$K_a = 10^{-9}$, and $k_{-1} = 10^{10}\ M^{-1}\ \text{sec}^{-1}$ (approximately diffusion controlled). From this plot it can be seen that the behavior is as predicted from the mechanism proposed by Harley-Mason.[14] At high pH eq 5 reduces to $k_{obsd} = k_1$, corresponding to a rate-limiting deprotonation reaction and a pH-independent rate constant. This behavior has recently been confirmed by use of a rotating disk electrode.[20]

One point of considerable interest is clear from the data of Table II. The cyclization rate (k_2) of adrenaline is 140 times faster than that of noradrenaline. These results are very interesting in the light of the recent studies of Walaas and Walaas.[4] In this work oxidation of reduced phosphopyridine nucleotides (DPNH and TPNH) was accomplished in a medium containing ceruloplasm and various catecholamines as substrates. Noradrenaline gave a considerably higher rate of oxidation than did adrenaline. It was suggested that adrenaline showed a greater tendency toward indolization and adrenochrome formation and hence lesser oxidative activity in the enzymatic oxidation of the DPNH. In a further study of the chemical transformation of catecholamines by ultraviolet irradiation, Walaas[5] again found much greater tendency toward ring closure with adrenaline. The importance of the uncyclized oxidation product of noradrenaline has been discussed by Walaas.[5] The present results on the relative cyclization rates are in perfect accord with the finding of Walaas. In this connection, the slow cyclization rate found for dopamine is of interest. The applications of the electrochemical techniques to melanization reactions would appear to be quite useful. The relative rate of adrenaline *vs.* isoproterenol is that expected from steric effects outweighing electronic effects.

(20) P. A. Malachesky, L. S. Marcoux, and R. N. Adams, *J. Phys. Chem.*, 70, 4068 (1966).

Acknowledgments. This work was supported in part by the Petroleum Research Fund administered by the American Chemical Society and by the General Research Fund of the University of Kansas. We are indebted to Professors E. Smissman and M. P. Mertes for valuable discussions during the course of this work.

Oligonucleotide Syntheses on Insoluble Polymer Supports. I. Stepwise Synthesis of Trithymidine Diphosphate

L. Russell Melby and Donald R. Strobach

Contribution No. 1249 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898.
Received September 12, 1966

Abstract: An *insoluble* cross-linked polystyrene with pendant monomethoxytrityl chloride groups was prepared and condensed with thymidine to obtain a polymer containing approximately 500 μmoles of bound thymidine per gram of polymer. Condensation of the thymidine derivative with 3′-O-acetylthymidine 5′-phosphate (pTOAc) in the presence of dicyclohexylcarbodiimide (DCC) resulted in 54% conversion of bound thymidine to the dinucleoside phosphate. Deacetylation of the latter polymer and subsequent condensation with pTOAc and DCC gave the trinucleoside diphosphate derivative from which thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine was isolated in 38% conversion based on polymer-bound dinucleoside phosphate.

The success achieved by Merrifield in stepwise synthesis of polypeptides on insoluble polymer supports[1] and the procedural advantages which the method affords prompted us to investigate the application of similar procedures to oligonucleotide synthesis. Reports of related studies in other laboratories have recently appeared.[2-4]

