[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXV.[1] The Stepwise Synthesis of Specific Deoxyribopolynucleotides (5).[2] Further Studies on the Synthesis of Internucleotide Bond by the Carbodiimide Method. The Synthesis of Suitably Protected Dinucleotides as Intermediates in the Synthesis of Higher Oligonucleotides[3]

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The condensation of 3′-O-acetylthymidine-5′ phosphate with 5′-O-trityl- and di-*p*-methoxytritylthymidine in the presence of dicyclohexylcarbodiimide (DCC) was further investigated. Use of stoichiometric amounts of the two components gave a 75% yield of the internucleotide bond. The addition of a second molar equivalent of the nucleotide after the yield of the condensation product had leveled off made little difference while the addition of a corresponding amount of the nucleoside increased the yield to near quantitative. The formation of labile side products, which evidently arise from the addition of DCC to the thymine ring, was observed during the above condensations. Kinetic studies of internucleotide bond synthesis using different suitably protected deoxyribonucleoside-5′ phosphates and suitably protected deoxyribonucleosides showed similar rates and final yields of the products, except when very bulky groups were present in the nucleotide component. Syntheses are described of many unprotected deoxyribonucleotidyl-(3′ → 5′)-deoxyribonucleosides and of the corresponding suitably protected derivatives which serve as intermediates in the synthesis of higher mixed oligonucleotides. The dinucleotide 5′-O-phosphoryl-N^6-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine was prepared by phosphorylation of N^6-anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine with a mixture of DCC and β-cyanoethyl phosphate followed by an alkaline treatment. The preparation of N-acetyldeoxyadenosine-5′ phosphate from N,3′-O-diacetyldeoxyadenosine-5′ phosphate is described. Cleavage of P^1,P^2-3′-O-acetylthymidine-5′ pyrophosphate by reaction with hydroacrylonitrile and DCC has been observed.

Investigations of the formation of thymidylyl-(3′ → 5′)-thymidine by the condensation of 3′-O-acetylthymidine-5′ phosphate and 5′-O-tritylthymidine have been reported previously.[2b,4b] The condensing agent which so far has proved the most satisfactory is dicyclohexylcarbodiimide.[2b,4,5] In the present work, the formation of thymidylyl-(3′ → 5′)-thymidine from the appropriate components has been investigated further and the kinetic studies of the internucleotide bond synthesis have been extended to include all combinations of suitably protected deoxyribonucleosides[1] and protected deoxyribonucleoside-5′ phosphates.[6-8] These studies were considered necessary because differences in the reactivity of the 3′-hydroxyl group in different nucleosides were indicated by the observations previously recorded in literature.[9] The present results show that the carbodiimide method is uniformly efficient for the synthesis of internucleotide bonds. Furthermore, the general suitability of the different protecting groups developed for the synthetic work has been demonstrated by preparing practically all combinations of dinucleotides containing only the 3′-hydroxyl group free for lengthening the oligonucleotide chains. A brief report of a part of this work has already appeared.[10]

Studies of Internucleotide Bond Synthesis.—The kinetics of internucleotide bond formation using 0.2 *M* concentration of each of 3′-O-acetylthymidine-5′ phosphate and 5′-O-di-*p*-methoxytritylthymidine[1,11] are shown in Table II (see Experimental). The results are similar to those reported elsewhere[2b,12] except that the final yield was higher (75% instead of 65%). In more recent experiments[12] using pyridine which has been carefully freed from traces of strongly basic amines[13] the final yields using stoichiometric amounts of the two components have been even higher (85–90%). Three further observations made in connection with the above experiment (line 1 of Table II) were: (1) There was no difference in the results when the reaction was carried out at higher concentration (1 *M* in place of 0.2 *M*) of the reactants. (2) Addition of water to the reaction mixture, when the reaction had leveled off, followed by re-evaporation and retreatment with DCC in dry pyridine made little difference in the result. (3) During the kinetic studies using analysis by paper chromatography it was noted that in the initial stages the proportion of the symmetrical P^1,P^2-dithymidine-5′ pyrophosphate to thymidine-5′ phosphate was high and it decreased as the diester (thymidylyl-(3′ → 5′)-thymidine) concentration increased.[15]

Previously, it was established that when a 100% excess of one of the components (nucleoside or the nucleotide) is used in the standard condensation, the yield of the internucleotide bond was quantitative with respect to the lesser component.[2d,5,16] In attempts to understand this effect, three parallel experiments were started using stoichiometric amounts of the two components. After the reaction had leveled off, one reaction mixture was then supplied with an additional amount of the nucleoside, the second with an equivalent amount of the nucleotide. The somewhat unexpected

(1) Paper XXIV: H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963).

(2) Previous papers which deal with this topic: (a) ref. 1; (b) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958); (c) *ibid.*, **81**, 4647 (1959); (d) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962).

(3) This work has been supported by grants from the National Science Foundation, Washington, D. C., the National Cancer Institute of the National Institutes of Health, Bethesda, Md., and the Life Insurance Medical Research Fund, New York, N. Y.

(4) (a) For a comparison of different reagents in polymerization studies, see H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *J. Am. Chem. Soc.*, **84**, 414 (1962); (b) for purposes of stepwise synthesis a detailed survey has been made by T. M. Jacob and H. G. Khorana, *Chem. Ind.* (London), 932 (1962), and *J. Am. Chem. Soc.*, in press.

(5) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(6) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).

(7) R. K. Ralph and H. G. Khorana *ibid.*, **83**, 2926 (1961).

(8) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

(9) Thus while dibenzylphosphorochloridate phosphorylated the 3′-hydroxyl group in 5′-O-tritylthymidine satisfactorily [A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 956 (1953)] and the same reagent was also used for the phosphorylation of 5′-O-trityldeoxycytidine [A. M. Michelson and A. R. Todd, *ibid.*, 34 (1954)] the 3′-hydroxyl group in protected purine deoxyribonucleosides could not be phosphorylated with the same reagent [D. H. Hayes, A. M. Michelson, and A. R. Todd, *ibid.*, 808 (1955)].

(10) H. Schaller and H. G. Khorana, *Chem. Ind.* (London), 669 (1962).

(11) Dimethoxytrityl is the abbreviation for di-*p*-methoxyphenylphenylmethyl group.

(12) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, in press.

(13) The higher yields obtained in current work do not affect the main conclusions or the arguments presented below. Similarly, the previous discussion on the mechanism of internucleotide bond synthesis[14] remains unaffected.

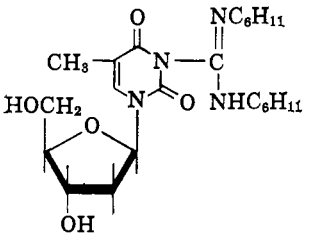
(14) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).

(15) In the earlier study of the condensation of 5′-O-tritylthymidine-3′ phosphate with 3′-O-acetylthymidine, when the yield of the diester never exceeded 43%,[2d] the proportion of the pyrophosphate to free nucleotide was similarly high.

(16) H. G. Khorana, *J. Cellular Comp. Physiol.*, **54**, Suppl. 1, 5 (1959).

result was that the addition of the nucleoside increased the yield to near quantitative with respect to the nucleotide while the addition of the extra mole of the nucleotide had little effect. The conclusion was drawn tentatively from the above findings that it was the hydroxyl group of the nucleoside which was somehow rendered unavailable for the condensation reaction. Attempts were therefore made to detect intermediates which could contain the nucleoside component in "bound" form. These attempts aimed at demonstration of some interaction between the nucleoside component and DCC[17] and while, as described below, previously undetected labile intermediates were found, no satisfactory explanation for the nucleoside "removal" was obtained.

Condensation products obtained under parallel conditions but by using an increasing excess of DCC (1.7–13.3 molar equiv.) were examined after minimal treatments with aqueous pyridine and acetic acid. Paper chromatography in solvents A and B showed the presence of ultraviolet absorbing materials other than the previously characterized[2b] products of the reaction. The properties of these products, which are labile and mostly disappear under the usual conditions of work-up,[18] are given in the Experimental section. From these properties, these compounds are tentatively concluded to be the adducts of DCC with the thymine ring, one possible site for the addition reaction being N-3 as shown in I for thymidine.



