Rate Law. All the alkylation reactions studied in this paper followed the simple second-order rate law $-d(Co^I)/dt = k^{2nd}(RX)(Co^I)$. In every case the first-order dependence on alkyl halide was checked by varying the concentration of RX by at least a factor of 4, and in many cases by a factor of 20. In one instance, the reaction of *n*-propyl chloride with a 3×10^{-4} *F* solution of cobaloximes, varying the concentration of RX from 0.02 to 0.92 *F* had no effect on the second-order rate constant calculated from the above expression (this experiment also provides additional evidence for the insensitivity of these reactions to medium effects, since the most concentrated solution was about 10% halide in methanol). The first-order dependence on $(Co^I)^-$ is shown by the linearity of the rate plots, which are straight over at least a factor of 4 in the concentration of Co(I) and are often linear for four half-lives.

Product Analysis

The reactions studied in most cases yield isolable organocobalamins or -cobaloximes. An identification of the products formed was necessary in certain doubtful cases, *i.e.*, the reaction of vitamin B_{12s} with secondary alkyl halides or those of cobaloximes with tertiary alkyl halides.

a. Reaction of Isopropyl Iodide with Vitamin B_{12s}. The *sec*-alkyl halide was very carefully purified to eliminate any possible contamination by primary halides.

Its reaction with vitamin B_{12s} under various conditions is slow, yielding vitamin B_{12r} and yellow corrin reduction products which are also obtained on prolonged reduction of vitamin B_{12a} with sodium borohydride. Organocobalamins could not be detected among the reaction products.

b. Reaction of *t*-Butyl Chloride with Cobaloxime(I). Solutions of cobaloxime(I) were prepared by reducing chloro(pyridine)cobaloxime with $NaBH_4$ in methanol. After destroying the excess of borohydride with acetone, carefully distilled *t*-butyl chloride was added under argon. After 1 hr the blue-green solution of the cobaloxime(I) was brown, and crystals of pyridine–cobaloxime(II) (recognized by its color and oxygen sensitivity in the methanolic suspension) had separated. Glpc analysis of the gas phase revealed the presence of isobutylene. No organocobaloxime could be detected in the reaction solution. In the cobalamin series optical absorption spectra were run to identify the reaction products. The initial reaction product with *t*-butyl chloride resembles vitamin B_{12r}. On prolonged standing under reducing conditions yellow, air-stable, reduced corrins are formed.

Acknowledgment. We thank Dr. R. J. Windgassen and J. W. Sibert for performing some of the kinetic measurements and other assistance.

Synthesis of Oligothymidylates *via* Phosphotriester Intermediates[1]

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Abstract: A procedure is described for the large-scale preparation of β-cyanoethyl ester derivatives of 5′-O-monomethoxytrityl TpT, TpTpT, and TpTpTpT. The essential feature is a double phosphorylation, the first step of which involves reaction of a terminal 3′-OH of a nucleoside with β-cyanoethyl phosphate and mesitylenesulfonyl chloride and the second step, condensation of the resulting phosphodiester with thymidine in the presence of triisopropylbenzenesulfonyl chloride. The products are separated by chromatography on silica gel with ethyl acetate and tetrahydrofuran. They may be converted in high yield to the corresponding demethoxytritylated derivatives and thence to TpT, TpTpT, and TpTpTpT, respectively, by successive treatment with aqueous acetic acid and ammonium hydroxide.

With the synthesis of 5′-O-trityldeoxycytidylyl-(3′–5′)-thymidine and related compounds two features were introduced in the methodology of oligonucleotide syntheses: (1) the nucleotide chains were constructed on an insoluble polymer support, which facilitated the separation of intermediates from solvents and soluble by-products; and (2) the nucleosides were joined by reactions designed to yield β-cyanoethyl phosphotriester links ($ROP(O)(OR')OCH_2CH_2CN$), which in a final alkaline treatment could be converted to phosphodiester salts.[2] The present set of papers reports the further elaboration of these techniques. In the first paper are described experiments adapting the β-cyanoethyl phosphotriester method to the synthesis of oligothymidylate derivatives in solutions in the absence of a polymer support. These experiments were stimulated both by the wish to elucidate the chemistry of the triester intermediates and by the hope that the homogeneous solution approach could be developed into a useful method in its own right. The second paper reports research on new blocking groups designed to meet the specific demands imposed by the phosphotriester approach, and the third paper describes procedures for synthesizing β-cyanoethyl phosphotriester derivatives of all four common nucleosides, making use of the new blocking groups.

Phosphotriester Method. The procedure for synthesizing oligonucleotides directly by condensation of 3′-O-protected nucleoside 5′-phosphates, or oligonucleotides bearing 5′-phosphomonoester groups, with nucleotides or oligonucleotides possessing a free ter-

(1) Part XIII in a series of nucleotide chemistry. A preliminary account has been published: R. L. Letsinger and K. K. Ogilvie, *J. Am. Chem. Soc.*, **89**, 4801 (1967). For part XII see R. L. Letsinger, P. S. Miller, and G. W. Grams, *Tetrahedron Letters*, 2621 (1968).

This research was supported by the Division of General Medical Sciences, National Institutes of Health (GM 10265).

(2) R. L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.*, **87**, 3526 (1965); *ibid.*, **88**, 5319 (1966).