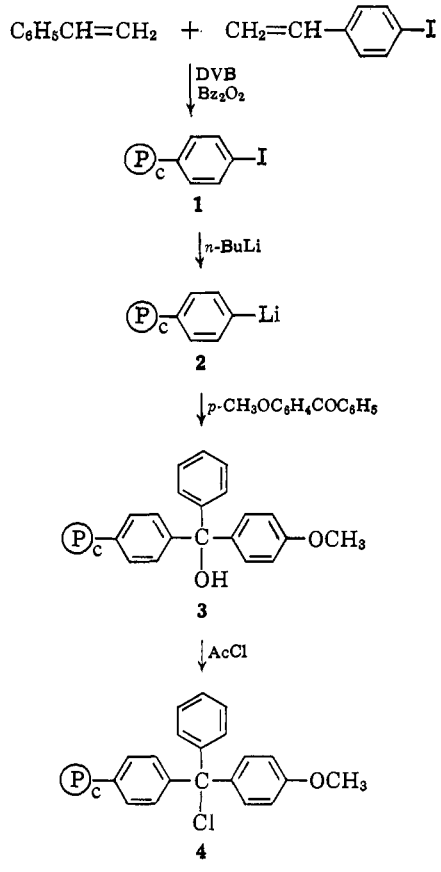
The support polymer under study in this laboratory consists of an *insoluble* styrene–divinylbenzene bead copolymer containing trityl chloride or 4-methoxytrityl chloride functional groups to which nucleosides are subsequently attached by trityl ether formation. Suitably protected nucleotides are then condensed with the polymer-bound nucleoside. The insolubility and form of this type of support confer physical and chemical characteristics which differentiate it from the soluble supports reported by Hayatsu and Khorana[3] and by Cramer, *et al.*[4] On the other hand, Letsinger and Mahadevan[2] utilized an insoluble popcorn polymer to which they attached amino-containing nucleosides through amide formation with polymer-borne carbonyl chloride groups.

Methoxytrityl Chloride Polymer Synthesis (Chart I). The synthesis of the supporting polymer was similar to procedures reported by Braun and Seelig[5] and is summarized in Chart I. Thus, a mixture of styrene and *p*-iodostyrene (mole ratio 4:1) containing 1% by weight of divinylbenzene (DVB) was polymerized in aqueous polyvinyl alcohol with benzoyl peroxide (Bz_2O_2) initiator to obtain cross-linked iodo copolymer (**1**) in the form of beads approximately 75 to 150 μ in diameter. The iodine content of the polymer corre-

(1) R. B. Merrifield, *Science*, 150, 178 (1965).
(2) R. L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.*, 87, 3526 (1965).
(3) H. Hayatsu and H. G. Khorana, *ibid.*, 88, 3182 (1966).
(4) F. Cramer, R. Helbig, H. Hettler, K. H. Scheit, and H. Seliger, *Angew. Chem. Intern. Ed. Engl.*, 5, 601 (1966).

(5) (a) D. Braun and E. Seelig, *Chem. Ber.*, 97, 3098 (1964); (b) D. Braun, *Angew. Chem.*, 73, 197 (1961).

Chart I. Cross-Linked Polystyrene with Pendant 4-Methoxytrityl Groups[a]



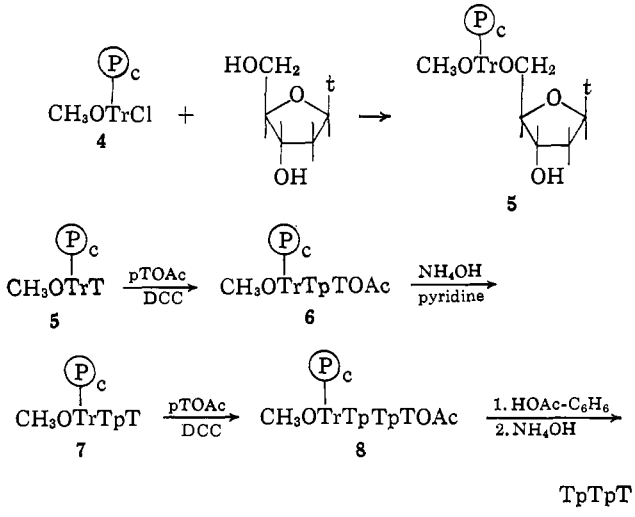
[a] Ⓟc– = cross-linked polystyrene backbone.

sponded to about 1600 μmoles/g and the degree of cross-linking was such that the polymer absorbed more than five times its weight of solvents such as benzene, pyridine, or dimethylformamide (DMF) before acquiring fluid mobility.