I

It should be noted that similar adducts have previously been obtained in this Laboratory during work with the water-soluble carbodiimides.[19] The formation of these adducts was found to be specific for the keto-bases, namely uridine and guanosine, and this, presumably, also is the case for the formation of adducts using DCC.[20] While some differences in the rates of formation and in the stabilities of the adducts from the water-soluble carbodiimides and DCC are to be expected, the over-all properties are similar.

That the formation of the labile intermediates discussed above cannot provide the explanation for the "binding" of the nucleoside component follows from the results in Table III (Experimental) which gives the proportion of the labile products corresponding to the nucleoside (designated T-x), the product (designated TpT-x),[21] and the nucleotide (designated pT-x) as a function of DCC concentration in one parallel set of experiments. Although the labile products increased with the increase in the DCC concentration used, there was no diminution in the final yield of the internucleotide bond. On the contrary, a slight increase in the yield of the desired product may have occurred (Table III).

Included in Table II are kinetic studies of the formation of the internucleotide bond by reaction of each one of the four major deoxyribonucleoside-5′ phosphates, with most of the protected deoxyribonucleosides. The first major conclusion of this study was that the carbodiimide method was uniformly effective, the reaction rates being very similar throughout. Secondly, the results provided abundant support for the conclusion reached earlier[14] that while the size of the protecting groups in the nucleoside component has little effect, the size of the groups in the nucleotide component has, in contrast, a marked effect on the rate and final yield of the phosphodiester bond. The most dramatic examples were the condensations involving N,5′-O-bis-dimethoxytrityldeoxyguanosine and N,3′-O-bis-dimethoxytrityldeoxyguanosine-5′ phosphate. The former reacted with 3′-O-acetylthymidine-5′ phosphate at the usual rate and the final yield of the product was in the expected range. In contrast, the condensation of the bis-dimethoxytrityldeoxyguanosine-5′ phosphate with 5′-O-dimethoxytritylthymidine (see Experimental) gave only a 12% final yield of the desired product. The data of Table II also give further general support to the conclusion that the yields of the internucleotide bonds decreased perceptibly with increase in the size of the nucleotide (thymidine-5′ phosphate to N-anisoyldeoxycytidine-5′ phosphate to the protected purine nucleotides).

Preparation of Dinucleotides and Their Protected Derivatives.—Selected procedures are described in the Experimental section for the preparation and isolation of many of the unprotected dinucleoside phosphates containing different nucleosides and nucleotides. The R_f's of all of the products prepared are listed in Table I. In addition to the tests for homogeneity, the products were characterized by degradation with spleen and venom phosphodiesterases as described in earlier papers. It was thus established in all cases that the internucleotide bonds synthesized were exclusively of the $C_{3'}$–$C_{5'}$ type. A further feature of the present study was the use of a reaction medium alternative to pyridine, namely, dimethylformamide in the presence of pyridinium Dowex-50 ion exchange resin (*cf.* ref. 8). The latter medium was used in the synthesis of thymidylyl-(3′ → 5′)-deoxyguanosine (see Experimental) and in the work reported in the accompanying papers.[22,23]

The protecting groups now available for the different functional groups in different nucleotides are illustrated in the syntheses recorded here and listed in Table II. The groups used are such that all of the nucleotides and nucleosides can be manipulated not only to prepare any of the possible dinucleotides but also to be able to expose selectively the 3′-hydroxyl end for chain elongation by reaction with the 5′-phosphate end group of mono- or oligonucleotides. Illustrations of the selective unblocking of the 3′-hydroxyl groups are provided in the present work by the preparation of 5′-O-di-*p*-methoxytritylthymidylyl-(3′ → 5′)-thymidine (II) and 5′-O-monomethoxytritylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (III).[24]

The protected deoxyribonucleoside-5′ phosphates, which serve well for work in the stepwise synthesis of oligonucleotides, contain an acetyl group in the 3′-hydroxyl position because the latter group requires the

(17) It is recalled that, previously, all attempts to demonstrate an addition reaction between the hydroxyl group of a nucleoside and the carbodiimide (formation of an isourea ether) have failed. The point is fully discussed in footnote 19 of ref. 2d.

(18) *Cf.* T. M. Jacob and H. G. Khorana, ref. 12, where some additional observations on these labile products are also reported.

(19) P. T. Gilham, *J. Am. Chem. Soc.*, **84**, 687 (1962).

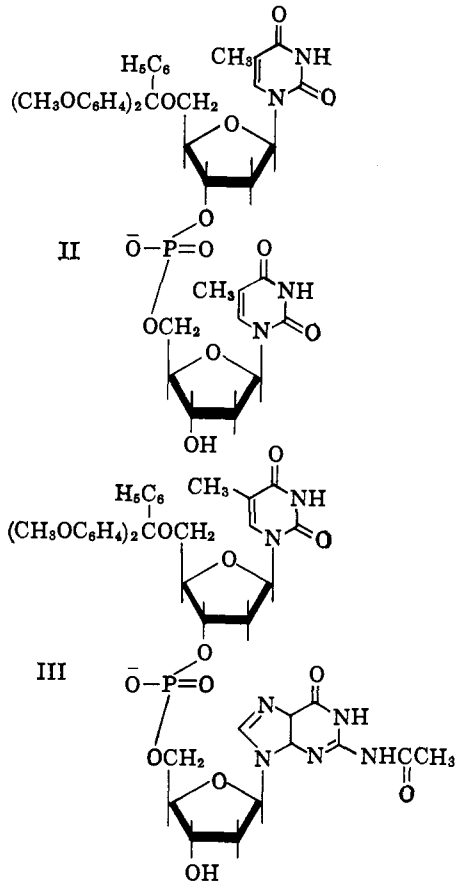
(20) This point is now under further investigation in this Laboratory.

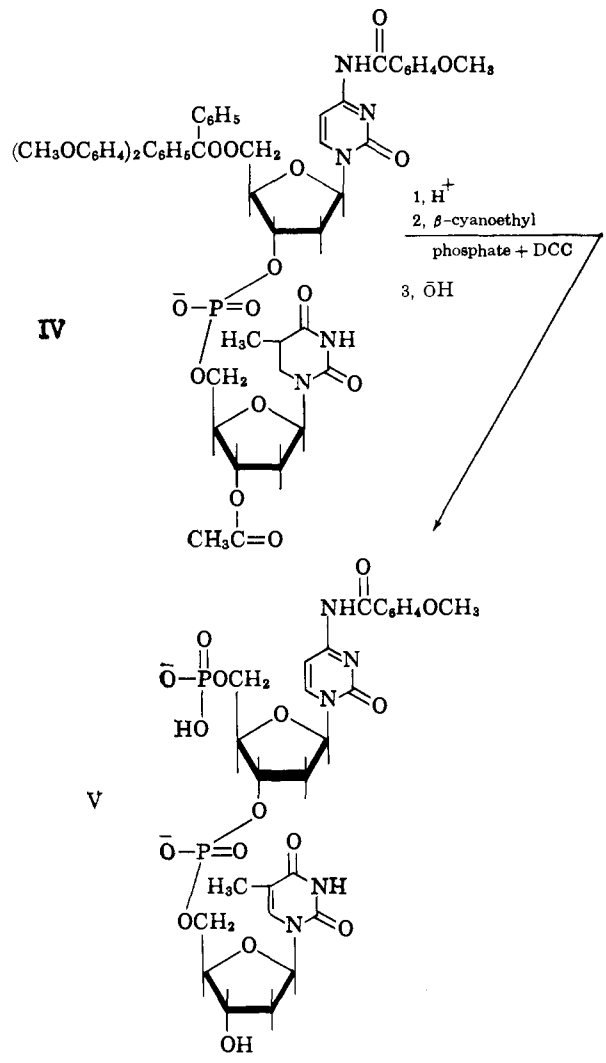
(21) The presence of an ultraviolet-absorbing product with identical properties (R_f in solvent A and hydrolyzability during work up to thymidylyl-(3′ → 5′)-thymidine) was also recorded in the early work: P. T. Gilham and H. G. Khorana (Table I of ref. 2b).

(22) G. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963).

