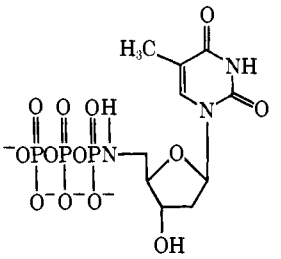
Enzymatic Synthesis of Polydeoxyribonucleotides Possessing Internucleotide Phosphoramidate Bonds[1]

Sir:

With the demonstration that small, chemically synthesized oligonucleotides possessing phosphoramidate links ($ROP(=O)(O^-)NHCH_2R'$) are stable in neutral and in alkaline solutions in the presence of nucleoside triphosphates,[1] an investigation of the feasibility of enzymatically synthesizing high molecular weight phosphoramidate analogs of nucleic acids appeared attractive. Aside from the intrinsic interest in the chemical and biological properties of such analogs, these substances could prove useful in studying base sequences in DNA.[2] We report in this communication that DNA polymerase I of *Escherichia coli*[3] in the presence of ϕX174 DNA as a template will indeed utilize 5′-amino-5′-deoxythymidine 5′-*N*-triphosphate (d_NTTP), along with deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP), to give high molecular weight polynucleotides possessing phosphoramidate links.

5′-Aminonucleoside triphosphates have not previously been described. A convenient route to these compounds was provided by the observation of Feldman and Thilo that simple aliphatic amines react readily with sodium trimetaphosphate in aqueous solution to give the amine triphosphate.[4] Accordingly, we found that 5′-amino-5′-deoxythymidine[5] (d_NT, 0.1 mmol) and sodium trimetaphosphate hexahydrate[6] (0.1 mmol) in 1 ml of water at pH 9.5 afford a single product detectable by paper chromatography and electrophoresis (80% conversion within 6 hr at room temperature). In confirmation of the structure as 5′-amino-5′-deoxythymidine 5′-*N*-triphosphate, expected from the method of synthesis, the substance yielded 5′-amino-5′-deoxythymidine quantitatively on mild acid hydrolysis and moved like dTTP on electrophoresis at pH 10.5 and on chromatography in several different solvent systems.[7] Furthermore, the ultraviolet spectrum (λ_{max} 267 mm) for a purified sample was the same as that of dTTP and the phosphorus–thymine ratio was 2.9 ± 0.1.[8] In alkaline solution (pH 8 and higher), d_NTTP is stable for several days; however, it hydrolyzes to d_NT within less than 1 min at pH 6.



d_NTTP

The enzymatic reactions were carried out at 15° in 0.5 ml of aqueous solution (pH 9.0) containing 100 nmol each of ^{3}H-dATP (1.25 mCi/mmol), dCTP, and dGTP, 25 nmol of ϕX174 DNA as template,[9] 24 nmol of degraded calf thymus DNA as primer, 20 units of DNA polymerase I (6200 units/mg), 4 μmol of $MgCl_2$ (or 2.5 μmol of $MnCl_2$), 0.6 μmol of β-mercaptoethanol, 35 μmol of sodium tetraborate as buffer, and the indicated thymidine derivative.[10] The reactions were followed by removing 50-μl aliquots at intervals, precipitating the polynucleotides with 15% aqueous trichloroacetic acid,[11] collecting the precipitate by filtration, and measuring the radioactivity in the collected precipitate with a liquid scintillation counter.

Data for reactions carried out in the presence of $MgCl_2$ are presented in Figure 1. Polymerizations for the complete system (+dTTP) and the incomplete system (−dTTP) are indicated by curves 1 and 2, respectively. It was found that neither d_NT nor sodium trimetaphosphate inhibit polymerization in the complete system. The key experiment (curve 3) is that in which d_NTTP, prepared by incubating a solution of d_NT and sodium trimetaphosphate, was added in place of dTTP. Net synthesis occurred, though at a reduced rate relative to synthesis in the system containing dTTP.

As shown in Figure 2, $MnCl_2$ was somewhat more effective than $MgCl_2$ in promoting polymerization in the system containing d_NTTP, and was less effective than $MgCl_2$ for the reaction utilizing dTTP. No polymerization was observed in the absence of a divalent metal ion. In other control studies it was found that the template DNA, the primer, and each of the other nucleoside triphosphates (dCTP, dGTP, and dATP)[12] are essential for net synthesis when d_NTTP is employed. It therefore seems likely that here, as in syntheses with the natural triphosphates, strands complementary to the template are produced. Zone sedimentation through alkaline CsCl gradients revealed that the products from reaction of d_NTTP are comparable in molecular weight to the polynucleotide produced in the complete system.

