Allomerization of deuteriochlorophyll a (120 mg) was carried out in essentially the same way, using 18 ml of methanol. The product corresponding chromatographically to 10-hydroxydeuteriochlorophyll a was isolated (51 mg) and found to have the resonances associated with the 10-hydroxyl and the δ protons (see Table I).

Chlorophyll b (105 mg) was dissolved in methanol (17.3 ml). Dissolution of the chlorophyll b was much slower than for a. After 3 days, the solution was processed as described above for the chlorophyll a allomerization. Again, there were two major fractions, but they did not separate as well as the products did in the a series. Nmr spectra clearly indicated that the product obtained in lowest yield (34 mg) was the same as the enzymatically produced 10-hydroxychlorophyll b. The nmr spectrum of the second major product (54 mg) was consistent with the 10-methoxy lactone formulation (see Table II).

Allomerization of deuteriochlorophyll b was analogous to that of chlorophyll b.

Spectra and Molecular Weights. Nmr spectra were recorded on a Varian HA-100 spectrometer by techniques previously described.[5] All nmr spectra were recorded on tetrahydrofuran-d_8 solutions. Chemical shifts are given in δ, ppm, downfield from hexamethylsiloxane (HMS). Infrared spectra were recorded on a Beckman IR-7; approximately 10% w/w solutions were used in an Irtran cell.[4] Absorption spectra in the visible and ultraviolet were recorded on a Cary 14 spectrophotometer. Molecular weights were measured on a Mechrolab vapor phase osmometer.[4]

Studies on Polynucleotides. LXXII.[1] Deoxyribooligonucleotide Synthesis on a Polymer Support[2]

H. Hayatsu and H. G. Khorana

Contribution from the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received February 10, 1967*

Abstract: The concept of carrying out stepwise deoxyribooligonucleotide synthesis on a polystyrene support, which permits condensation reactions in completely homogeneous organic medium such as pyridine, has been successfully developed. A portion of the total phenyl groups in polystyrene was derivatized to yield monomethoxytrityl chloride groups. Condensation reactions with deoxyribonucleosides afforded polystyrene-supported 5′-monomethoxytrityl deoxyribonucleosides. Previously developed methods involving condensations with protected deoxyribonucleoside 5′-phosphates were now used for internucleotide bond synthesis. After condensations, polymer-supported deoxyribooligonucleotides were separated from the excess of reagents and mononucleotide by precipitation from an aqueous medium. Methods for the removal of protecting groups were adapted for work with polymer-supported compounds. The compounds prepared by the new method include: thymidylyl-(3′→5′)-thymidine, thymidylyl-(3′→5′)-deoxycytidine, thymidylyl-(3′→5′)-deoxyadenosine, thymidylyl-(3′→5′)-deoxyguanosine, and a trinucleotide, thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine. The yield at each internucleotide bond synthesis ranged between 88 and 96%.

Chemical synthesis of polynucleotides containing defined nucleotide sequences has formed the subject of extended investigations in recent years and the methods developed have been used successfully in the synthesis of a large variety of ribo- and deoxyribopolynucleotides.[3] In all of the synthetic work hitherto reported, the products obtained after each condensation step to form internucleotide bonds are separated by time-consuming procedures involving mainly column chromatography. Marked rapidity in synthetic work would result if after the condensation reactions the product is present in a form readily separable from the remainder of the reaction components. This concept, while having been expressed in literature from time to time,[4] has recently been developed with striking success by Merrifield for the synthesis of polypeptides.[5] In the Merrifield procedure, a polypeptide chain is built up in a stepwise manner from one end while it is linked by a covalent bond at the other end to an insoluble polymeric support. Shemyakin and co-workers have more recently reported an alternative approach to polypeptide synthesis in which the polymer carrying the growing peptide chain is soluble in the medium of reaction, and therefore the repetitive condensations are performed in completely homogeneous phase.[6]

Clearly, it would be desirable to develop the use of similar concepts for work in the polynucleotide field and in this paper we report on an approach to deoxyribooligonucleotide synthesis by the use of a soluble polymer as a support. In concept, therefore, the approach is similar to that used previously by Shemyakin and co-workers in the peptide field. Preliminary reports of this work have already appeared.[7] A number of other laboratories have also reported activity in this area. Thus, Letsinger and Mahadevan[8] reported an

(1) Paper LXXI in this series is by R. D. Wells and J. Blair, *J. Mol. Biol.*, in press.

(2) This work has been supported by grants from the Life Insurance Medical Research Fund (Grant No. G-62-54), the National Science Foundation (Grant No. GB-3342), and the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178).

(3) Selected references which give literature citations are: H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965); R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *ibid.*, **88**, 819 (1966); and H. G. Khorana, H. Büchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *ibid.*, **89**, 2154 (1967).

(4) I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, **35**, 873 (1966).

(5) For a review see R. B. Merrifield, *Science*, **150**, 178 (1965).

(6) M. M. Shemyakin, Y. A. Ovchinnikov, A. A. Kinyushkin, and I. V. Kozhevnikova, *Tetrahedron Letters*, 2323 (1965).

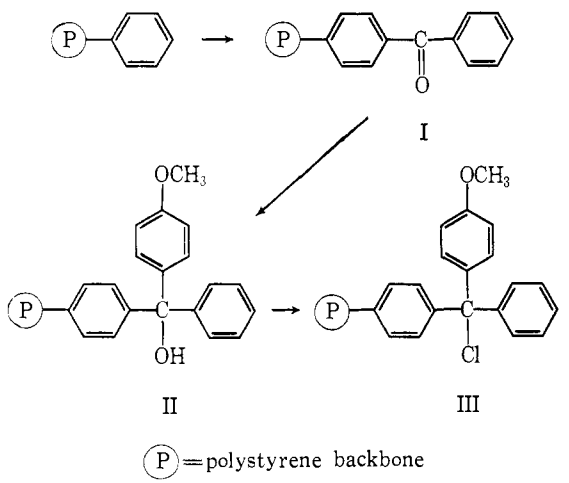
(7) H. Hayatsu and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 3182 (1966); Abstracts, 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966, p 59C.

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

approach which uses a popcorn type of insoluble polymer as the support. Cramer and co-workers[9] have briefly outlined an approach essentially identical with that reported here while a similar approach but using an insoluble polymer as the support has been developed by Melby and Strobach.[10]

Preparation of Polymer Support. Polystyrene having *p*-methoxytrityl groups as a part of its structure was chosen as the support for deoxyribooligonucleotide synthesis. Considerations for this choice were as follows. While a number of ways may be conceived for covalently linking the first nucleoside or nucleotide to a polymer support, a major consideration was the chemical principles and protecting groups which are in current use for deoxypolynucleotide synthesis. The use of a methoxytrityl group as a point of linkage of the first deoxyribonucleoside to a polymer was therefore an attractive possibility. Secondly, methoxytrityl derivatives of polystyrene and the protected oligo- and polynucleotides built onto this polymer were all expected to be freely soluble in anhydrous pyridine, which is used most often as the medium of reaction in polynucleotide synthesis. Thirdly, the introduction of the methoxytrityl groups was expected to be straightfoward using the reaction sequence shown in Chart I. Finally,

Chart I. Preparation of Polystyrene-Supported *p*-Methoxytrityl Chloride



the extent of derivatization of the phenyl groups in polystyrene to form the benzophenone as well as the subsequent formation of the methoxytrityl derivatives could be easily controlled by varying the reaction conditions, thus enabling a wide study of this type of polymer support.

