Incorporation of 5′-Amino-5′-deoxythymidine 5′-Phosphate in Polynucleotides by Use of DNA Polymerase I and a ϕX174 DNA Template†

Robert L. Letsinger,* John S. Wilkes,‡ and Lawrence B. Dumas

ABSTRACT: An aqueous solution of 5′-amino-5′-deoxythymidine 5′-triphosphate, prepared by incubation of equimolar solutions of 5′-amino-5′-deoxythymidine and sodium trimetaphosphate, stimulates synthesis of acid-precipitable polynucleotides in a system containing single-strand ϕX174 DNA template, random oligonucleotide primers, dATP, dCTP, dGTP, *Escherichia coli* DNA polymerase I, and either magnesium or manganese ion. Approximately onefold synthesis on the template can be achieved and each of the indicated reagents is essential for extensive synthesis. The reaction is slower than the corresponding reaction of dTTP as a consequence of a lower V_{max} and a higher K_m for the amino analogue. That aminodeoxythymidine phosphate is incorporated into the synthetic polynucleotides was shown by a double-labeling experiment with [^{14}C]dATP and [^{32}P]-5′-amino-5′-deoxythymidine 5′-triphosphate and by the unusually high lability of the phosphoramidate polynucleotides toward acid. The phosphoramidate polynucleotides range in size from about 100 nucleotide units to well over a thousand nucleotide units, and the size is increased by addition of DNA ligase to the system. These experiments indicate that synthetic polynucleotides in which oligonucleotide blocks have been joined by means of phosphoramidate bonds should prove useful as primers for enzymatic syntheses with DNA polymerase I.

Polydeoxyribonucleotides can be joined by use of DNA ligase and a template polynucleotide which is complementary to and overlaps the terminal nucleotide sequences of the two fragments to be connected. This procedure has been used effectively to synthesize double-strand polynucleotides from short synthetic fragments (Khorana et al., 1972). For the synthesis of single-strand polynucleotides, however, the technique is hampered by the necessity to prepare a specific template for each juncture that is made. A chemical procedure that would enable one to couple synthetic oligonucleotides to unprotected synthetic or natural polynucleotides without the need for a template would greatly facilitate the synthesis of single-strand polynucleotides. Unfortunately the prospects for achieving such couplings via natural phosphodiester bonds seem poor since the reactivity of the terminal hydroxyl groups involved in the reaction is comparable to that of the amino groups in the nucleoside bases. As an alternative, a study of the feasibility of utilizing phosphoramidate links in polynucleotide synthesis was undertaken in this laboratory (Letsinger and Mungall, 1970) with the hope of exploiting the high nucleophilicity of aliphatic amines relative to alcohols, the amino groups of purine and pyrimidine bases, and phosphate anions. Several chemical aspects of this approach have been reported (Mungall et al., 1974, 1975; Greene and Letsinger, 1975). Especially encouraging was the observation that the internucleoside phosphoramidate link (ROP(O)NHR′) is sufficiently stable in neutral and alkaline aqueous buffer solutions to survive conventional conditions employed in the enzymatic synthesis and isolation of polynucleotides. The present paper addresses two questions of critical importance for this technique. (1) Will polynucleotides that possess a number of phosphoramidate links bind to complementary polynucleotide strands? (2) If so, will the polynucleotides with phosphoramidate links serve as primers for enzymatic synthesis? Our approach has been to ask yet another question. Will DNA polymerase I in the presence of a suitable template and other necessary reactants accept the amino analogue 5′-amino-5′-deoxythymidine 5′-triphosphate in place of thymidine 5′-triphosphate and yield high molecular weight polynucleotides that possess phosphoramidate links? A positive answer to this question would provide positive answers to the other two.

We describe in this paper the synthesis of 5′-amino-5′-deoxythymidine 5′-triphosphate and studies of the enzymatic reactions of this substrate with *Escherichia coli* DNA polymerase I in the presence of poly[d(A-T)] and ϕX174 DNA templates. A preliminary report of a portion of this material has been published (Letsinger et al., 1972).

Experimental Section

General Procedures. Paper chromatography was carried out on Whatman 3MM paper with solvent A (isopropyl alcohol–NH_4OH–H_2O, 7:1:12) and solvent F (*n*-propyl alcohol–NH_4OH–H_2O, 11:2:11). Electrophoretic separations were conducted for 1 h at 2000 V on Whatman 3MM paper at pH 7.2 (0.05 M sodium phosphate buffer). Linear sucrose or cesium chloride sedimentations were carried out with a Beckman Model L2-65 B or an International Equipment Corp. Model B-60 centrifuge by the general method of Burton and Sinsheimer (1965). Convex sucrose gradients for isokinetic sedimentations were prepared as described by Noll (1970). DEAE-Cellulose[1] (Bio-Rad Laboratories, Cellex D) columns

† Contribution from the Departments of Chemistry and of Biochemistry and Molecular Biology of Northwestern University, Evanston, Illinois 60201. *Received December 29, 1975.* This research was supported by a grant (GM 10265) from the Division of General Medical Sciences of the National Institutes of Health.

‡ This work is taken from a dissertation submitted by J.S.W. to Northwestern University in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Chemistry, University of Colorado at Denver, Colorado.

[1] Abbreviations used are: d(NH_2)T, 5′-amino-5′-deoxythymidine; (5′-NH)-dTTP, 5′-amino-5′-deoxythymidine 5′-triphosphate; DEAE-cellulose, diethylaminoethylcellulose; TEAB, triethylammonium bicarbonate.

TABLE I: Chromatographic and Electrophoretic Properties.

	dTTP	(5′-NH)-dTTP	d(NH_2)T
Chromatography[a]			
Silica gel TLC, solvent F	0.24	0.22	0.53
DEAE-Cellulose TLC, 0.05 M LiCl	0.30	0.30	0.65
Paper (3MM), solvent F	0.26	0.31	0.66
Paper (3MM), solvent A	0.01	0.01	0.46
Electrophoresis[b]			
Paper (3MM), pH 7.2	1.00	0.94	−0.67

[a] R_f values. [b] Mobility relative to dTTP.