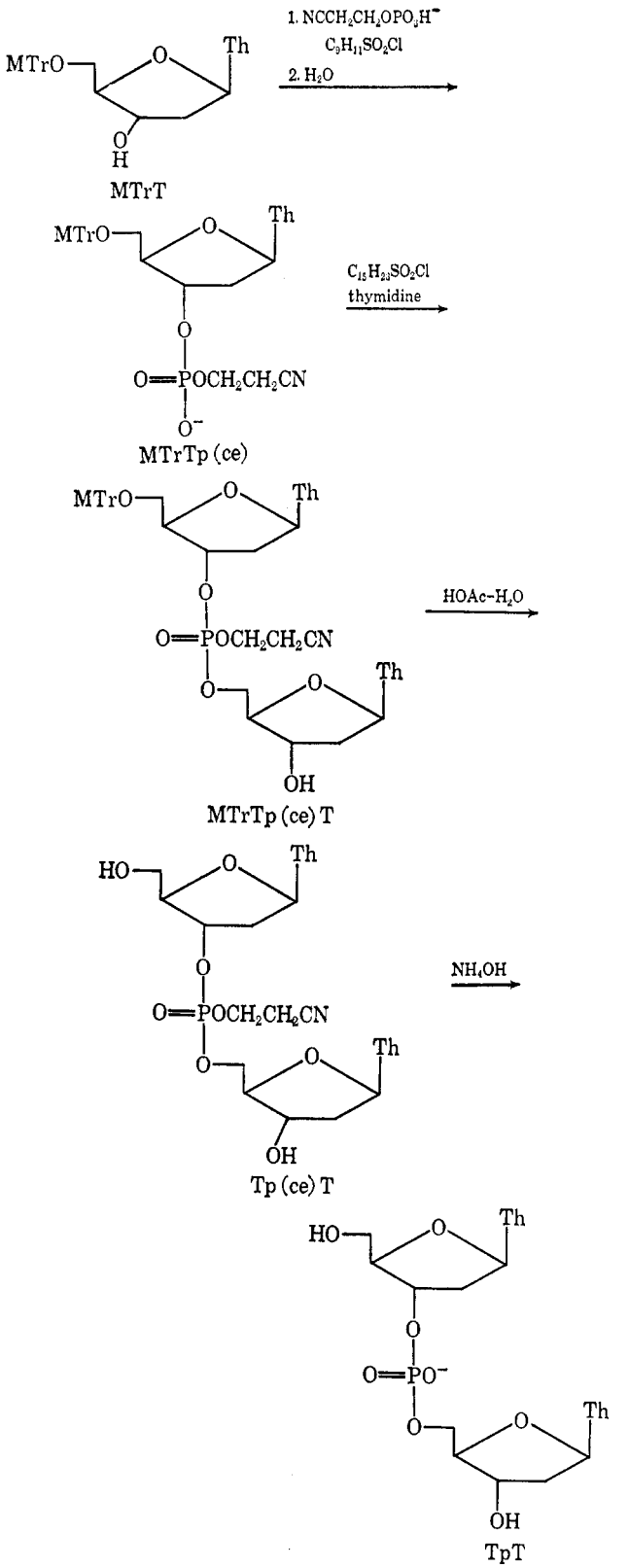
minal 3′-hydroxyl group has proved highly successful for preparation of small quantities of oligonucleotides of defined sequence for use in biochemical studies.[3] This approach is not readily adapted, however, to the synthesis of large quantities of oligonucleotides and modified derivatives desirable for many chemical investigations. At each stage in the synthesis the products are separated as salts by chromatography on DEAE-cellulose with aqueous buffer solutions, an efficient but a very time-consuming operation. To maintain high yields in the condensation step increasingly large excesses of the 5′-O-phosphomonoester component must be employed as the length of the oligonucleotide chain is increased.[4] This requirement severely limits the size of the oligonucleotide phosphomonoester component which can be used in a practical way in a chemical coupling reaction. Finally, condensation reactions involving the phosphodiester anions of the oligonucleotide chain may yield products which undergo chain fission, yielding shorter chain oligonucleotides possessing unnatural 3′–3′ phosphodiester linkages.[5]

In an attempt to obviate these difficulties, which stem from the presence of anionic sites in the oligonucleotide backbone, we have investigated a synthetic approach in which these sites are masked by β-cyanoethyl groups throughout the course of the building sequence. As uncharged molecules, the phosphotriesters produced as intermediates would be expected to be soluble in organic solvents and amenable to conventional techniques for separating and characterizing organic molecules. In addition, masking the *endo*-P–O⁻ groups would prevent formation of pyrophosphate bonds responsible for chain fission and binding of the phosphomonoester component.

β-Cyanoethyl was selected as a blocking group for P–O⁻ since the requisite reagents were readily available[6,7] and the final unblocking step could be effected under very mild alkaline conditions.[8] Initial experiments involving reactions conducted on a polymer support indeed indicated that the condensation of a β-cyanoethyl phosphodiester derivative of a nucleoside with another nucleoside would proceed satisfactorily;[2] however, the presence of the polymer support precluded the isolation and characterization of the phosphotriesters.[9]

The general synthetic scheme developed for derivatives of thymidine is outlined in Chart I. This involves phosphorylation of the 3′-OH of a nucleoside or oligonucleotide derivative with pyridinium mono(β-cyanoethyl) phosphate[10] and mesitylenesulfonyl chloride followed by condensation of the phosphodiester with a nucleoside in the presence of an arenesulfonyl chloride.

Chart I



Th = $C_5H_5N_2O_2$; MTr = p-$CH_3OC_6H_4C(C_6H_5)_2$–

(3) See H. Kossel, M. W. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2148 (1967); H. G. Khorana, H. Buchi, T. M. Jacob, H. Kossel, S. A. Narang, and E. Ohtsuka, *ibid.*, **89**, 2154 (1967), and related papers.

(4) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).

(5) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, **87**, 2956 (1965).

(6) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(7) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963).

(8) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963).

(9) It is of interest that the first synthesis of a dinucleoside monophosphate possessing the natural 3′–5′ phosphodiester link (A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2632 (1955)) involved formation of a benzyl phosphotriester derivative. This substance was not isolated as such but was converted directly to the diester by hydrolysis with hot aqueous hydrochloric acid. The yield was low and the hydrolytic conditions required to remove the benzyl group were far too severe for use with oligonucleotides containing purine bases.

F. Eckstein and I. Rizk, *Angew. Chem. Intern. Ed. Engl.*, **6**, 949 (1967) have recently described the synthesis of the β,β,β-trichloroethyl ester of 5′-O-(*p*-monomethoxytrityl)thymidylyl-(3′–5′)-3′-O-acetylthymidine. The β,β,β-trichloroethyl group was removed by treatment with zinc and hot acetic acid.