Polystyrene of average molecular weight 270,000 was subjected to a Friedel–Crafts reaction with benzoyl chloride and aluminum chloride. The amounts of the reagents used were such that approximately half of the phenyl groups of the polystyrene could be converted into the benzophenone derivative. Under the reaction conditions used probably optimal conversion to the ketone took place. As judged by the increase in weight of the product isolated, at least 30% of the total phenyl groups had in fact been derivatized. The ultraviolet and infrared absorption characteristics of the product were as expected for the benzophenone derivative I. This product was brought into reaction with the Grignard reagent, *p*-methoxyphenylmagnesium bromide, to give the trityl alcohol derivative II. Because of the insolubility of I in ether, benzene was used as the solvent for it, the Grignard reagent being introduced as its ethereal solution. Although the reaction mixture was heterogeneous, the reaction appeared to be extremely rapid. The product isolated after work-up gave a coloration on acidification which is characteristic of the *p*-methoxytrityl cation.[11] Treatment of II with acetyl chloride gave the trityl chloride III which was isolated as a fluffy white powder. Determination of the active trityl chloride groups as measured by the capacity of the preparation to react with methanol in pyridine showed a halide content of 0.4 mmole/g of the derivatized polymer. It would therefore appear that in the above preparation, out of every 100 styrene units, 25–35 have benzophenone groups and 5–7 have trityl chloride groups. The trityl content in this polymer was lower than that expected from the amount of the Grignard reagent used (2 mmoles/g of the benzophenone). In several repetitions of the Grignard reaction, the amounts of the reagent used were varied.[12] One preparation carried out using 3 mmoles of the Grignard reagent per gram of the polymer gave a trityl chloride with higher capacity to react with thymidine (see below). While in all these experiments the Grignard step was not easy to control because of the heterogeneity of the reaction mixture, the aim was to convert only a portion of the benzophenone groups to trityl groups.

All the work on deoxyribooligonucleotide synthesis described herein has been carried out with trityl chlorides which started with a polymer containing a high benzophenone content as described above. In more recent experiments, a polymer derivative with a low (3%) ketone content has been used. At the Grignard reaction step a homogeneous solution is obtained and a large excess of the reagent is used so as to convert essentially all of the ketone groups to the trityl groups. The resulting preparation of polystyrene methoxytrityl chloride appears to offer distinct advantages in synthetic work aimed at the construction of longer polynucleotide sequences. The preparation of the polymer-supported trityl chloride with the low ketone content is included in the Experimental Section. Synthetic work using these modified polymers will be reported upon in a forthcoming paper.

Deoxyribooligonucleotide Synthesis. The typical steps used are shown in Chart II. The first step was the attachment through a covalent linkage of deoxyribonucleosides to the derivatized polymer. Ⓟ-Tr-(OCH_3)-Cl[13] was allowed to react with an excess of dry thymidine in pyridine. This treatment was followed

(9) F. Cramer, R. Helbig, H. Hettler, K. H. Scheit, and H. Seliger, *Angew. Chem.*, **78**, 640 (1966).

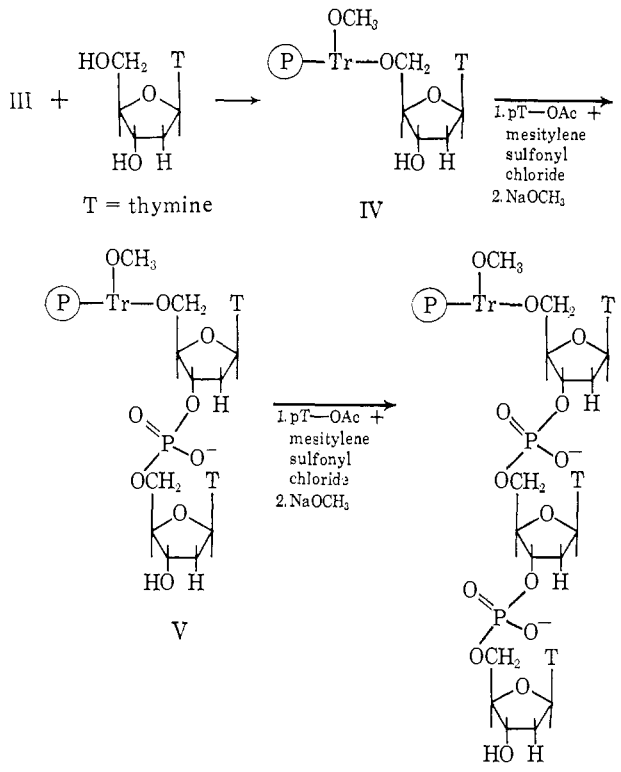
(10) L. R. Melby and D. R. Strobach, *J. Am. Chem. Soc.*, **89**, 450 (1967).

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

(12) In one experiment in which a larger amount (5 mmoles/g of I) of the Grignard reagent was used the product was found to have an extremely high viscosity and a low solubility in organic solvents such as chloroform or benzene.

(13) Abbreviations for protected mononucleotides are as have been used in previous papers [see, *e.g.*, H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3841 (1963)]. The polymer-supported methoxytrityl chloride is abbreviated Ⓟ-Tr(OCH_3)-Cl, and the oligonucleotides supported on this polymer are abbreviated in the usual way; thus Ⓟ-Tr(OCH_3)-T for polymer-supported methoxytritylthymidine, Ⓟ-Tr(OCH_3)-TpT for the corresponding thymidylylthymidine, and so on.

Chart II. Deoxyribooligonucleotide Synthesis Using Polystyrene-Supported Methoxytrityl Chloride (III)



by the addition of methanol so as to convert unreacted trityl chloride groups to the corresponding methyl ether. Since a large excess of the nucleoside was used, it was not necessary to protect the 3′-hydroxyl group for ensuring selective reaction with the 5′-hydroxyl group. The thymidine content of the polymer-supported methoxytritylthymidine was determined by release of thymidine with acid (see below) and extraction into an aqueous medium. Using a preparation of the polymer-supported methoxytrityl chloride, which showed a chloride content of 0.4 mmole/g of the polymer derivative, the thymidine content was found to be 60 μmoles/g of the Ⓟ-Tr(OCH$_3$)-T (IV). Thus, in this case, only about 15% of the total trityl chloride groups had accepted thymidine. This result suggests that there is considerable steric hindrance around the trityl groups in the polymer.

Using several other preparations of Ⓟ-Tr(OCH$_3$)-Cl, in which the Grignard reaction was carried out by using a larger proportion of the reagent, the thymidine content after reaction with an excess of thymidine was higher. Thus, in three preparations of Ⓟ-Tr(OCH$_3$)-T, thymidine content varied between 200 and 340 μmoles/g of the polymer derivative.