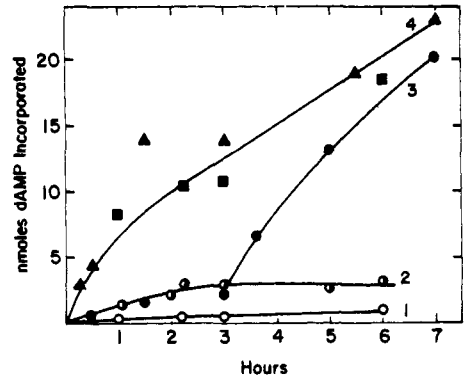


FIGURE 1: Synthesis of poly [d(A-T)] at 15 °C. In addition to DNA polymerase, $MgCl_2$, and buffer (see Experimental Section), each mixture (0.5 ml) contained [^{3}H]dATP (0.2 mM, 12.5 Ci/mol), poly [d(A-T)] (0.1 mM), and either dTTP (0.2 mM, curve 4; ▲ and ■ represent data from the two separate experiments), or (5′-NH)-dTTP (1 mM, curve 2, ◑), or no thymidine component (curve 1, ○). For experiment 3 (●), dTTP (0.2 mM) was added at 3 h to a reaction duplicating 2.

and phosphocellulose columns were prepared by the method of Tener (1967). All columns, lyophilization glassware, and reaction vessels used in the polymerization reactions were treated with Siliclad (Scientific Products Div. of American Hospital Supply Corp.) prior to use. Hydrolyses with snake venom phosphodiesterase (Worthington Biochemical Corp.) were carried out by the method of Ralph et al. (1963).

Materials. dATP and dCTP labeled with ^{3}H or ^{14}C were obtained from Schwarz/Mann. 5′-Amino-5′-deoxythymidine was prepared by the method of Horwitz et al. (1962) as modified by Letsinger and Mungall (1970). Sodium trimetaphosphate was synthesized from tri-*n*-butylammonium phosphate and dicyclohexylcarbodiimide by the method of Dr. T. Meyers (personal communication). It was homogeneous on TLC on DEAE-cellulose (developed with 0.1 M NaCl and visualized with reagent of Haynes and Isherwood, 1949). Preparation by the method of Ondik and Gryder (1960) also yielded material suitable for use in the synthesis of the aminodeoxythymidine triphosphate; however, in this case the sodium trimetaphosphate was accompanied by two minor components (probably ortho- and pyrophosphate). 5′-Methylamino-5′-deoxythymidine was synthesized by Geoffrey Greene and 5′-amino-2′,5′-dideoxyadenosine was provided by Steven Jacobs. *E. coli* DNA polymerase I (2930 units/mg) was isolated as described by Dumas et al. (1971) (one unit incorporates 1 nmol of dAMP into acid-insoluble polynucleotide on a ϕX174 DNA template in 0.5 h at 37 °C). ϕX174 amber 3 DNA was obtained by the method of Guthrie and Sinsheimer (1963). DNA ligase was purchased from Miles Labotatories, Inc. Oligonucleotides for use as primers were obtained by DNase digestion of calf thymus DNA as described by Dumas et al. (1971) and were stored frozen at −20 °C. Analytical fractionation on Sephadex G-100 showed a single peak centered in the nucleotide and small oligonucleotide range and no material in the high molecular weight range (>100 nucleotides).

5′-Amino-5′-deoxythymidine 5′-N-Triphosphate (5′-NH)-dTTP. 5′-Amino-5′-deoxythymidine (50 mg, 0.2 mmol) and sodium trimetaphosphate hexahydrate (44 mg, 0.2 mmol) were dissolved in water (0.2 ml) and allowed to stand for 24 h at room temperature. Analysis of a portion of the reaction mixture (high-pressure, anion-exchange chromatography) revealed that about half of the d(NH_2)T had been converted to (5′-NH)-dTTP.

The concentration of (5′-NH)-dTTP used in an enzymatic reaction was calculated from the absorbance units of (5′-NH)-dTTP present in a standard solution and the mixing volumes. For analysis, the aminotriphosphate was precipitated with methanol, and a sample (50 mg) was dissolved in 10 ml of 1 mM sodium tetraborate (pH 10) and applied to a Bio-Gel P-2 (2.5 × 40 cm) column. Elution with 1 mM sodium tetraborate solution (66 ml/h) afforded (5′-NH)-dTTP in fractions 13–21.

The ultraviolet spectrum of (5′-NH)-dTTP was indistinguishable from that for dTTP. The phosphorus/thymine ratio (determined on a sample purified on the Bio-Gel column) was 2.9 ± 0.1, in agreement with the expected value. For this analysis the molar concentration of thymine residues was determined from the absorbance at 267 nm (ϵ_{267} was taken to be 7800 at pH 12) and the concentration of phosphorus was determined by the method of Chen et al. (1965). Further support for the structural assignment was provided by the similarity of the chromatographic and electrophoretic properties of (5′-NH)-dTTP and dTTP (Table I). Finally, the fact that the phosphate group was linked to the aminodeoxythymidine through a P–N bond was indicated by quantitative conversion to d(NH_2)T on short exposure to 15% aqueous acetic acid.

[^{32}P](5′-NH)-dTTP. Sodium trimetaphosphate hexahydrate (5.7 mg) and d(NH_2)T (10 mg) were dissolved in 50 μl of a solution containing approximately 100 μCi of ^{32}P-labeled sodium trimetaphosphate (prepared by Gene R. Petersen). After 24 h the reaction mixture was lyophilized, redissolved in 50 μl of 0.05 M triethylammonium bicarbonate (pH 9), and precipitated with methanol. For determination of the radiochemical purity, a sample was dissolved in 0.1 ml of 0.05 M TEAB. Chromatography on a DEAE-cellulose plate with 0.02 M LiCl showed two spots, corresponding to d($NH)_2$T (R_f 0.85) and (5′-NH)-dTTP (R_f 0.64). An autoradiogram revealed four radioactive materials: (5′-NH)-dTTP at R_f 0.64, trimetaphosphate at R_f 0.40, and two unidentified inorganic phosphates at R_f 0.95 and 0.17. Assay of the radioactivity by liquid scintillation counting showed that 22% of the total radioactivity was in the (5′-NH)-dTTP, 33% in trimetaphosphate, and 45% in the two unidentified phosphates.