The iodo polymer, as a suspension in benzene, reacted with excess *n*-butyllithium to form a lithio polymer (**2**) which was not isolated, but was treated *in situ* with 4-methoxybenzophenone and then with a mixture of acetic and hydrochloric acids to liberate the methoxytrityl alcohol polymer **3**. The infrared spectrum of this polymer (Nujol mull) exhibits characteristic bands at 2.95, 6.62, 7.70, 8.0, and 12.2 μ. The methoxytrityl alcohol polymer was virtually free of iodine, suggesting almost quantitative reaction with *n*-butyllithium, but considerable reduction had presumably taken place since conversion to the trityl alcohol was incomplete.[6] Treatment of the alcohol polymer with excess acetyl chloride in boiling benzene formed the methoxytrityl polymer **4** whose chlorine content corresponded to approximately 730–760 μmoles/g.[7] The infrared absorption spectrum is similar to that of the alcohol precursor but lacks the 2.95-μ hydroxylic absorption. Both trityl derivatives developed a deep red-brown color when warmed with 72% perchloric acid[8] and exhibited yellowish-white fluorescence under 3660-A ultraviolet irradiation, both qualities being characteristic of methoxytrityl chloride itself. The methoxytrityl chloride polymer is moderately stable to atmospheric moisture, but to ensure its integrity it was stored and handled in a drybox.

Polymer-Supported Thymidine (5, Chart II).[9] Reaction of the monomethoxytrityl chloride polymer with excess thymidine (T) in anhydrous pyridine or DMF–pyridine mixtures at room temperature for periods of 24–48 hr formed methoxytritylthymidine polymer (**5**) containing up to 490–550 μmoles of bound thymidine per gram of polymer, representing 75–85% conversions based on polymer-bound chloride.[10] Furthermore, by using a pyridine–benzene solvent mixture (1:2, v/v) conversions of over 95% were achieved to obtain polymer containing 610–620 μmoles of T/g. The thymidine content of the product was determined by exhaustive hydrolysis of the polymer in boiling acetic acid–hydrochloric acid. This procedure quantitatively liberated thymine which was assayed by ultraviolet spectral analysis of the hydrolysate. The infrared spectrum of the thymidine polymer exhibits a strong band at 5.92 μ characteristic of thymidine carbonyl group absorption.

Chart II. Insoluble Polymer-Supported Oligonucleotide Synthesis[a]



[a] T = thymidine; t = thymine.

Polymer-Supported Thymidylyl-(3′→5′)-thymidine 3′-O-Acetate (6, Chart II). Reaction of the methoxytritylthymidine polymer **5** with 3′-O-acetylthymidine 5′-phosphate[11] (pTOAc) and dicyclohexylcarbodiimide (DCC) in anhydrous pyridine–dimethylacetamide (DMAc) solvent mixture gave the supported thymidylyl-(3′→5′)-thymidine 3′-O-acetate (TpTOAc)[12] derivative **6**. Treatment of the polymer with an acetic acid–water–benzene solution (32:8:10, v/v) at room temperature for 5 hr liberated TpTOAc as well as some unreacted thymidine. The TpTOAc was deacetylated to form free thymidylyl-(3′→5′)-thymidine (TpT) which was

(6) For example, methoxyl determination showed functionality much below that expected for quantitative conversion.

(7) This estimation was based on combustion analysis for chlorine. However, the chloride was quantitatively released from the polymer by hydrolysis in aqueous pyridine at room temperature during 18 hr. The chlorine content varied to a modest degree among several preparations.

(8) Also noted by Hayatsu and Khorana.[8]

(9) The symbol –Ⓟc will be used to denote the cross-linked poystyrene backbone according to the convention of R. L. Letsinger, *et al.* (see ref. 2), and CH_3OTr– to denote the pendant methoxytrityl group.

