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Studies on Polynucleotides. XIII.[1] Stepwise Synthesis of Deoxyribo-oligonucleotides. An Alternative General Approach and the Synthesis of Thymidine Di-, Tri- and Tetranucleotides Bearing 3′-Phosphomonoester End Groups[2]

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Phosphorylation of 5′-*O*-tritylthymidine with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide (DCC) followed by alkaline treatment of the product (II) gave 5′-*O*-tritylthymidine-3′ phosphate. Acid treatment of II gave β-cyanoethylthymidine-3′ phosphate (X). Condensation of 5′-*O*-tritylthymidine-3′-phosphate with 3′-*O*-acetylthymidine in the presence of DCC gave 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine (VIII). 5′-*O*-Tritylthymidylyl-(3′→5′)-thymidine-3′-phosphate (IX) was prepared by phosphorylation of VIII with a mixture of β-cyanoethylphosphate and DCC and, preferably, by condensation of 5′-*O*-tritylthymidine-3′ phosphate with β-cyanoethyl thymidine-3′ phosphate (X). Condensation of the protected dinucleotide, IX, with 3′-*O*-acetylthymidine gave 5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (XI). Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine-3′ phosphate was prepared by the condensation of the protected dinucleotide (IX) with β-cyanoethyl thymidine-3′ phosphate. Condensation of the same protected dinucleotide (IX) with thymidylyl-(3′ → 5′)-3′-*O*-acetylthymidine (XVII) followed by alkaline treatment gave 5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (XVIII) from which by phosphorylation with a mixture of β-cyanoethyl phosphate and DCC, the tetranucleotide thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine-3′ phosphate was prepared. The nature of some of the side products formed in the above condensations is discussed.

Introduction

Progress has been made in recent years on the chemical synthesis of the C_3'–C_5' linked deoxyribopolynucleotides.[3,4] The studies reported have been concerned with the polymerization of either unprotected or suitably protected mononucleotides[5] or with the stepwise synthesis of deoxyriboöligonucleotides.[6,7] For stepwise synthesis, the reactions of suitably protected nucleotides bearing 5′-phosphomonoester groups with protected nucleosides or nucleotides bearing free 3′-hydroxyl groups were studied first.[6] This work then was extended to reactions of suitably protected mononucleotides with components containing a 3′-hydroxyl group as well as a preformed internucleotide bond, and syntheses of thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine and thymidylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxycytidine were realized.[7] From these and the studies on polymerization of mononucleotides, several types of problems inherent in the synthesis of higher mixed polynucleotides became evident. Two major problems are, first, that of suitable protecting groups for the amino groups on the heterocyclic rings and the primary and secondary hydroxyl groups in the sugar moieties, and second, that of the reactivity of the preformed phosphodiester bonds during the activation of an appropriate phosphomonoester group in one component for phosphorylation of a hydroxylic group present in the second component. While promising methods for the protection of different functions now exist[5b,8–10] and their application in the problems of mixed polynucleotide synthesis is under investigation, the present work reports on a rather systematic study of the stepwise synthesis of internucleotide bonds by condensation of protected mono- and di-nucleotide fragments. Detailed studies of the scope of the methods and the nature of side products formed are important since, clearly, in undertaking the stepwise synthesis of polynucleotide chains, it would be advantageous to be able to condense preformed oligonucleotides. The present work, initial to the synthesis of mixed polynucleotides, has been carried out with oligonucleotides derived from thymidine only.

Synthesis of Protected Dinucleoside Monophosphate and Dinucleotide.—Except for the polymerization of thymidine-3′ phosphate to form C_3'–C_5' linked thymidine polynucleotides,[11] the approach used in all the previous work has been to condense the 5′-phosphomonoester group of a mononucleotide with the 3′-hydroxyl group of a second component. In the present work, the alternative general approach has been investigated according to which the 3′-phosphomonoester group of a nucleotide is activated to phosphorylate the 5′-hydroxyl group of a second component. The sequence of reactions used for the synthesis of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine[6] (VIII) is shown in Chart I.

Phosphorylation of 5′-*O*-tritylthymidine (I) with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide (DCC),[12] followed by a mild alkaline treatment of the initial product (II) gave III, which was isolated pure in a yield of 88%. It is of interest that when the work-up after phosphorylation involved a direct alkaline treatment instead of an aqueous pyridine treatment, a small amount of a side product was produced, to which structure IV is tentatively assigned. This substance which was isolated pure by fractional crystalliza-

(1) Paper XII. H. G. Khorana, J. P. Vizsolyi and R. K. Ralph, *J. Am. Chem. Soc.*, **84**, 414 (1962). (Preceding paper.)

(2) This work has been supported by grants from the National Institutes of Health, U. S. Public Health Service and the Life Insurance Medical Research Fund, New York.

(3) H. G. Khorana in E. Chargaff and J. N. Davidson, eds., "The Nucleic Acids," Vol. III, Academic Press, Inc., New York, N. Y., 1960, p. 105.

(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.

(5) See *e.g.*; (a) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961). (b) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).

(6) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

(7) P. T. Gilham and H. G. Khorana, *ibid.*, **81**, 4647 (1959).