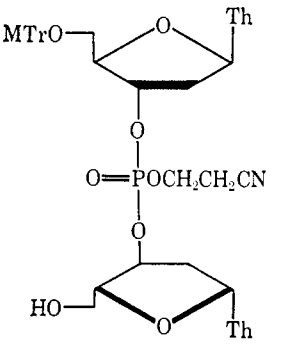
(10) Hereafter this substance is referred to simply as β-cyanoethyl phosphate.

In a preliminary small-scale reaction to test the effectiveness of the phosphorylation steps, 26 mg of 5′-O-(*p*-monomethoxytrityl)thymidine was converted to the phosphotriester, which without isolation was treated successively with 80% aqueous acetic acid to cleave the methoxytrityl group and with concentrated ammonium hydroxide to eliminate the β-cyanoethyl group. Paper chromatography revealed thymidylylthymidine (TpT, 78% by spectrophotometric assay) as the sole nucleotidic material. Since no thymidine 3′-phosphate was found, the conversion of the β-cyanoethyl ester of 5′-O-(*p*-monomethoxytrityl)thymidine 3′-phosphate (MTrTp(ce)) to the corresponding ester of 5′-O-(*p*-monomethoxytrityl)thymidylylthymidine (MTrTp(ce)T)[11] may in fact have been quantitative.

The reaction was then scaled up to permit isolation of the intermediates. For this purpose 36 g of 5′-O-(*p*-monomethoxytrityl)thymidine was phosphorylated with excess β-cyanoethyl phosphate. Following treatment with water, the product (MTrTp(ce)) was extracted into chloroform, transferred to pyridine, and subjected to reaction with onefold excess of thymidine in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride. Work-up of this mixture and elution of the products from a silica gel column with ethyl acetate and tetrahydrofuran afforded 39 g (64%) of MTrTp(ce)T. This compound was isolated as a white powder and characterized by elemental analysis, spectral data, and hydrolytic degradation.

The chemical properties of MTrTp(ce)T proved to be quite satisfactory for an intermediate in an extended synthesis. The β-cyanoethyl phosphotriester was stable in dry pyridine, the solvent in which the reactions are conducted (no change occurred over a period of 1 week at room temperature), and in neutral organic solvents in the presence of silica gel, the medium employed in separating the reaction products. In addition, the triester was stable in aqueous acetic acid; therefore MTrTp(ce)T could be unblocked at the 5′-oxygen without disturbing the β-cyanoethyl group. Thus, when MTrTp(ce)T was heated in 80% aqueous acetic acid at reflux for 10 min, it was converted to *p*-monomethoxytritanol and Tp(ce)T, which was isolated in 90% yield. No further change occurred when the heating period was extended to 3 hr. On the other hand, the β-cyanoethyl phosphotriester was extremely sensitive to aqueous alkali. Tp(ce)T lost the β-cyanoethyl group within seconds when treated with ammonium hydroxide at room temperature; chromatography and electrophoresis indicated that thymidylylthymidine (TpT) was produced quantitatively in this reaction. In 50% aqueous pyridine the phosphotriester was much more stable. The half-time for conversion of Tp(ce)T to TpT in aqueous pyridine at room temperature was approximately 1 week.

Activated MTrTp(ce) could in principle attack thymidine at either the 5′-OH, to give the 3′–5′ isomer of MTrTp(ce)T, or at the 3′-OH, to give the 3′–3′ linked isomer. For determination of the relative amounts of these two isomers in fact produced, the TpT obtained on removing the blocking groups from MTrTp(ce)T was subjected to the action of snake venom phosphodiesterase, an enzyme reported to act specifically on oligonucleotides possessing a free terminal 3′-hydroxyl group.[12] Ninety six per cent of the TpT was hydrolyzed, and the products were thymidine 5′-phosphate and thymidine (pT/T = 0.97) as expected for degradation of thymidylyl-(3′–5′)-thymidine. The undegraded material is considered to be thymidylyl-(3′–3′)-thymidine.



MTrTp(ce)(3′–3′)T

The reliability of the enzymatic assay was checked with a sample of thymidylyl-(3′–3′)-thymidine synthesized independently from 5′-O-(*p*-monomethoxytrityl)thymidine and 5′-O-acetylthymidine by the sequence outlined in Chart I with 5′-O-acetylthymidine replacing thymidine. The yield of thymidylyl-(3′–3′)-thymidine was only 24% (by spectrophotometric assay), in accordance with the general observation that reactions at the 3′-OH of thymidine are less favored than those at the 5′-OH. Thymidylyl-(3′–3′)-thymidine indeed proved to be indistinguishable from thymidyl-(3′–5′)-thymidine on paper electrophoresis and paper chromatography in several solvent systems and it was completely resistant to snake venom phosphodiesterase. Of interest also is the observation that the 3′–3′ isomer is not attacked by spleen phosphodiesterase.[13]

The next aspects of the synthetic procedure considered were: (1) would the yield in the condensation to form the phosphotriester be maintained as the number of nucleotide units in the chain was increased and (2) could the 3′–5′ linked derivatives be obtained free of the 3′–3′ linked material.

The first question was answered by extending the chain to the tetranucleoside triphosphate stage. Repetition of the phosphorylation and condensation steps with MTrTp(ce)T in place of MTrT yielded the bis-β-cyanoethyl ester of 5′-O-(*p*-monomethoxytrityl)thymidylylthymidylylthymidine, MTrTp(ce)Tp(ce)T. This compound in turn was converted by the same sequence to the tris-β-cyanoethyl ester of 5′-O-(*p*-monomethoxytrityl)thymidylylthymidylylthymidylylthymidine, MTrTp(ce)Tp(ce)Tp(ce)T. Since the yields based on the phosphotriester precursors were 49 and 57%, respectively, it is clear that the reactions proceed satisfactorily with the higher molecular weight substrates even though the ratio of the nucleoside to the oligonucleotide component is maintained constant (2:1).