(10) Percentage conversions take into account the weight gain of the polymer resulting from substitution of Cl by thymidine.

(11) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(12) Abbreviations are as previously defined by E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, **87**, 2956 (1965); see also H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

chromatographically identical with an authentic sample[13] and was completely hydrolyzed to Tp and T by spleen phosphodiesterase.[14] This hydrolysis verified the exclusiveness of 3′→5′ phosphodiester bonds in the product and thus proved the 5′-O attachment of thymidine to the polymer. The TpT was also completely degraded to pT and T by snake venom phosphodiesterase.[15]

The yield of isolated TpT corresponded to 220 μmoles/g of polymer, representing 54% conversion of polymer-bound thymidine.[16]

The release of TpTOAc by the acetic acid–water–benzene reagent revealed a marked lability of the methoxytrityl ether bond associated with bound TpTOAc relative to the equivalent bond in unelaborated thymidine polymer. Thus, treatment of the polymer with the acetic acid reagent for 5 hr liberated substantially all of the TpTOAc and some thymidine equivalent to 251 μmoles of initially bound thymidine per gram of polymer. On the other hand, the thymidine precursor polymer liberated about 60 μmoles of T/g under identical conditions. As yet we have no adequate explanation for this trityl ether labilization subsequent to nucleotide condensation, but we are pursuing its study.

Polymer-Supported TpTpTOAc (8, Chart II). The dinucleoside phosphate 3′-O-acetate polymer **6** was deacetylated by treatment with concentrated ammonium hydroxide in pyridine for 3 days. Ammonium ion and occluded ammonium hydroxide were removed from the deacylated product by ion exchange with pyridinium acetate since cations derived from strong bases have been shown to inhibit DCC-promoted phosphorylations.[17] The derived TpT polymer **7** was condensed with pTOAc and DCC in pyridine to obtain the trinucleoside diphosphate polymer **8** from which TpTpT was isolated in amounts corresponding to 29 μmoles/g, representing 38% conversion of polymer-bound TpT. The trinucleoside diphosphate was accompanied by TpT and thymidine. The constitution of the TpTpT was confirmed by spleen and venom phosphodiesterase hydrolysis.

Addendum. We are continuing our study of this insoluble polymer support system for oligonucleotide synthesis with derivatives containing deoxycytidine, -adenosine, and -guanosine residues. This developing work will be reported in subsequent papers.

Experimental Section

General Methods. Paper chromatography was carried out by the descending technique using Whatman No. 40 paper for quantitative work and Whatman No. 1 paper for qualitative work. Solvent systems used were: A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2, v/v); B, ethanol–1 *M* ammonium acetate (pH 7.5) (7:3, v/v); and C, 1-butanol–acetic acid–water (5:2:3, v/v).

Ultraviolet spectra were determined on a Cary Model 11 recording spectrophotometer. The expression OD_{267} unit (or OD_{264} unit) is defined as that amount of substance in 1 ml of solution which gives an optical density of 1.00 through a 1-cm path length at the indicated wavelength.

Pyridinium 3′-O-acetylthymidine 5′-phosphate was prepared according to the method of Khorana and Vizsolyi,[11] purified by ether precipitation,[18] and stored as a 0.2–0.3 *M* solution in purified anhydrous pyridine.[17] Its purity was checked by chromatography in solvent B.