It was important to establish (1) that the attachment of thymidine to the polymer was through a covalent linkage, (2) that the linkage was acid labile as expected for a trityl ether, and (3) to develop suitable nonaqueous conditions for the acid-catalyzed release of the nucleoside. (The conventional method, aqueous acetic acid, for this purpose was not applicable because of the insolubility of the polymer in both water and glacial acetic acid.) In accordance with expectation, thymidine linked to the polymer was stable to repeated precipitation of Ⓟ-Tr(OCH$_3$)-T from aqueous medium and to alkaline treatment. Only by an acidic treatment was thymidine released in the free form. A variety of acidic conditions was investigated for this purpose. With very small amounts of trifluoroacetic acid in chloroform (1:6000, v/v) the release was complete in 10 min at room temperature; with 0.1% (v/v) dichloroacetic acid in chloroform it was complete in 1 hr; and with 20% acetic acid in chloroform (v/v) it was complete in 2 days.

For the analogous attachment of other deoxyribonucleosides to the polymer derivative, the N-protected compounds, N-benzoyldeoxyadenosine,[11] N-anisoyldeoxycytidine,[11] and N-benzoyldeoxyguanosine[14] were used. In one preparation of Ⓟ-Tr(OCH$_3$)-dA^{Bz}, deoxyadenosine content was found to be 35 μmoles/g of the polymer derivative. The experience with other nucleosides is very limited so far. One preparation of Ⓟ-Tr(OCH$_3$)-dC^{An} had a deoxycytidine content of 8.4 μmoles/g and the attachment of deoxyguanosine has so far been effected only in a very small amount (1 μmole/g of the polymer).

For internucleotide bond synthesis, a study was first performed on the rate of condensation of 3′-O-acetylthymidine 5′-phosphate (pT-OAc) with Ⓟ-Tr(OCH$_3$)-T. A large excess (about 40-fold with respect to thymidine on the polymer) of the protected mononucleotide and a proportionate excess of the condensing agent, mesitylenesulfonyl chloride, was used in order to maintain a concentration of activated phosphomonoester group comparable to those used in extensive previous syntheses. The condensation product Ⓟ-Tr(OCH$_3$)-TpT (V) was isolated simply by pouring the reaction mixture in an aqueous medium followed either by filtration or by centrifugation. The extent of reaction was determined by paper chromatographic analysis after releasing the nucleotidic products from the polymer with trifluoroacetic acid. As seen in Table II, the yield (93%) of TpT reached a plateau within 2 hr.

Applying similar reaction conditions, the reaction of Ⓟ-Tr(OCH$_3$)-T with the other three protected deoxyribonucleoside 5′-phosphates gave the protected products, Ⓟ-Tr(OCH$_3$)-TpA^{Bz}-OAc, Ⓟ-Tr(OCH$_3$)-TpC^{An}-OAc, and Ⓟ-Tr(OCH$_3$)-TpG^{Ac}-OAc. The yields in all cases were nearly quantitative.

From previous work, the removal of N-protecting groups, especially that of the benzoyl group on adenine, is required before the acidic treatment to cleave the trityl ether linkage. Treatment of the polymer-supported protected dinucleotides with either methanolic ammonia or aqueous ammonia could obviously not be used. Benzylamine, however, was found to dissolve the polymer and this amine was effective in removing the protecting groups. This method was actually used in the case of Ⓟ-Tr(OCH$_3$)-dA^{Bz} and Ⓟ-Tr(OCH$_3$)-TpA^{Bz}-OAc and the results were satisfactory.[15]

(14) Unpublished work of Dr. H. Weber in our laboratory. The N-benzoyldeoxyguanosine was prepared by benzoylation of deoxyguanosine with benzoyl chloride followed by selective de-O-benzoylation[11] of the fully acylated product. We thank Dr. Weber for a generous gift of this compound.

(15) In unpublished work, Dr. H. Weber, in our laboratory, has shown that when an alkylamine such as *n*-butylamine is used to remove the N-anisoyl group from N-anisoyldeoxycytidine, a side product is formed. A similar side product has also been observed when benzylamine was used to remove the N-anisoyl group. The benzylamine procedure therefore cannot be used when N-anisoyldeoxycytidine is present in the oligonucleotide chain to be constructed on the polymer support.

The next requirement for oligonucleotide chain elongation was the selective removal of the acetyl group at the 3′-hydroxyl end. A new procedure using organic solvents was developed for the purpose. In model experiments, treatment of the protected mononucleotides, pT-OAc, d-pA^{Bz}-OAc, d-pC^{An}-OAc, and d-pG^{Ac}-OAc, with a mixture of dimethyl sulfoxide, pyridine, and 1 *M* sodium methoxide in methanol was found to give at room temperature complete de-O-acetylation in 2 min. Under these conditions, the N-protecting groups were completely stable for 10 min, the maximum time period tested. Using this method, Ⓟ-Tr(OCH$_3$)-TpT was prepared from the corresponding 3′-*O*-acetyl derivative and was subjected to a repeat condensation with pT-OAc. The product Ⓟ-Tr(OCH$_3$)-TpTpT-OAc was treated with alkali and after precipitation was treated with acid to release the oligonucleotidic material. The latter was analyzed by a combination of the techniques of paper chromatography and paper electrophoresis. The yield at this second step of internucleotide bond synthesis was 88%.

All of the products, TpT, d-TpC, d-TpA, d-TpG, and TpTpT, were checked for their purity in paper chromatography (two solvents) and paper electrophoresis and were completely susceptible to the action of spleen phosphodiesterase, thus showing the exclusive presence of C$_3$′–C$_5$′ internucleotidic linkages in them.

Discussion

The approach described incorporates all of the principles previously developed for construction of deoxyribopolynucleotide chains containing predetermined sequences. Thus, in brief, the 5′-hydroxyl group of the terminal nucleoside is protected by a methoxytrityl group and chain elongation occurs by successive condensations with the 3′-hydroxyl group. Further, the use of a soluble polymer support such as developed here appears to be theoretically preferable to the use of an insoluble polymer support. Using the soluble polymer support, it would be hoped that the repetitive condensation steps would always be carried out in completely homogeneous medium and therefore no sharp or sudden deviation at any stage in the pattern of kinetics or yields would be expected. In contrast, in work with insoluble polymers the yields obtained as a function of polynucleotide chain length could be unpredictable. One conceivable concern about the use of soluble polymer support would be that as the size of the growing polynucleotide chain increases, the polymer may show a change in solubility properties. This would obviously be determined by the ratio of the total nucleotidic material carried by the polymer to the amount of the polystyrene backbone. It is, in fact, from a consideration of this reason that although it was possible to attach much higher amounts of thymidine to Ⓟ-Tr(OCH$_3$)-Cl, the oligonucleotide syntheses described were carried out with a polymer derivative having a thymidine content of about 50 μmoles/g. It can be seen that if the chain built onto this polymer were the size of a decanucleotide, the increase in its weight would be about 20%.

A prerequisite for success of the concept of polymer support synthesis is that the yield at every repetition of internucleotide bond synthesis be as close to 100% as possible. The yields obtained in the present work were high (88–96%) but further improvement along this line would be desirable. Further work with this approach in the synthesis of longer oligonucleotide chains will certainly provide additional critical evaluation of the efficiency in repetitive condensations.

Finally, specific improvements and modifications at different steps, especially removal of N- and O-protecting groups, continue to be made. Further, the experience with the release of oligonucleotide chains containing purine nucleosides by acidic treatment is limited so far. It is possible that the methoxytrityl group may have to be replaced by the dimethoxytrityl group, which would be more acid sensitive. This modification could in principle be introduced simply by carrying out a Friedel–Crafts reaction with anisoyl chloride in place of benzoyl chloride. Work along the various lines indicated will be reported in subsequent papers.