MTrTp(ce)Tp(ce)T reacted readily with 80% aqueous acetic acid to give Tp(ce)Tp(ce)T, which was isolated in

(11) In this notation MTr refers to *p*-monomethoxytrityl, T to the thymidine portion of the molecule, and p(ce) to phosphoryl with one β-cyanoethyl group (see Chart I).

(12) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

(13) Spleen phosphodiesterase, which requires a free 5′-OH, converts thymidylyl-(3′–5′)-thymidine to thymidine 3′-phosphate and thymidine: (a) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **236**, 1144 (1961); (b) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1983 (1963).

Table I. Chromatographic and Spectral Properties of β-Cyanoethyl Phosphotriester Derivatives

Derivative	Tlc[a]	Pc solvent[b] A	F	B	C	Spectral data (in EtOH) λ_{max} ($10^{-3}\epsilon$)	λ_{min} ($10^{-3}\epsilon$)
MTrTp(ce)T	0.74	0.64	0.89		0.80	267 (18.3)	245 (11.9)
MTrTp(ce)Tp(ce)T	0.60	0.48	0.82		0.70	267 (30.8)	244 (17.6)
MTrTp(ce)Tp(ce)Tp(ce)T	0.54	0.20	0.73		0.65	266 (37.3)	242 (20.5)
Tp(ce)T	0.65	0.37	0.63	0.65	0.66	267 (18.7)	234 (6.62)
Tp(ce)Tp(ce)T	0.35	0.13	0.54	0.47	0.63	267 (29.1)	233 (7.02)
Tp(ce)Tp(ce)Tp(ce)T	0.30	0.048	0.40	0.05	0.48	267 (35.2)	236 (11.4)

[a] Silica slides; tetrahydrofuran. [b] Pc = paper chromatography. [c] See Experimental Section for solvent compositions. Since solvents A and F were ammonical the β-cyanoethyl groups were lost in these solutions and the R_f values are those of the NH_4^+ salts of the corresponding phosphodiesters.

96% yield and was characterized by elemental analysis. On treatment with ammonium hydroxide Tp(ce)-Tp(ce)T afforded TpTpT, 94% of which was completely degradable by snake venom phosphodiesterase to thymidine 5′-phosphate and thymidine (pT/T = 2.28). A similar series of reactions with MTrTp(ce)Tp(ce)-Tp(ce)T gave successively Tp(ce)Tp(ce)Tp(ce)T (90% yield), TpTpTpT, and thymidine 5′-phosphate and thymidine (pT/T = 3.3; 84% yield of the TpTpTpT was totally degradable enzymatically, *i.e.*, contained only 3′–5′ phosphodiester links).

The problem of eliminating the 3′–3′ isomer was resolved by exploiting the fact that *p,p′*-dimethoxytrityl chloride reacts with the primary hydroxyl group in a deoxyribonucleoside much faster than with the secondary hydroxyl group. When the mixture of isomers of MTrTp(ce)T was treated for a short time with *p,p′*-dimethoxytrityl chloride in pyridine, the 3′–3′ isomer, which has an exposed primary hydroxyl group, was converted to the dimethoxytrityl derivative while the 3′–5′ isomer, which has a free secondary hydroxyl group, remained largely unaffected. These compounds were then separated by chromatography on silica gel. With the mixture consisting of 96% 3′–5′ and 4% 3′–3′MTrTp(ce)T, a 79% recovery of pure 3′–5′ MTrTp(ce)T was realized by this procedure. The TpT formed on removal of the blocking groups was completely degradable with both snake venom and spleen phosphodiesterase.

The experiments described in this paper show that the β-cyanoethyl phosphotriester derivatives of thymidine can be synthesized and isolated readily in large-scale quantities. Since the blocking groups can be removed efficiently, the procedure provides a convenient route to compounds of the type TpT, TpTpT, TpTpTpT, etc., as well as to the protected, uncharged derivatives. Data on the chromatographic and spectral properties of the compounds which have been prepared are summarized in Table I. It may be noted that the mobility of the *p*-monomethoxytrityl derivatives on silica gel decreases as the number of thymidine units increases. Since the incremental change is not great, especially as one progresses to higher molecular weight compounds, difficulty in separating the homologous members might be expected. An important feature of the two-step phosphotriester synthetic scheme is that the isolation of pure compounds does not in fact require the separation of these homologs since the oligonucleotide component bearing the 3′-hydroxyl group reacts quantitatively with β-cyanoethyl phosphate and mesitylenesulfonyl chloride. Therefore none of this reactant is present in the product mixture that is subsequently separated on silica gel.

Experimental Section

General Methods. Paper chromatography was carried out by the descending technique on Whatman 3 MM paper. The solvent systems were: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2); solvent B, 1-butanol–acetic acid–water (5:2:3); solvent C, 1 *M* ammonium acetate–ethanol (3:7) adjusted to pH 7.5; solvent F, 1-propanol–concentrated ammonium hydroxide–water (55:10:35). The solvents were prepared on a volume basis. Thin layer chromatography was carried out by the ascending technique with Eastman Chromagram Sheets 6060 (silica gel with fluorescent indicator) unless otherwise indicated. Paper electrophoresis was performed using Whatman 3 MM paper in a Savant Flat Plate electrophoretic chamber with a Savant Model HV power supply operated at 2000 V for 1 hr; the solution was buffered at pH 8.0 with 0.0033 *M* potassium dihydrogen phosphate and 0.063 *M* disodium hydrogen phosphate. Nucleosides and their derivatives were observed on paper strips and thin layer sheets by fluorescence in ultraviolet light (Mineralite). Compounds containing *p*-monomethoxytrityl groups were detected by spraying the papers or thin layer sheets with 10% perchloric acid and drying them in a stream of warm air.[14] Silica gel (60–200 mesh) from Davidson Chemical Co was used for column chromatography. The eluent was monitored by thin layer chromatography.