(23) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

(24) The location of the N-acetyl group in the guanine ring is shown to be at N^2-position but this has not been proved (*cf.* ref. 8)





mildest of alkaline conditions for removal. Protected nucleotides containing this group are readily prepared by acetylation, for example, of N-benzoyldeoxyadenosine-5′ phosphate[22] and of N-anisoyldeoxycytidine-5′ phosphate (present work). In the case of deoxyguanosine-5′ phosphate, the N-acetyl group has adequate stability and the N,3′-O-diacetyl derivative can directly be used. In the case of deoxyadenosine-5′ phosphate also it now seems feasible to use N,3′-O-diacetyldeoxyadenosine-5′ phosphate in an analogous manner (see Experimental).[25]

The di-*p*-methoxytrityl group has been used extensively in the present work for protecting the 5′-hydroxyl group. Its acidic lability is such that it can be removed under conditions which are safe throughout except for the glycosyl bond in N-benzoyldeoxyadenosine. Only in the latter case is it necessary to remove first the N-benzoyl group with ammonia and then the dimethoxytrityl group by an acidic step. In the present work, the selective removal of the dimethoxytrityl group was used in preparation of the dinucleotide 5′-O-phosphoryl-N^6-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine (V) from IV by the steps: (1) acidic treatment, (2) phosphorylation with a mixture of β-cyanoethyl phosphate and DCC, and (3) selective removal of the cyanoethyl group by alkaline treatment. This route gave high yield of the dinucleotide and is preferable to the alternative one which involved the condensation of N-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate with 3′-O-acetylthymidine-5′ phosphate (see Experimental).

During the present work, an approach investigated for the selective cleavage of the P^1,P^2-dialkyl pyrophosphates, compounds which are often obtained as side products during both the polymerization work and the stepwise synthesis, involved their reaction with DCC in the presence of hydroacrylonitrile. (The approach previously developed for the purpose involved treatment with an excess of acetic anhydride in dry pyridine.[26]) The major product from P^1,P^2-dithymidine-5′ pyrophosphate was thymidine-5′ β-cyanoethyl phosphate which, if necessary, could readily be converted to the parent nucleotide. A neutral nucleotidic product (see also the following papers[22,33]) was obtained as a side product. The neutral ester, presumably the dicyanoethyl ester, could be converted practically quantitatively to the mono-β-cyanoethyl nucleotide on being kept at slightly alkaline pH. Thus the method is of practical utility for cleavage of the pyrophosphates and, when followed by an alkaline treatment, gives the phosphomonoesters.

Concluding Remarks.—The comprehensive studies now reported establish the carbodiimide method as a general and powerful method for the synthesis of internucleotide bonds. The yields obtained were consistently high except in special cases when very bulky groups were used in the nucleotide component. The labile side products noted would not be expected to cause any significant interference. Prolonged aqueous pyridine or ammoniacal treatment is effective in causing their disappearance. Nor is the evidence that the nucleoside component may become "bound" in some manner of any serious concern. Yields of 75% of the product were obtained using stoichiometric amounts

(25) Previously,[7] at pH 11.3, the removal of N- and O-acetyl groups was found to be competitive but, as is firmly established now in all of the cases studied, selectivity in removal of the 3′-O-acyl group is more readily achieved by working in high alkalinity.

(26) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959); H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1961).

of the two components and in more recent work yields of 90% are being obtained.[12] Only when the internucleotide bond formation is unusually sluggish does the unknown side reaction become serious. Often, in such cases when an excess of one of the components is used at the start, then the yield of the internucleotide bond is quantitative.

Experimental

General Methods.—Solvents for paper chromatography and the other general technique used were as described in an accompanying paper.[1] The R_f's of different compounds on paper chromatograms are given in Table I.

TABLE I

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	Solvent A	Solvent B	Solvent C
Thymidine (T)	0.68	0.60	
Thymidylyl-(3′ → 5′)-3′-O-acetyl thymidine (TpT-acetyl)	.45		
3′-O-Acetylthymidine-5′ phosphate (pT-acetyl)	.12		
P¹,P²-Di-3′-O-acetylthymidine-5′ pyrophosphate	.20		
T-x	.78		
Acetyl TpT-x	.87	.79	
Acetyl pT-x	.90–0.94		
Thymidylyl-(3′ → 5′)-thymidine (TpT)	.39		
Deoxyadenylyl-(3′ → 5′)-thymidine (d-ApT)	.34	.22	0.41
Deoxycytidylyl-(3′ → 5′)-thymidine (d-CpT)	.32	.21	.44
Deoxyguanylyl-(3′ → 5′)-thymidine (d-GpT)	.22	.19	.39
Deoxycytidylyl-(3′ → 5′)-deoxycytidine (d-CpC)	.28	.18	.38
Deoxyguanylyl-(3′ → 5′)-deoxycytidine (d-GpC)	.17	.15	.33
Deoxycytidylyl-(3′ → 5′)-deoxyguanosine (d-CpG)	.16	.13	.35
Thymidylyl-(3′ → 5′)-deoxyguanosine (d-TpG)	.24	.24	.39
Deoxyguanylyl-(3′ → 5′)-deoxyguanosine (d-GpG)	.10	.11	.29
Thymidylyl-(3′ → 5′)-deoxyadenosine (d-TpA)	.36	.27	.41
5′-O-Di-*p*-methoxytritylthymidylyl-(3′ → 5′)-thymidine	.75		
N-Anisoyldeoxycytidylyl-(3′ → 5′)-thymidine	.47		.65
N-Anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine	.58		
N-Anisoyl-5′-O-di-*p*-methoxytrityldeoxycytidylyl-(3′ → 5′)-thymidine			.90
N,O⁶-Bis-di-*p*-methoxytrityldeoxyguanylyl-(3′ → 5′)-thymidine			.92
5′-O-Monomethoxytritylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine	.70		.87
5′-O-Phosphoryldeoxycytidylyl-(3′ → 5′)-thymidine (d-pCpT)	.05		.25
5′-O-Phosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine	.16		.41
5′-O-Cyanoethylphosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine	.49		.72

Condensation of 3′-O-Acetylthymidine-5′ Phosphate with 5′-O-Tritylthymidine or 5′-O-Di-*p*-methoxytritylthymidine. General Method.—A mixture of 3′-O-acetylthymidine-5′ phosphate (0.2 mmole), 5′-O-tritylthymidine (0.2 mmole), and DCC (1 mmole) in dry pyridine (1 ml.) was kept at room temperature for different lengths of time with exclusion of moisture. Aliquots (0.025–0.05 ml.) were removed, added to an excess of 50% aqueous pyridine (about 0.5 ml.) and the mixture kept for several hours at room temperature; DCC was extracted[27] with petroleum ether, the aqueous pyridine solutions evaporated with water, and the latter operation repeated to remove all pyridine. Acetic acid (1 ml. of 80%) was added and the mixture heated at 100° for 15 min. In the case that 5′-O-di-*p*-methoxytritylthymidine was used, the treatment with 80% acetic acid was carried out at room temperature for 0.5–1 hr. The acetic acid was removed by evaporation and the residue was treated with a mixture of water and ether. The ether layer was separated off and the aqueous layer evaporated repeatedly to ensure complete removal of acetic acid. Concentrated ammonium hydroxide was added and the solution kept at room temperature for at least 1 hr. Samples were chromatographed in solvent A. The various ultraviolet-absorbing spots and appropriate blanks were eluted with water and the optical densities determined at 267 mμ. The yields of thymidylyl-(3′ → 5′)-thymidine as a function of time are included in Table II.

When the same synthesis was carried out at 1 *M* concentration of the two components (5′-O-di-*p*-methoxytritylthymidine was the nucleoside used), the rate and the final yields were similar to those obtained at 0.2 *M* concentration of the reactants.

In one experiment, after a 3-day reaction period, an excess of water was added and after keeping overnight and extraction of the excess of DCC, the mixture was rendered anhydrous by repeated evaporation of pyridine; DCC (1 mmole) was added and the reaction mixture again examined for the yield of thymidylyl-(3′ → 5′)-thymidine. No increase in yield over that obtained initially after 3 days (about 80%) was detected.

