP or dATPP, deoxyadenosine triphosphate; dCTP or dCTPP, deoxycytidine triphosphate; dGTP or dGTPP, deoxyguanosine triphosphate; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; P*, P³²-labeled phosphate; P_i, inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane; dTTP, dTPPP, thymidine triphosphate.

The purpose of this report is to describe in detail the methods for the partial purification and assay of the enzyme from *E. coli* and for the preparation of the substrates for the reaction. In order to facilitate reference in this report, the enzyme responsible for deoxyribonucleotide incorporation is designated as "polymerase." The succeeding report will present evidence for the net synthesis of the DNA and other general properties of the system.

EXPERIMENTAL

Preparation of Substrates

Preparation of P³²-Labeled Deoxyribonucleotides—DNA was isolated from *E. coli* cells grown to a limit on P³²-orthophosphate. The DNA was then degraded to 5'-mononucleotides, which were separated by anion exchange chromatography. 100 ml. of glycerol-lactate medium (8) containing 8 × 10⁻⁴ M phosphate with a specific radioactivity of about 0.3 mc. per μmole was inoculated with 0.1 ml. of a 7 hour broth culture of *E. coli* strain B. After 18 hours growth at 37°, the cells were harvested and washed twice with 10 ml. portions of a solution containing 0.5 per cent each of KCl and NaCl. The packed cells were suspended in 6 ml. of alcohol-ether (3:1), incubated at 37° for 30 minutes, centrifuged, and resuspended in 6 ml. of the alcohol-ether solution. After centrifugation, the cells were dried over KOH *in vacuo*. The dry powder was then suspended in 3.0 ml. of 1 N NaOH, and left for 15 hours at 37°. The turbid, viscous solution was chilled to 0° and treated with 1.0 ml. of 5 N perchloric acid. The precipitate, collected by centrifugation, was suspended in 1.75 ml. of water and dissolved by the addition of 0.25 ml. of 1 N NaOH. This solution contained approximately 5 μmoles of purine deoxynucleotides, as judged by deoxypentose estimation, and from 90 to 100 per cent of the deoxypentose of the original alkaline digest. The DNA was precipitated from solution by the addition of 0.23 ml. of 5 N perchloric acid, washed with 2 ml. of cold water, and then redissolved in 2 ml. of 0.12 N NaOH. This reprecipitation was repeated until the radioactivity in the perchloric acid supernatant solution was less than 0.5 per cent of that in the dissolved precipitate. Usually two to three reprecipitations were sufficient. The precipitate was suspended finally in 1.0 ml. of water, the pH adjusted to approximately 7.5 with 1 N NaOH, and the volume brought to 2.0 ml.

Digestion with pancreatic DNase was carried out at 37° in a mixture containing 80 μmoles of Tris buffer, at pH 7.5, 10 μmoles of MgCl₂, and 300 μg. of crystalline pancreatic DNase (Worth-

ington Biochemical Corporation) in a final volume of 2.25 ml. Aliquots removed at intervals from the digestion mixture were precipitated with an equal volume of 1 N perchloric acid in the presence of thymus DNA which was added as carrier. 90 to 100 per cent of the radioactivity was rendered acid-soluble after approximately 3 hours.

The polydeoxyribonucleotides of the DNase digest were degraded to mononucleotides by a snake venom phosphodiesterase purified free of mononucleotidase (9). The incubation mixture (10 ml.) consisted of the DNase digest, 180 μ moles of magnesium acetate, 600 μ moles of glycine buffer (at pH 8.5), and 50 units of snake venom phosphodiesterase (an amount which releases mononucleotides from a pancreatic DNase digest of thymus DNA at a rate of 50 μ moles per hour). Formation of deoxyribonucleotide was measured in aliquots by the release of inorganic orthophosphate upon incubation with purified 5'-nucleotidase (10). Conversion of the DNase digest to deoxyribonucleotides was usually complete within 3 hours. The reaction mixture was heated in a boiling water bath for 2 minutes; a flocculent precipitate was separated by centrifugation, washed with 10 ml. of water, and then discarded. The supernatant fluid and wash were combined and adsorbed on a column of Dowex 1 (chloride form, 2 per cent cross-linked, 6.0×1.0 cm.²). The elution rate was 0.5 ml. per minute. Deoxycytidylate was eluted between 12 and 15 resin bed volumes of 0.002 N HCl, after which the eluant was changed to 0.01 N HCl. Deoxyadenylate appeared in the next 5 to 6 resin bed volumes. The eluant was changed to 0.01 N HCl containing 0.05 M KCl, and then deoxyguanylate and thymidylate were eluted together in approximately 3 resin bed volumes. The tubes containing the latter fractions were pooled, neutralized, and reappplied to a Dowex 1 column (chloride form, 2 per cent cross-linked, 10.0×1.0 cm.²). Deoxyguanylate was eluted between 15 and 20 resin bed volumes of 0.01 N HCl, and thymidylate was eluted between 30 and 36 resin bed volumes. Approximately 1.5 μ moles of each deoxyribonucleotide, with a specific radioactivity of about 0.3 mc. per μ mole, were obtained under these conditions.

Enzymatic Synthesis of Deoxynucleoside 5'-Triphosphates—The P³²-labeled deoxyribonucleotides isolated as described above and the unlabeled nucleotides obtained from the California Foundation for Biochemical Research were converted to the triphosphates by enzymes partially purified from *E. coli*. The preparation and assay of these kinases, and the synthesis and isolation of the triphosphates are described below.