Infrared spectra were obtained with a Beckman Model IR 5 recording spectrophotometer. All samples were prepared in KBr disks. Ultraviolet spectra were obtained on a Cary 11 recording spectrophotometer; individual absorbance values were measured at 267 mμ on a Gilford spectrophotometer, the extinction coefficients used in calculating yields being 9700 for thymidine, 18,500 for TpT, 25,800 for TpTpT, and 34,000 for TpTpTpT.[15] The term OD unit refers to the extinction of the nucleotidic material in 1 ml of neutral solution in a quartz cell with a 1-cm light path.

Melting points were determined on a Fisher-Johns melting point block and are uncorrected. The melting points of the β-cyanoethyl phosphotriester derivatives generally were not sharp, possibly as a consequence of diastereoisomerism stemming from the asymmetric phosphorus atoms. Elemental analyses were performed by Micro-Tech laboratories, Skokie, Ill. Samples submitted to them were prepared by precipitation from tetrahydrofuran with hexane followed by heating in a drying pistol over P_2O_5 at the temperature of refluxing acetone.

Reagents and Chemicals. Reagent grade pyridine was distilled from *p*-toluenesulfonyl chloride, redistilled from calcium hydride, and stored over Linde Molecular Sieve. Pyridinium mono-β-cyanoethyl phosphate was prepared from the barium salt by passage over a column of Dowex 50 resin (pyridinium form). The clear solution containing the pyridinium salt was first concentrated to a small volume and then lyophilized to a gum. The gum was dissolved in pyridine and diluted to a known volume and stored in a sealed flask under refrigeration.

Nucleosides were purchased from Nutritional Biochemical Corp. or CalBiochem. 5′-O-(*p*-Monomethoxytrityl)thymidine[7] (mp 103–105°); 5′-O-acetylthymidine[16] (mp 150°); mesitylenesulfonyl chloride[17] (mp 155°), and 2,4,6-triisopropylbenzenesulfonyl chloride[14] (mp 95–96°) were prepared as described in the literature.

Enzyme Assays. In general the procedure described by Ralph, *et al.*, was followed.[13b]

(14) R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 829 (1966).
(15) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).
(16) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).
(17) C. Wang and S. G. Coehn, *ibid.*, **79**, 1924 (1957).

a. Spleen Phosphodiesterase. Lyophilized spleen phosphodiesterase (10–15 units) obtained from Nutritional Biochemicals Corp. was dissolved in 1 ml of 0.01 *M* sodium pyrophosphate buffer (adjusted to pH 6.5 with phosphoric acid). An aliquot (0.1 ml) of this solution, 0.2 ml of 0.05 *M* ammonium acetate (adjusted to pH 6.5 with acetic acid), and 0.1 to 1 mg of the nucleotide substrate were incubated together at 37° for 5 hr. The solution was then applied to Whatman 3 MM paper as a band 3 cm long and developed with solvent F. Nucleoside and nucleotide bands were cut out, eluted with water, and diluted to a volume of 10 ml. Absorbances were measured at 267 mμ and were corrected for absorption due to extraneous materials eluted from the paper, buffer, etc. For this purpose blanks were cut from the paper horizontally adjacent to the nucleotide spots and were treated the same as the sample material. The absorbance of the blank (0.07–0.11 absorbance units) was generally <10% of that of the nucleotide sample.

b. Snake Venom. Two hundred units of lyophilized snake venom phosphodiesterase obtained from CalBiochem was dissolved in 1 ml of tris(hydroxymethyl)aminomethane buffer (adjusted to pH 9.2 with 0.1 *N* hydrochloric acid). An aliquot (0.2 ml) of the enzyme solution was added to the nucleotide material (~1 mg) in a small test tube and incubated at 37° for 7 hr. The solution was then worked up in the same manner as described for the spleen enzyme.

General Procedures. All reactions of more than 0.2 mmol of the limiting reagent were carried out in tightly stoppered ground glass joint flasks. Reactants were dried by evaporation of pyridine at reduced pressure, air being readmitted to the sample through a column (50 × 1 cm) of anhydrous magnesium perchlorate. Highest yields in the phosphorylation steps were obtained when the minimum volume of pyridine required to give a homogeneous solution was used. Extractions of the salts of the phosphate derivatives into chloroform usually resulted in emulsions which would clear on standing for a period of 0.5 hr. Particularly difficult emulsions were broken by centrifugation or by addition of sodium chloride.

Reactions of less than 0.2 mmol of the limiting reagent were carried out in Pyrex tests tubes (10 ml, screw cap type) sealed with serum caps. Extractions were carried out in the test tubes and emulsions were always broken by centrifugation. Solvent was added or withdrawn with pipets. All reactions were run at room temperature unless otherwise stated.

Small-Scale Synthesis of Thymidylylthymidines. 5′-O-(*p*-Monomethoxytrityl)thymidine (26 mg, 0.05 mmol) and β-cyanoethyl phosphate (0.1 mmol) were dried by evaporation of pyridine (four 1-ml portions). Mesitylenesulfonyl chloride (44 mg, 0.2 mmol) and pyridine (0.2 ml) were added and the resulting solution was stirred for 6 hr. After the addition of water (0.2 ml), stirring was continued for 16 hr. The solvents were removed at reduced pressure and the residue was dissolved in chloroform (1.5 ml). Following extraction with water (two 0.3-ml portions), the chloroform solution was treated as described below.

a. One-half of the above chloroform solution was evaporated to a gum which was dried by evaporation of pyridine (four 1-ml portions). Thymidine (12 mg, 0.05 mmol), triisopropylbenzenesulfonyl chloride (23 mg, 0.075 mmol), and pyridine (0.3 ml) were added, and the solution was stirred for 25 hr. Following addition of water (0.3 ml), stirring was continued for 4 hr, and the solvent was removed at reduced pressure. The residue was dissolved in 80% aqueous acetic acid (1 ml) and heated on a steam bath for 20 min, whereupon the solvent was removed at reduced pressure and the residual gum was dissolved in a minimum volume of dioxane. Ammonium hydroxide was added and the solution was stirred for 4 hr and diluted to 5 ml. A portion was chromatographed on Whatman paper in solvent F. Products appeared at R_f^F 0.62 (TpT), 0.78 (thymidine), and 0.96 (possibly sulfonylated nucleoside; electrophoretic mobility at pH 8.0 was 0.00). The TpT band was eluted with water and the yield (78% based on the initial amount of 5′-O-monomethoxytritylthymidine) was determined spectroscopically at 267 mμ. This product was largely degraded (>90%) to thymidine and thymidine 5′-phosphate by snake venom phosphodiesterase.