Enzymatic Polymerizations. A set of "standard condition" was adopted which served as the basic conditions for exploring the reaction variables. Unless otherwise noted, these conditions were used for the reactions described in Figures 1–7. Deviations are indicated explicitly in the legends for the figures. Standard conditions for the phosphoramidate synthesis are defined as 15 °C at pH 9.0 for aqueous solutions containing [^{3}H]dATP (0.2 mM, 1 Ci/mol), dCTP, and dGTP (0.2 mM each), ϕX174 DNA (50 μM in nucleotide units), calf thymus oligonucleo-

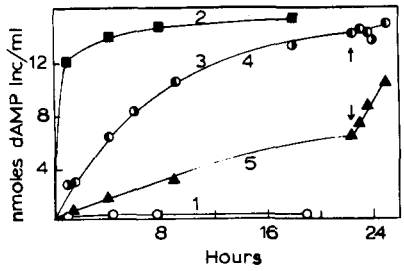


FIGURE 2: Synthesis of phosphoramidate polynucleotides on a ϕX174 DNA template at 15 °C. Standard conditions (Experimental Section) were altered for experiments 1, 2, and 3 by use of [^{14}C]dATP (0.2 mM, 1 Ci/mol) and changes in the thymidine component: experiment 1 (○) had no thymidine component, experiment 2 (■) had dTTP (0.2 mM), and experiment 3 (◑) had (5′-NH)-dTTP (2 mM); the pH was 9 and the volume was 1 ml in each case. Experiments 4 (◐) and 5 (▲) were carried out under "standard conditions" (use of (5′-NH)-dTTP with [^{3}H]dATP as marker) except that experiment 5 was buffered at pH 7.5 with potassium phosphate (60 mM) and experiment 4 with borate at pH 9. After 22 h, dTTP was added to experiments 4 and 5 (to 5 nmol/ml).

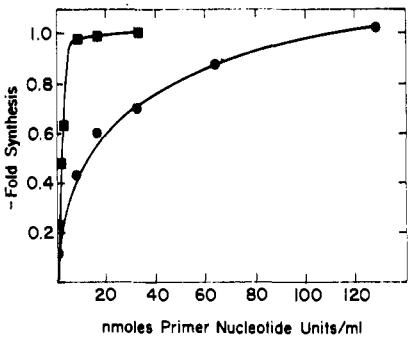


FIGURE 3: Dependence on primer concentration. "Standard conditions" were used for the synthesis of phosphoramidate polynucleotides (●), but the amount of primer oligonucleotides from calf thymus DNA was varied. The same conditions were used for the synthesis of polynucleotides with all natural links (■), except that dTTP (0.2 mM) was used in place of the amino analogue and the solutions were buffered at pH 7.5 with potassium phosphate (0.3 M). The fraction onefold synthesis at a given primer concentration was calculated from the amount of dAMP incorporated in 5.5 h for the reactions with dTTP (■) and 24 h for the system with (5′-NH)-dTTP (●).

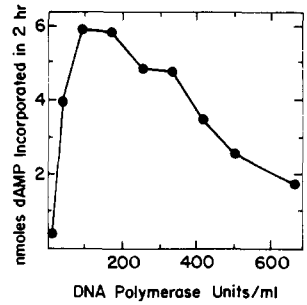


FIGURE 4: Dependence on enzyme concentration. For each point a reaction mixture (0.1 ml) was incubated for 2 h at 15 °C; then radioactivity in acid-precipitable polymer was determined. "Standard conditions" were employed, except that the primer concentration was 128 μM in nucleotide units and the enzyme concentration was varied as indicated.

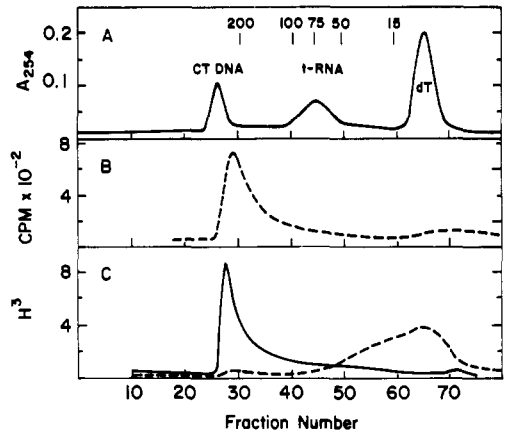


FIGURE 5: Acid hydrolysis of polynucleotides. (A) Calibration curve. (B) Polynucleotides with all natural phosphodiester links were prepared in the presence of $MnCl_2$ (5 mM). After removal of low molecular weight material on Sephadex G-100, a solution of the polynucleotides was made 15% in acetic acid, incubated 12 h, made alkaline (NaOH), and analyzed on a Sephadex G-100 column. (C) Phosphoramidate polynucleotides were obtained by the procedure used in B, with (5′-NH)-dTTP (2 mM) in place of dTTP and a 20-h reaction time; then they were treated with acid for 2 min (—) or for 12 h (- - -) and analyzed as in B.

tides (64 μM in nucleotide units), *E. coli* DNA polymerase I (84 units/ml), $MgCl_2$ (10 mM), sodium tetraborate (60 mM), 2-mercaptoethanol (1.2 mM), and (5′-NH)-dTTP (2.0 mM). In each case the last component to be added was the DNA polymerase; reactions were timed from that point. Acid insoluble products were analyzed by a modification of the method of Josse and Kornberg (1962). Samples (20 or 50 μl) were removed at appropriate intervals and added to 1.0 ml of cold 15% trichloroacetic acid in 0.02 M sodium pyrophosphate. Calf thymus DNA (0.20 ml of 1 mg/ml solution) was added as a carrier. The mixture was allowed to stand 10 min at 0 °C, filtered through a Whatman GF/A class fiber filter, and washed with cold 5% trichloroacetic acid and methanol.