The Effect of Addition of an Excess of One Component on the Yield of Thymidylyl-(3′ → 5′)-thymidine.—A set of three reaction mixtures were set up by the general procedure described above. They contained the reaction components as follows: no. 1, 3′-O-acetylthymidine-5′ phosphate (0.1 mmole), 5′-O-tritylthymidine (0.1 mmole), DCC (105 mg., 0.5 mmole) in 0.5 ml. of dry pyridine. Numbers 2 and 3 each contained 3′-O-acetylthymidine-5′ phosphate (0.2 mmole), 5′-O-tritylthymidine (0.2 mmole), and DCC (210 mg., about 1 mmole) in 1 ml. of pyridine. The three reaction mixtures were kept sealed at room temperature for 3 days. After this time, a small aliquot (0.05 ml. about 10% of total) was removed from reaction mixture 1. This aliquot was worked up as described above and the yield of thymidylyl-(3′ → 5′)-thymidine was found to be 72%, as calculated on the remaining thymidine and 74% as based on the nucleotide. To the remainder of this reaction mixture was added more DCC (50 mg.) and pyridine (0.5 ml.) and the mixture was kept for a further period of 2 days. The yield of thymidylyl-(3′ → 5′)-thymidine determined after this time was unchanged (73.5% as based on the nucleoside).

To reaction mixture 2 was added, after the initial 3 days, 5′-O-tritylthymidine (112 mg., 0.2 mmole), pyridine (1 ml.), and DCC (100 mg.). To reaction mixture 3 was added a dry solution in pyridine (1 ml.) of 3′-O-acetylthymidine-5′ phosphate (0.2 mmole) and DCC (100 mg.). Both reaction mixtures 2 and 3 were also kept for 2 days and then worked up as described above. The yield of thymidylyl-(3′ → 5′)-thymidine in reaction mixture 2 was 98% as based on the nucleotide while the yield from reaction mixture 3 was 77% as based on the nucleoside.

Detection of Labile Products in the Synthesis of Thymidylyl-(3′ → 5′)-thymidine.—A stock solution of 3′-O-acetylthymidine-5′ phosphate (1 mmole) and 5′-O-di-*p*-methoxytritylthymidine (590 mg., 1 mmole) in dry pyridine (11 ml.) was prepared; 2-ml. aliquots were removed from this solution and the mixture of the two components in these solutions rendered anhydrous by co-evaporation with pyridine. Reaction mixtures were set up by addition of varying amounts of DCC: reaction mixture 1 contained 65 mg. (1.7 molar equiv. as based on the nucleotide), 2 contained 95 mg. (2.5 molar equiv.), 3 contained 200 mg. (5.3 molar equiv.), and 4 contained 500 mg. (13.3 molar equiv.) of the reagent. The sealed reaction mixtures were kept at room temperature for 4 days; to each water was added and the mixtures kept overnight. The solvent was evaporated and the residues re-evaporated after addition of water to remove pyridine completely. Acetic acid (5 ml. of 80%) was added to each; after 1 hr. at room temperature the solvent was removed by evaporation (repeat evaporation with water). Finally, the residues were dissolved in a 1:1 mixture of methyl alcohol and water (5 ml. total). The pH was adjusted to near neutrality with ammonia and the solutions stored at 2°. The products were examined by paper chromatography in solvents A and B. In both solvents ultraviolet-absorbing materials were observed in addition to the known and previously characterized products (3′-O-acetylthymidine-5′ phosphate, the corresponding pyrophosphate, thymidylyl-(3′ → 5′)-3′-O-acetylthymidine, and thymidine). The R_f's are given in Table I and the proportions of the additional products are given in Table III. The product

(27) In some early experiments this step was omitted, DCC then being hydrated to the corresponding urea during subsequent treatment with aqueous acetic acid.

TABLE II

RATE OF FORMATION OF INTERNUCLEOTIDE (C_3'–C_5') BOND BY CONDENSATION OF PROTECTED NUCLEOTIDES WITH DIFFERENT PROTECTED NUCLEOSIDES (STOICHIOMETRIC AMOUNTS, 0.1–0.2 *M*)

Protected nucleotide	Protected nucleoside	Product isolated								
3′-O-Acetylthymidine-5′ phosphate	5′-O-Di-*p*-methoxytritylthymidine	Thymidylyl-(3′ → 5′)-thymidine	Time, hr.	0.75	1.5	3	7.5	22	72	
			Yield,[a] (a) %	8	18	32	54	65	78	
			(b) %	7	17	30	50	64	76	
3′-O-Acetylthymidine-5′ phosphate	N^6-Anisoyl-5′-O-di-*p*-methoxytrityl-deoxycytidine	N^6-Anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine	Time, hr.	0.5	1.5	4.5	16	40	67	90
			Yield,[a] (a) %	3	18	39	63	69	77	
			(b) %	3	18	42	62	68	76	80
		Deoxycytidylyl-(3′ → 5′)-thymidine	Time, hr.	90						
			Yield,[a] (a) %	75						
			(b) %	77						
3′-O-Acetylthymidine-5′ phosphate	N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine	Deoxyadenylyl-(3′ → 5′)-thymidine	Time, hr.	0.75	2.17	4.25	11.5	22	54	81
			Yield, (a) %	7.5	24	38	59	66	75	72
			(b) %	7.5	18	43	57	64	75	75
3′-O-Acetylthymidine-5′ phosphate	N,O^6-Bis-di-*p*-methoxytrityldeoxyguanosine	Deoxyguanylyl-(3′ → 5′)-thymidine	Time, hr.	2	4	10	40	72		
			Yield,[b] %	22	37	52	63	67		
N^6-Anisoyl-3′-O-acetyldeoxycytidine 5′ phosphate	N,O^6-Bis-di-*p*-methoxytrityldeoxyguanosine	Deoxyguanylyl-(3′ → 5′)-deoxycytidine	Time, hr.	5.5	18	72				
			Yield,[b] %	44	60	68				
N,O^3-Diacetyldeoxyguanosine-5′ phosphate	N,O^6-Bis-di-*p*-methoxytrityldeoxyguanosine	Deoxyguanylyl-(3 → 5′)-deoxyguanosine[c]	Time, hr.	79						
			Yield,[a] (a) %	55						
			(b) %	56						
N,O^3-Diacetyldeoxyguanosine-5′ phosphate	5′-O-Di-*p*-methoxytritylthymidine	Thymidylyl-(3′ → 5′)-deoxyguanosine[d]	Time, hr.	94						
			Yield,[a] (a) %	59						
			(b) %	64						
$N,O^{3'}$-Diacetyldeoxyadenosine-5′ phosphate	5′-O-Di-*p*-methoxytritylthymidine	Thymidylyl-(3′ → 5′)-deoxyadenosine	Time, hr.	2	5.5	19	72			
			Yield,[a] (a)				75			
			(b) %	21	46	63	71			
N^6-Anisoyl-3′-O-acetyldeoxycytidine-5′ phosphate	N^6-Anisoyl-5′-O-di-*p*-methoxytrityldeoxycytidine	Deoxycytidylyl-(3′ → 5′)-deoxycytidine	Time, hr.	5.5	72					
			Yield, (a) %		63					
			(b) %	42	62					

[a] Yields given in the line (a) are based on the nucleotide, those in the line (b) are on the nucleoside. [b] Yields given here are based on the nucleotide, since the nucleoside recovery was not quantitative because of a small amount of cleavage of the glycosyl bond; the same glycosyl bond in deoxyguanosine-5′ phosphate as well as in dinucleotides appeared to be stable under the conditions used for removal of the dimethoxytrityl group. [c] This product was isolated by chromatography on a DEAE–cellulose column (*cf.* text). [d] See text for the modified conditions used.

designated T-x gave thymidine (T) after treatment with concentrated ammonia for about 12 hr. at room temperature; the product designated TpT-x gave thymidylyl-(3′ → 5′)-thymidine (TpT) after similar treatment. The product designated pT-x after corresponding ammoniacal treatment gave thymidine-5′ phosphate (pT). Additional properties of these products were:

TpT-x was neutral at pH 7.1 and at pH 3.5 as determined by paper electrophoresis. The ultraviolet absorption spectrum characteristics were: pH 7, λ_{max} 269 mμ, λ_{min} 244 mμ; pH 10, λ_{max} 267.5 mμ, λ_{min} 244 mμ. The compound was resistant to the action of spleen phosphodiesterase for 5 hr. using conditions which caused complete degradation of thymidylyl-(3′ → 5′)-thymidine (TpT) in 2 hr. Treatment[28] with 0.3 *N* sodium hydroxide at room temperature for 7.5 hr. caused complete conversion to TpT. In 5 *N* ammonia at room temperature for 7.5 hr. conversion to TpT was to the extent of 75%. No change occurred on keeping it at pH 3.5, pH 1, and in aqueous pyridine.[28] During the alkaline treatment, an insoluble material, presumably dicyclohexylurea, separated.