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

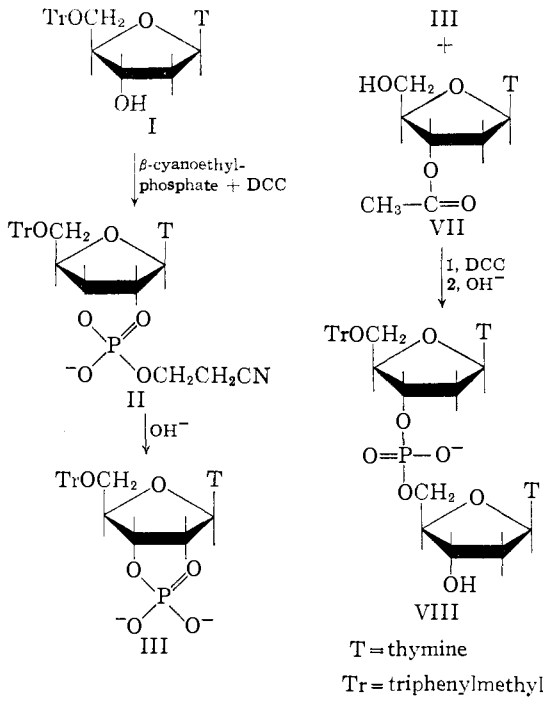
(9) M. Smith, D. H. Rammler, I. Goldberg and H. G. Khorana, *ibid.*, **84**, 430 (1962).

(10) B. Lerch and H. G. Khorana, unpublished work.

(11) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

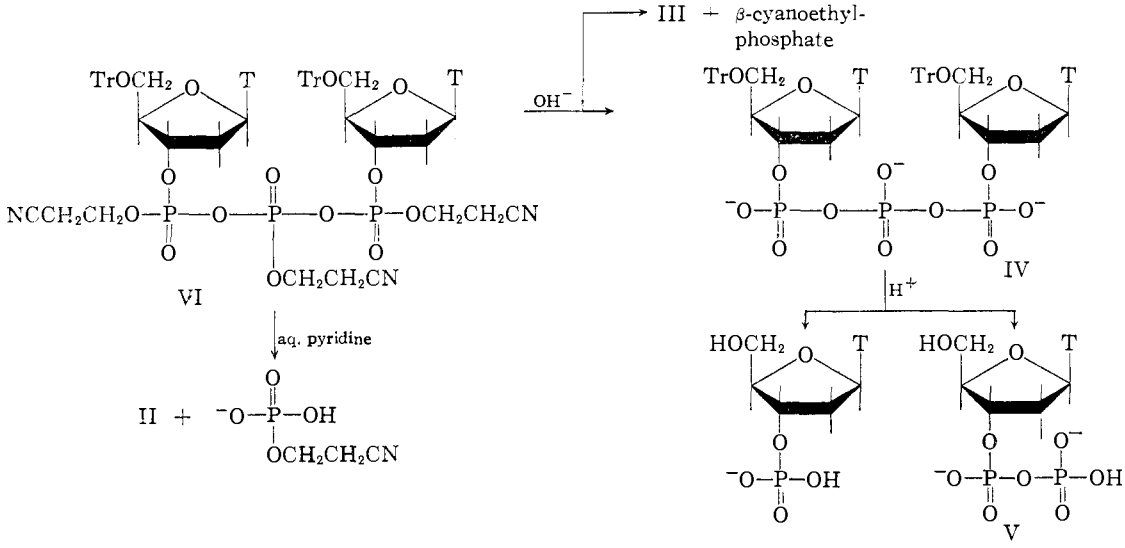
(12) G. M. Tener, *ibid.*, **83**, 159 (1961).

CHART I.—SYNTHESIS OF 5-*O*-TRITYLTHYMIDYLYL-(3′ → 5′)-THYMIDINE



tion had a thymidine to phosphorus ratio of 2:3. It was labile to acid giving after a brief treatment, thymidine-3′ phosphate, a new nucleotide, evidently thymidine-3′ pyrophosphate (V) and some inorganic phosphate. The ratio of labile phosphorus to total phosphorus in IV was 1:3. The identification of IV is of interest from the standpoint of the mechanism of phosphodiester bond synthesis by the activation of phosphomonoesters. As has been previously suggested, the active phosphorylating species may be poly- or meta- phosphates.[4,6,7,13] The initial product giving rise to IV would be VI, which on being kept in aqueous pyridine hydrolyzes as shown to II and β-cyanoethyl phosphate. With alkali, the initial major reaction is again the cleavage of the pyrophosphate bonds to form 5′-*O*-tritylthymidine-3′ phosphate (III) and β-cyanoethyl phosphate but, in addition, preferential elimination of the β-cyanoethyl group occurs to give some of IV.