Iodo Polymer 1. Into a 1-l., three-necked flask equipped with a mechanical stirrer and reflux condenser was placed 2.5 g of polyvinyl alcohol[19] and 250 ml of deaerated water, and the mixture was stirred under a nitrogen atomosphere until the alcohol had dissolved. A solution of 16.0 g (0.154 mole) of freshly distilled styrene, 9.0 g (0.04 mole) of *p*-iodostyrene,[20] 0.25 ml of divinylbenzene, and 0.25 g of benzoyl peroxide was added to the flask; the suspension was stirred under nitrogen and heated with a water bath (80–90°) for 5 hr. The snow-white bead polymer was collected and washed thoroughly with water and ethanol (or methanol). The dried polymer was suspended in 400 ml of benzene and agitated overnight to remove undesirable low molecular weight contaminants. The polymer was collected, washed with a large volume of ethanol, dried at 100° *in vacuo*, and sieved. The portion passing 100 mesh but retained on 200 mesh sieve[21] (19.5 g, 77% yield) was used in subsequent reactions. *Anal.* Calcd for $C_{40}H_{39}I$: C, 74.3; H, 6.1; I, 19.6. Found: C, 72.7; H, 6.0; I, 20.4.

The iodine content corresponded to 1610 μmoles/g. The infrared spectrum (Nujol mull) of the polymer exhibits a characteristic band at 12.2 μ assignable to *para*-disubstituted benzene. This band was absent in polystyrene homopolymer.

Methoxytrityl Alcohol Polymer 3. To a suspension of 65 g of the iodo polymer (equivalent to *ca.* 0.1 mole of iodine) in 1 l. of anhydrous benzene was added 140 ml of 1.6 *M* *n*-butyllithium in *n*-hexane[22] (0.22 mole), and the mixture was stirred under nitrogen at room temperature for 24 hr. During this time the polymer changed from swollen, translucent beads to an opaque white granular material. Most of the liquid was removed by suction through a filter stick and the solid was washed in the flask with two 500-ml portions of benzene, removing each wash in the same manner. To this material was added 1 l. of anhydrous benzene and 25 g (0.12 mole) of 4-methoxybenzophenone, and the mixture was stirred under nitrogen at room temperature for 24 hr. A mixture of 200 ml of glacial acetic acid and 50 ml of 6 *N* hydrochloric acid was added cautiously, and stirring was continued for 24 hr. The dark brown product was collected and washed on the filter successively with 1 l. each of benzene, ethanol, 3 *N* hydrochloric acid, and ethanol. The granular orange product was suspended in a mixture of 1500 ml of a benzene–ethanol mixture (2:1, v/v) and warmed gently on a steam bath until the polymer assumed a light yellow color. It was collected, washed with a large volume of ethanol, and vacuum dried at 100°. The yield was 67 g. *Anal.* Found: I, 0.26 and 0.23.

The infrared spectrum (Nujol mull) exhibits bands at 2.95, 8.0, and 12.2 μ assignable, respectively, to –OH, ═COC–, and *para*-disubstituted benzene, as well as other characteristic bands at 6.62 and 7.70 μ.

Methoxytrityl Chloride Polymer 4. A suspension of *ca.* 60 g of the alcohol polymer **3** in 1 l. of benzene and 100 ml of acetyl chloride was boiled under reflux for 12 hr with exclusion of moisture. The product was collected in a dry atmosphere, washed with 1 l. of dry benzene and 1 l. of dry petroleum ether, and dried under high vacuum over phosphorus pentoxide at room temperature. It was stored and handled in a drybox. The product, a slightly yellow free-flowing solid, was obtained in essentially quantitative yield. *Anal.* Found: Cl, 2.6 and 2.6.

The chloride content of the polymer corresponded to *ca.* 730 μmoles/g.

A 1-g portion of the polymer was agitated for 20 hr in a mixture of 20 ml of pyridine containing 100 μl of water. The product was washed with pyridine, benzene, and hexane, and dried. It was virtually free of chlorine. *Anal.* Found: Cl, 0.12, 0.14; methoxyl, 1.8, 1.8.