1. *Assay of Deoxynucleotide Kinase*—This assay measures the conversion of a P³²-labeled deoxynucleotide that is susceptible to the action of semen phosphatase to a form that is resistant to the phosphatase. Adsorption on Norit is used to distinguish between the phosphate that is liberated from the nucleoside and that which is bound to it. The assay consists of three stages (Scheme I).

In a control incubation, from which enzyme was omitted in Stage I, no radioactivity was found adsorbed to the Norit, whereas with an excess of the deoxynucleotide kinase, all the radioactivity was accounted for in the Norit precipitate. A unit of enzyme was defined as the amount that converts 100 μ moles of deoxynucleotide to a phosphatase-resistant form in 1 hour under these assay conditions. The reaction rate was proportional to the amount of enzyme added; with 0.005, 0.01, and 0.02 ml. of streptomycin fraction (Table I), there were obtained 3.92, 4.33, and 4.38 units per ml., respectively.

Stage	Reagent	Reaction
I	Deoxynucleotide kinase	Deoxynucleoside-P* $\xrightarrow{\text{ATP}}$ deoxynucleoside-P*-P-(P)
II	Phosphatase (monoesterase) in excess	Deoxynucleoside + P _i no change
III	Norit, in excess	Radioactivity unadsorbed Radioactivity adsorbed

SCHEME I

In Stage I, the incubation mixture (0.25 ml.) contained 2 μ moles of ATP, 4 μ moles of MgCl₂, 16 μ moles of P³²-labeled deoxynucleotide (0.5 to 1×10^6 c.p.m. per μ mole), 0.05 to 0.2 unit of enzyme, and 10 μ moles of Tris buffer (pH 7.5). After incubation at 37° for 20 minutes, 0.5 ml. of water was added, the tube was immersed in boiling water for 2 minutes, and then was chilled in an ice bath. In Stage II, 4 μ moles of MgCl₂ (0.04 ml.), 100 μ moles of sodium acetate buffer, at pH 5.0, (0.1 ml.), 0.2 μ mole of unlabeled deoxynucleotide (0.02 ml.), and 50 units of human semen phosphatase (0.02 ml.) (11) were added to the mixture, which was then incubated at 37°; after 15 minutes it was chilled. In Stage III, 0.1 ml. of cold 2 N HCl and 0.15 ml. of a Norit suspension (20 per cent packed volume) were added and shaken for 2 to 3 minutes with the incubation mixture. The Norit precipitate was collected by centrifugation, washed three times with 2.5 ml. portions of cold water, and suspended in 0.5 ml. of 50 per cent ethanol containing 0.3 ml. of concentrated ammonium hydroxide per 100 ml. The entire suspension was plated and counted.

2. *Purification of Deoxynucleotide Kinase*—An enzyme preparation (partially purified from extracts of *E. coli*) catalyzes the phosphorylation, by ATP, of deoxyadenylate, deoxyguanylate, deoxycytidylate, and thymidylate to yield the corresponding triphosphates (Table I). The purification procedures were carried out at 0° to 4°. Bacterial extract (60 ml. of Fraction I as described later for "polymerase") was diluted with 30 ml. of glycyglycine buffer (0.05 M, pH 7), and 27 ml. of 5 per cent streptomycin sulfate² was added slowly as the solution was stirred. The suspension was allowed to stand for 5 minutes and then was centrifuged for 10 minutes at $10,000 \times g$. The supernatant solution (streptomycin fraction, 115 ml.) was next treated with calcium phosphate gel (12) as follows: 66 ml. of gel (15 mg. of solids per ml.) was centrifuged and the supernatant fluid was discarded. The streptomycin fraction (110 ml.) was mixed with the packed gel; after 5 minutes, the suspension was centrifuged, and the supernatant solution was collected (calcium phosphate gel fraction, 113 ml.). This fraction (108 ml.) was added to the packed residue from 45 ml. of alumina C γ gel (13) (15 mg. of solids per ml.). The gel was thoroughly dispersed, and, after 5 minutes, was collected by centrifugation. The enzyme was then eluted from the gel with 54 ml. of potassium phosphate buffer (0.066 M, pH 7.4) (alumina C γ gel fraction, 52 ml.).

The alumina C γ fraction showed no significant loss of activity over a 4 month period when stored at -10° . The specific

² We are extremely grateful to Merck and Company, Inc., for generous gifts of this substance.

activity (units per mg. of protein) of a typical enzyme preparation, using equivalent levels of deoxyadenylate, deoxyguanylate, deoxycytidylate, and thymidylate as substrates, was 8.8, 7.1, 4.0, and 6.8, respectively. Although this preparation served as a kinase for all the deoxynucleotides, specific kinases for each of the deoxynucleotides can be obtained by further purification.³

3. *Enzymatic Preparation of the Triphosphates: dGTP, dCTP or dTTP*—The reaction mixture (90 ml.) consisted of the following: Tris buffer, at pH 7.5, 3 mmoles; MgCl₂, 300 μmoles; cysteine, 50 μmoles; adenosine diphosphate, 35 μmoles; acetyl phosphate, 175 μmoles; a deoxynucleoside 5'-phosphate, 35 μmoles; acetokinase, 5 units (14); and the deoxynucleotide kinase (alumina C_γ gel fraction), 300 units. After incubation at 37° for 2 hours, the mixture was heated for 2 minutes in a boiling water bath, and was then immediately chilled; the precipitated proteins were removed by filtration.

dATP—The incubation mixture was the same as that described above, except that glycylglycine buffer, at pH 7.4, was used in place of Tris.

Although the chromatographic procedures to be described effectively separated the excess ATP in the reaction mixture from dGTP, dCTP and dTTP, they did not resolve it from dATP. ATP was therefore destroyed in the dATP mixture before chromatography.