Experimental Section[16]

Materials and Methods. Polystyrene used throughout this work was a preparation with an average molecular weight of 270,000 with atactic configuration of the skeleton. This preparation (lot no. 683) and several others with different molecular weights were kindly supplied by Dr. E. T. Dumitru of Dow Chemical Co., Midland, Mich. Reagent grade carbon disulfide was dried over calcium chloride prior to use. Dry pyridine was prepared by distillation of reagent grade pyridine over potassium hydroxide and keeping the distilled portion over Linde Molecular Sieve Type 4A. Benzoyl chloride and acetyl chloride were redistilled before use. Reagent grade benzene was dried by keeping it over calcium hydride for more than a week. Cyclohexane was dried over sodium sulfate. Chloroform, which was used as solvent for the acidic treatment of polymer-supported nucleotidic materials, was prepared by treatment of reagent grade chloroform with aqueous sodium carbonate, washing with water, and drying over sodium sulfate. Mesitylenesulfonyl chloride was recrystallized from pentane and stored in a desiccator over phosphorus pentoxide.

Paper chromatography was carried out by the descending technique using the following solvent systems: solvent 1, 2-propanol–concentrated ammonia–water (7:1:2, v/v); solvent 2, 1-butanol–acetic acid–water (5:2:3, v/v); solvent 3, ethanol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v).

Paper electrophoresis was performed using 0.03 *M* potassium phosphate buffer, pH 7.1, at 80–100 v/cm for 20–30 min. For checking of purity of nucleotidic compounds by paper chromatography or paper electrophoresis, at least 3 OD units (260 mμ) of the compound was used per spot. R_f's and electrophoretic mobilities of different compounds are listed in Table I.

Table I. R_f Valus of Compounds in Paper Chromatography and in Paper Electrophoresis

Compound	Paper chromatography Solvent system 1	2	Paper electrophoresis at pH 7.1[a]
Thymidine	0.67	0.67	0
Thymidine 5′-phosphate	0.13	0.35	1.0
Thymidylyl-(3′→5′)-thymidine	0.41	0.35	0.45
Thymidylyl-(3′→5′)-deoxycytidine	0.34	0.33	0.47
Thymidylyl-(3′→5′)-deoxyadenosine	0.40	0.37	0.47
Thymidylyl-(3′→5′)-deoxyguanosine	0.22	0.26	0.46
Thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine	0.17	0.27	0.75

[a] Relative mobilities compared to those of thymidine 5′-phosphate and thymidine.

(16) OD refers to the absorbance value obtained for a 1-ml solution of a compound measured in a quartz cuvette with a 1-cm light path. The number in subscript indicates the wavelength at which the measurement was taken.

All evaporations were carried out under reduced pressure at room temperature. For internucleotide bond synthesis great care was taken to exclude moisture from the reaction mixtures. For this purpose any release of vacuum or opening of the reaction vessel was carried out in the atmosphere of a drybox in which relative humidity was less than 10%. Adequate stirring was always maintained during precipitation of the polymer or polymer-supported nucleotidic material which was carried out by pouring a dilute solution of the polymer into a large volume of a second liquid medium in which the polymer was insoluble.

Spleen phosphodiesterase digestion of the oligodeoxynucleotides was carried out according to the previously reported procedure.[17]

The calculated OD_{280}/OD_{260} ratios are based on the assumption that the absorption spectra of the oligonucleotides are the sum of the spectra of constituent nucleosides.[18]

Removal of Protecting Groups. Removal of 3′-O-acetyl groups in polymer-supported oligonucleotides was carried out in a mixture of dimethyl sulfoxide–pyridine and 1 *M* sodium methoxide in methanol. The conditions used are described in individual experiments. In model experiments with protected mononucleotides (d-pC^{An}-OAc, d-pA^{Bz}-OAc, d-pG^{Bz}-OAc, and d-pG^{Ac}-OAc) when a 5-mg sample was treated with a mixture of dimethyl sulfoxide (0.25 ml), pyridine (0.20 ml), and 1 *M* sodium methoxide in methanol (0.05 ml) at room temperature, de-O-acetylation was found to be complete in 2 min, and in a period up to 10 min, no de-N-acylation was observed for any of the protected derivatives.

Removal of the N-protecting groups from polymer-supported nucleosides and oligonucleotides was carried out by keeping their solution in benzylamine at room temperature for periods shown in individual experiments.

Preparation of Monomethoxytrityl Chloride Derivative of Polystyrene (Ⓟ-Tr(OCH_3)-Cl). Preparation I. a. Benzophenone Derivative Ⓟ-COC_6H_5. To a mechanically stirred suspension of anhydrous aluminum chloride (6.8 g) in carbon disulfide (100 ml) was added a solution of polystyrene (10 g) and benzoyl chloride (6 ml, 51 mmoles)[19] in carbon disulfide (50 ml). The reaction was commenced by gentle warming in a glycerol bath when a vigorous evolution of hydrogen chloride was observed. Soon a very viscous solution resulted and then red polymeric material separated out from the reaction mixture. The mixture was heated under reflux for 3 hr. At the end of the period the evolution of hydrogen chloride was almost undetectable. After cooling, the mixture was poured into ice water (1.5 l.) along with chloroform (200 ml), and this mixture was stirred for 2.5 hr. The red semisolid complex which was initially present had now completely dissolved and only the two liquid phases were present. The mixture was allowed to stand until the phases separated, and the lower organic layer was collected. The aqueous phase was extracted with chloroform (150 ml). The combined organic phase was then washed with aqueous sodium carbonate and water. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in benzene (180 ml) and the solution poured into pentane (2 l.) under stirring. The precipitate was collected by filtration and washed with pentane. The product was dried under vacuum over phosphorus pentoxide to give 13.2 g of a white powder. Theoretical yield (assuming quantitative utilization of benzoyl chloride and quantitative recovery of the resulting product) is 15.4 g. Therefore, as judged by the increase in weight, at least 30%[20] of the benzene residues were converted to benzophenone derivatives. The ultraviolet absorption spectrum showed λ_{max} 256–262 mμ in dioxane and the infrared spectrum taken in $CHCl_3$ showed an absorption band at 6.1 μ.