The condensation of 5′-*O*-tritylthymidine-3′ phosphate (III) with two equivalents of 3′-*O*-acetylthymidine (VII) in the presence of DCC[14] gave after a four day reaction and subsequent alkaline treatment an excellent yield of the di-nucleoside phosphate (VIII). The virtually quantitative yield obtained with respect to III when an excess of 3′-*O*-acetylthymidine was used parallels the previous findings on the condensation of 3′-*O*-acetylthymidine-5′ phosphate with 5′-*O*-tritylthymidine using similar ratios of the two components.[3,4] It was, however, surprising that when a 1:1 molar ratio of the two components was used, the yields obtained in the two sets of experiments were significantly different. In the reaction between 5′-*O*-tritylthymidine-3′ phosphate and 3′-*O*-acetylthymidine the internucleotide bond formation levelled off at about 45%,[15,16] whereas in the experiment between 3′-*O*-acetylthymidine-5′ phosphate and 5′-*O*-acetylthymidine the yield was around 60%.[6] It should be noted that the yield in the latter experiment when 5′-*O*-tritylthymidine was used in place of 5′-*O*-acetylthymidine was again of the same order and was in fact higher (66%) when



(13) M. Smith, J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(14) A comparative study of some activating agents in the polymerization of mononucleotides is reported in the preceding paper.[1] In the present work, a parallel experiment was carried out on the condensation of 5′-*O*-tritylthymidine-3′ phosphate and 3′-*O*-acetylthymidine using trichloroacetonitrile. [F. Cramer and G. Weimann, *Chem. and Ind. (London)*, 42 (1960).] After work-up including an acidic treatment, only P^1, P^2-dithymidine-3′ pyrophosphate was obtained and none of the thymidylyl-(3′ → 5′)-thymidine was observed up to 64 hr. at room temperature.

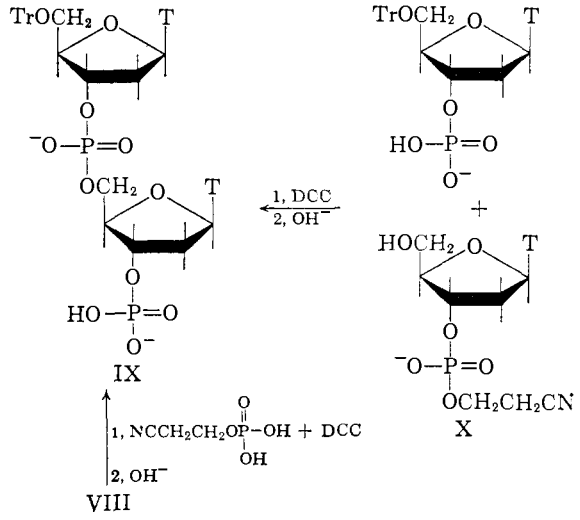
(15) A further amount of DCC was added after 170 hr. to ensure an excess of the reagent. No increase in yield was observed.

(16) More recent experiments have shown this relatively low yield to be due to the bulky trityl group in 5′-*O*-tritylthymidine-3′ phosphate. For a detailed study of the internucleotide bond formation using thymidine-3′ phosphate containing different protecting groups in the 5′-position, see G. Weimann and H. G. Khorana, *Chem. and Ind. (London)*, in press.

an acidic step was given at the end to remove the 5′-*O*-trityl group.[6]

The synthesis of the protected dinucleotide, 5′-*O*-tritylthymidyl-(3′→5′)-thymidine-3′ phosphate (IX), was investigated next. In the first approach, (Chart II) VIII was phosphorylated with an excess of a mixture of β-cyanoethyl phosphate and DCC and after work-up including an alkaline treatment the desired IX was obtained pure in a yield of 68%.[17]

Chart II.—Synthesis of 5′*O*-tritylthymidylyl-(3′ → 5′)-thymidine-3′ phosphate.



An alternative, more direct approach to the dinucleotide IX involved the condensation of III with X (Chart II). The latter component, which was prepared in an excellent yield by mild acidic treatment of II, may be regarded as a nucleotide in which the phosphomonoester group is "protected" by conversion to a diester. Since the condensation of III with X in the presence of DCC represents a simple model of the reactions involving activation of a phosphomonoester in the presence of a phosphodiester bond, the reaction was investigated in considerable detail. The results of three parallel experiments using 1:1, 2:1 and 1:2 molar proportions of III and X are shown in Fig. 1. The finding of practical interest was that the yield of the desired dinucleotide IX was approximately the same in all of the experiments, a result which is in contrast with those obtained in parallel experiments on the condensation of a protected nucleotide with a protected nucleoside. Nevertheless, the yield, nearly the maximum, obtained by using a 1:1 ratio of the two components was considerably higher (55–60% as against 45%), than that obtained in condensation of III with the simpler 3′-*O*-acetylthymidine under similar conditions. It may be concluded that the hydroxylic component, when it also carries a phosphate group, as is the case in X, is joined to the activated intermediates derived from III by means of a pyrophosphate bond and that by virtue of this the phosphorylation of the 5′-hydroxyl group in

(17) An excess of DCC (three-fold) over β-cyanoethyl phosphate was used. When only one mole of DCC was used per mole of β-cyanoethyl phosphate, the yield of the phosphorylated product was only 23%, even though β-cyanoethyl phosphate was present in a 5-fold excess over the dinucleoside phosphate.