T-x.—During elutions with water and rechromatography on paper chromatograms, there was evidence of partial breakdown to thymidine. Treatment with alkali or ammonia caused breakdown to thymidine. The compound appeared to be more labile than TpT-x described above.

pT-x[29] was also obtained in a yield of about 20% by the reaction of 3′-O-acetylthymidine-5′ phosphate with 5 equivalents of DCC in dry pyridine for 3 days. In a much shorter period (4 hr.) only a trace of this compound was detectable. The mobility at pH 7.1 (paper electrophoresis) was 0.36 relative to pT. On treatment with ammonia (9 *N*) for 10 hr. at room temperature it gave pT.

Study of Rate of Formation of Internucleotide Bonds by Condensation of Different Protected Nucleotides with Protected Nucleosides.—The general method was as described above for the condensation of 3′-O-acetylthymidine-5′ phosphate with 5′-O-di-*p*-methoxytritylthymidine. After addition of water to stop the reaction, the treatment depended upon the nature of the protecting groups and the products to be isolated. Most often the first treatment was with an excess of concentrated ammonium hydroxide for appropriate periods (2 days for removal of N-protecting groups, 1 hr. for O-acetyl groups). After evaporation of the ammoniacal solutions the residues were treated with 80% acetic acid at room temperature for 1–3.5 hr. (1 hr. for the removal of O-di-*p*-methoxytrityl group, 3.5 hr. for the removal of N-di-*p*-methoxytrityl group in the guanine ring). Acetic acid was then removed by evaporation and the products examined by paper chromatography in solvent A. In the case of N^6-anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine the ammoniacal treatment was omitted and after the acetic acid treatment the product was estimated after paper chromatography in the neutral solvent C.

The components, protected nucleotides and nucleosides, of reactions, the rate of formation, and the yields of different products are given in Table II.

Thymidylyl-(3′ → 5′)-deoxyguanosine. (a) **From $N,O^{3'}$-Diacetyldeoxyguanosine-5′ Phosphate and 5′-O-Di-*p*-methoxy-**

TABLE III

PERCENTAGES OF LABILE COMPOUNDS IN THYMIDYLYL-(3′ → 5′)-THYMIDINE SYNTHESIS AS A FUNCTION OF DCC CONCENTRATION

Reaction mixture	1	2	3	4
DCC (molar equiv.)	1.7	2.5	5.3	13.3
T-x and T + T-x	39%	44%	61%	71%
TpT-x[a] and TpT + TpT-x	9.8%	19%	42%	43%
pT-x[a] and pT + pT-x + pyrophosphate	6%	47%	51%	60%
TpT[b] (total yield, %)	70.7[c]	72.2[c]	78.5[c]	80.1[c]
	70.5[d]	73.0[d]	77.7[d]	84.4[d]

[a] These products initially carried 3′-O-acetyl groups. [b] This is the total (final) yield after ammoniacal treatment of the reaction products when the only products were T, pT, pyrophosphate, and TpT. [c] Yield as based on the nucleoside. [d] Yield as based on the nucleotide.

(28) These stability tests were carried out on a sample which had been stored frozen in water for about 1 year. The possibility of rearrangement to a different compound cannot be ruled out (see observations of T. M. Jacob and H. G. Khorana (forthcoming paper) on a similar compound).

(29) In the total reaction mixtures, some P^1P^2-dithymidine-5′ pyrophosphate is also present. A derivative of this pyrophosphate corresponding to pT-x has also been detected.

tritylthymidine.—A mixture of pyridinium N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate (0.14 mmole), 5′-O-di-*p*-methoxytritylthymidine[30] (0.142 mmole), dry pyridine (0.7 ml.), and DCC (170 mg.) was kept at room temperature for 79 hr. The gum which had separated immediately on the addition of DCC persisted after this time and freshly distilled dimethylformamide (0.5 ml.) was then added. Continued shaking at room temperature resulted in a homogeneous solution except for the crystalline dicyclohexylurea. After a total of 15 hr. water was added and the mixture kept overnight. It was then evaporated, the residue washed with ether, and the residue evaporated from water again to remove residual pyridine. The residue was then dissolved in 5 ml. of 80% acetic acid for 1 hr. The solution was then evaporated and the residue shaken with a mixture of water and ether. The ether layer was removed and the aqueous layer evaporated. The residue was kept in concentrated ammonium hydroxide for 2 days at room temperature and then applied to 4 strips (9 in. wide) of Whatman 3 MM paper. Paper chromatography in solvent A showed the major product (R_f 0.3) to be thymidylyl-(3′ → 5′)-deoxyguanosine. The total optical density units (256 mμ) of this product recovered after elution with water were 1910 and, discounting any hypochromicity for this compound, this yield corresponded to 0.092 mmole (65%). In addition to the major desired product, other bands detected in order of increasing mobilities were: deoxyguanosine-5′ phosphate (R_f 0.065), a weak band (R_f 0.15) corresponding to P^1,P^2-di-deoxyguanosine-5′ pyrophosphate, another very weak band (R_f 0.60), a strong band corresponding to thymidine, and a weak band[31] (R_f 0.84).

Thymidylyl-(3′ → 5′)-deoxyguanosine showed in water λ_{max} at 256 mμ with an inflection at 264 mμ. It was completely degraded by spleen and venom phosphodiesterases under the standard conditions described to the expected nucleotide and nucleoside.

(b) **From N,O$^{3'}$-Bis-di-*p*-methoxytrityldeoxyguanosine-5′ Phosphate and 5′-O-Di-*p*-methoxytritylthymidine.**—A reaction mixture was set up using pyridinium N,O$^{3'}$-bis-di-*p*-methoxytrityldeoxyguanosine-5′ phosphate[8] (0.1 mmole), di-*p*-methoxytritylthymidine (0.1 mmole), dry pyridine (0.8 ml.), and DCC (159 mg.). After 3 days at room temperature the mixture was worked up as described above under (a) except that the treatment with 80% acetic acid at room temperature was for 4 hr. and further so that the subsequent ammonia step was omitted. The isolation was carried out as above by preparative paper chromatography. The yield of thymidylyl-(3′ → 5′)-deoxyguanosine was 0.012 mmole (12%) disregarding hypochromicity in the product. Lyophilization of the total eluate of the desired product gave 9 mg. of a dry powder (ammonium salt).

Deoxyguanylyl-(3′ → 5′)-deoxyguanosine.—An anhydrous pyridine (0.7 ml.) solution of pyridinium N,3′-O-diacetyldeoxyguanosine-5′ phosphate (0.141 mmole), N,5′-O-bis-di-*p*-methoxytrityldeoxyguanosine (125 mg., 0.143 mmole), and DCC (150 mg.) was kept at room temperature for 79 hr. Water (about 0.5 ml.) was then added followed by the addition of minimal amount of pyridine to redissolve the turbidity that resulted. The solution was kept at room temperature overnight after extraction with cyclohexane. It was then evaporated and the residual pyridine removed after addition of water. The residue was kept in 80% acetic acid (5 ml.) at room temperature for 4 hr. The mixture was then evaporated, the residue taken up in a mixture of water and ether. After separation the aqueous layer was evaporated and the residue kept in concentrated ammonium hydroxide for 1 day and after subsequent evaporation of most of ammonia and removal of the insoluble dicyclohexylurea, the bulk[32] of the product was applied to the top of a DEAE–cellulose (carbonate form) column (23 × 2 cm.). The column was washed with water until no further ultraviolet-absorbing material was eluted (total amount eluted at this stage, 600 optical density units at 252 mμ). Further elution was carried out using a linear gradient of triethylammonium bicarbonate (pH 7.5). The mixing vessel and the rservoir contained, respectively, 1 l. of water and an equal volume of 0.25 *M* salt. A flow rate of 13–15 ml./20 min. fraction was maintained. After a small peak (170 optical density units, fractions 42–56), the desired product was eluted in fractions 59–72. The pooled fractions contained a total of 1720 optical density units (252 mμ) of this product. (The unreacted deoxyguanosine-5′ phosphate (610 optical density units, 252 mμ) was eluted on continued elution as the third peak.) The yield of the dinucleoside phosphate, disregarding hypochromicity, was 51% of the total optical density units recovered from the column. This material was homogeneous in solvents A and C (see table of R_f's) and as judged by paper electrophoresis at pH 7.1. It was completely degraded by spleen and venom phosphodiesterases under the standard conditions used. The ultraviolet absorption characteristics in water were: λ_{max} at 252 mμ, ratio of $\epsilon_{260\ m\mu}/\epsilon_{280}$ was 1.65, that of deoxyguanosine-5′ phosphate being 1.5.