***b.* *p*-Monomethoxytrityl Alcohol Ⓟ-Tr(OCH_3)-OH.** A Grignard reagent was prepared from *p*-bromoanisole (0.51 ml) and magnesium (100 mg) using dry ether (5 ml) as a solvent and by heating the mixture under reflux for 3 hr under exclusion of moisture. This Grignard reagent was added to a vigorously stirred solution of Ⓟ-COC_6H_5 (2 g) in dry benzene (50 ml). A yellow precipitate appeared immediately and stirring was continued for 30 min at room temperature. Concentrated hydrochloric acid (20 ml) was added to decompose the solid complex, and the mixture was stirred for 10 min. The resulting brown organic phase was separated and washed successively with water, aqueous sodium carbonate, and again water. If an emulsion resulted, which was often the case during these washings, it was broken by adding a small amount of ethanol to the mixture. The organic phase was finally dried over sodium sulfate and evaporated to dryness. The dry residue was then dissolved in benzene (40 ml), and the product was precipitated by pouring the solution into pentane (500 ml). The precipitate was collected by filtration and washed with pentane. The powder obtained after drying weighed 2 g. When a solution of this material in chloroform or benzene was treated with 60% aqueous perchloric acid or trifluoroacetic acid, an orange coloration characteristic of monomethoxytrityl derivatives[11] was observed in the organic phase (λ_{max} 256–262 mμ in dioxane).

c. *p*-Monomethoxytrityl Chloride Derivative Ⓟ-Tr(OCH_3)-Cl. A solution of Ⓟ-Tr(OCH_3)-OH (1.2 g) in a mixture of cyclohexane (4 ml) and acetyl chloride (8 ml) was heated under reflux for 30 min. After this time, evolution of hydrogen chloride, which was vigorous in the first 10 min of heating, had mostly subsided. After cooling, the reaction mixture was concentrated under reduced pressure to remove most of the acetyl chloride. The concentrated solution (one-third to one-fourth of the original volume) was diluted with dry benzene (30 ml) and the solution poured into pentane (0.5 l.) under stirring. The precipitate thus formed was collected by centrifugation and washed with pentane (three 50-ml portions). The white powder after drying weighed 1.1 g and gave a positive copper flame test.

Estimation of active chloride was performed in the following manner. A solution of the trityl chloride (Ⓟ-Tr(OCH_3)-Cl, 70 mg) in a mixture of pyridine (4 ml) and methanol (0.4 ml) was heated at 70–75° (bath temperature) for 1 hr. After cooling, the solution was poured into aqueous 2% sodium nitrate (75 ml) under stirring. The polymer thus precipitated was removed by filtration and the filtrate was concentrated to a volume of approximately 5 ml under reduced pressure to remove most of the pyridine and methanol. To the concentrated solution, water (45 ml) and 0.1 *M* potassium chromate (1 ml) were added. This solution was then titrated with freshly prepared 0.04 *M* silver nitrate. The consumption of the silver nitrate solution by this test solution was 0.70 ml whereas a blank (75 ml of 2% aqueous sodium nitrate) reached an end point on addition of no more than 0.05 ml of the silver nitrate solution. From this titration the active chloride was calculated to be 0.4 mmole/g of the derivatized polymer.

In another run of active chloride assay, which was carried out for another lot of Ⓟ-Tr(OCH_3)-Cl prepared from polystyrene in a similar fashion as described above, 300 mg of the Ⓟ-Tr(OCH_3)-Cl consumed 2.50 ml of 0.04 *M* silver nitrate, this value corresponding to 0.33 mmole of active chloride/g of the polymer.

Monomethoxytrityl Chloride Derivative of Polystyrene (Ⓟ-Tr(OCH_3)-Cl). Preparation II. A second preparation in which the proportion of benzophenone groups was much reduced was prepared as follows.

a. Benzophenone Derivative. To a stirred suspension of aluminum chloride (450 mg, 3.3 mmoles) in carbon disulfide (180 ml) was added a solution of polystyrene (9.4 g, 90 mmoles in styrene units) and benzoyl chloride (0.36 ml, 3 mmoles) in carbon disulfide (70 ml). The mixture was heated under reflux with stirring under exclusion of moisture for 18 hr. The reaction mixture was then cooled and the solution washed with water (two 200-ml portions), aqueous sodium carbonate (two 100-ml portions), and water (two 100-ml portions) successively. The organic phase was dried with sodium sulfate and evaporated to dryness. The residual oil was taken up in benzene (150 ml) and the benzene solution poured into pentane (3 l.). The precipitate was collected by filtration, washed with pentane, and dried. The white solid was precipitated again by pouring its dry benzene solution into an excess of pentane. This product was again collected and dried, yield being 8.9 g.

b. Monomethoxytrityl Alcohol Derivative. A Grignard reagent was prepared by heating under reflux a mixture of *p*-bromoanisole (0.63 ml, 5 mmoles), magnesium (125 mg, 5 g-atoms), and a small piece of iodine in dry ether (5 ml) for 7 hr under exclusion of moisture. The Grignard reagent in which a small amount (26 ml) of magnesium was still present was then added dropwise into a vigorously stirred solution of the ketone (3 g of the preceding preparation corresponding approximately to 1 mmole of the ketone) in dry benzene (100 ml). A yellow coloration immediately resulted and an increase in viscosity of the reaction mixture was observed. In the transfer of the Grignard reagent, the pieces of magnesium which remained in the original reaction vessel were washed with dry ether

(17) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962).

(18) The spectral data of nucleosides used here were those reported in Schwarz BioResearch, Inc. Catalog, 1966, p 58.

(19) This amount corresponds to the benzoylation of 50% of the total of phenyl groups in 10 g of polystyrene.

(20) This figure does not take into account any loss during recovery of the product. Probably the per cent of benzene groups converted to benzophenone is higher.

(two 4-ml portions), and the ether washings were added to the reaction mixture. The reaction mixture was then heated under reflux for 20 min under exclusion of moisture. After cooling, concentrated hydrochloric acid (10 ml) was added giving a red mixture. After stirring for several minutes, when the color changed to yellow, the organic phase was separated and washed successively with water (two 100-ml portions), aqueous sodium carbonate (two 100-ml portions), and water (two 100-ml portions). The organic phase was then dried and evaporated to dryness. The residual oil was dissolved in benzene (100 ml) and the resulting solution poured into pentane (2 l.). The precipitate thus formed was collected by filtration, washed with pentane, and dried. A yellowish fluffy powder (2.92 g) was obtained as the product. A chloroform solution of this material, on addition of 70% perchloric acid, exhibited orange coloration indicating the presence of mono-*p*-methoxytrityl groups in the product.

c. **Monomethoxytrityl Chloride.** The above trityl alcohol (2 g) was treated with a mixture of cyclohexane (10 ml) and acetyl chloride (20 ml). After heating the solution under reflux for 4.5 hr, the mixture was diluted with dry benzene (70 ml) and the diluted solution was poured into anhydrous pentane (2 l.) under exclusion of moisture. The resulting fine precipitate was collected by centrifugation, washed with pentane, and dried under vacuum over phosphorus pentoxide. The yield was 1.8 g.

Polymer-Supported 5′-O-Monomethoxytritylthymidine (Ⓟ-Tr-(OCH_3)-T). Preparation I of the polymer-supported monomethoxytrityl chloride was used in this and the subsequent preparations. A solution of Ⓟ-Tr(OCH_3)-Cl (0.4 mmole of Cl/g) (500 mg) and thymidine (70 mg)[21] in pyridine (6 ml) was allowed to stand at room temperature for 12 hr and then heated at 65–75° (bath temperature) for 0.5 hr. Methanol (1 ml) was added and the mixture heated at 75° for 0.5 hr. Pyridine (5 ml) was then added and the solution poured into water[22] (200 ml) under stirring. The product (436 mg) was collected by filtration, washed with water, and dried.

The amount of thymidine in this product was determined by treatment with 10% (v/v) trifluoroacetic acid in chloroform at 0° for 2 min followed by extraction of thymidine into 0.5 *N* aqueous ammonium hydroxide and measurement of OD at 267 mμ after neutralization. Thymidine content was thus found to be 6 μmoles/100 mg of the product. A control experiment in which a chloroform solution of Ⓟ-Tr(OCH_3)-T without addition of trifluoroacetic acid was extracted with 0.5 *N* ammonia in a similar manner gave no ultraviolet-absorbing material in the ammoniacal extract.