Methoxytritylthymidine Polymer 5. A. To a solution of 100 mg (415 μmoles) of thymidine[23] in 10 ml of anhydrous pyridine[17]

(13) Prepared by the method of P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 6212 (1958).
(14) W. E. Razzel and H. G. Khorana, *J. Biol. Chem.*, 236, 1144 (1961).
(15) W. E. Razzel and H. G. Khorana, *ibid.*, 234, 2105 (1959).
(16) The conversion figure takes into account the weight gain of the polymer in going from bound thymidine to pyridinium TpTOAc.
(17) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 86, 1630 (1964), and footnote 17 therein.
(18) T. M. Jacob and H. G. Khorana, *ibid.*, 87, 2971 (1965).
(19) Elvanol® 52-22, a medium viscosity grade of polyvinyl alcohol, was obtained from the Electrochemicals Department, E. I. du Pont de Nemours and Co., Inc., Wilmington, Del. 19898.
(20) Ionac Chemical Co., Birmingham, N. J.
(21) A.S.T.M. specifications.
(22) Foote Mineral Co., Exton, Pa.

was added 500 mg of monomethoxytrityl chloride polymer **4** (equivalent to 365 μmoles of chloride), and the mixture was agitated for 48 hr at room temperature. The insoluble polymer **5** was collected and washed exhaustively with dry pyridine until the washings were free of thymidine. A small portion (*ca.* 50 mg) of the polymer was removed under anhydrous conditions, washed with ethanol, and vacuum dried at 60°. A sample (10.0 mg) was refluxed overnight in glacial acetic acid–6 *N* hydrochloric acid (1:1, v/v, 10 ml) to liberate thymine. The hydrolysate was evaporated to dryness on a rotary evaporator, and the residue was shaken with 25.0 ml of 0.01 *N* hydrochloric acid and filtered. Ultraviolet spectral analysis of the filtrate indicated a thymine content of 4.94 μmoles (39.0 OD_{264} units using ε 7900) corresponding to 494 μmoles of T/g of polymer or 77.5% conversion of chloride.

To verify the absence of occluded thymidine a 20-mg portion of the polymer was agitated with 3 ml of pyridine for 20 hr. The extract was completely devoid of thymidine as judged by ultraviolet spectral analysis.

The infrared spectrum of the thymidine polymer exhibits a strong, sharp, slightly asymmetric band at 5.95 μ characteristic of thymidine carbonyl absorption.

B. In 10 ml of anhydrous pyridine was dissolved 400 mg (1650 μmoles) of thymidine, and 20 ml of dry benzene was added to the solution (the solution remained homogeneous). Two grams of the chloride polymer (equivalent to 1455 μg-atoms of Cl) was added, and the mixture was agitated for 40 hr. A white crystalline solid deposited on the wall of the reaction vessel, presumably pyridine hydrochloride. The polymer was collected, washed, and dried as described above, to obtain 2.13 g of product. Thymine analysis indicated 610 μmoles of T/g. The product was shown to be free of occluded thymidine.

TpTOAc Polymer 6. To the remaining pyridine-swollen thymidine polymer **5** from procedure A (*ca.* 450 mg, equivalent to 220 μmoles of bound thymidine) was added 900 μmoles of 3′-O-acetylthymidine 5′-phosphate[11] in 4 ml of anhydrous pyridine, 1.8 g of dicyclohexylcarbodiimide, and 4 ml of anhydrous dimethylacetamide, and the suspension was agitated for 5 days at room temperature. The polymer beads were collected and washed exhaustively with pyridine, then with ethanol, and the product (**6**) was dried under vacuum at room temperature. Exhaustive hydrolysis with acetic acid–hydrochloric acid indicated a thymine content of 706 μmoles/g (58.5 OD_{264} units from 10.47 mg of polymer). To liberate thymidylyl-(3′→5′)-thymidine a portion of the dried polymer (10 21 mg) was agitated in 2 ml of a solution of 80% aqueous acetic acid saturated with benzene ($HOAc–H_2O–C_6H_6$, 32:8:10, v/v) for 5 hr at room temperature. The hydrolysate was filtered, concentrated, treated with concentrated ammonium hydroxide for 1 hr, and subjected to paper chromatography in solvent A. Two bands were resolved, one with chromatographic mobility identical with that of authentic TpT[13,24] and the second corresponding to thymidine (representative R_f 0.67). The yield of isolated TpT (41.6 OD_{267} units or 2.25 μmoles using ε 18,500[13]) corresponded to 220 μmoles/g of polymer or 54% conversion of polymer-bound thymidine. The isolated thymidine amounted to 31 μmoles/g.