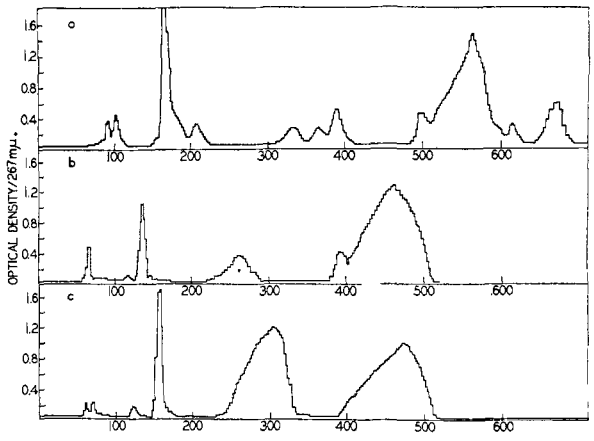


Fig. 1.—Synthesis of 5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidine-3′ phosphate (IX). (a) Using 2:1 molar ratio of thymidine-3′ β-cyanoethyl phosphate and 5′-*O*-tritylthymidine-3′ phosphate, the material in fractions 510–600 was pure dinucleotide (IX); (b) and (c), elution patterns obtained by using, respectively, 1:1 and 1:2 molar ratio of thymidine-3′ β-cyanoethyl phosphate and 5′-*O*-tritylthymidine-3′ phosphate. The elution patterns were obtained by chromatography of total products on DEAE-cellulose (bicarbonate) columns using triethylammonium bicarbonate gradient for elution. Conditions for (a) were slightly different from those for (b) and (c), for which identical conditions were used.

the "bound" X becomes more facile than that of the corresponding group in 3′-*O*-acetylthymidine.

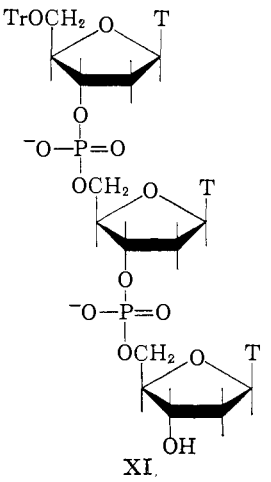
While the failure to increase the yield of the dinucleotide, using an excess of one of the components of the reaction over the second is not clear because of the complexity of the mechanism of the diester bond synthesis, it can be seen from Fig. 1 that the elution pattern was the most complex when an excess of X was used. A major cause of the side products formed was the lack of complete stability of X itself in the presence of DCC.

Treatment of X with DCC in dry pyridine followed by an alkaline treatment gave small amounts of thymidine-3′,5′ cyclic phosphate, thymidylyl-(3′→5′)-thymidylic-(3′) acid and other products emerging later than the dinucleotide from a DEAE-cellulose column (Table II, Experimental). In spite of the minor side reactions obtained, X is a useful intermediate, particularly if an excess is avoided, and the route using it is preferred for the preparation of practical quantities of IX.[18]

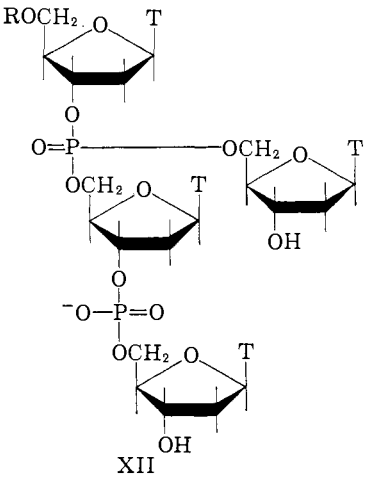
The Synthesis of Tri- and Tetra-nucleotides.—The condensations of IX with the protected nucleoside, 3′-*O*-acetylthymidine, the protected mononucleotide, β-cyanoethyl thymidine-3′ phosphate (X), and the protected dinucleoside phosphate thymidylyl-(3′→5′)-3′-*O*-acetylthymidine (XVII) have been studied.

The reaction of IX with DCC and 4 molar equivalents of 3′-*O*-acetylthymidine followed by a mild alkaline treatment showed the complete dis-

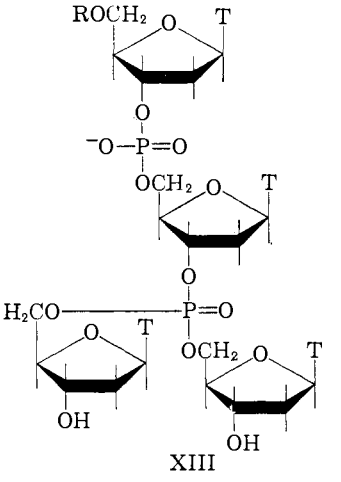
(18) More recent experiments show that the cyanoethyl group is completely removed from barium β-cyanoethyl thymidine-3′ phosphate and related compounds in about one min. at 0° in 2 *N* sodium hydroxide. These conditions appear to be selective enough for the removal of this group in the presence of some other protecting groups such as N-benzoyl groups on the purine or pyrimidine rings.[6,8,9]