4. *Selective Destruction of ATP*—ATP may be selectively degraded in the presence of deoxyribonucleotides by periodate and then alkaline treatment, by Whitfeld's procedure for oligoribonucleotides (15). According to this author, the procedure should result first in an oxidation of the ribose and then a cleavage of the adenine from the nucleotide. The dATP reaction mixture containing 35 μmoles of dATP was treated with 175 μmoles of sodium metaperiodate at 25°. Spectrophotometric measurement of periodate reduction (16) indicated that under these conditions 90 per cent of the theoretical amount was consumed within 4 minutes and that the reaction was essentially complete within 8 minutes. After incubation for 30 minutes, the excess periodate was destroyed by adding 200 μmoles of glucose and incubating the mixture for 30 minutes at 25°. The pH was then adjusted to 10 with glycine buffer (1 M, at pH 10.2), and the solution was incubated for 12 to 16 hours at 37°. Prior to treatment with alkali, it appeared that about 10 per cent of the ATP remained, as judged by hexokinase assay (17); however, the slow rate of reaction with hexokinase as compared with an ATP control (ATP added after the periodate was destroyed by glucose) suggests that it was a periodate-oxidation product of ATP which reacted with hexokinase or an associated kinase. After treatment with alkali, ATP was completely destroyed; less than 1 per cent remained, as determined by the hexokinase assay. Such an exposure of ATP to alkali without prior periodate oxidation resulted in losses of only 5 per cent or less. A

³ Further purification procedures developed by Dr. Jerard Hurwitz have shown distinct kinases for deoxyguanylate, deoxycytidylate, and thymidylate. The presence in *E. coli* of a kinase for deoxyadenylate (very likely adenylate kinase) which is distinguishable from these other kinases has been suggested (30). Heating of the alumina C_γ gel eluate at 100° for 3 minutes at pH 7.4 permitted the survival of some kinase activity for deoxyadenylate, whereas that for the other deoxynucleotides was completely destroyed. Phosphorylation of deoxynucleotides by enzyme preparations from animal (31, 32) and other bacterial cells (33) has been reported.

TABLE I
Purification of deoxynucleotide kinase

Fraction	Units	Protein	Specific activity*
	<i>total</i>	<i>mg./ml.</i>	<i>units/mg. protein</i>
Extract (Fraction I)	918	14.8	1.0
Streptomycin fraction	1210	3.4	3.1
Calcium phosphate gel fraction	836	1.6	4.8
Alumina C _γ gel eluate	633	1.8	6.8

* Using thymidine 5'-phosphate as a substrate.

more sensitive assay of the removal of ATP involved the use of ATP labeled with C¹⁴ in the adenine. After periodate and alkaline treatment, the mixture was made 1 N with respect to HCl, and passed over a column of Dowex 50-H⁺ resin. Only 0.6 per cent of the radioactivity passed through the column, indicating that 99.4 per cent of the ATP had been degraded to adenine, which is quantitatively adsorbed by this resin, whereas ATP is not adsorbed. Comparable results have been obtained for adenosine 5'-phosphate and presumably should be expected for other derivatives of adenosine. When applied to uridine nucleotides, this procedure did not result in a quantitative cleavage of the pyrimidine base from the nucleotide. Determinations by the orcinol method (18) of the DATP isolated after ion exchange chromatography have indicated 0.04 to 0.05 μmole of orcinol-reactive material per μmole of DATP; the significance of this determination is not clear.

5. *Isolation of Triphosphates*—The chilled incubation mixtures were adsorbed on Dowex 1 columns (2 per cent cross-linked, chloride form, 10 cm. × 3.8 cm.²), and the individual triphosphates were eluted as symmetrical peaks. All fractions were neutralized or made slightly alkaline with NH₄OH immediately upon collection.

dATP—Elution was begun with 0.01 N HCl-0.08 M LiCl. When the adenine resulting from the periodate degradation of ATP had been removed completely, dATP was eluted with 0.01 N HCl-0.2 M LiCl. The fractions between 2 and 4 resin bed volumes represented a yield of dATP of approximately 60 per cent, based on the amount of deoxyadenylate initially added to the reaction mixture.

dGTP—Following the elution of ATP from the column with 0.01 N HCl-0.1 M LiCl, the dGTP was eluted with 0.01 N HCl-0.2 M LiCl. This appeared between 11 and 16 resin bed volumes of effluent with a yield of about 75 per cent.

dCTP—The column was first washed with 0.01 N HCl-0.05 M LiCl to remove any mono- or diphosphates present in the reaction mixture. dCTP was eluted with 0.01 N HCl-0.08 M LiCl. The fraction between 4 and 12 resin bed volumes of effluent represented a yield of triphosphate of about 60 per cent.

dTTP—After removal of ATP from the column, the dTTP was eluted with 0.02 N HCl-0.2 M LiCl, and appeared between 10 and 18 resin bed volumes of eluate, and about 75 per cent of the starting thymidylate was recovered as the triphosphate.