N^6-Anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine.—An anhydrous pyridine solution (8 ml.) of 5′-O-di-*p*-methoxytrityl-N^6-anisoyldeoxycytidine (0.450 g., 0.68 mmole), 3′-O-acetylthymidine-5′ phosphate (0.7 mmole), and DCC (1.5 g.) was kept sealed at room temperature for 4 days. Water (1 ml.) and pyridine (2 ml.) were added and the insoluble dicyclohexylurea removed by filtration and washed with pyridine (20 ml.). The total filtrate was extracted twice with a 1:1 mixture of cyclohexane and ether and the aqueous pyridine solution evaporated to about 3 ml. An equal volume of pyridine was further added and the solution re-evaporated to about 3 ml. This solution was now made up to 25 ml. with pyridine (stock solution).

An aliquot (2 ml.) was kept in ethanolic ammonia (3 ml. of concentrated ammonia + 2 ml. of ethyl alcohol) for 2 days at room temperature. The turbid solution was then evaporated to dryness and the residue taken up in 5 ml. of 80% acetic acid. After 2 hr. at room temperature, the acetic acid was removed by evaporation and the gummy residue shaken with a mixture of water and ether. The aqueous layer was chromatographed on a 9-in. wide strip of Whatman 3 MM paper in solvent A. A yield of deoxycytidylyl-(3′ → 5′)-thymidine in the above reaction was thus determined to be 76% [770 optical density units (269 mμ) of the product from the 2-ml. aliquot of the above stock solution].

A portion (10 ml.) of the above pyridine stock solution was evaporated to about 1 ml. after the addition of a small amount (0.02 ml.) of concentrated ammonia. Water (2 ml.) was added and the solution re-evaporated to remove residual pyridine. The residue was taken up in 10 ml. of 80% acetic acid and after 1.5 hr. at room temperature the acetic acid was removed by evaporation. After an ether extraction the product was chromatographed on 4 strips (9 in. wide) of Whatman 3 MM paper in solvent C. N^6-Anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine was the major product and its yield after elution with water was 3840 optical density units (302 mμ). This yield corresponded to 0.157 mmole (58% of theory) as based on the extinction of N-anisoyldeoxycytidine at 302 mμ. This product was passed through a column of pyridinium Dowex-50 ion exchange resin and the total effluent lyophilized twice to ensure complete removal of pyridinium acetate. The lyophilized powder was dissolved in dry pyridine for use in the succeeding experiment.

The spectral characteristics of the product as eluted from a chromatogram in solvent C were: in water, λ_{max} 302 and 275 mμ, λ_{min} 295 and 236 mμ; in acid, λ_{max} 312 and 275 mμ; λ_{min} 294 and 238 mμ, $\epsilon_{302}/\epsilon_{274}$ 0.93 (in water), $\epsilon_{312}/\epsilon_{275}$ 1.03 (in acid). The R_f's in different solvents are given in Table I.

5′-O-Di-*p*-methoxytritylthymidylyl-(3′ → 5′)-thymidine.—An anhydrous pyridine (5 ml.) solution of 3′-O-acetylthymidine-5′ phosphate (1 mmole), 5′-O-di-*p*-methoxytritylthymidine (1.2 g., 2 mmoles), and DCC (1.0 g.) was kept for 3 days at room temperature. Water (1 ml.) was then added and the mixture extracted with cyclohexane (10-ml. portions) twice. More pyridine (5 ml.) was added and the mixture left at room temperature overnight. The solution was then treated with 2 *N* sodium hydroxide (10 ml.) for 5 min. at room temperature. The alkali was removed by treatment with pyridinium Dowex-50 ion exchange resin and the total solution (aqueous pyridine wash) after removal of the resin was further diluted with pyridine (about 25 ml.). Ammonium bicarbonate (1 mmole) was added and the total solution was concentrated at low temperature. The concentrate was applied to the top of a DEAE–cellulose (bicarbonate) column (45 × 3 cm. diam.) in the cold room (2°). The unreacted nucleoside was eluted by passing 2 l. of 25% aqueous ethyl alcohol through the column. Subsequent elution with 2 l. of 0.5 *M* ammonium bicarbonate (pH 8.2) in 25% ethyl alcohol gave 5′-O-di-*p*-methoxytritylthymidylyl-(3′ → 5′)-thymidine. Most of this product appeared within the first 410 ml. of the eluate. The recovery in the combined fractions was 19,300 O.D. units (268 mμ). Pyridinium Dowex-50 ion exchange resin (about 250 ml.) was added as well as 50 ml. of pyridine. After thorough mixing for about 10 min. the solution was filtered, diluted with an equal volume of pyridine, and concentrated at low temperature (Dry Ice–acetone trap). The product was stored in dry pyridine. It was homogeneous on paper chromatography in solvent A (R_f 0.74); λ_{max} 268 mμ, λ_{min} 253 mμ, with a broad shoulder at 220–240 mμ (in water–methyl alcohol, 1:1).

5′-O-Monomethoxytritylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine.—A mixture of pyridinium N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate (1.3 mmoles), 5′-O-monomethoxytritylthymidine (1.45 mmoles), anhydrous pyridinium Dowex-50 ion exchange resin (8% cross linked, 500 mg.), DCC (1.1 g.), pyridine (11 ml.), and dimethylformamide (2 ml.) was kept at room temperature for 3 days. Water (1 ml.) was then added and the mixture extracted with ether (this extraction removed the excess

(30) In all the experiments involving di-*p*-methoxytrityldeoxynucleosides, the protected nucleotide was first rendered anhydrous by evaporation of its solution in pyridine before the addition of the protected nucleoside. This was necessary to avoid any loss of the di-*p*-methoxytrityl group.

(31) This presumably was T-x, the labile derivative of thymidine described above in thymidylyl-(3′ → 5′)-thymidine synthesis.

(32) A test run of an aliquot on a paper chromatogram in solvent A showed lack of separation of the desired product from unreacted deoxyguanosine-5′ phosphate and the corresponding pyrophosphate.

of the reagent and the unreacted nucleoside). More pyridine (5 ml.) was added and after keeping overnight the mixture was cooled to 0° and treated with 10 ml. of 2 *N* sodium hydroxide for 2 min. at 2°. An excess of pyridinium Dowex-50 ion exchange resin was added to neutralize the alkali, the resin was removed by filtration and washed with aqueous pyridine. The total solution was evaporated at low temperature after addition of 2 mmoles of ammonium bicarbonate. The concentrate (about 30 ml.) was made up to 50 ml. (10 ml. of ethyl alcohol, the remainder being water). Chromatography of an aliquot in solvent C showed in addition to the desired product a small amount of the nucleotide. The total material was applied to the top of a DEAE–cellulose (carbonate) column (70 × 3.8 cm.). The column was washed with 250 ml. of 20% aqueous ethyl alcohol and elution was then carried out with a linear gradient of ammonium bicarbonate. The reservoir contained 2 l. of 0.3 *M* salt and the mixing vessel an equal volume of 0.03 *M* salt. The column was maintained at about 15° by circulating cold water in a jacket; 20-ml. fractions were collected. After some minor fore peaks, the major product began to appear in fractions 49 on. At fraction 120 when the salt concentration in the effluent was 0.2 *M* and the concentration of the product was still increasing, the gradient was discontinued and 0.3 *M* ammonium bicarbonate solution (20% ethyl alcohol, 600 ml.) was passed through the column to complete the elution. Examination of selected fractions (49–120) by paper chromatography in solvent A showed contamination of the desired product with N-acetyldeoxyguanosine-5′ phosphate. The fractions 50–60 and 84–155 contained only 5′-O-di-*p*-methoxytrityl-thymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine and were combined (total O.D. 27,700, 260 mμ). Fractions 61–83 contained a total of 5700 O.D. units (260 mμ) and by further partition chromatography on a cellulose column afforded 2500 O.D. units of the pure protected dinucleoside phosphate. The above pooled solution of the product was treated with an excess of pyridinium Dowex-50 ion exchange resin (to exchange ammonium ions with pyridinium ions), diluted with an equal volume of pyridine, and concentrated under reduced pressure (Dry Ice–acetone trap). The bulk of the material was stored as a solution in pyridine. A part of the above product could be lyophilized as the ammonium salt without any detectable decomposition. The R_f's are given in Table I. The ultraviolet absorption characteristics as eluted with 25% ethyl alcoholic ammonium bicarbonate solution were: λ_{max} 262 mμ, λ_{min} 243 mμ, shoulder at 235 mμ, $\epsilon_{262}:\epsilon_{243}:\epsilon_{235}$ = 1.32:1:1.08.