XI

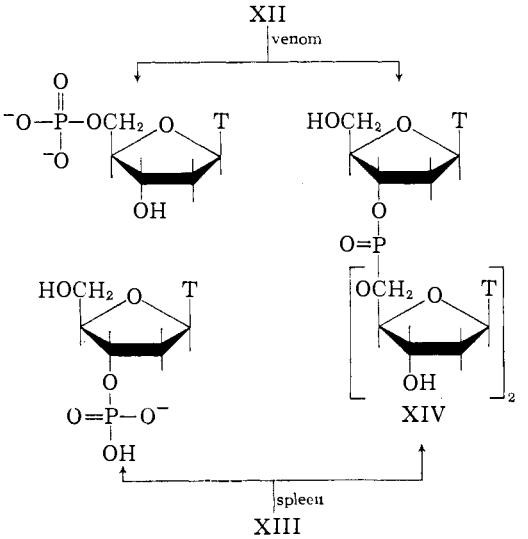


XII



XIII

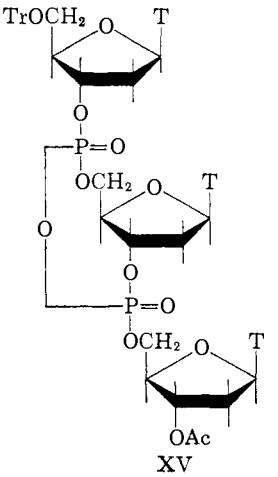
appearance of IX. The major product (49%) isolated after chromatography was the desired XI. It was homogeneous and was characterized by direct comparison with a sample synthesized earlier[7] and by degradation with the spleen phosphodiesterase after removal of the 5′-*O*-trityl group. The enzymic degradation proceeded to completion to give thymidine-3′ phosphate and thymidine in the molar ratio of two to one. A side product eluted from the DEAE-bicarbonate column before IX and XI has been tentatively concluded to be a mixture of XII and XIII. Thus its position of elution from the DEAE-cellulose column and its low mobility on paper electrophoresis indicated only one negative charge. The product gave after removal of the trityl group a new product which was partly degraded by venom phosphodiesterase to give thymidine-5′ phosphate and a neutral substance. Assuming the structure XII (R = H) for the substance being attacked by the venom phosphodiesterase, the products would be thymidine-5′ phosphate and the neutral di-thymidine-5′ thymidine-3′ phosphate (XIV). The isomeric product (XIII, R =



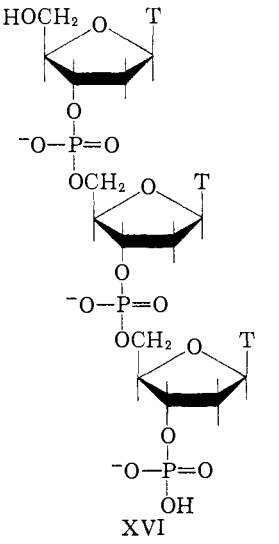
XIII

H) in the mixture was probably resistant to the venom phosphodiesterase but, as expected, was degraded by the spleen phosphodiesterase to give thymidine-3′ phosphate and again the neutral (XIV) as shown in the annexed formulae. Further evidence was obtained by alkaline degradation of the trityl-containing substances (XII and XIII; R = trityl). The products thus obtained are listed in Table III (Experimental). These products, which were identified by paper chromatography and paper electrophoresis, were all as expected for the starting material to be a mixture of XII and XIII. Thus, alkaline cleavage occurred at the tertiary phosphate linkages and the expected sets of degradation products matched fairly well in concentration.

The formation of XII and XIII presumably implies that an anhydride of the type XV or an equivalent tetra-substituted pyrophosphate derived from the phosphodiester linkages can serve as a