The triphosphates were concentrated by precipitation as the barium salts, then metathesized with Dowex 50-K⁺ resin. A typical preparation was carried out in the following manner. The ion exchange fractions were pooled (285 ml.). Glycine buffer (1 N, at pH 10.2) was added to a final concentration of 0.01 M, and the solution was adjusted to pH 8.5 by the dropwise

TABLE II
Analysis of the deoxynucleoside triphosphates

Compound	Absorbance ratios*		Base	Deoxy- pentose	Acid- labile P†	Total P
	$\lambda_{250}/\lambda_{260}$	$\lambda_{280}/\lambda_{260}$				
dTTP.....	0.64 (0.64)	0.72 (0.72)	1	‡	1.90	3.18
dTTP§.....	0.63	0.74	1	‡	1.98	3.26
dCTP.....	0.44 (0.43)	2.14 (2.12)	1	‡	1.92	3.20
dATP.....	0.77 (0.79)	0.14 (0.15)	1	0.99	1.82	3.00
dGTP.....	1.14 (1.15)	0.66 (0.68)	1	1.03	1.78	2.97

* The values cited in the literature for the corresponding 5'-monophosphates (20) are given in parentheses for comparison. dTTP and dCTP were determined at pH 2 and dATP and dGTP at pH 7.

† Hydrolysis was for 10 minutes at 100° in 1 N HCl. In the case of dATP and dGTP, 0.65 μ mole of P per μ mole of base was subtracted in order to correct for the hydrolysis of the phosphate linked to the deoxyribose; this correction, based on the hydrolysis rate of deoxyadenylate and deoxyguanylate, is only an approximation. Hydrolysis of dATP and dGTP for 30 minutes yielded 93 to 94 per cent of the total P.

‡ Deoxypentose determinations of dTTP and dCTP by the cysteine-sulfuric acid method (19) did not yield reproducible values.

§ Chemically synthesized.

addition of 4 N LiOH. 2 ml. of a saturated solution of barium bromide and 285 ml. of cold ethanol were added. After 30 minutes at 0°, the precipitate was collected by centrifugation, and was dried in a vacuum desiccator over KOH for about an hour.

The barium salt was dispersed in about 2 ml. of cold water, and 1 ml. (packed volume) of washed Dowex 50-K⁺ resin was added. The suspension was shaken for 15 minutes at 0°, then adjusted to a pH of about 6 with 0.5 N HCl, and again shaken for 15 minutes. The suspension was then filtered through a sintered glass funnel, and the resin was washed with cold water. The combined filtrate and washings were neutralized with 1 N KOH. The recovery of the triphosphates from the column effluents by this procedure ranged from 90 to 100 per cent. Analyses of the four deoxynucleoside triphosphates are presented in Table II.

6. *Chemical Synthesis of dTTP*—dTTP was also prepared by chemical synthesis* according to the method described by Hall and Khorana (21) for uridine triphosphate. The calcium salt of thymidine 5'-phosphate (1 gm., California Foundation for Biochemical Research) was first converted to the pyridine salt on Dowex 50 pyridine, taken to dryness, and then treated with proportions of phosphoric acid, pyridine, water, and dicyclohexylcarbodiimide, as previously described (21) (Table I). The mono-, di-, tri- and higher polyphosphates of thymidine were separated by ion exchange chromatography with recoveries of 14, 22, 40 and 24 per cent, respectively, and dTTP was finally collected as a barium salt after the chromatogram eluates were adsorbed on and eluted from Norit (Table II).

Preparation of Deoxyribonucleic Acids

P³²-Labeled Bacteriophage DNA—Lysates of T2r⁺ were prepared in the glycerol-lactate medium (8). 200 ml. of medium

* This preparation was synthesized in the laboratory of Dr. H. G. Khorana, to whom we are indebted for guidance and hospitality.

containing 0.60 μ mole of inorganic orthophosphate per ml. (specific activity, 10 μ c. per μ mole) were inoculated with 0.1 ml. of an 18 hour culture of *E. coli* strain B and then incubated at 37°. When the culture reached a level of 2×10^8 cells per ml., bacteriophage at a multiplicity of 1 was added. Lysis was usually complete in 8 to 10 hours with titers of about 4×10^{10} infectious particles per ml. The bacteriophage was purified as described by Herriott (22), with omission of the filtration through Super-cel, and finally taken up in 0.5 ml. of 0.15 M NaCl. In order to disrupt the bacteriophage by osmotic shock (23), 180 mg. of NaCl was dissolved in the suspension, to which 25 ml. of distilled water was added rapidly, with mixing. The mixture stood for 1 hour at 25° and was then centrifuged for 90 minutes at $20,000 \times g$ to remove intact phage and debris. The supernatant solution, designated P³²-phage DNA, contained 0.16 μ mole per ml. of phosphorus, and had a molar extinction coefficient, $E(P)$, on this basis, of 6900 at 260 m μ .

Calf Thymus DNA—This was prepared by the method of Kay, Simmons and Dounce (24). A solution of 0.5 mg. per ml. had an extinction number at 260 m μ of about 7.0.