Determination of the thymidine content of the above preparation of polymer-supported 5′-O-methoxytritylthymidine was carried out after a repeat of the precipitation procedure. The latter involved pouring a pyridine solution of Ⓟ-Tr(OCH_3)-T into an excess of 2% aqueous sodium chloride solution. Thymidine content was found to be unchanged. A sample (42 mg) of Ⓟ-Tr(OCH_3)-T was treated with a mixture (1 ml) of dimethyl sulfoxide–pyridine–1 *M* sodium methoxide in methanol (5:4:1, v/v) at room temperature for 1 hr. On pouring the solution into water and examining the supernatant aqueous phase, no thymidine release in the medium was detected. Thymidine was thus concluded to be bound to the polymer with a covalent bond which is labile in acid but not in neutral or alkaline media.

Polymer-Supported 5′-O-Monomethoxytrityl-N-benzoyldeoxyadenosine. The preparation of Ⓟ-Tr(OCH_3)-Cl used in this experiment was the one that resembled preparation I described above in ketone content but contained a much higher trityl chloride content as judged by reactivity toward thymidine. Thus, this sample of methoxytrityl chloride when treated with an excess of dry thymidine showed a methoxytritylthymidine content of 20–34 μmoles/100 mg of the polymer-supported methoxytritylthymidine. A solution of this preparation of Ⓟ-Tr(OCH_3)-Cl (258 mg) and N-benzoyldeoxyadenosine (300 mg) in pyridine (5 ml) was allowed to stand at room temperature for 3 days. Pyridine (3 ml) and methanol (0.4 ml) were then added, and the solution was allowed to stand at room temperature further for 17 hr. The solution was then poured into 2% aqueous sodium chloride solution (250 ml). The precipitate was collected by filtration and washed with water, methanol, and pentane successively. The dried product weighed 247 mg.

(21) In other experiments, to increase thymidine content, 50–100 mg of dry thymidine per milliliter of pyridine was used.

(22) In later experiments water containing 2% sodium chloride was used instead of pure water to avoid the possible formation of colloidal solutions.

The amount of deoxyadenosine in this product was determined after removal of the N-benzoyl group with benzylamine (3 days). The product was precipitated, dried, and treated with 20% acetic acid in chloroform for 48 hr at room temperature. Paper chromatography (solvent 1) showed that this acid treatment caused 9% cleavage of the glycosidic linkage in deoxyadenosine. The amount of deoxyadenosine on the polymer was found to be 3.5 μmoles/100 mg.

Rate of Release of Thymidine from Ⓟ-Tr(OCH_3)-T by Treatment with Acid. i. **With Trifluoroacetic Acid.** Ⓟ-Tr(OCH_3)-T (20 mg which contained 0.5 μmole of thymidine) was dissolved in dry chloroform (5 ml). To this solution, trifluoroacetic acid solution [1 ml of 0.1% (v/v)] in chloroform was added and the reaction mixture kept at room temperature (25°) in a stoppered flask. Aliquots (1 ml) were removed from time to time and treated with 0.05–0.1 ml of triethylamine. Thymidine was extracted into water (two 1-ml portions), the water phase being separated by centrifugation. The p*H* of the aqueous phase was adjusted to 4–6 with 1 *N* hydrochloric acid and then optical density determinations were performed at 267 mμ. The solutions obtained from all of the aliquots showed ultraviolet absorption spectra characteristic of thymidine. Release of thymidine as a function of time was as follows: 2 min, 57%; 5 min, 84%; 10 min, 100%; 30 min, 105%.

ii. **With Dichloroacetic Acid.** Using a solution of Ⓟ-Tr-(OCH_3)-T (50 mg containing 1.25 μmoles of thymidine) in 10 ml of 0.1% dichloroacetic acid in chloroform the rate determination was carried out in a similar manner as for trifluoroacetic acid described above. Time of reaction and release of thymidine were as follows: 0 min (before the addition of acid), 0%; 5 min, 40%; 10 min, 62%; 20 min, 82%; 40 min, 94%; 1 hr, 100%; 1.5 hr, 96%; 3.5 hr, 101%. A control solution of P-Tr(OCH_3)-T in chloroform to which no acid was added showed no liberation of thymidine after 1.5 hr.

iii. **With Acetic Acid.** A solution of Ⓟ-Tr(OCH_3)-T (50 mg containing 2.5 μmoles of thymidine) in 20% acetic acid in chloroform (v/v; 5.5 ml) was allowed to stand at room temperature in a tightly stoppered flask. Aliquots (1 ml) were withdrawn from time to time and thymidine liberated was extracted with water (two 2-ml portions). The aqueous phase was concentrated under reduced pressure and submitted to paper chromatography using solvent system 1. The single ultraviolet-absorbing spot observed corresponded to thymidine. The spot was eluted with water and the amount of thymidine estimated. A blank solution was prepared by eluting a blank area, parallel in R_f, of approximately the same size as the thymidine spot. Time of reaction and release of thymidine were: 5 hr, 54%; 24 hr, 85%; 48 hr, 99%; 72 hr, 100%.

Kinetic Study of Internucleotide Bond Synthesis. A mixture of Ⓟ-Tr(OCH_3)-T (300 mg containing 7.5 μmoles of thymidine) and pyridinium pT-OAc (132 mg, 300 μmoles) was dissolved in pyridine (3 ml) and the solution evaporated to dryness under reduced pressure. The evaporation with pyridine was repeated two additional times, and the final residue was dissolved in pyridine (2 ml). To this solution mesitylenesulfonyl chloride (128 mg, 600 μmoles) was added followed by addition of more pyridine (1 ml). The reaction mixture was allowed to stand at room temperature. Aliquots (0.6 ml) were taken up from time to time under exclusion of moisture and mixed with pyridine (1 ml) and water (0.1 ml). The aqueous pyridine solutions of aliquots were kept overnight and were each treated with more pyridine (1.5 ml) followed by dimethyl sulfoxide (4 ml) and 1 *M* sodium methoxide in methanol (0.8 ml). The mixtures were allowed to stand at room temperature for 10 min, then poured into 2% aqueous sodium chloride solution (50 ml) under stirring. The resulting precipitates were collected by centrifugation and washed with water and methanol successively. The products were then dried *in vacuo* over phosphorus pentoxide and submitted to analysis of the condensation reaction. For analysis a part of the product in every case was treated with 0.2% (v/v) trifluoroacetic acid in chloroform (2 ml) at room temperature for 10 min. Pyridine (0.1 ml) was added to stop the reaction and the solution evaporated to dryness. The residue was dissolved in pyridine (2 ml) and the solution poured into water (40 ml). The aqueous mixture including the colloidal precipitate was concentrated to a small volume and the precipitate was removed by centrifugation. The supernatant solution was evaporated and the residue submitted to paper chromatographic analysis. The results are given in Table II.

Thymidylyl-(3′→5′)-thymidine (TpT). A mixture of Ⓟ-Tr-(OCH_3)-T (190 mg, containing 9 μmoles of thymidine) and pyridinium pT-OAc (80 mg, 180 μmoles) was dissolved in pyridine (3 ml). The pyridine solution was evaporated to dryness under reduced pressure.