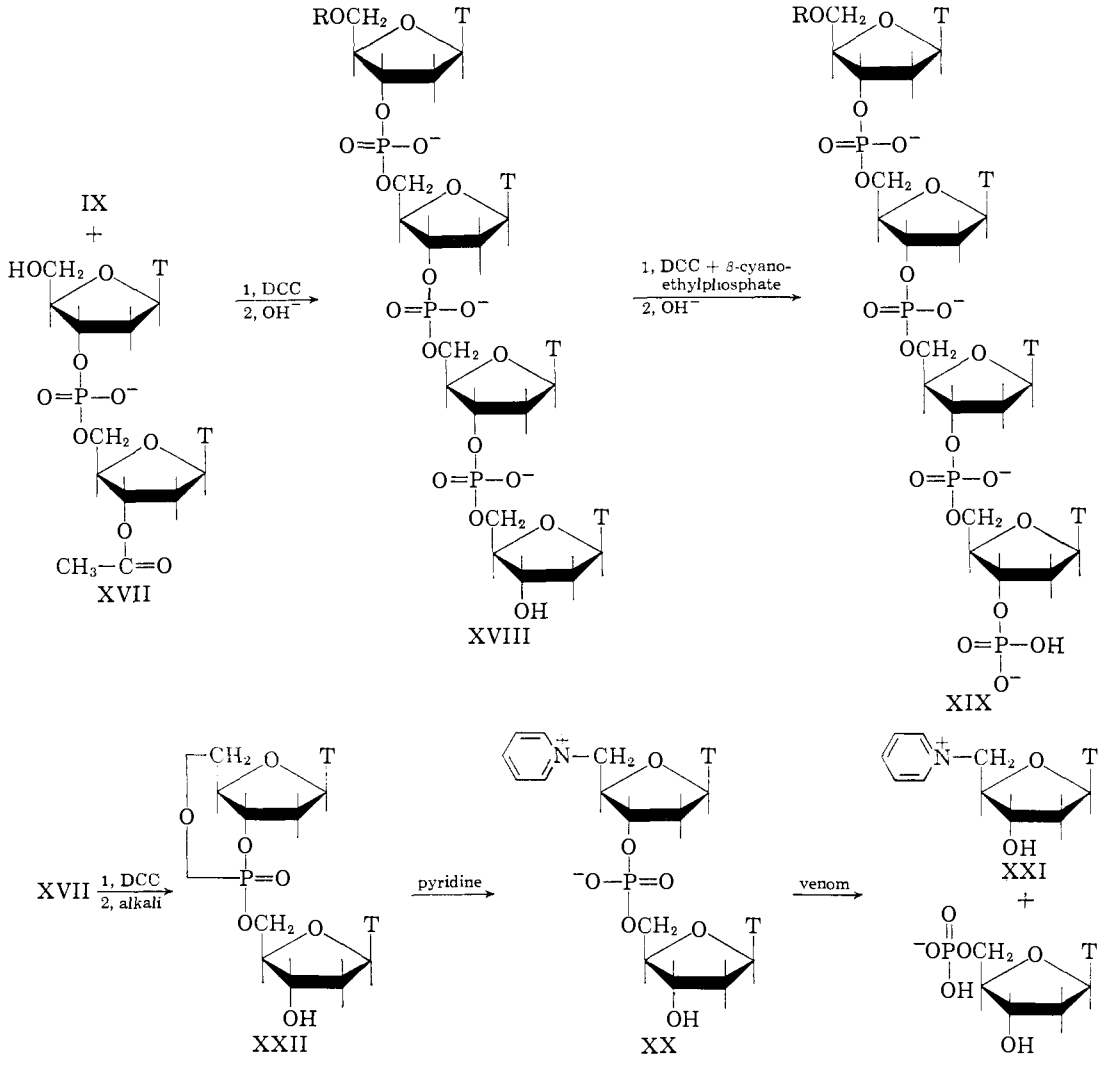
XV



XVI

phosphorylating agent if an excess of the component bearing the 5′-hydroxyl group is present. The reaction, however, is expected to be slow and its extent would be insignificant if an excess of the hydroxylic component was avoided.[18a]

(18a) The synthetic samples of thymidylyl-(3′→5′)-thymidine, the trinucleotide (XVI) and tetranucleotide (XIX), have been checked carefully for complete degradation with spleen phosphodiesterase. They were thus shown to contain exclusively C_3'–C_5' linkages. If during the synthetic reactions, any tertiary phosphate linkages, as in



The condensation of IX with 1.5 molar equivalents of β-cyanoethyl thymidine-3′ phosphate in the presence of DCC proceeded satisfactorily and, after successive alkaline and acidic treatments to remove the protecting groups, the trinucleotide XVI was isolated pure in a yield of 44% and characterized. As seen in Table IV (Experimental), a number of side products were obtained, some of which were identified.

The reaction of IX with thymidylyl-(3′→5′)-3′-*O*-acetylthymidine (XVII) gave after work-up the desired XVIII (R = trityl) which was purified by chromatography on a DEAE-cellulose column. XVIII, obtained in 23% yield, was homogeneous on paper chromatography and paper electrophoresis and after detritylation gave thymidylyl-(3′→5′-thymidyl-(3′ → 5′)-thymidyl-(3′ → 5′)-thymidine (XVIII; R = H), which was also homogeneous. The latter on degradation with a venom phosphodiesterase preparation gave thymidine-5′ phosphate and thymidine in the expected ratio. The product, XVIII (R = H), also was completely degraded by the splenic phosphodiesterase to give thymidine-3′ phosphate and thymidine. The two enzymic degradations confirmed that the synthetic product consisted entirely of C_3'–C_5' internucleotide bonds.

The trityl compound (XVIII; R = trityl) was phosphorylated by a mixture of β-cyanoethyl phosphate and DCC and the main product isolated after successive alkaline and acidic treatments was the tetranucleotide (XIX). In the small scale experiment performed the yield was about 40%. The product was homogeneous and it was completely degraded by the spleen phosphodiesterase to thymidine-3′ phosphate. Phosphorylation with a mixture of β-cyanoethyl phosphate and DCC can thus be used to introduce phosphomonoester groups at the termini of oligonucleotide chains.

In the synthesis of XVIII from IX and XVII a number of minor side products were encountered (Table V, Experimental). Two such products were evidently derived from IX, one by detritylation and another by the dephosphorylation of the phosphomonoester group. The clue to the formation of another side product was obtained by studying the reaction of XVII alone with DCC in anhydrous pyridine. After a four-day reaction followed by a mild alkaline treatment two new minor products

XII and XIII, had been formed, these would then degrade during the subsequent alkaline treatments to give some C_5'–C_5' linked compounds. The latter would be resistant to spleen phosphodiesterase. The complete degradation with this enzyme ensured that no such complication occurred.