Enzyme Assays

Assay of "Polymerase"—This assay measures the conversion of acid-soluble P³²-labeled deoxynucleoside triphosphates into an acid-insoluble product. The incubation mixture (0.3 ml.) contained 0.02 ml. of glycine buffer (1 M, pH 9.2), 0.02 ml. of MgCl₂ (0.1 M), 0.03 ml. of 2-mercaptoethanol (0.01 M), 0.02 ml. of thymus DNA (0.5 mg. per ml.), 0.01 ml. of dATP (0.5 μ mole per ml.), 0.02 ml. of dGTP (0.5 μ mole per ml.), 0.01 ml. of dCTP (0.5 μ mole per ml.), 0.01 ml. of dTP³²PP⁵ (0.5 μ mole per ml., 1.5×10^6 c.p.m. per μ mole), and 0.005 to 0.05 unit of enzyme. Dilutions of the enzyme for assay were made in Tris buffer (0.05 M, pH 7.5) containing 0.1 mg. per ml. of thymus DNA. After incubation at 37° for 30 minutes, the tube was placed in ice, and 0.2 ml. of a cold solution of thymus DNA (2.5 mg. per ml.) was added as carrier. The reaction was stopped, and the DNA was precipitated by the immediate addition of 0.5 ml. of ice-cold 1 N perchloric acid. After 2 to 3 minutes, the precipitate was broken up thoroughly with a snug-fitting glass pestle, 2 ml. of cold distilled water were added, and the precipitate was thoroughly dispersed. After centrifugation for 3 minutes at $10,000 \times g$, the supernatant fluid was discarded. The precipitate was dissolved in 0.3 ml. of 0.2 N NaOH, the DNA was reprecipitated by the addition of 0.40 ml. of cold 1 N perchloric acid, 2.0 ml. of cold water were added, and the precipitate was thoroughly dispersed. After centrifugation, this precipitate was again dissolved, reprecipitated again and recentrifuged. Finally, the precipitate was dissolved by the addition of 0.2 ml. of 0.1 N NaOH, the entire solution was pipetted into a shallow dish, dried, and the radioactivity measured.

Controls for the crude enzyme fractions (I to III) were incubation mixtures to which the enzyme fraction was added after completion of the incubation period, but just before the perchloric acid was added. With more purified fractions (Fractions IV to VII), an incubation mixture lacking Mg⁺⁺ or one of the deoxynucleoside triphosphates served as well. Precipitates obtained from control incubation mixtures contained less than 0.10

⁵ dCP*PP, dAP*PP, and dTTP labeled in carbon-2 of thymine have also been used in routine assays during the course of enzyme purification, with essentially identical results.

per cent of the total radioactivity added, and, in most assays they contained radioactivity in the order of 2 per cent of the experimental values. A unit of enzyme was defined as the amount causing the incorporation of 10 μ moles of the labeled deoxynucleotide into the acid-insoluble product during the period of incubation. The specific activity was expressed as units per mg. of protein.

With the exception of Fraction I, proportionality of enzyme addition, with the amount of labeled substrate incorporated into the product, was obtained. With Fraction IV, for example, the addition of 0.5, 1.0, and 1.5 μ g. of the enzyme preparation yielded specific activities of 21.0, 18.7 and 20.5, respectively. Assays of Fraction I are only an approximation, and the levels of activity assayed should not exceed 0.02 unit; even so, augmentation of incorporation by as much as 50 per cent was obtained at times with the addition of ATP (0.0025 M) and the deoxynucleotide kinase (0.7 unit of alumina C γ gel eluate fraction).

With respect to the optimal pH for the assay, the rate was most rapid at about 8.7; at pH 6.5, 8.0 and 10.0, the respective rates were 15, 70 and 15 per cent of that observed at pH 8.7. The pH usually achieved in the assay mixture was in the neighborhood of 8.7 to 9.0. It is noteworthy that high concentrations of salt interfere with the assay; for example, NaCl at a final concentration of 0.2 M produced a 97 per cent inhibition. The use of fluoride to inhibit the action of phosphatases in the assay of crude enzyme fractions is limited by the inhibitory action of fluoride on the "polymerase" (0.05 M KF produced a 90 per cent inhibition).

Assays of DNase—There is an abundance of DNase activity in cell-free extracts of *E. coli* which degrades both the DNA added initially to the assay mixture and the newly synthesized DNA. As will be described in a later section, there are indications for the existence of at least 3 distinct DNases. One activity (or activities) to be designated tentatively as "DNase A", degrades calf thymus DNA with a pH optimum near 8.5, whereas it does not degrade it at all at pH 10. Although the cleavage of bacteriophage DNA is only one-tenth as rapid, the use of this substrate labeled with P³² provided a sensitive assay when this enzyme activity is reduced to trace concentrations. Another activity, designated "DNase B", is characterized by its effective action on the enzymatically synthesized DNA even at pH 10. Immunological and fractionation procedures, to be described later, suggest that there are at least two distinct enzymes in this group. The assays of DNase A on thymus and bacteriophage DNA and of DNase B on enzymatically prepared DNA are described below.

1. *Assay of DNase A on Thymus DNA*—This assay depends on the degradation of DNA to acid-soluble fragments which are measured spectrophotometrically. The reaction mixture (0.3 ml.) contained 0.1 ml. of thymus DNA (0.5 mg. per ml.), 0.02 ml. of Tris buffer (1 M, pH 7.5), 0.02 ml. of MgCl₂ (0.1 M) and about 4 units of enzyme. After incubation for 30 minutes at 37°, 0.2 ml. of a thymus DNA solution (2.5 mg. per ml.) was added as carrier, followed by 0.5 ml. of cold 1 N perchloric acid. After 5 minutes at 0°, the precipitate was removed by centrifugation. The supernatant solution was diluted with an equal volume of distilled water, and the optical density at 260 μ was determined. One unit of enzyme was defined as the amount causing the production of 10 μ moles of acid-soluble DNA polynucleotides in 30 minutes (assuming a molar extinction coefficient, $E(P)$, of 10,000). The assay showed proportionality

between the substrate split and the enzyme added at levels of 1 to 8 units. With the addition of 0.02, 0.05, and 0.10 ml. of a 1:10 dilution of an enzyme fraction (the ammonium sulfate fraction preceding "polymerase" Fraction VI), 405, 362 and 394 units per ml., respectively, were obtained. The activity of this enzyme at pH 7.5 is approximately twice that observed at pH 9.