Table II. Kinetic Study of the Internucleotide Bond Synthesis Using Ⓟ-Tr(OCH_3)-T and pT-OAc[a]

Reaction time, hr	Weight of the product submitted to analysis, mg	Amt of products obtained by paper chromatographic analysis: T		TpT		Recovery of nucleosidic and nucleotidic material, μmoles/300 mg[b]	Extent of internucleotide bond synthesis, %
		$OD_{267\ m\mu}$	μmole	$OD_{267\ m\mu}$	μmole		
0.5	35	3.54	0.37	8.62	0.45	7.0	55
1	39	1.62	0.17	15.15	0.79	7.4	82.5
2	34	0.52	0.05	13.99	0.73	7.0	93
4	35	0.39	0.04	12.59	0.65	6.2	94
10	38	0.21	0.02	14.20	0.74	6.0	97

[a] For details of procedure see text. [b] The starting material contained 7.5 μmoles of thymidine/300 mg.

Evaporation with pyridine was repeated four more times to remove any trace of moisture. The final residue was dissolved in pyridine (1 ml), and to the solution were added mesitylenesulfonyl chloride (77 mg, 0.360 mmole) and more pyridine (1.5 ml). The reaction mixture, which was homogeneous, was stirred using a magnetic stirrer at room temperature. After 1.5 hr, at which stage the reaction mixture was highly viscous, more pyridine (2 ml) was added and stirring was continued. After a total period of 3.5 hr, pyridine (2.5 ml) and water (0.4 ml) were added under cooling in ice. The solution was stirred for 15 hr at room temperature and was then poured into aqueous 2% sodium chloride solution (500 ml) under stirring. The resulting precipitate was collected by filtration, washed with water, and dried over phosphorus pentoxide under reduced pressure. This material was then dissolved in a mixture of pyridine (8 ml) and dimethyl sulfoxide (10 ml). To the solution was added 1 *M* sodium methoxide in methanol (2 ml). The slightly turbid solution was allowed to stand at room temperature for 5 min, and then more pyridine (8 ml) was added to obtain a completely clear solution. After a total period of 15 min, the solution was quickly (within 2 min) poured into aqueous 2% sodium chloride solution (500 ml). The precipitate was collected by filtration and washed with water. The product was dried *in vacuo* over phosphorus pentoxide overnight to give 178 mg of a slightly colored powder.

Subsequent treatment of this product (21 mg) with trifluoroacetic acid in chloroform followed by chromatographic analysis (solvent 1) gave only two spots, corresponding to thymidylyl-(3′→5′)-thymidine (22 OD_{267} units, 96%) and thymidine (0.46 OD_{267} unit, 4%) as the products. The recovery of TpT from the total Ⓟ-Tr(OCH_3)-TpT therefore corresponded to 10 μmoles (110% of that present in the starting material). The TpT was chromatographically (solvents 1 and 2) and electrophoretically pure. R_f values are listed in Table I. The ultraviolet spectrum showed λ_{max} 267 mμ and λ_{min} 234 mμ in water; OD_{280}/OD_{260} = 0.71 (calculated ratio, 0.72). Upon treatment with spleen phosphodiesterase the preparation (5.5 OD_{267} units) was completely hydrolyzed to thymidylic acid and thymidine, the ratio of thymidylic acid to thymidine being 0.99.

Thymidylyl-(3′→5′)-deoxycytidine (d-TpC). Ⓟ-Tr(OCH_3)-T (110 mg containing 21 μmoles of thymidine) and pyridinium d-pC^{An}-OAc (125 mg, 183 μmoles) were treated with pyridine (2 ml) in the presence of trihexylamine (0.06 ml) using mesitylenesulfonyl chloride (70 mg, 327 μmoles) as the condensing agent. The reaction mixture was stirred with a magnetic stirrer at room temperature for 1.5 hr. At this stage more pyridine (1 ml) was added because of the formation of a thick viscous solution. Stirring was continued further for 1 hr and then pyridine (4 ml) and water (0.4 ml) were added under ice cooling. The mixture was stirred further for 16 hr and was then poured into 2% aqueous sodium chloride solution (150 ml). The precipitate was collected by filtration and washed with water. The wet product, which was obtained in the form of a wet cake, weighed 170 mg. For analysis, a sample (22 mg) was immediately weighed and treated with 5 ml of 0.2% trifluoroacetic acid in chloroform at room temperature for 20 min. Pyridine (1 ml) was added to the solution and the solution evaporated to dryness under reduced pressure. The residue was dissolved in pyridine (3 ml) and the solution poured into water (20 ml). The precipitate thus formed was removed by centrifugation and the supernatant evaporated to dryness. The residue was treated with concentrated ammonium hydroxide (4 ml) for 24 hr and submitted to paper chromatographic analysis (solvent 1). Compounds detected and estimated on the chromatogram were: d-TpC, 35 OD_{260} units; T, 1.73 OD_{260}; and d-pC, 0.45 OD_{260}. Thus, the extent of reaction (T to d-TpC) was 91%, and the actual recovery as based on the amount of thymidine present in the starting material was 88%.

The preparation of d-TpC was chromatographically (solvents 1 and 2) and electrophoretically pure. R_f values are shown in Table I. The ultraviolet spectrum showed λ_{max} 268 (pH 8) and 273 mμ (pH 1) and λ_{min} 233 (pH 8) and 235 mμ (pH 1); OD_{280}/OD_{260} = 0.81 (pH 8) and 1.25 (pH 1) [calculated ratio, 0.83 (pH 8) and 1.31 (pH 1)]. Six OD_{260} units of d-TpC were digested with spleen phosphodiesterase. Complete hydrolysis was observed on chromatographic analysis and the products were Tp and dC, the molar ratio being 1.06.

Thymidylyl-(3′→5′)-deoxyadenosine (d-TpA). A solution of Ⓟ-Tr(OCH_3)-T (300 mg, containing 18 μmoles of thymidine) and pyridinium d-pA^{Bz}-OAc (200 mg, 360 μmoles) in pyridine (4 ml) was rendered anhydrous by repeated evaporation of its pyridine solution. To the dry residue, pyridine (4 ml) and mesitylenesulfonyl chloride (170 mg, 800 μmoles) were added, and the solution was allowed to stand at room temperature for 3.5 hr. Then, more pyridine (4 ml) and water (0.4 ml) were added under ice cooling. The mixture was allowed to stand at room temperature for 18 hr and then poured into 2% aqueous sodium chloride solution (250 ml) under stirring. The resulting precipitate was collected by filtration, washed with water, methanol, and pentane successively, and dried *in vacuo* over phosphorus pentoxide overnight. The yield of the dry powder was 304 mg.