When the products released by acetic acid were chromatographed directly without ammonia treatment TpTOAc was isolated (R_f 0.55 in solvent C *vs.* R_f 0.42 for TpT).

TpTpTOAc Polymer 8. A TpTOAc polymer similar to **6** (*ca.* 300 mg, TpTOAc content 78 μmoles/g) was deacetylated by agitating it for 3 days at room temperature with 10 ml of pyridine–concentrated NH_4OH (3:1, v/v). The polymer was collected and washed with 10% acetic acid in pyridine for 30 min, and then with dry pyridine and DMAc. The swollen polymer was treated with 240 μmoles of pTOAc and 0.24 g of DCC in a solvent mixture of 1.2 ml of dry pyridine and 2 ml of DMAc for 4 days and worked up as described above. Aqueous acetic acid–benzene hydrolysis of 16.6 mg of polymer followed by ammonia treatment and paper chromatography in solvent A afforded three components corresponding to TpTpT (R_f 0.21, 13.5 OD_{267} units), TpT (21.6 OD_{267} units), and thymidine (6.6 OD_{267} units). The TpTpT isolated from polymer **8** thus corresponded to 29 μmoles/g,[25] representing 38% conversion of TpT.

The R_f value for TpTpT in solvent A varied between 0.21 and 0.25 among various runs and the mobility referred to that of pT (R_{pT}) varied between 1.65 and 1.85. In solvent C the R_f was 0.24 and the R_{pT} 0.69.[26]

Enzymic Hydrolyses. Spleen phosphodiesterase hydrolyses were conducted by treating lyophilized oligonucleotide preparations (*ca.* 0.5 μmole) with 30 μl of enzyme solution[27] and incubating at 37° for 5–6 hr. The hydrolysate was chromatographed in solvent A to reveal complete hydrolysis of TpTpT to Tp and T in a mole ratio of 1.75:1 in one experiment and 2.0:1 in another. In a similar manner TpT was completely hydrolyzed to Tp and T. Snake venom phosphodiesterase[28] (25 μl of solution/0.2 μmole of oligonucleotide, 5–6 hr) also completely degraded TpTpT and TpT to pT and T.

Acknowledgment. We wish to thank Miss Eleanor Applegate for technical assistance, and Misses B. K. Londergan and L. E. Williams and Messrs. B. R. Stevens and A. Vatvars for elemental analyses. Helpful discussions with Dr. R. E. Benson are also gratefully acknowledged.

(23) P-L Biochemicals, Inc., Milwaukee, Wis.

(24) The R_f for TpT in solvent A varied between 0.32 and 0.45 among several runs. In solvent C the R_f was 0.40–0.42.

(25) Using ε 28,100 for TpTpT, *i.e.*, the sum of those for pT (9600) and TpT (18,500).

(26) For TpTpT ref 14 reports R_f 0.20, R_{Tp} 1.35 in solvent A, and R_f 0.20, R_{Tp} 0.67 in solvent C. G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962), report R_f 0.23, R_{Tp} 1.75 in solvent A.

(27) Worthington Biochemical Corp., Freehold, N. J. The lyophilized enzyme (10–15 units) was dissolved in 1 ml of 0.03 *M* succinate hydrochloride buffer, pH 6.5. The solution was stored at −20°.

(28) Calbiochem, New York, N. Y. The lyophilized enzyme from Russels viper was dissolved in 0.15 *M* Tris–HCl buffer, pH 8.9, to a concentration corresponding to 800 units of enzyme/ml.