2. *Assay of DNase A on Bacteriophage DNA*—This assay depends on the degradation of DNA to acid-soluble fragments which are determined by measurement of radioactivity. The incubation mixture (0.30 ml.) contained 0.02 ml. of glycine buffer (1 M, pH 8.5), 0.01 ml. of MgCl₂ (0.10 M), 0.03 ml. of P³²-phage DNA (0.15 μ mole of P per ml.; specific activity 5×10^6 c.p.m. per μ mole of P), and 0.01 to 0.10 unit of enzyme. After incubation for 30 minutes at 37°, 0.20 ml. of thymus DNA (2.5 mg. per ml.) was added as carrier, and then 0.50 ml. of 0.5 N perchloric acid was added. After standing 2 to 3 minutes in ice, the precipitate was removed by centrifugation, and 0.5 ml. of the supernatant fluid was transferred to a shallow dish, and the radioactivity was determined. An incubation mixture from which enzyme was omitted served as a control for nonenzymatic hydrolysis of bacteriophage DNA. The proportionality of the amount of enzyme added to the amount of substrate hydrolyzed was obtained when 2 to 20 per cent of the substrate was converted to acid-soluble material. The unit of enzyme activity is the same as that defined in the thymus DNA assay.

3. *Assay of DNase B*—This assay also measures the release of radioactive acid-soluble fragments from DNA. The incubation mixture (0.3 ml.) contained 0.03 ml. of P³²-labeled, enzymatically prepared, DNA⁶ (0.3 μ mole per ml., 2×10^6 c.p.m. per μ mole of DNA nucleotide), 0.03 ml. of glycine buffer (1 M, at pH 9.2), 0.02 ml. of MgCl₂ (0.1 M), 0.03 ml. of 2-mercaptoethanol (0.01 M), and about 0.05 unit of enzyme. After incubation of the mixture for 30 minutes at 37°, carrier DNA and cold perchloric acid were added, as in the assay for DNase A. After removal of the precipitate by centrifugation, 0.2 ml. of the supernatant solution was plated, and the radioactivity was determined. The enzyme unit is the same as for DNase A. The proportionality between the amount of substrate hydrolyzed and the amount of enzyme added was observed from 0.05 to 0.25 unit of enzyme. With the addition of 0.02, 0.05, and 0.10 ml. of a 1:100 dilution of an enzyme fraction ("polymerase" Fraction VI), 340, 260 and 257 units per ml., respectively, were obtained.

Other Methods

Determinations of phosphate, pentose and protein, procedures for ion exchange chromatography and measurements of radioactivity have been cited previously (25). The efficiency of the

⁶ The reaction mixture consisted of 0.15 μ mole each of dTTP, dGTP, dATP and dCTP (the latter having a specific radioactivity of 20 μ c. per μ mole), 0.33 ml. of thymus DNA (2.5 mg. per ml.), glycine buffer, at pH 9.2 (330 μ moles), MgCl₂ (330 μ moles) and Fraction VI (20 units) in a final volume of 5 ml. After incubation at 37° for 1 hour, the incubation mixture was chilled and 0.55 ml. of 4 N trichloroacetic acid was added, and the suspension was centrifuged. The precipitate was dissolved in 1.5 ml. of cold 0.02 N NaOH, and then was treated with 1.5 ml. of cold 1 N perchloric acid and 2 ml. of water. The precipitate was collected by centrifugation, dissolved, reprecipitated, and finally dissolved in 1.0 ml. of 0.02 N NaOH. 0.1 N HCl was added dropwise to adjust the pH to 7.5, and cold water was added to bring the volume to 3.3 ml. Such a preparation had a specific activity of 2.5×10^6 c.p.m. per μ mole of DNA nucleotide.

TABLE III
Purification of "polymerase"

Fraction no.	Step	Units		Protein mg./ml.	Specific activity units/mg. protein
		per ml.	total		
I	Sonic extraction.....	2.0	16,800	20.0	0.1
II	Streptomycin.....	13.0	19,500	3.0	4.3
III	DNase, dialysis.....	12.1	18,100	1.80	6.7
IV	Alumina gel.....	15.4	12,300	0.78	19.8
V	Concentration of gel eluate.....	110	9,900	4.90	22.4
VI	Ammonium sulfate.....	670	6,030	8.40	80.0
VII	Diethylaminoethyl cellulose*.....	120	3,600	0.60	200.0†

* This step was actually carried out many times on a smaller scale (see the text), and these values are calculated for the large-scale procedure.

† In some runs, values as high as 400 have been obtained.

gas-flow counter was approximately 50 per cent. Deoxypentose of purine deoxynucleotides was determined by the diphenylamine method of Dische (26).

Purification of "Polymerase"

Growth and Harvest of Bacteria—*E. coli* strain B or ML30, was grown in a medium containing 1.1 per cent K_2HPO_4 , 0.85 per cent KH_2PO_4 , 0.6 per cent Difco yeast extract, and 1 per cent glucose. Cultures, usually 60 liters, were grown with vigorous aeration in a large growth tank,⁷ and were harvested about 2 hours after the end of exponential growth. The cultures were chilled by the addition of ice, the cells collected in a Sharples supercentrifuge, washed once in a Waring Blendor by suspension in 0.5 per cent NaCl-0.5 per cent KCl (3 ml. per gm. of packed cells), centrifuged, and then stored at -12° . The yield of packed wet cells was approximately 8 gm. per liter of culture, and cells stored as long as 1 month were used. All subsequent operations were carried out at 0° to 3° unless otherwise specified.