N⁶-Anisoyl-3′-O-acetyldeoxycytidine-5′ Phosphate.—Pyridinium N-anisoyldeoxycytidine-5′ phosphate[6] (2 mmoles) was rendered anhydrous by repeated evaporation of dry pyridine solution. Partial crystallization of the product occurred and, subsequently, on addition of pyridine (50 ml.), a completely clear solution was not obtained. Shaking of the sealed reaction mixture after addition of 5 ml. of acetic anhydride gave a clear solution within 10 min. The solution was kept in the dark for 2–5 hr. and then methyl alcohol (10 ml.) was added. After a further 4 hr. at room temperature the solution was evaporated to about 5 ml. and ether (100 ml.) and water (5 ml.) were added. After separation of the layers, the aqueous layer was again extracted with ether (20 ml.) and then lyophilized after dilution with more water (200 ml.). A pale yellow powder was thus obtained. It was immediately dissolved in dry pyridine and the solution stored at −15°. The yield as determined spectrophotometrically was 97%. The product was homogeneous by paper chromatography in solvent C (R_f 0.64; that of the starting material, 0.53).

Synthesis of other Deoxyribonucleotidyl-(3′ → 5′)-deoxyribonucleosides.—The general method for condensation of different protected nucleotides with protected nucleosides was as described above under "Study of Rate of Internucleotide Bond Synthesis." All the condensations were carried out at about 0.2-mmole scale using 0.2 *M* solution in dry pyridine. The yields of products given below were calculated disregarding any hypochromicity.

Deoxyadenylyl-(3′ → 5′)-thymidine.—The isolated yield from 0.195 mmole of each of the two components was 1991 optical density units (261 mμ, 0.081 mmole). The product had in water λ_{max} 261 mμ, λ_{min} 230 mμ (R_f's in Table I).

Deoxyguanylyl-(3′ → 5′)-thymidine.—The isolated yield from a 0.2-mmole reaction was 2530 optical density units (257 mμ, 0.122 mmole); λ_{max} in water 257 mμ, λ_{min} 228 mμ.

Deoxyguanylyl-(3′ → 5′)-deoxycytidine.—The isolated yield of 1520 optical density units (256 mμ, 0.077 mmole) was low due to losses during work-up; λ_{max} 256 mμ, λ_{min} 225 mμ with a shoulder at 268 mμ.

Thymidylyl-(3′ → 5′)-deoxyadenosine.—The isolated yield was 2012 optical density units (261 mμ, 0.082 mmole); λ_{max} 261 mμ, λ_{min} 230 mμ.

Deoxycytidylyl-(3′ → 5′)-deoxycytidine.—The isolated yield was 2500 optical density units (280 mμ at pH 1, 0.096 mmole); λ_{max} 271 mμ, λ_{min} 248 mμ, in water; λ_{max} 279 mμ, λ_{min} 240 mμ, in acid.

Deoxycytidylyl-(3′ → 5′)-deoxyguanosine.—N,O³′-Diacetyldeoxyguanosine-5′ phosphate (0.72 mmole), N⁶-benzoyl-5′-O-dimethoxytrityldeoxyguanosine (220 mg., 0.35 mmole), Dowex-50 (pyridinium) ion exchange resin (200 mg.), dimethylformamide (1 ml.), pyridine (1 ml.), and DCC (2.5 mmoles) were kept at room temperature for 3 days. One-fifth of the total mixture was worked up in the standard way. The yield of deoxycytidylyl-(3′ → 5′)-deoxyguanosine was 89%. A little unreacted deoxycytidine (3%) was recovered.

5′-O-Phosphoryl-N⁶-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine. Method A.—The bulk (0.14 mmole) of the pyridine solution of N⁶-anisoyldeoxycytidylyl-(3′ → 5′)-3′-O-acetylthymidine prepared above was evaporated and treated in dry pyridine (1 ml.) with a mixture of pyridinium β-cyanoethyl phosphate (0.7 mmole) and DCC (0.300 g., 1.4 mmoles) for 64 hr. at room temperature. Water (2 ml.) was then added and the mixture kept for 1 day. Dicyclohexylurea was filtered off and washed with aqueous ethyl alcohol (1:1). The total filtrate was evaporated to about 15 ml. and the solution cooled to 0°. Cold 2 *N* sodium hydroxide (10 ml.) was added and the solution kept at 0° for 6 min.[33] An excess of pyridinium Dowex-50 ion exchange resin was added to neutralize the alkali and after removal of the resin by filtration (water wash) the filtrate was made up to 100 ml. with water. The total optical density at 302 mμ was 2720.[34]

A portion (one-fifth of the total) of the above was concentrated to about 5 ml. and retreated with 2.5 ml. of 2 *N* sodium hydroxide at 0° for 10 min. After neutralization with pyridinium Dowex-50 ion exchange resin, removal of the resin, and concentration of the total filtrate, the products were separated on two 9-in. wide strips of Whatman 3 MM paper in solvent A. The different ultraviolet-absorbing bands were eluted with water and their concentrations as determined spectrophotometrically were: 5′-O-phosphoryldeoxycytidylyl-(3′ → 5′)-thymidine (R_f 0.05), 6.5 μmoles; 5′-O-phosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine (R_f 0.17), 16.5 μmoles; deoxycytidylyl-(3′ → 5′)-thymidine (R_f 0.31), 0.7 μmole; N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine (R_f 0.71), 1.6 μmoles, and another unidentified product (R_f 0.5), 0.3 μmole. The yield in the above phosphorylation reaction (the total of N-anisoyldinucleotide and the dinucleotide d-pCpT) was 84%.

The remainder (four-fifths) of the stock solution obtained above was concentrated under reduced pressure[35] and directly applied to the top of a DEAE–cellulose (carbonate form) column (2.5 × 30 cm.). Elution was carried out using a linear salt gradient, the mixing vessel containing 1 l. of water and the reservoir 1 l. of 0.5 *M* triethylammonium bicarbonate (pH 7.5). Fractions of about 13 ml./10 min. were collected and examined for absorption both at 270 and 302 mμ, in order to select for the protected dinucleotide having the characteristic N-anisoyldeoxycytidine absorption at 302 mμ.[6] (For correct 302 mμ/274 mμ extinction ratio, see the preceding experiment.) The following four peaks with absorption at 302 mμ were obtained: peak I (fractions 36–42), 110 O.D. units, N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine; peak II (fractions 44–53), 300 O.D. units, mainly[36] 5′-O-cyanoethylphosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine; peak III (fractions 56–61), 120 O.D. units, unidentified; peak IV (fractions 66–89), 1040 O.D. units, 5′-O-phosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-thymidine. On treatment with concentrated ammonia the sole product formed was 5′-O-phosphoryldeoxycytidylyl-(3′ → 5′)-thymidine (d-pCpT).

Method B.—To an anhydrous mixture of pyridinium N⁶-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate[23] (0.6 mmole), 3′-O-acetylthymidine-5′ phosphate (1.0 mmole), pyridinium Dowex-50 ion exchange resin (0.5 g.), pyridine (5 ml.), dimethylformamide (0.5 ml.), DCC (1.0 g.) was added and the reaction mixture shaken at room temperature. After 3 days, water (3 ml.) was added and the excess of the reagent extracted with chloroform (2 × 20 ml.). To the aqueous layer, pyridine (5 ml.) was added and the solution left at room temperature for 5 hr. The volume was adjusted with water to 20 ml. and cooled at 0°. An equal volume of 2 *M* sodium hydroxide was then added and the mixture kept at 0° for 20 min. after which time the reaction was stopped by the rapid addition of an excess of pyridinium Dowex-50 ion exchange resin. The resin was removed by filtration and washed with water (total volume about 150 ml.). The total filtrate was evaporated after addition of ammonium bicarbonate (5 mmoles) to 25 ml. The precipitate which ap-

(33) This treatment was later found to be insufficient for the removal of the cyanoethyl group and the treatment was repeated on a portion (see below).