b. The remaining half of the chloroform solution was treated exactly as above except that 5′-O-acetylthymidine (15 mg, 0.05 mmol) was used in place of thymidine. The products of the reaction had the following R_f's in solvent F: 0.38, 0.48 (Tp, 10% yield), 0.64 (TpT), 0.70, 0.78 (thymidine), and 0.94. The yield of Tp-(3′–3′)-T determined spectroscopically (267 mμ) was 24% based on 5′-O-monomethoxytritylthymidine (0.025 mmol). This product was unaffected by snake venom and spleen enzymes under the standard conditions.

β-Cyanoethyl Ester of 5′-O-(*p*-Monomethoxytrityl)thymidylylthymidine (MTrTp(ce)T). 5′-O-(*p*-Monomethoxytritylthymidine (36 g, 70 mmol) and β-cyanoethyl phosphate (70 mmol) were dried by evaporation of pyridine (two 50-ml portions). Mesitylenesulfonyl chloride (30.5 g, 140 mmol) and pyridine (140 ml) were added and the solution was stirred for 6 hr. After the reaction solution was cooled in an ice bath, cold water (140 ml) was added and stirring was continued for 17 hr. More water (140 ml) was added and the solution was extracted with chloroform (two 400-ml portions plus one 300-ml portion). The combined chloroform extracts were washed with water (500 ml), dried over sodium sulfate, and concentrated to a gum at reduced pressure. The gum was dried by evaporation of pyridine (three 50-ml portions). Triisopropylbenzenesulfonyl chloride (42.5 g, 140 mmol) and pyridine (140 ml) were added, and the solution was stirred for 1.5 hr. Thymidine (33.9 g, 140 mmol) was added and stirring was continued for 14 hr. Cold water (140 ml) was added and after 1.5 hr the very viscous aqueous solution was extracted with chloroform (two 300-ml portions plus one 100-ml portion). The combined chloroform extracts were washed with water (four 500-ml portions), dried over sodium sulfate, and made up to a volume of 900 ml.

Two-thirds of the chloroform solution was concentrated and applied to a column (100 × 3 cm) of silica gel in ethyl acetate. The column was eluted first with ethyl acetate (8.5 l) and then with tetrahydrofuran (4 l.). The first 500 ml of eluent contained some of the desired product along with impurities. Pure MTrTp(ce)T was obtained in the tetrahydrofuran fractions. Mixed fractions were rechromatographed in the same manner and a total of 27.2 g of MTrTp(ce)T was obtained. Similar chromatography of the remaining one-third of the chloroform solution yielded 12.1 g of this substance. The total yield of MTrTp(ce)T was 39.3 g (64.5%). An analytical sample prepared by precipitating the product from a tetrahydrofuran solution with hexane became a clear melt at 126–128° (softening from 115°). The thin layer chromatographic properties of this material were: R_f 0.08 (ethyl acetate), 0.74 (tetrahydrofuran), 0.70 (dioxane), and 0.79 (ethanol). The principal bands in the infrared spectrum occurred at 2.91, 4.42 (w), 5.91, 7.78, 9.72, and 14.2 mμ.

Anal. Calcd for $C_{43}H_{46}N_5O_{13}P$: C, 59.24; H, 5.32; N, 8.03; P, 3.55. Found: C, 59.49; H, 5.47; N, 7.96; P, 3.33.

β-Cyanoethyl Derivative of Thymidylylthymidine (Tp(ce)T). MTrTp(ce)T (2.62 g, 3 mmol) was heated in 40 ml of 80% acetic acid at reflux for 10 min. The solvent was removed at reduced pressure and the last trace of acetic acid was removed by evaporation of ethanol. The residue was dissolved in chloroform and applied to a column (50 × 3.5 cm) of silica gel in ethyl acetate. The column was eluted first with ethyl acetate (700 ml) and then with tetrahydrofuran (2.1 l.). Tp(ce)T was obtained by concentration of the tetrahydrofuran fractions and precipitation with hexane: yield, 1.62 g (90%). An analytical sample softened at 110° and became a clear melt by 115°. The thin layer chromatographic properties of the product were R_f 0.05 (ethyl acetate), 0.65 (tetrahydrofuran), 0.68 (dioxane), and 0.77 (ethanol).

Anal. Calcd for $C_{23}H_{30}N_5O_{12}P \cdot 1H_2O$: C, 44.74; H, 5.22; N, 11.34; P, 5.02. Found: C, 44.72; H, 4.99; N, 11.17; P, 5.20.

Thymidylylthymidine (TpT). Tp(ce)T (50 mg) was dissolved in tetrahydrofuran, applied to a sheet of Whatman 3 MM paper, and developed with solvent F. In this ammonia cal solvent the β-cyanoethyl group was rapidly cleaved, and a single fluorescent product was obtained. It was eluted from the paper with water and identified as TpT by its chromatographic (Table I) and electrophoretic properties and by hydrolysis with snake venom phosphodiesterase.

Approximately 65 OD units of the TpT was treated with snake venom phosphodiesterase by the standard procedure. The work-up yielded 32 OD units of pT (R_f^F 0.45), 33 OD units of thymidine (R_f^F 0.77), and 2.7 OD units of TpT (R_f^F 0.65); pT/T = 0.97. The TpT recovered, representing 4% of the starting material, was not degraded by further treatment with the enzyme.