Results

(5′-NH)-dTTP was prepared by the reaction of sodium trimetaphosphate with 5′-amino-5′-deoxythymidine in aqueous solution (Letsinger et al., 1972). This synthetic approach was based on the observation of Feldman and Thilo (1964) that simple aliphatic amines are converted to the amine triphosphates on reaction with the trimetaphosphate ion. The reaction has also been used to prepare the phosphoramidate analogue of ATP (Trowbridge et al., 1972; Wilkes et al., 1973).

Aqueous solutions of (5′-NH)-dTTP are unstable. Three types of reactions were observed. (a) In acidic media (5′-NH)-dTTP hydrolyzed irreversibly to d(NH_2)T. The half-life was less than a minute at pH 6 but was of the order of 12 h at pH 7. (b) In alkaline solutions reversible conversion of (5′-NH)-dTTP to d(NH_2)T and the trimetaphosphate ion occurred, analogous to the reaction of methylamine triphosphate reported by Feldman and Thilo (1964). Under our conditions ca. 40% of the nucleoside was present as the triphosphate at equilibrium. As a consequence, d(NH_2)T developed in fractions containing (5′-NH)-dTTP isolated from chromatography columns. This reaction occurred slowly even in frozen solutions. (c) In alkaline solution slow hydrolysis at phosphorus–oxygen bonds also occurred. Solutions at pH 9 at equilibrium were stable for days at room temperature; however, some breakdown of (5′-NH)-dTTP was observed over a period of several weeks. The facile hydrolysis of the product to d(NH_2)T in acidic solutions and the electrophoretic and chromatographic properties indicated that this substance was 5′-amino-5′-deoxythymidine 5′-diphosphate.

(5′-NH)-dTTP is hydrolyzed by snake venom phosphodiesterase. Under conditions for which 93% of a sample of dTTP was hydrolyzed to thymidine 5′-phosphate, 33% of a sample of (5′-NH)-dTTP was degraded to a new substance that resembled thymidine 5′-monophosphate in chromatographic and

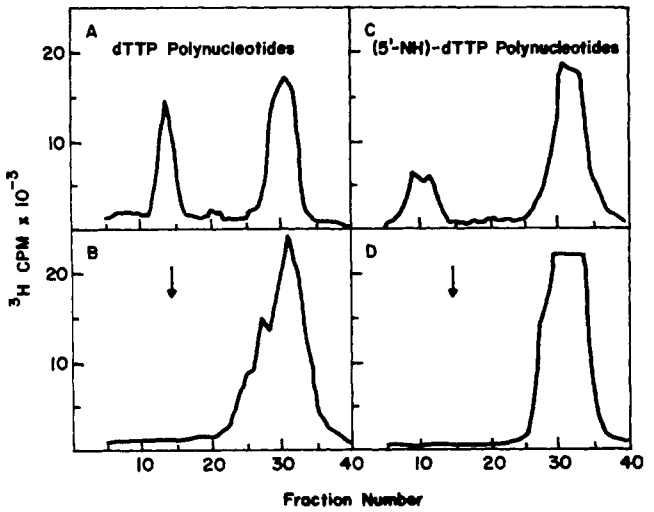


FIGURE 6: Association of products with template. Sedimentation in neutral and alkaline sucrose gradients, respectively, for polynucleotides prepared with dTTP (patterns A and B) and with (5′-NH)-dTTP (patterns C and D). In each case, the reaction used to prepare the polynucleotides was carried to onefold synthesis of the template. The arrow marks the position at which the ϕX174 DNA template sediments (18 S). The total radioactivity in each fraction was measured.

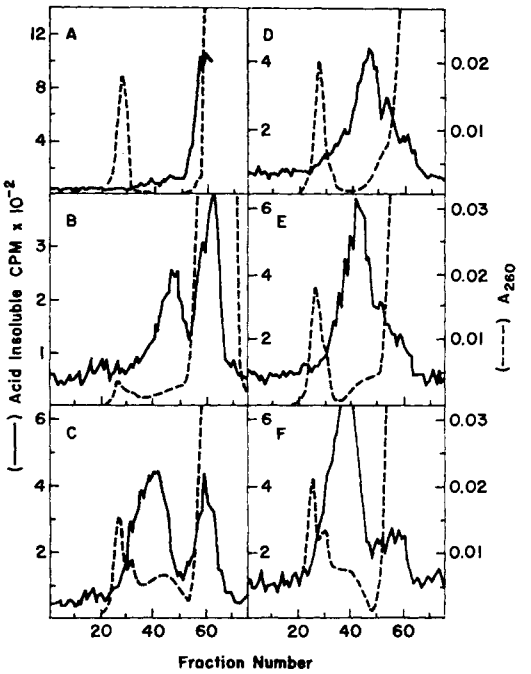
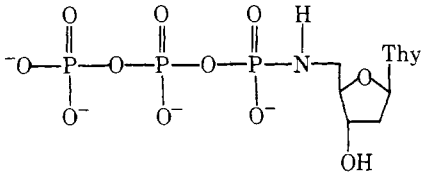


FIGURE 7: Joining of phosphoramidate polynucleotides by DNA ligase. Polynucleotides were synthesized under standard conditions but with [^{14}C]dATP (0.2 mM, 5 Ci/mol), (5′-NH)-dTTP (1 mM), and either 128 μM (high primer) or 16 μM (low primer) calf thymus oligonucleotides in 1-ml solutions. Three 0.2-ml aliquots were withdrawn from each reaction after incubating 20 h at 15 °C. To one portion from each (C and F) was added 5 nmol of dTTP; then all portions were incubated for 2 h at 15 °C. To B, C, E, and F were added 14 nmol of ATP and 1 unit of DNA ligase (from T_4-infected *E. coli*). The reactions were incubated for 0.5 h at 15 °C, terminated by addition of 4 μmol of EDTA, and analyzed by sedimentation on alkaline (0.1 M NaOH) 1.2 to 1.35 g/ml linear CsCl gradients. The tubes were eluted from the bottom. (The absorbance scale for B is 0.02, 0.04, and 0.06 absorbance units.) (A, B, and C) High primer concentration with no ligase, added ligase, and added ligase and dTTP, respectively. (D, E, and F) Low primer concentration with no ligase, added ligase, and added ligase and dTTP, respectively.