(34) The difference between the total amount used and recovered seems to be due to partial loss of the N-anisoyl group. This probably occurred because of the partly ethanolic solvent used for removal of the cyanoethyl group. The alcohol, presumably, provided the very powerful base, ethoxide ion.

(35) A paper chromatogram in solvent A confirmed that a small portion of the product had lost the N-anisoyl group.

(36) The contaminant appeared to be the dinucleotide without the N-anisoyl group. This is evidenced by the fact that the material in this peak on treatment with concentrated ammonia gave only the dinucleotide (d-pCpT).

peared was removed by centrifugation and the supernatant was applied at 2° on top of a column (3.5 × 45 cm.) of DEAE-cellulose (carbonate). Elution was carried out in the cold by a linear gradient of ammonium bicarbonate (pH 8.5) solution (3 l. of water in the mixing vessel and an equal volume of 0.3 *M* salt in the reservoir); 20-ml. fractions were collected at a flow rate of 2.6 ml./min. After a pyridine peak (20–30 fractions), mononucleotides appeared in fractions 45–145. The desired dinucleotide was present in fractions 172–196. Fractions 177–194 were pooled and evaporated in the standard way to give 6500 O.D. units (302 mμ, 0.29 mmole, 48%) of pure dinucleotide. The product was pure by paper electrophoresis and in solvent C and identical with the sample prepared by method A.

N-Acetyldeoxyadenosine-5′ Phosphate.—N,O$^{3'}$-Diacetyldeoxyadenosine-5′ phosphate was prepared as described previously.[37] The rate of removal of the N-acetyl group was followed by measuring the decrease in ultraviolet absorption at 290 mμ in 1 *N* sodium hydroxide (adenosine has virtually no absorption at 290 mμ, whereas the N-acetyl derivative has λ_{max} 286 mμ in alkali). The half-life of the N-acetyl group was thus found to be 160 min. at room temperature. For preparation of N-acetyldeoxyadenosine-5′ phosphate, N,O$^{3'}$-diacetyldeoxyadenosine-5′ phosphate was kept in 1 *N* sodium hydroxide at 0° for 3 min. N-Acetyldeoxyadenosine-5′ phosphate, the sole product, was isolated by the procedure described for the preparation of N-acetyldeoxyguanosine-5′ phosphate[8]; R_f's in solvent C: N-acetyldeoxyadenosine-5′ phosphate, 0.51; 3′-O-acetyldeoxyadenosine-5′ phosphate,[37] 0.46; N,O^3-diacetyldeoxyadenosine-5′ phosphate, 0.62; deoxyadenosine-5′ phosphate, 0.35.

Cleavage of P^1,P^2-3′-O-Acetylthymidine-5′ Pyrophosphate by Reaction With Hydroacrylonitrile and DCC.—P^1,P^2-3′-O-Acetylthymidine-5′ pyrophosphate (0.2 mmole) was treated in dry pyridine (2 ml.) with hydroacrylonitrile (0.29 ml., 2 mmoles) and DCC (2 mmoles) for 3 days at room temperature. Water (1 ml.) was then added and the mixture extracted twice with 10-ml. portions of ether. The aqueous layer was made up to 10 ml. with pyridine and the solution kept at room temperature. Chromatography of the solution in solvent C at the start showed the major product to be 3′-O-acetylthymidine-5′ β-cyanoethyl phosphate (R_f 0.63), but in addition a minor faster traveling product (R_f 0.86) was also present. The latter product lacked any phosphoryl dissociation as indicated by its zero mobility on paper electrophoresis (pH 7.1). Chromatography of the aqueous pyridine solution after 1 week showed the disappearance of the neutral side product, the yield of the desired β-cyanoethyl thymidine-5′ phosphate being quantitative.

In another experiment, the neutral product was present initially in the amount of 18%. On maintaining the total reaction mixture in dilute aqueous ethanolic ammonia solution at around pH 8.5 at room temperature, the neutral product could be hydrolyzed to give the cyanoethyl thymidine-5′ phosphate as the sole product.

Alkaline Hydrolysis of 3′-O-Acetylthymidine-5′ β-Cyanoethyl Phosphate.—A solution of this ester (0.2 mmole; see foregoing preparation) in 5 ml. of pyridine was treated at 0° with an equal volume of 1 *M* sodium hydroxide. Aliquots were removed at different intervals and treated with an excess of pyridinium Dowex-50 ion exchange resin in the cold, the neutralization being complete within 0.5 min. The products were analyzed by paper chromatography in solvent C using Whatman 40 paper. The half-life of the acetyl group was too short to be measured (under 1 min.), while the half-life of the cyanoethyl group was about 5 min.

In another experiment, the same reactions were studied in 1 *M* sodium hydroxide at 0°. The half-life of the cyanoethyl group was about 2.5 min.

(37) Prepared by acetylation of deoxyadenosine-5′ phosphate with acetic anhydride–pyridine for a period of about 1 hr.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXVI.[1] The Stepwise Synthesis of Specific Deoxyribopolynucleotides (6).[2] The Synthesis of Thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine and of Polynucleotides Containing Thymidine and Deoxyadenosine in Alternating Sequence[3]

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5′-O-Dimethoxytritylthymidylyl-(3′ → 5′)-N-benzoyldeoxyadenosine was prepared by the condensation of N-benzoyl-3′-O-acetyldeoxyadenosine-5′ phosphate with 5′-O-dimethoxytritylthymidine in the presence of dicyclohexylcarbodiimide (DCC), followed by alkaline treatment. Condensation of this protected dinucleoside phosphate with 5′-O-phosphorylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-3′-O-acetylthymidine followed by a work-up inclusive of chromatography gave a 12% isolated yield of the pentanucleotide thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine. Condensation of thymidine-5′ β-cyanoethyl phosphate with N-benzoyl-3′-O-acetyldeoxyadenosine-5′ phosphate followed by an alkaline treatment gave the protected dinucleotide 5′-O-phosphorylthymidylyl-(3′ → 5′)-N-benzoyldeoxyadenosine in 30–40% yield. The treatment of the latter in dry pyridine with DCC for 6 days followed by a work-up inclusive of ammoniacal treatment gave a polymeric mixture containing polynucleotides with alternating thymidine and deoxyadenosine residues. Products up to the dodecanucleotide d-pT-(pApT)$_5$-pA were characterized, higher polynucleotides being present in detectable amounts. The synthesis of 5′-O-dimethoxytritylthymidylyl-(3′ → 5′)-thymidine-3′ phosphate and the corresponding unprotected dinucleotide is recorded. 5′-O-Trimethylacetylthymidylyl-(3′ → 5′)-3′-O-acetyl-N-benzoyldeoxyadenosine was prepared.

In continuation of the work reported in the two accompanying papers,[1,2d] the synthesis of larger oligonucleotide chains by condensation of preformed "blocks" of di- and trinucleotides[4,5] was undertaken and the results are presented in this and the succeeding paper.[6] The synthesis of the pentanucleotide thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine and of polynucleotides containing thymidine and deoxyadenosine residues in alternating sequence, which were prepared by polymerization of the suitably protected thymidine–deoxyadenosine dinucleotide, are reported in this paper. The following paper deals mainly with parallel investigations of polynucleotides containing deoxycytidine and deoxyguanosine.[6] A preliminary report of some of this work has been published.[7]

Thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (VI).—

(1) Paper XXV: H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3828 (1963).

(2) Previous papers which deal directly with this topic: (a) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958); (b) *ibid.*, **81**, 4647 (1959); (c) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962); (d) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (e) H. Schaller and H. G. Khorana, ref. 1.

(3) This work has been supported by grants from the Life Insurance Medical Research Fund, New York, N. Y., the National Science Foundation, Washington, D. C., and the National Cancer Institute of the National Institutes of Health, Bethesda, Md.

(4) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(5) The only previous study along these lines involved thymidine oligonucleotides. Notably, the synthesis of a tetranucleotide by condensation of 5′-O-tritylthymidylyl-(3′ → 5′)-thymidine-3′ phosphate with thymidylyl-(3′ → 5′)-3′-O-acetylthymidine was recorded (G. Weimann and H. G. Khorana, ref. 2c).

(6) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

(7) H. Schaller, G. Weimann, and H. G. Khorana, *ibid.*, **85**, 355 (1963).