The experiment was repeated with 10 OD units of TpT and 20 units of snake venom phosphodiesterase. The products were again pT (5.1 OD units), thymidine (5.2 OD units), and TpT (0.50 OD units); pT/T = 0.98.

The Bis(β-cyanoethyl) Derivative of 5′-O-(*p*-Monomethoxytrityl)thymidylylthymidylylthymidine (MTrTp(ce)Tp(ce)T). MtrTp(ce)T (10 g, 11.5 mmol) and β-cyanoethyl phosphate (15 mmol) were dried by evaporation of pyridine (four 25-ml portions). Mesitylenesulfonyl chloride (5.1 g, 23 mmol) was added, and the reagents were dissolved in pyridine (20 ml) and stirred for 6 hr

Cold water (20 ml) was added and stirring was continued for 15 hr. After removing the solvents at reduced pressure, the residue was dissolved in chloroform (100 ml) and the solution was washed with saturated aqueous sodium chloride (150 ml). Three layers formed; the bottom two layers were combined and evaporated at reduced pressure. The resulting gum was dried by evaporation of pyridine (three 20-ml portions) and then stirred with thymidine (5.6 g, 23 mmol), triisopropylbenzenesulfonyl chloride (6.95 g, 23 mmol), and pyridine (20 ml) for 24 hr. After addition of 20 ml of water the solution was stirred for 18 hr and extracted with chloroform (three 100-ml portions). The combined chloroform extracts were washed with water (four 25-ml portions); then the chloroform was evaporated and the residual gum was taken up in 10 ml of tetrahydrofuran.

One milliliter of this solution was applied to a silica gel column (60 × 3.5 cm) in tetrahydrofuran. The column was eluted with tetrahydrofuran (2.75 l.). Impurities were obtained in the first 75 ml of eluent and MTrTp(ce)Tp(ce)T (R_f 0.60, THF) was obtained in the next 2 l. When the product had been washed through the column, another 1 ml of the stock solution was applied to the top of the same column and eluted as before. This process was repeated until all of the material had been separated. The total yield of MTrTp(ce)Tp(ce)T obtained in this manner was 6.48 g (49%). The melting point of an analytical sample was 135–139° (softening from 128°); thin layer chromatographic properties were: R_f 0.05 (ethyl acetate), 0.60 (tetrahydrofuran), 0.76 (dioxane), and 0.73 (ethanol). The paper chromatographic properties are listed in Table I.

Anal. Calcd for $C_{56}H_{62}N_8O_{20}P_2 \cdot 1H_2O$: C, 53.93; H, 5.17; N, 8.99. Found: C, 53.77; H, 5.06; N, 8.87.

The Bis(β-cyanoethyl) Derivative of Thymidylylthymidylylthymidine (Tp(ce)Tp(ce)T). MTrTp(ce)Tp(ce)T (1.02 g) was heated at reflux in 80% aqueous acetic acid (30 ml) for 15 min and the solution was then cooled and poured onto 150 ml of ice water. The aqueous solution was extracted with hexane (200 ml) and then lyophilized to a powder. This powder was dissolved in tetrahydrofuran, filtered from some insoluble material, and precipitated with hexane. The product was collected by centrifugation. The supernatent liquid was discarded and the remaining solid was dried to yield 770 mg (96%) of Tp(ce)Tp(ce)T as a white powder. The melting point of an analytical sample was 124–126° (softening from 120°); the thin layer chromatographic properties were R_f 0.00 (ethyl acetate), 0.35 (tetrahydrofuran), 0.58 (dioxane), and 0.66 (ethanol).

Anal. Calcd for $C_{36}H_{46}N_8O_{19}P_2 \cdot 1H_2O$: C, 44.36; H, 4.96; N, 11.49. Found: C, 44.42; H, 4.83; N, 10.86.

Thymidylylthymidylylthymidine (TpTpT). Tp(ce)Tp(ce)T (10 mg) was dissolved in tetrahydrofuran and applied to Whatman paper and eluted with solvent F. The only nucleotidic product was TpTpT (R_f^F 0.51). It was extracted from the paper with water. Approximately 60 OD units of TpTpT was treated with the snake venom enzyme. Work-up yielded 33.3 OD units of pT (R_f^F 0.38), 1.3 OD units of TpT (R_f^F 0.61), 1.4 OD units of TpTpT (R_f^F 0.51), and 18 OD units of thymidine (R_f^F 0.74), *i.e.*, 94% of the TpTpT was completely degraded to pT and T (pT/T = 2.11). Hydrolysis of 30 OD units of TpTpT with the spleen enzyme yielded Tp (R_f^F 0.48, 19 OD units), TpT (R_f^F 0.72, 1.5 OD units), TpTpT (R_f^F 0.66, 0.9 OD unit), and thymidine (R_f^F 0.79, 8 OD units). In this case 87% of the TpTpT was completely degraded to Tp and T (Tp/T = 2.28).

Thymidylylthymidylylthymidine 3′-Phosphate (TpTpTp). MTrTp(ce)Tp(ce)T (10 mg, 0.0081 mmol) and β-cyanoethyl phosphate (0.016 mmol) were dried by evaporation of pyridine (two 1-ml portions). Mesitylenesulfonyl chloride (10 mg, 0.056 mmol) and pyridine (0.2 ml) were added and the solution was stirred for 6 hr. Water (0.2 ml) was added and after stirring for 15 hr the solvents were removed at reduced pressure and the residue was heated in 80% acetic acid (1 ml) on a steam bath for 15 min. The acetic acid was removed at reduced pressure and the residue was stirred with ammonium hydroxide (2 ml) for 30 min. The solution was applied to Whatman paper and eluted in solvent F, in which the major product TpTpTp (R_f^F 0.27) separated cleanly from three minor components (R_f^F 0.36, 0.57, 0.87). Elution with water gave TpTpTp in 85% yield (determined spectrophotometrically).