electrophoretic properties. Although this substance was not characterized in detail, this information in conjunction with the fact that the product readily yielded d(NH_2)T on treatment with acid indicates that the hydrolytic product was 5′-amino-5′-deoxythymidine 5′-monophosphate. This assignment is further supported by analogy to the reactions of the triphosphate derived from 5′-amino-5′-deoxyadenosine (Wilkes et al., 1973).



(5′-NH)-dTTP

Poly[d(A-T)] Template. The initial experiments on the utilization of (5′-NH)-dTTP by DNA polymerase I were carried out with a poly[d(A-T)] template. The conditions (Figure 1) are conventional except that a high pH (9) was employed to avoid loss of (5′-NH)-dTTP by acid-catalyzed hydrolysis and a high concentration of (5′-NH)-dTTP was used to favor incorporation of this nucleotide analogue. Control reactions with dATP and with a mixture of dATP and dTTP behaved normally; very little reaction occurred when only dATP was present (curve 1; incorporation corresponded to 2% of the template present), whereas extensive polymerization occurred when both dATP and dTTP were present (curve 4; 35% of onefold synthesis in 6 h). With a mixture of (5′-NH)-dTTP and dATP some polymer formation was observed (curve 2); however, the reaction leveled off after synthesis corresponding to ca. 6% of the template. Further reaction could not be induced by adding more (5′-NH)-dTTP. That d(NH_2)T, trimetaphosphate, and (5′-NH)-dTTP do not inhibit DNA polymerase I was demonstrated by two experiments. (a) When dTTP was added to the system containing (5′-NH)-dTTP after the reaction had leveled off (3 h), a rapid reaction set in (curve 3). (b) Parallel experiments in which dTTP and trimetaphosphate were added to a system containing dATP and dTTP also exhibited rates characteristic of the control. The observation that neither d(NH_2)T nor the trimetaphosphate inhibits DNA polymerase I is important since both substances are always present in reaction systems containing the aminonucleoside triphosphate.

ϕX174 DNA Template. For further experimentation, single-strand (+) circular ϕX174 DNA was selected as a template. This polynucleotide is a well-defined molecular species and extensive information on its role in in vitro polymerization systems is available (Kornberg, 1974). Furthermore, since it contains four nucleotides in approximately equal amounts, cumulative geometrical distortions induced by phosphoramidate links at any one nucleotide would be less than in poly[d(A-T)].

As shown by the data in Figure 2, ϕX174 DNA serves as a much better template than poly[d(A-T)] for syntheses involving (5′-NH)-dTTP. Reactions 1–3 were run in parallel at pH 9 with [^{14}C]dATP as a marker. Curve 2 represents a control with the full complement of natural nucleoside triphosphatase along with template DNA, oligonucleotide primers, and magnesium ion. Polymerization was rapid and terminated, as expected, after onefold synthesis on the ϕX template. Curve 1 represents a second control in which dTTP was omitted from the system; very little reaction occurred under these conditions. Finally, curve 3 presents data for a reaction in which (5′-NH)-dTTP replaced dTTP in the complete system. It is apparent that the aminonucleotide reagent stimulates extensive synthesis of radioactive, acid-precipitable polymer. The rate

was less than in the case of the four natural substrates; however, synthesis corresponding to about 90% of the template was achieved within 24 h.

Effect of pH. Experiments 4 (pH 9) and 5 (pH 7.5) reported in Figure 2 were carried out to examine the effect of pH on the reaction of the amino analogue. The radioactive marker for these reactions was [^{3}H]dATP. It may be noted that the synthesis utilizing (5′-NH)-dTTP was considerably faster at pH 9 than at pH 7.5. As a test of completeness of reaction in these cases, dTTP was added to each at the end of 22 h. The very limited synthesis thereby induced at pH 9.0 is consistent with the idea that most of the template had been transcribed in the prior reaction. The extensive synthesis induced at pH 7.5 demonstrates both that the template had been only partially transcribed during the 22-h period and that the enzyme was still functional after the extensive reaction period.

Primer Concentration. A marked difference was observed in the amount of oligonucleotide primers required to induce onefold synthesis in the system with natural nucleoside triphosphates and that containing (5′-NH)-dTTP. The data are presented in Figure 3 in terms of the fraction of onefold synthesis on the template within a given time period as a function of the concentration of nucleotides in the primer. The time period was 5.5 h for the system with all natural triphosphates and was 24 h for that with the phosphoramidate analogue. In the latter case the conversions in the reactions approached limiting values within the 24-h period even at the lower primer concentrations, indicating that onefold synthesis could not be achieved at low primer concentrations even for much longer reaction periods.

Enzyme Concentration. The dependence of the initial rate on the enzyme concentration is indicated in Figure 4. Synthesis increased with increasing enzyme concentration in the low concentration range, as expected; however, the amount of polymer remaining at the end of the 2-h periods decreased when high concentrations of enzyme were employed. The decrease was not investigated further, but it probably stems from nuclease activity that becomes prominent at the high enzyme levels.