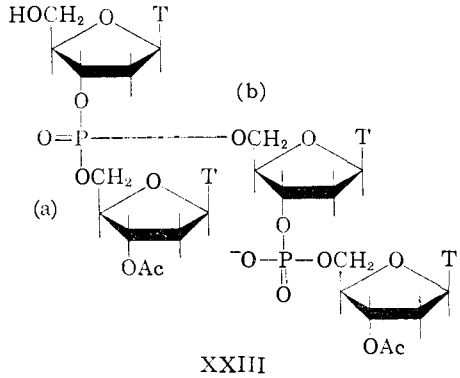
were detected. Investigation led to the tentative identification of one of these products as XX. Thus it had zero mobility on paper electrophoresis at *p*H 7.5 and at *p*H 3.5. It moved much slower than the deacetylated starting material (XVII) on paper chromatograms in Solvent A. The substance was degraded by venom phosphodiesterase to give thymidine-5′ phosphate, and a second product (tentative structure XXI) which behaved as a cation on paper electrophoresis and showed an ultraviolet spectrum additive of those of a pyridinium cation and a thymidine chromophore.

Reduction of XX with sodium borohydride gave an ultraviolet spectrum similar to that of thymidine. From this evidence the structure XX seems highly probable for the side product, although the alternative possibility in which the pyridinium cation is located at the 3′-carbon and the phosphodiester linkage involves the two 5′-hydroxyl groups cannot be ruled out.

The formation of XX probably involves[19] the intermediate XXII and the nucleophilic attack of pyridine on the cyclic tertiary ester group. This attack would be likely to occur at the 5′-carbon in preference to the secondary 3′-carbon. Some analogy is provided by the predominant formation of nucleoside 3′-phosphates on alkaline ring opening of the nucleoside-3′,5′ cyclic phosphates.[20]

It is recalled that during the polymerizations of the mononucleotides, minor by-products possessing spectra composite of the pyridinium cation and the deoxyribonucleosides were obtained.[5,11] There also was evidence of higher homologs, namely, oligonucleotide chains containing a pyridinium chromophore at one terminus. The present findings provide an explanation of the origin of the minor side products of this class. Thus they would arise by the further activation of the terminal phosphodiester bond and the subsequent formation of a cyclic tertiary phosphate with the hydroxyl group on the terminal nucleoside.

(19) Two other possible routes to the formation of XX may be considered. The first is the activation of the 5′-hydroxyl group of XVII by the formation of an isourea ether between XVII and DCC. This mechanism is unlikely because, as pointed out earlier (ref. 5a, footnote 25), the formation of XXI in reaction mixtures containing 3′-*O*-acetylthymidine and DCC in a pyridine medium has not been observed. In fact none of XXI was observed even when 3′-*O*-acetylthymidine and DCC were kept in pyridine in the presence of an acid catalyst such as pyridinium *p*-toluene sulfonate (unpublished experiment of Dr. A. F. Turner). A second possibility for the formation of XX is *via* an intermolecularly formed tertiary ester such as XXIII. In this case the nucleophilic attack by pyridine would be expected to occur equally well at sites a and b. Attack at site a would give rise to the acetylated form of XXI. The absence of XXI among the products of reaction of XVII with DCC renders this mechanism for the formation of XX unlikely.



XXIII

(20) M. Smith, G. I. Drummond and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).

Concluding Remarks.—The present work has shown that a suitably protected dinucleotide can be used as a starting material in the synthesis of oligonucleotides. Thus the stepwise synthesis need not be limited to lengthening of oligonucleotide chains in units of one at a time. However, side products were encountered during the condensations and further work is necessary to improve the yields and determine the optimal conditions for the desired reactions. Further work also is aimed at the synthesis of oligonucleotides containing different mononucleotides.

Comparing the present approach of condensing the 3′-phosphoryl group of a suitably protected nucleotide with the 5′-hydroxyl group of a second component with the previous approach in which a 5′-phosphoryl group was condensed with the 3′-hydroxyl group, the latter would appear to be preferable. This is so in view of (1) the lower yield obtained in the simple model condensation of 5′-*O*-tritylthymidine-3′ phosphate with 3′-*O*-acetylthymidine as compared with the yield obtained during condensation of 3′-*O*-acetylthymidine-5′ phosphate with 5′-*O*-tritylthymidine (see, however, ref. 16) (2) the lack of complete stability of the components bearing the 5′-hydroxyl group for example cyanoethyl thymidine-3′ phosphate under the conditions of the reaction and (3) the ready availability of deoxyribonucleoside-5′ phosphates compared with the relative inaccessibility of the -3′ phosphates. However, direct comparisons between the two approaches using parallel experiments are as yet not available and current work aims at further closer examination of the two approaches in the stepwise synthesis of polynucleotides.

Experimental

Methods.—Paper chromatography was performed by the descending technique using Whatman chromatographic paper No. 1, 40 or 3 MM. The solvents used routinely were: isopropyl alcohol–concentrated ammonia–water (7–1–2, v./v.) (Solvent A); *n*-butyl alcohol–acetic acid–water (5–2–3) (Solvent B); *n*-propyl alcohol–concentrated ammonia–water (55–10–35) (Solvent C); ethyl alcohol–1 *M* ammonium acetate, *p*H 7.5 (5–2) (Solvent D); *n*-butyl alcohol–water (86–14) (Solvent E). Chromatography also was performed on the commercially available diethylaminoethyl cellulose paper (DEAE-20) using 0.1–0.5 *M* aqueous triethylammonium bicarbonate (*p*H 7.5) for the development. The R_f's of different compounds and oligonucleotides using paper chromatography are listed in Table I.