For analysis a portion (103 mg) of the product was treated with benzylamine (4 ml) at room temperature for 36 hr. The benzylamine solution was diluted with dioxane (6 ml) and poured into 2% aqueous sodium chloride solution (125 ml). The precipitate thus obtained was collected by centrifugation and washed with water, methanol, and pentane successively. The dried material weighed 99 mg. A portion (40 mg) of this product was then treated with a mixture of acetic acid (0.6 ml) and chloroform (2.4 ml) at room temperature for 41 hr. The solution was evaporated to dryness under reduced pressure, and the residue was dissolved in benzene and evaporated again to dryness to remove almost all of the acetic acid. The remaining oily material was dissolved in pyridine (2 ml) and the solution poured into water (40 ml). The precipitate which resulted was removed by centrifugation, and the supernatant, after concentration, was submitted to paper chromatography. The products detected on the chromatogram were as follows: d-TpA, 31.7 OD_{260} units (86%);[23] thymidine, 1.35 OD_{260} units (10%); adenine, 0.79 OD_{260} unit (4%);[23] deoxyadenylic acid, 0.34 OD_{260} unit; thymidylic acid, 0.47 OD_{260} unit (4%).[23] Thus the total recovery of the nucleotidic compounds was 76% as based on the amount of thymidine in the starting material.

The d-TpA was found pure in chromatography (solvents 1 and 2) and in paper electrophoresis. R_f values are given in Table I. The ultraviolet spectrum showed λ_{max} 260 mμ and λ_{min} 230 mμ in water; OD_{280}/OD_{260} = 0.36 (calculated ratio, 0.36). On digestion with spleen phosphodiesterase the d-TpA (6.5 OD_{260} units) was completely hydrolyzed to give Tp and dA, the ratio being 1.04.

Thymidylyl-(3′→5′)-deoxyguanosine (d-TpG). The internucleotide bond synthesis was carried out between Ⓟ-Tr(OCH_3)-T (1.03 g, containing 25 μmoles of thymidine) and pyridinium d-pG^{Ac}-OAc (520 mg, 1 mmole) in pyridine (10 ml) using mesitylenesulfonyl chloride (420 mg, 2 mmoles) as the condensing agent. The reaction time was 3.5 hr. During this time, a viscous gel had resulted

(23) Adenine and thymidylic acid would have been produced from d-TpA during the work-up procedures. Thus the extent of internucleotide bond synthesis was believed to be approximately 90%.

which, after addition of pyridine (15 ml) and water (1.2 ml) and shaking, quickly went into solution. This aqueous pyridine solution was kept at room temperature for 17 hr. For analysis, an aliquot (1.0 ml) was poured into 2% aqueous sodium chloride solution (20 ml). The precipitated product from the aliquot was collected by filtration, washed with water, methanol, and pentane successively, and then dried to a slightly colored powder (33 mg). A portion (21 mg) of this product was treated with 0.1% trifluoroacetic acid in chloroform (3 ml) at room temperature for 6 min followed by treatment with concentrated ammonia for 20 hr. Paper chromatographic analysis (solvent 1) showed the following products: d-TpG, 9.1 OD_{260} units (94%); thymidine, 0.26 OD_{260} unit (6%); deoxyguanylic acid, 0.3 OD_{260} unit; and a compound traveling with R_f 0.3, 0.36 OD_{260} unit. Thus the recovery of the nucleotidic material was 94% as based on the amount of thymidine in the starting material. The d-TpG thus isolated from the paper chromatogram was checked for its purity by paper chromatography (solvents 1 and 2) and by paper electrophoresis. R_f values are given in Table I. The ultraviolet spectrum showed λ_{max} 254 mμ and 227 mμ in water, OD_{280}/OD_{260} = 0.67 (calculated ratio, 0.69). On digestion with spleen phosphodiesterase the d-TpG (6 OD_{260} units) was completely hydrolyzed to form thymidylic acid and deoxyguanosine, the ratio of these two products being 1.03.

To the main portion of the reaction mixture, which had been kept cold for 2 days while the aliquot was being worked up, pyridine (7 ml) and dimethyl sulfoxide (40 ml) were added. This solution was next made alkaline by addition of 1 *M* sodium methoxide in methanol (8 ml). After being kept at room temperature for 10 min, the solution was poured into 2% aqueous sodium chloride solution (2 l.). This transfer was completed in 5 min. The fine precipitate which formed was collected by filtration and washed with water, methanol, and pentane successively. The lightly colored powder weighed 1.00 g after drying overnight *in vacuo* over phosphorus pentoxide.

Thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (TpTpT). Polymer-supported 5′-monomethoxytritylthymidylyl-(3′→5′)-thymidine[24] (125 mg which contained 7.4 μmoles of nucleotidic material composed of thymidylyl-(3′→5′)-thymidine, 96%, and thymidine, 4%) was dissolved together with pyridinium 3′-O-acetylthymidine 5′-phosphate (200 mg, 450 μmoles) in pyridine (2.5 ml). The solution was rendered anhydrous by repeated evaporation with pyridine. After final evaporation with pyridine, the residue was dissolved in pyridine (2 ml), and to the solution was added mesitylenesulfonyl chloride (200 mg, 900 μmoles) followed by more pyridine (0.5 ml). The solution was allowed to stand at room temperature for 2.75 hr. Pyridine (4.5 ml) and water (0.4 ml) were then added under ice cooling and the aqueous pyridine solution was left at room temperature for 17 hr. It was then poured into 2% aqueous sodium chloride solution (200 ml). To collect the resulting precipitate, most of the liquid was carefully sucked off after centrifugation and the resulting loosely packed product was now collected by filtration and washed with water. The semidried material was dissolved in pyridine (4 ml). To this solution were added dimethyl sulfoxide (5 ml) and 1 *M* sodium methoxide in methanol (1 ml). The solution was allowed to stand at room temperature for 12 min and then poured into 2% aqueous sodium chloride solution. The product thus precipitated was collected by centrifugation followed by filtration, as described above, and washed with water. Drying under vacuum over phosphorus pentoxide for a day gave a slightly colored powder (105 mg) as the product.

A portion of this material (21 mg) was treated with 1% (v/v) trifluoroacetic acid in chloroform (2 ml) at 0° for 15 min. The solution was then evaporated to dryness under reduced pressure, and the residue was rendered acid free by repeated evaporation with benzene. The oily residue was then taken up in pyridine (2 ml) and the pyridine solution poured into water (20 ml). The precipitate was removed by filtration and the filtrate concentrated and submitted to paper chromatography (solvent 1). The compounds detected on the chromatogram were: TpTpT, 33.8 OD_{267} units (84%); TpT, 3.28 OD_{267} units (12%); and thymidine, 0.55 OD_{267} unit (4%). The TpTpT fraction, upon paper electrophoresis, was shown to be contaminated with thymidylic acid (1.7%) and a material (1.9%) traveling slightly ahead of TpTpT. Thus, the extent of reaction from TpT to TpTpT was 88%, and the actual recovery of the total nucleotidic material was 95% as based on the amounts present in the starting material.

The TpTpT thus purified was homogeneous upon paper chromatography (solvents 1 and 2). The R_f values are given in Table I. The ultraviolet spectrum showed λ_{max} 267 mμ and λ_{min} 234 mμ in water; OD_{280}/OD_{260} = 0.70 (calculated ratio, 0.72). The preparation (10 OD_{260} units) was digested with spleen phosphodiesterase. A complete hydrolysis was observed as examined by paper chromatography (solvent 1) and the products, thymidylic acid and thymidine, were found to be in the ratio of 2.16:1.

(24) This was present, presumably, as the sodium salt since the alkaline treatment for removal of the 3′-O-acetyl group involved sodium methoxide and subsequent precipitation was from aqueous sodium chloride.