Requirement for Nucleoside Triphosphates and Template. If (5′-NH)-dTTP serves as a replacement for dTTP in the enzymatic reaction, the polymerization should require the presence of the other three nucleoside triphosphates and a DNA template. To test this point a series of reactions was carried out in which either one of the nucleoside triphosphates or the ϕX174 DNA was omitted from the full reaction system. A control with (5′-NH)-dTTP, dCTP, dGTP, [^{3}H]dATP, template, and primer proceeded in the normal fashion and afforded radioactive polynucleotides (21 000 cpm). When dCTP, or dGTP, or the ϕX174 template was omitted from the system, no significant polymerization occurred (less than 1000 cpm in the precipitable polymer). Similarly, when [^{14}C]dCTP was used as a marker and dATP was omitted, the reaction did not go.[2] Accordingly, the nucleoside triphosphate and template requirements for the reaction utilizing (5′-NH)-dTTP are the same as those for the normal enzymatic reaction.

Concentration of (5′-NH)-dTTP. The dependence of the initial rate on the concentration of (5′-NH)-dTTP was established from a set of reactions in which the concentration of the amino analogue was varied from 4.8×10^{-5} to 4.8×10^{-3} M, while the concentration of the other three nucleoside triphosphates was maintained at 2×10^{-4} M. The initial rates were measured by the extent of incorporation of [^{14}C]dAMP in acid-precipitable polymer in a 2-h period (other conditions were "standard" except that the concentration of primer oligonucleotides was 128 μM). At very high concentrations, the amino analogue inhibited the polymerization; however, in the range of 6×10^{-4} to 4.8×10^{-5} M, the reciprocal of the initial velocity was linear with the reciprocal of the concentration of (5′-NH)-dTTP. The values of K_m and V_{max} derived from this graph are 1.5×10^{-4} M and 2.5 nmol/h, respectively.

For comparison, a corresponding reaction series was examined in which the concentration of dTTP was varied from 3.15×10^{-6} to 5.25×10^{-5} M, while the concentrations of dATP, dCTP, and dGTP were held at 2×10^{-4} M. The reaction time was 0.5 h. A plot of these data gave $K_m = 2.4 \times 10^{-6}$ M and $V_{max} = 14$ nmol/h. Therefore, the slowness of the reaction of (5′-NH)-dTTP relative to dTTP reflects both a relatively unfavorable K_m (by a factor of about 60) and a relatively unfavorable V_{max} (by a factor of about 6) for the analogue reaction.

Divalent Metal Ion. DNA polymerase I will function when magnesium ion is replaced by manganese ion in in vitro systems, although the specificity for the deoxyribonucleoside triphosphates is somewhat reduced (Van de Sande et al., 1972). Manganese ion can also fulfill the divalent metal ion requirement in systems involving (5′-NH)-dTTP. The rate of the reaction of the amino analogue in the presence of manganese ion (2.5 mM) was comparable to that for the reaction stimulated by magnesium ion. Additional experiments revealed, as in the reactions with magnesium ion, that each of the other three nucleoside triphosphates (dATP, dCTP, and dGTP) and template DNA are required for extensive synthesis in the manganese–amino analogue system.

Other Aminonucleosides. A few experiments were carried out with 5′-methylamino-5′-deoxythymidine and with 5′-amino-2′,5′-dideoxyadenosine to explore the generality of incorporation of aminonucleosides in DNA. In each case the aminonucleoside was incubated with trimetaphosphate in water, as in the preparation of (5′-NH)-dTTP. The electrophoretic patterns of the resulting solutions were similar to that for the reaction of d(NH_2)T, indicating that the aminonucleosides had been successfully converted to the corresponding triphosphates. Under the conditions used for enzymatic incorporation of aminodeoxythymidine phosphate, no reaction of the *N*-methylamino analogue was observed. On the other hand, the triphosphate derived from 5′-amino-2′,5′-dideoxyadenosine stimulated synthesis on a ϕX174 template to the same extent as (5′-NH)-dTTP, as judged by radioactivity incorporated in acid-precipitable polymer. In this experiment, [^{14}C]dCTP was employed as the radioactive substrate. Polymerization even proceeded when both dTTP and dATP were replaced by the corresponding 5′-amino analogues; however, the rate was only about half that observed for mixtures containing one of the amino analogues along with the three other natural deoxyribonucleoside triphosphates.

Reaction of [^{32}P]-(5′-NH)-dTTP. In order to measure directly the incorporation of the aminothymidine analogue into the phosphoramidate polynucleotides, labeled [^{32}P]-(5′-NH)dTTP was prepared from d(NH_2)T and [^{32}P]trimetaphosphate. Since the isotope effect for attack at the phosphorus atoms of the trimetaphosphate should be very small, it was assumed that the radioactive phosphorus was equally distributed among the α, β, and γ phosphorus atoms. Incorporation of one aminothymidine monophosphate unit therefore corresponds to incorporation of one-third of the radioactivity in the triphosphate.

[2] This experiment was performed by David Robinson and Gene R. Petersen.

A double-label experiment was performed in which enzymatic polynucleotide synthesis was monitored by incorporation of both ^{14}C from [^{14}C]dATP and ^{32}P from [^{32}P]-(5′-NH)-dTTP. As shown in Table II (experiment 3), both isotopes were indeed incorporated in the new polynucleotides. Moreover, the extent of polynucleotide synthesis calculated from the acid-insoluble radioactivity corresponding to each isotope agreed within the accuracy of the experiment (7.0 and 7.6 nmol/h), indicating satisfactory incorporation of the amino analogue. For this calculation the mole fractions of deoxyadenosine monophosphate and thymidine monophosphate in the (+) strand of ϕX174 DNA were taken to be 0.246 and 0.328, respectively (Sinsheimer, 1959). Experiment 1 is a control in which no synthesis occurred since the enzyme was omitted. It provides a measure of the background cpm in the filter paper assay. Experiment 2 is a control which indicates the extent of spillover of counts from the ^{14}C channel into the ^{32}P channel of the scintillation counter. When both dTTP and [^{32}P]-(5′-NH)-dTTP were present simultaneously (experiment 4), the reaction proceeded at about the rate for dTTP and no uptake of ^{32}P in the synthetic polymer could be observed. This competition experiment shows that incorporation of thymidine phosphate is strongly favored over incorporation of aminothymidine phosphate, in agreement with the conclusion drawn from the study of the dependence of rate on substrate concentration. It should be noted, however, that as a consequence of the considerable level of background counts in the ^{32}P channel, the incorporation of a few percent of the aminothymidine phosphate would not have been detected.