Tris-β-cyanoethyl Ester of 5′-O-(*p*-Monomethoxytrityl)thymidylylthymidylylthymidylylthymidine (MTrTp(ce)Tp(ce)Tp(ce)T). The procedures for preparing this compound and for hydrolyzing it were essentially the same as for preparing MTrTp(ce)Tp(ce)T from MTrTp(ce) and for hydrolyzing MTrTp(ce)Tp(ce)T. The quantities of reagents used in the synthetic sequence were: MTrTp(ce)Tp(ce)T, 1.23 g, 1 mmol; β-cyanoethyl phosphate, 1.5 mmol; mesitylenesulfonyl chloride, 0.540 g, 2.5 mmol; thymidine, 0.484 g, 2.0 mmol; and 2,4,6-triisopropylbenzenesulfonyl chloride, 0.756 g, 2.5 mmol. In the purification one-half of the crude products in tetrahydrofuran solution was applied to a silica gel column (60 × 3.5 cm) and eluted with tetrahydrofuran (3.4 l.). The first 600 ml contained only impurities, the next 400 ml contained a mixture including the desired product, and the final 2.4 l. contained only MTrTp(ce)Tp(ce)T. The remaining half of the stock solution was separated in the same way, and the mixed fractions were rechromatographed. Precipitation with hexane afforded 912 mg (57%) of MTrTp(ce)Tp(ce)Tp(ce)T as a white powder melting at 144–146° (softening from 140°); thin layer chromatographic properties, R_f 0.00 (ethyl acetate), 0.54 (tetrahydrofuran), 0.70 (dioxane), and 0.69 (ethanol).

Anal. Calcd for $C_{69}H_{78}N_{11}O_{27}P_3 \cdot 1H_2O$: C, 51.65; H, 5.03; N, 9.60. Found: C, 51.32; H, 4.99; N, 8.73.

The Tris(β-cyanoethyl) Derivative of Thymidylylthymidylylthymidylylthymidine (Tp(ce)Tp(ce)Tp(ce)T). MTrTp(ce)Tp(ce)Tp(ce)T (350 mg, 0.219 mmol) was dissolved with heating in 80% aqueous acetic acid (5 ml) and the solution was refluxed for 12 min. Following removal of the solvents, the residue was dissolved in tetrahydrofuran containing just enough water to effect solution and the solution was applied to a silica gel column (45 × 2 cm). Elution with tetrahydrofuran in 50-ml fractions yielded monomethoxytritanol in the second fraction and Tp(ce)Tp(ce)Tp(ce)T in fractions 3–8. On concentration and precipitation with hexane, 260 mg (90%) of Tp(ce)Tp(ce)Tp(ce)T was obtained, mp 145–148° (softening from 140°); thin layer chromatographic properties: R_f 0.00 (ethyl acetate), 0.30 (tetrahydrofuran), 0.53 (dioxane), 0.50 (ethanol).

Anal. Calcd for $C_{49}H_{62}N_{11}O_{26}P_3 \cdot 1H_2O$: C, 44.18; H, 4.84; N, 11.57. Found: C, 44.53; H, 4.93; N, 10.69.

Thymidylylthymidylylthymidylylthymidine (TpTpTpT). Tp(ce)Tp(ce)Tp(ce)T (20 mg) was dissolved in tetrahydrofuran and applied to Whatman paper and developed with solvent F; TpTpTpT (R_f 0.40) was eluted from the nucleotidic spot with water and characterized by paper chromatography and electrophoresis. On treatment of approximately 27 OD units of TpTpTpT with the snake venom enzyme and chromatography on paper with solvent F, four bands were obtained: R_f 0.40 (pT and TpTpTpT), 0.51 (TpTpT, 0.54 OD unit), 0.62 (TpT, 0.65 OD unit), and 0.74 (thymidine, 5.6 OD units). The band at 0.40 was eluted with water and run on paper electrophoresis at pH 8.0. Two distinct bands appeared at R_m^{Tp} 0.68 (TpTpTpT, 1.75 OD units) and R_m^{Tp} 1.0 (pT, 18.7 OD units). These results show that 84% of the original tetranucleotide was degraded to pT and T (pT/T = 3.3).

The TpTpTpT (2 OD units) was treated with spleen enzyme and the results were obtained as above with the Tp and TpTpTpT products being separated by electrophoresis (pH 8.0). In this manner 1.04 OD units of Tp (R_f^F 0.41, R_m^{Tp} 1.0), 0.15 OD units of TpT (R_f^F 0.62), 0.1 OD units of TpTpT (R_f^F 0.51), 0.06 OD units of TpTpTpT (R_f^F 0.41, R_m^{Tp} 0.68), and 0.6 OD units of thymidine (R_f^F 0.74) were obtained. Thus 79% of the starting TpTpTpT was degraded to Tp and T (Tp/T = 3.8).

Removal of the 3′–3′ Isomer from MTrTp(ce)T Using Dimethoxytrityl Chloride. MTrTp(ce)T (2.8 g, containing 4% 3′–3′ isomer) and *p,p′*-dimethoxytrityl chloride (507 mg, 1.5 mmol) were dissolved in pyridine (3 ml). After 22 hr the solution was concentrated to a gum, which was dissolved in a minimum volume of tetrahydrofuran and applied to a silica gel column (50 × 3.5 cm) made up in ethyl acetate. The column was eluted with ethyl acetate–tetrahydrofuran mixtures, beginning with 1 l. of pure ethyl acetate. The tetrahydrofuran content was increased by 100 ml for each 1 l. of solvent until the mixture was 70% tetrahydrofuran; thereafter 1 l. of tetrahydrofuran was used. Fractions of 500 ml were collected. The first five did not contain any nucleotide material. Fractions 6, 7, and 8 each contained a large amount of 3′–3′ isomer (as determined by snake venom assay of the derived TpT). A total of 288 mg of material was contained in these three fractions. Fractions 9–18 contained MTrTp(ce)T (2.24 g, 79% recovery) consisting only of 3′–5′ isomer as determined by the snake venom assay (the assay yielded 5.12 OD units of pT (R_f^F 0.40) and 5.23 OD units of thymidine (R_f^F 0.74) with pT/T = 0.98).