Preparation of Extract—Cells were suspended in .05 M glycylglycine buffer, at pH 7.0 (4 ml. per gm. of packed cells), and were disrupted by treatment for 15 minutes in a Raytheon 10-KC sonic oscillator. The suspension was centrifuged for 15 minutes at $12,000 \times g$, and the slightly turbid supernatant liquid was collected. The protein content was determined and adjusted to a concentration of 20 mg. per ml. by addition of the same glycylglycine buffer (Fraction I) (Table III).

Streptomycin Precipitation—8400 ml. of Fraction I, obtained from 2 kilos of packed cells, were treated in the following manner. To a 525 ml. batch were added 525 ml. of Tris buffer (0.05 M, at pH 7.5), then slowly, with stirring, 81 ml. of 5 per cent streptomycin sulfate² were added. After 10 minutes, the precipitate was collected by centrifugation at $10,000 \times g$. This precipitation with streptomycin was carried out four times on this scale, and the precipitates were collected in the same centrifuge cups. The precipitates, with potassium phosphate buffer (0.05 M, at pH 7.4) added to a total volume of 430 ml., were homogenized in a Waring Blendor for 30 minutes at low speed. This suspension was centrifuged for 2 hours at $78,000 \times g$ in a

⁷ Available from Rinco Instruments Company, Greenville, Illinois.

Spinco model L centrifuge, and the supernatant fluid was collected (Fraction II).

Deoxyribonuclease Digestion—To 1500 ml. of Fraction II (derived from 8400 ml. of Fraction I) were added 15 ml. of 0.3 M $MgCl_2$ and 1.5 ml. of pancreatic deoxyribonuclease (100 μ g. per ml.). This mixture was incubated at 37° for about 5 hours, until 85 to 90 per cent of the ultraviolet-absorbing material was rendered acid-soluble.⁸ A considerable amount of protein settled out during the digestion, but it was not removed at this time. The digest was dialyzed for 16 hours against 24 liters of Tris buffer (0.01 M, at pH 7.5), then centrifuged for 5 minutes at $10,000 \times g$, and the supernatant fluid was collected (Fraction III).

Alumina C γ Gel Adsorption and Elution—Enough aged alumina gel (13) (195 ml. containing 15 mg. dry weight per ml.) was added to 1500 ml. of Fraction III in order to adsorb 90 to 95 per cent of the enzyme. The mixture was stirred for 5 minutes, and then centrifuged. The supernatant fluid was discarded and the gel washed with 400 ml. of potassium phosphate buffer (0.02 M, at pH 7.2). The gel was then eluted twice with 400 ml. portions of potassium phosphate buffer (0.10 M, at pH 7.4) in order to remove the enzyme, and the eluates were combined (Fraction IV).

Concentration of Alumina C γ Eluate—To 800 ml. of Fraction IV were added 16 ml. of 5 N acetic acid and then 480 gm. of ammonium sulfate. After 10 minutes at 0° , the resulting precipitate was collected by centrifugation (30 minutes, $30,000 \times g$) and dissolved in 90 ml. of potassium phosphate buffer (0.02 M, at pH 7.2) (Fraction V).

Ammonium Sulfate Fractionation—To 90 ml. of Fraction V were added 9 ml. of potassium phosphate buffer (1 M, at pH 6.5) and 0.90 ml. of a 0.10 M solution of 2-mercaptoethanol. 24.7 gm. of ammonium sulfate were added, and after 10 minutes at 0° , the precipitate was removed by centrifugation at $12,000 \times g$ for 10 minutes.⁹ To the supernatant fluid an additional 9.6 gm. of ammonium sulfate were added, and, after 10 minutes at 0° , the precipitate which formed was collected by centrifugation at $12,000 \times g$ for 10 minutes. This precipitate was dissolved in 9 ml. of potassium phosphate buffer (0.02 M, at pH 7.2) (Fraction VI).

Diethylaminoethyl Cellulose Fractionation—A column (11 \times 1 cm.) was prepared from diethylaminoethyl cellulose (27) which had previously been equilibrated with K_2HPO_4 (0.02 M). 1.2 ml. of Fraction VI was diluted to 8.0 ml. with 0.02 M K_2HPO_4 and passed through the column at a rate of 12 ml. per hour. The column was washed with 3.0 ml. of the same buffer, and then eluted (flow rate of 9 ml. per hour) with pH 6.5 potassium phosphate buffers as follows: 8 ml. of 0.05 M, 10 ml. of 0.10 M, 3 ml. of 0.20 M, and finally 4 ml. of 0.20 M. Approximately 60 per cent of the enzyme applied to the adsorbent was obtained in the last elution with 0.20 M buffer (Fraction VII).

Samples of Fraction VII have been further purified by another treatment with diethylaminoethyl cellulose or by treatment with a phosphocellulose adsorbent (27). Specific activities of 250 to 350 have thus been obtained.

⁸ The optical density at $260 m\mu$ was determined before and after precipitation with an equal volume of cold 1 N perchloric acid.

⁹ This precipitate dissolved in 9 ml. of potassium phosphate buffer (0.02 M, at pH 7.2) has been designated AS 1 and used as an antigen in studies cited below.