Acid Hydrolysis. Independent evidence that the polynucleotides formed in the enzymatic reactions utilizing (5′-NH)-dTTP contained phosphoramidate links was obtained by controlled acid hydrolysis of the polymers. This experiment rests on the high lability of internucleotide phosphoramidate links relative to phosphodiester bonds and other functional groups in the polynucleotides. The most sensitive links in natural polydeoxyribonucleotides are the purine glycoside bonds. Some breakdown of high molecular weight polynucleotides occurs in acidic solutions even under rather mild conditions; however, experiments with model compounds showed that the phosphoramidate links are sufficiently more sensitive to acid to provide a valid diagnostic test. Thus in 5% acetic acid in water at 20 °C the half-life for hydrolysis at the P–N bond of dTp(NH)T is 5.5 h, whereas the half-life for depurination of adenosine 5′-phosphate is 155 h (Mungall and Letsinger, unpublished research).

The pertinent experimental data are presented in Figure 5. Gel filtration through a Sephadex G-100 column was used to analyze the reaction mixtures (method of Hohn and Schaller, 1967, as modified by Hutchison and Edgell, 1971). As observed in B (Figure 5), treatment with 15% acetic acid (12 h) caused very little breakdown of the polynucleotides prepared from the four natural nucleoside triphosphates. In contrast, the acid treatment degraded the products from the amino analogue reaction to mononucleotides and oligonucleotides smaller than tRNA (dashed line, C in Figure 5). That the low molecular weight materials were indeed produced in the course of the acid treatment was demonstrated by analyzing the mixture just 5 min after acidification. In this case (solid line, C in Figure 5) the radioactive material eluted very largely in the high molecular weight fraction. The acid lability of the phosphoramidate polynucleotides was further confirmed by similar experiments with products from a reaction employing magnesium ion.

The Template–Product Complex. Association of the synthetic polynucleotides with template DNA was examined by means of sedimentation through neutral and alkaline sucrose density gradients. The method, basically that of Shildkraut et al. (1962), takes advantage of the faster sedimentation of synthetic polynucleotides when bound to ϕX174 DNA than when free. The synthetic (−) strand fragments sediment as a broad band at about 9–12 S in an alkaline gradient, in which they are separated from the template, but they sediment in a narrow band at a much greater rate (17 S) when annealed to the template (Dumas et al., 1971). The faster sedimenting peaks in experiments A and C in Figure 6 correspond to double-strand ϕX174 DNA. The radioactivity at the top of the sucrose gradients in these two experiments represents unincorporated substrate. It is clear from these data that the fragments containing phosphoramidate links, like the polynucleotides prepared with the four natural nucleoside triphosphates, are annealed to the template DNA in the neutral medium. Furthermore, as indicated in experiments B and D, the template–product complex is denatured in alkaline medium. The product DNA fragments are seen as leading shoulders on the substrate peaks near the top of these sucrose gradients.

TABLE II: Enzymatic Synthesis with [^{32}P]-(5′-NH)-dTTP.[a]

Expt	Nucleotides in Addition to [^{14}C]dATP, dCTP, dGTP	Cpm ^{14}C	Cpm ^{32}P	DNA Synthesized (nmol) per h Calcd from: ^{14}C	DNA Synthesized (nmol) per h Calcd from: ^{32}P
1.	[^{32}P]-(5′-NH)-dTTP (no enzyme)	58	530	0	0
2.	dTTP	8662	634	18.5	0
3.	[^{32}P]-(5′-NH)-dTTP	3326	4131	7.0	7.6
4.	[^{32}P]-(5′-NH)-dTTP + dTTP	8036	948	17.2	~0

[a] Pertinent concentrations are: dTTP, dCTP, dGTP, and [^{14}C]-dATP (0.05 Ci/mol), 0.033 mM each; [^{32}P]-(5′-NH)-dTTP (12 Ci/mol), 0.04 mM; $MnCl_2$, 2.5 mM. The mixtures (0.2 ml) were incubated 1 h at 37 °C and the acid-insoluble radioactivity was determined as described in the Experimental Section.

Effect of Ligase on Phosphoramidate Polynucleotides. To test the effect of ligase on the phosphoramidate polynucleotides, polymerization reactions were carried out with low and high (eightfold greater) concentrations of oligonucleotide primers. The extent of synthesis after 20 h corresponded to about 90% of the template ϕX174 DNA for the high primer reaction and 60% of the template for the low primer reaction. The products were separated by zone sedimentation in alkaline CsCl gradients and were analyzed both by absorbance at 260 nm and by radioactivity in acid-precipitable products (Figure 7). The peak for sedimentation of the radioactive fragments from the low primer reaction (D, fraction 45) corresponds to 9 S, and the leading edge of the band for products from the high primer reaction (A, fraction 56) falls at about 4 S. Circular ϕX174 DNA (18.4 S, Studier, 1965), which was used as a template, appears as a peak in the ultraviolet light assay at fraction 27 (A–F), and the full length linear molecules (16.1 S, Studier, 1965) appear as a shoulder at fraction 31 (C, F). The molecular weights of the 4S and 9S fragments are estimated to be 5×10^4 and 4×10^5, respectively, by the use of the equation of Studier (1965).

Incubation of the reaction mixtures for 30 min with DNA ligase after the synthesis with DNA polymerase I led to con-

siderable increase in average size of the polynucleotides (see B and E, Figure 7), the effect being most pronounced for the high primer reaction (9 S to 12 S for the products of the lower primer reaction and 4 S to 9 S for the products of the high primer reaction). An even greater increase in size was observed when dTTP was added 2 h prior to addition of DNA ligase, in expectation of completing sequences that may not have been transcribed by the amino analogue (C and F).