(1) Part XVII in a series on Nucleotide Chemistry; for XVI, see R. L. Letsinger and W. S. Mungall, *J. Org. Chem.*, **35**, 3800 (1970). This research was supported by a grant (GM10265) from the Division of General Medical Sciences of the National Institutes of Health.

(2) The P–N bonds could serve as sites for selective cleavage of polynucleotides prepared from DNA templates.

(3) This enzyme catalyzes the synthesis of polydeoxyribonucleotides from dTTP, dATP, dCTP, and dGTP in the presence of a template, primer, and Mg^{2+} or Mn^{2+}; A. Kornberg, *Science*, **163**, 1410 (1969).

(4) V. Feldman and E. Thilo, *Z. Anorg. Chem.*, **327**, 159 (1964). We are grateful to Dr. Leslie Orgel for calling this reference to our attention.

(5) J. Horwitz, A. Thomson, J. Urbanski, and J. Chua, *J. Org. Chem.*, 27, 3045 (1962). For an improved procedure see the Ph.D. Dissertation of W. S. Mungall, Northwestern University, 1970.

(6) H. Ondik and J. Gryder, *J. Inorg. Nucl. Chem.*, **14**, 240 (1960); R. N. Bell, *Inorg. Syn.*, **3**, 103 (1950).

(7) On Whatman 3 MM paper in solvent F (*n*-PrOH–NH_4OH–H_2O, 55:10:35), or cellulose thin-layer plates in solvent F; on DEAE-cellulose paper in 0.3 *M* ammonium bicarbonate at pH 8.5; and on a pellicular anion exchange resin eluted with 0.75 *M* potassium phosphate (pH 8.0).

(8) For this preparation a onefold excess of d_NT was used to convert a high fraction of the trimetaphosphate to product, and d_NTTP was separated from d_NT and trimetaphosphate by elution from Bio-Gel P2 with 10^{-3} *M* borate buffer. The thymine content was measured by the absorbance at 267 mm and phosphorus was determined by the method of P. S. Chen, T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

(9) The concentration of DNA is expressed as a concentration of nucleotide in DNA.

(10) The methods for the enzymatic reactions are the same as described by L. B. Dumas, G. Darby, and R. L. Sinsheimer, *Biochem. Biophys. Acta*, **228**, 407 (1971).

(11) Similar results were obtained when the polynucleotides were collected by filtration through DEAE-cellulose. This fact in conjunction with model experiments leads us to believe that no significant degradation occurs during the brief exposure to trichloroacetic acid employed for precipitation of the polynucleotides.

(12) The requirement for dATP was tested with a system containing ^{3}H-dCTP.

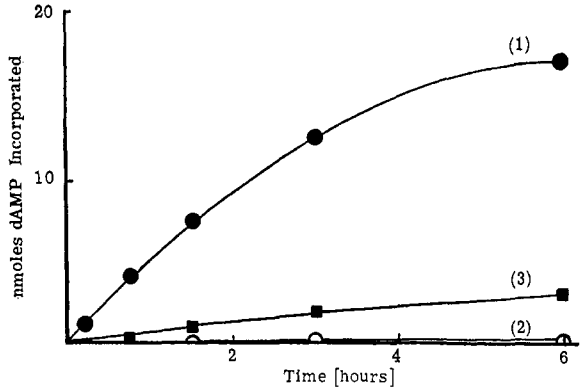


Figure 1. Synthesis of polynucleotides on ϕX174 DNA template in the presence of $MgCl_2$: curve 1 (●), complete system (+ 100 nmol of dTTP); curve 2 (○), incomplete system (−dTTP); curve 3 (■), test system (−dTTP, +d_NTTP from 1 μmol each of d_NT and sodium trimetaphosphate). Onefold synthesis corresponds to incorporation of 7.9 nmol of ^{3}H-dAMP fragments.

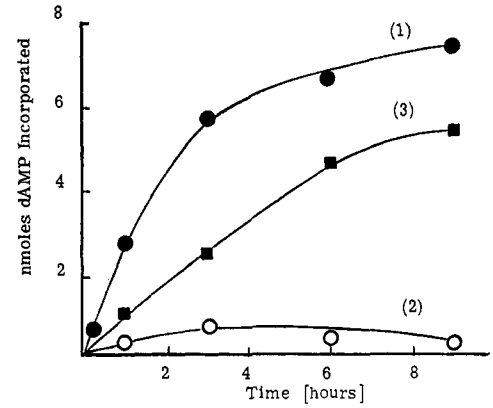


Figure 2. Synthesis of polynulceotides on ϕX DNA template in the presence of $MnCl_2$. The conditions were the same as for experiments in Figure 1, except $MnCl_2$ was used in place of $MgCl_2$.