Stability of "Polymerase"

With the exception of the alumina gel eluate (Fraction IV), which in some instances lost activity on storage, each of the enzyme fractions has been stored for at least several weeks at -12° without any significant loss in activity. When heated at pH 7.2 for 3 minutes at 80° , all the activity was destroyed, and, after 10 minutes at 60° , only a trace (less than 2 per cent) remained. Incubation for 10 minutes at 45° or for 30 minutes at 37° produced losses of 75 and 30 per cent, respectively, whereas similar incubations, but at pH 8.7, resulted in respective losses of 95 and 85 per cent. Since the assay of the enzyme is carried out for 30 minutes at 37° at a pH of about 8.7, it was of interest to determine the stabilizing components in the assay mixture. Among the constituents of the assay mixture only DNA was active in this regard. Complete protection against inactivation of the enzyme during incubation was provided at a level of 17 μg . per ml. Replacement of the DNA by bovine serum albumin (1 mg. per ml.), apurinic acid (100 μg . per ml.) (28), ribonucleic acid from crystalline turnip yellow mosaic virus¹⁰ (68 μg . per ml.), or thymus DNA treated with 1 N NaOH for 15 hours at 37° (37 μg . per ml.), resulted in losses in enzyme activity of 90, 70, 85, and 70 per cent, respectively. Thymus DNA heated for 3 minutes at 100° had about half the stabilizing effect of the untreated DNA.

Deoxyribonucleases in "Polymerase" Fractions

During the course of purification of "polymerase" the relative amounts of DNase were considerably reduced but DNase activity was not completely removed even from the best preparation (Table IV). Results of immunological studies not only supported the indications from fractionation data (Table IV) that DNase A and B were distinguishable, but also suggested that there are two distinct enzymes in the DNase B group.

Using an enzyme fraction rich in DNase A and B (AS 1 (Table IV), collected just prior to Fraction VI) as antigen, rabbit antisera were produced which neutralized 95 per cent of DNase B but only 50 per cent or less of DNase A, even with larger amounts of the sera. In addition, these sera neutralized less than 10 per cent of the DNase B in the adjacent enzyme fraction (AS 2). Inasmuch as the failure of the antisera to neutralize much of the DNase B in fraction AS 2 might have been due to an inhibitory substance in AS 2, equal amounts of fraction AS 1 and AS 2 (in terms of DNase B units) were mixed and then treated with the antisera; approximately 50 per cent of the DNase was neutralized, indicating the absence of such an inhibitor. In the most purified enzyme fractions (Fraction VII refractionated with diethylaminoethyl cellulose), DNase A activity was reduced to levels of 5 per cent, or less than that of "polymerase". DNase B, although persisting to a significant extent, was considerably reduced in activity by modifying the assay conditions of "polymerase" (last line of Table IV).

DISCUSSION

The extensive purification of the enzyme has not yet resulted in a homogeneous preparation, and it has not removed the last traces of DNase activities. Further enzyme purification is limited by the small yields of the purified fraction (1 kilo of *E. coli* yields less than 10 mg. of the purified enzyme), and the

TABLE IV
DNase activities in "polymerase" fractions

"Polymerase" fraction	Ratios of DNase to "polymerase"*		
	DNase A on thymus DNA	DNase A on phage DNA	DNase B on enzymatic DNA
Fraction I.....	†	†	34-62
Fraction V.....	1	0.1	1
Ammonium sulfate fractionation of Fraction V:			
AS 1.....	5	0.6	6
AS 2 (Fraction VI).....	1	0.1	1
AS 3.....	2	0.3	2
Ammonium sulfate fractionation of AS 3:			
AS 3a.....	1	0.4	2
AS 3b.....	26	2.1	6
Fraction VII.....	0.2	0.003	0.3
Fraction VII†.....			0.06

* "Polymerase" was determined as described in the text section on assays. Nucleases were determined with thymus DNA, bacteriophage DNA, or synthetic DNA as substrates. The ratios here express:

Nucleotide rendered acid-soluble, μmoles

Nucleotide rendered acid-insoluble, μmoles

† No accurate assay was possible because of the high DNA content of this fraction.

‡ Tested under conditions which differed from the standard assay (see the text) as follows: potassium phosphate buffer, at pH 7.4, replaced the glycine buffer, and the 2-mercaptoethanol was omitted.

indications are that there are at least three distinct nucleases to be considered. Preliminary studies of enzymes which appear to be comparable to the "polymerase" in extracts of other bacteria indicate lower activities than those found in *E. coli* extracts, and the DNA-synthesizing systems in acetone powder extracts of calf thymus gland or HeLa (tumor) cells appear to be only 1 to 2 per cent as active. It would appear that *E. coli*, with a generation time of only 20 minutes, is likely to prove at least as fertile a source of this enzyme as most other cells available for large-scale work.

In connection with the phosphorylation of the four deoxynucleotides (which commonly occur in DNA) to the triphosphate level by enzymes in *E. coli*, it is of interest to recall the observation (29) that no such kinase activity for deoxyuridine 5'-phosphate was detectable. This finding led to the suggestion that the lack of an enzyme to make deoxyuridine triphosphate might explain the lack of uracil in DNA. Current studies¹¹ which show that deoxyuridine triphosphate, prepared by chemical deamination of deoxycytidine triphosphate, can replace thymidine triphosphate and can be incorporated into the enzymatically synthesized DNA, furnish additional support for this suggestion.

SUMMARY

An enzyme which catalyzes the incorporation of deoxyribonucleotides from the triphosphates of deoxyadenosine, deoxy-

¹⁰ Gift from Dr. L. A. Heppel.

¹¹ Unpublished results.

guanosine, deoxycytidine and thymidine into deoxyribonucleic acid has been purified from cell-free extracts of *Escherichia coli* in excess of 2000-fold. The reaction mixture includes polymerized deoxyribonucleic acid and Mg^{++} .

The deoxynucleoside triphosphate substrates were synthesized from the deoxynucleotides by kinases partially purified from

Escherichia coli. Procedures for the preparation of P^{32} -labeled deoxynucleotides have also been described.

Acknowledgments—We gratefully acknowledge the grants from the Public Health Service and the National Science Foundation which made this work possible.

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