Discussion

The enzymatic reaction utilizing (5′-NH)-dTTP and a ϕX174 DNA template resembles in vitro reactions of natural nucleoside triphosphates in the requirement for metal ion (magnesium or manganese), primer oligonucleotides, and a full complement of nucleoside triphosphates. This fact suggests that the analogue reaction proceeds normally with respect to gross mechanistic features. Two differences in the course of these enzymatic reactions were noted, however. When dTTP was replaced by (5′-NH)-dTTP, the reaction rate was less and a higher concentration of oligonucleotide primers was needed to achieve onefold synthesis on the template DNA. The kinetics are consistent with the view that the enzymatic reaction proceeds in bursts in which dAMP, dCTP, and dGTP are added in fast steps and the aminothymidine phosphate is added in a slow, rate-determining step. This feature should prove advantageous in the utilization of phosphoramidate polynucleotides prepared enzymatically for DNA sequence studies. Evidence that aminodeoxythymidine phosphate is incorporated into the synthetic polynucleotides was obtained by two independent experiments. (1) A double-labeling experiment with [^{14}C]dATP and [^{32}P]-(5′-NH)-dTTP showed that both ^{14}C and ^{32}P are incorporated into acid-precipitable polynucleotides in amounts expected for synthesis of (−) ϕX174 DNA. (2) The polymers prepared by use of (5′-NH)-dTTP were found to be much more labile to acid than polymers prepared by use of dTTP.

Zone sedimentation in an alkaline CsCl gradient showed that the molecular weight of average size fragments of phosphoramidate polynucleotides prepared in a representative reaction was about 4×10^5 (~1300 nucleotide units). Accordingly, there must be on the order of 300 phosphoramidate bonds in typical fragments synthesized on the template. Gel chromatography of polynucleotides prepared in another reaction and denatured with sodium hydroxide (pH 13.5) revealed a small amount of material (~15%) in a range corresponding to 100–200 nucleotide units; the remainder eluted in fractions characteristic of larger fragments. Even the small fragments, therefore, must have had 20 or more phosphoramidate bonds. These results therefore provide an unequivocal answer to the question of the capability of phosphoramidate polynucleotides to serve as primers for DNA polymerase I. Since the chains are extended enzymatically to include many phosphoramidate links, these polynucleotides are serving as effective primers on a template that must have great diversity in the nucleotide sequences.

Direct evidence for existence of a complex between the synthetic phosphoramidate polynucleotides and the ϕX174 DNA template was obtained by zone sedimentation of the reaction products in neutral and alkaline sucrose gradients. In addition, the observations that DNA polymerase I extends the polynucleotide chains, that the reactions level off at onefold synthesis at 15 °C, and that DNA ligase joins the ends of the synthetic polynucleotides to yield higher molecular weight products support the conclusion that the phosphoramidate polynucleotides form a complex with the template.

References

Burton, A., and Sinsheimer, R. L. (1965), *J. Mol. Biol. 14,* 327.

Chen, P., Toribara, T., and Warner, H. (1965), *Anal. Chem. 28,* 1756.

Dumas, L. B., Darby, G., and Sinsheimer, R. L. (1971), *Biochim. Biophys. Acta 228,* 407.

Feldman, V., and Thilo, E. (1964), *Z. Anorg. Allg. Chem. 327,* 159.

Greene, G. L., and Letsinger, R. L. (1975), *Nucleic Acids Res. 2,* 1123.

Guthrie, G. D., and Sinsheimer, R. L. (1963), *Biochim. Biophys. Acta 72,* 290.

Haynes, C., and Isherwood, F. (1949), *Nature (London) 164,* 1107.

Hohn, T., and Shaller, H. (1967), *Biochim. Biophys. Acta 138,* 466.

Horwitz, J., Thompson, A., Urbanski, J., and Chua, J. (1962), *J. Org. Chem. 27,* 3045.

Hutchison, C., and Edgell, M. (1971), *J. Virol. 8,* 181.

Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237,* 1968.

Khorana, H. G., Agarwal, K., Buchi, H., Caruthers, M., Gupta, N., Kleppe, K., Kumar, A., Ohtsuka, E., Rajbhandary, U., Van de Sande, J., Sgaramella, V., Terao, T., Weber, H., and Yamada, T. (1972), *J. Mol. Biol. 72,* 209.

Kornberg, A. (1974), DNA Synthesis, San Francisco, Calif, W. H. Freeman.

Letsinger, R. L., and Mungall, W. S. (1970), *J. Org. Chem. 35,* 3800.

Letsinger, R. L., Wilkes, J. S., and Dumas, L. B. (1972), *J. Am. Chem. Soc. 94,* 292.

Mungall, W. S., Greene, G. L., Heavner, G. A., and Letsinger (1975), *J. Org. Chem. 40,* 1659.

Mungall, W. S., Greene, G. L., and Letsinger, R. L. (1974), *Nucleic Acids Res. 1,* 615.

Noll, H. (1970), Techniques of Protein Biosynthesis, II, Campbell, P. N., Ed., New York, N.Y., Academic Press, p 101.

Ondik, H., and Gryder, J. (1960), *J. Inorg. Nucl. Chem. 14,* 240.

Ralph, R. K., Connors, W. J., Schaller, H., and Khorana, H. G. (1963), *J. Am. Chem. Soc. 85,* 1983.

Shildkraut, C., Marmur, J., Fresco, J., and Doty, P. (1962), *J. Biol. Chem. 236,* PC2.

Sinsheimer, R. L. (1959), *J. Mol. Biol. 1,* 37.

Studier, F. (1965), *J. Mol. Biol. 11,* 373.

Tener, G. (1967), *Methods Enzymol. 12A,* 398.

Trowbridge, D., Yamamoto, D., and Kenyon, G. (1972), *J. Am. Chem. Soc. 94,* 3816.

Van de Sande, J., Loewen, P. C., and Khorana, H. G. (1972), *J. Biol. Chem. 247,* 6140.

Wilkes, J. S., Hapke, B., and Letsinger, R. L. (1973), *Biochem. Biophys. Res. Commun. 53,* 917.