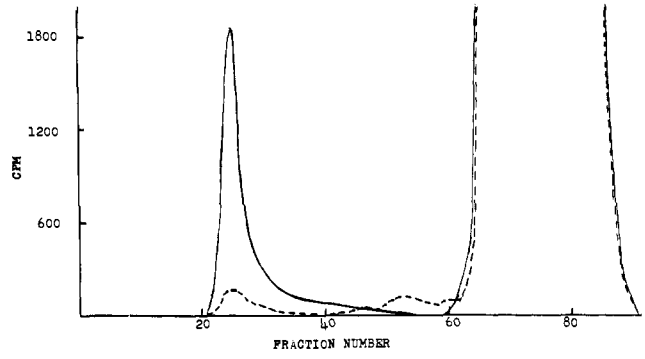


Figure 3. Separation on Sephadex from system containing d_NTTP, after treatment with 15% acetic acid for 5 min (——) and for 11 hr (- - - - -). The high CPM in fractions >62 stem primarily from residual, unreacted ^{3}H-dATP.

Studies with model compounds have shown that the cleavage of internucleotide phosphoramidate links in dilute acid solution is rapid relative to depurination of purine nucleotides. One would therefore expect polynucleotides from the enzymatic reactions to be unusually sensitive to acid if they contained phosphoramidate groups. As a test of the incorporation of the aminodeoxythymidine phosphate into the polynucleotides, samples of polymerization mixtures (from systems with nucleotide template to primer ratio 23/1 and with $MnCl_2$) were acidified with acetic acid (to 5% in acetic acid), allowed to stand 16 hr at room temperature, and precipitated with trichloroacetic acid as before. The insoluble material from the complete system (+dTTP) exhibited only slightly fewer counts per minute (5–10% fewer) than the polymer that had not been subjected to the long acid treatment. In contrast, the product from the reaction with d_NTTP showed a reduction of 40–50% in counts per minute, indicative of appreciable breakdown to low molecular weight oligonucleotides during the acid treatment. Additional evidence for lability of the phosphoramidate polymer was obtained by gel filtration on Sephadex G-100 with 1.0 *M* aqueous sodium chloride, which separates polynucleotides according to size.[13] Controls with calf thymus DNA, *E. coli* tRNA, and thymidine showed these three substances to be separated in well-resolved bands with maxima at fractions 23 (void volume), 45, and 70, respectively (1.1-ml fractions were collected). The mixtures from the enzymatic polymerizations were brought to 15% acetic acid by addition of acetic acid and incubated for 5 min and for 11 hr; then they were neutralized with sodium hydroxide and separated on the Sephadex column and the counts per minute in the fractions were determined. For the system containing dTTP, ^{3}H-labeled polynucleotide material came out in a band near the void volume in both cases (5-min and 11-hr reactions). A similar pattern was found for the products from the reaction of d_NTTP after the 5-min acid treatment; however, the pattern for the products from the d_NTTP reaction which had been treated with acid for 11 hr was quite different (see Figure 3). The amount of high molecular weight material was greatly reduced and appreciable material was found in the region between the band for tRNA and the band containing short oligonucleotides and unreacted nucleoside triphosphates. These experiments demonstrate that the polymers obtained from the systems containing d_NTTP contain acid-labile bonds (*i.e.*, phosphoramidate bonds) not found in the polynucleotides derived from the four natural nucleoside triphosphates.

(13) Th. Hohn and H. Schaller, *Biochem. Biophys. Acta*, 138, 466 (1967); C. A. Hutchison and M. H. Edgall, *J. Virol.*, 8, 181 (1971).

Robert L. Letsinger,* John S. Wilkes, Lawrence B. Dumas
Departments of Chemistry and Biological Sciences
Northwestern University
Evanston, Illinois 60201
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Mechanism of the α-Chymotrypsin-Catalyzed Hydrolysis of Specific Amide Substrates

Sir:

I wish to report further[1] evidence in support of an intermediate between the noncovalently bound enzyme–substrate complex and the acyl-enzyme in the chymotrypsin-catalyzed hydrolysis of specific amide substrates. This intermediate does not accumulate but exists in a low steady-state concentration. A change in rate-determining step from its formation to its breakdown with changing pH causes a kinetic pK_a in the pH–k_{cat} profile; k_{cat} varies with pH according to an ionization

(1) A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, 93, 7079 (1971).