Paper electrophoresis was carried out either in an apparatus similar to that described by Markham and Smith[21] or in a commercially available water-cooled apparatus. Buffers used were 0.05 *M* formate, *p*H 3.5, 1 *M* acetic acid *p*H 2.4 and 0.05 triethylammonium bicarbonate, *p*H 7.5.

The compounds containing the trityl group were located by the bright yellow color which developed when the chromatograms were sprayed with the acidic Hanes–Isherwood reagent[22] for phosphorus compounds and then heated. Another characteristic of the trityl containing compounds was

(21) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 52 (1952).

(22) (a) C. S. Hanes and F. R. Isherwood, *Nature*, **164**, 1107 (1949). (b) D. A. Applegarth and J. G. Buchanan [*J. Chem. Soc.*, 4706 (1960)] have noted similar color development with trityl compounds.

TABLE I

PAPER CHROMATOGRAPHY OF NUCLEOSIDE, NUCLEOTIDE AND OLIGONUCLEOTIDE DERIVATIVES

Substance	Solvent (Whatman no. 40) A	B	DEAE-cellulose paper[a]
Thymidine	0.67	0.65	0.74
3′-*O*-Acetylthymidine	.75[b]	.79	.77
5′-*O*-Tritylthymidine	.85	..	..
5′-*O*-Tritylthymidine-3′ phosphate	.51	..	..
5′-*O*-Tritylthymidylyl-(3′ → 5′)-thymidine	.72	..	..
5′-*O*-Tritylthymidylyl-(3′ → 5′)-thymidine-3′ phosphate	.27	..	..
5′-*O*-Tritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine	.49	..	..
5′-*O*-Tritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine	.30	..	..
P¹,P²-Di-(5′-*O*-tritylthymidine-3′) pyrophosphate	.75	..	..
Thymidine-3′ phosphate	.13	.37	.53
Thymidylyl-(3′ → 5′)-thymidine	.42	.32	.53
Thymidylyl-(3′ → 5′)-thymidine-3′ phosphate	.05	.19	.32
Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine	.23	.16	.36
Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine-3′ phosphate	.025	.11	.16
Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine	.07	.10	.19
Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine-3′-phosphate	..	.08	.10
Thymidine-3′,5′ cyclic phosphate	.42	..	.59
β-Cyanoethyl thymidine-3′ phosphate	.51	.43	.69
3′-*O*-Acetylthymidylyl-(3′ → 5′)-thymidine	.52[b]	.46	.54
P¹,P² Di-thymidine pyrophosphate	.22	..	.40

[a] Development was with 0.25 *M* triethylammonium bicarbonate for 3 hr. [b] Tailing was observed in this solvent system due to partial deacetylation.

noted in that irradiation for 0.5–1 hour with ultraviolet light (253 mμ) caused the disappearance of the spots which on subsequent viewing under 366 mμ radiation were found to have strong fluorescence. Compounds lacking trityl groups were not affected by this treatment.

Phosphorus was determined by King's method[23] or by the more sensitive method of Chen, *et al.*[24]

The spleen phosphodiesterase preparation was made according to Hilmoe.[25] The manner of its use has been described earlier.[5] The snake venom phosphodiesterase was prepared as previously described.[26] It was used as described earlier.[5]

β-Cyanoethyl Thymidine-3′ Phosphate.—5′-*O*-trityl thymidine (2 mmole) was brought into reaction with a mixture of pyridinium β-cyanoethyl phosphate[27] (from 1300 mg. of the crystalline barium salt: 4 mmole) and DCC (10 mmole) in anhydrous pyridine (10 ml.).[12] After three days at room temperature water (15 ml.) was added and the mixture kept for a further 5 hr. at room temperature. The insoluble dicyclohexylurea was filtered off and the clear filtrate evaporated *in vacuo* at 35°. Water (20 ml.) was added and the mixture was extracted twice with petroleum ether to remove unreacted DCC. The aqueous solution was again evaporated to dryness, the residue taken up in 20 ml. of 80% acetic acid and the resulting mixture heated at 100° for 10 minutes. The mixture was then evaporated *in vacuo* and the process of evaporation repeated several times, after addition of water, to remove acetic acid completely. The residue was finally taken up in 10 ml. of water and the clear solution obtained after removal of triphenylmethyl alcohol by filtration was passed through a column of Amberlite IR-120 (H^+) resin. The acidic effluent and washings were neutralized carefully to *p*H 7 with barium hydroxide (0.05 *M*) under ice-cooling and stirring. The addition of 2–3 volumes of ethyl alcohol caused the precipitation of the unreacted β-cyanoethyl phosphate, the recovery as the barium salt being practically quantitative (645 mg.). The clear supernatant was evaporated to dryness and the residue was dissolved in about 15 ml. of water. The desired product was precipitated by adding first ethyl alcohol until cloudiness appeared and then an excess of acetone. After keeping the mixture in the cold for several hours, the product was collected by centrifugation. It was redissolved in a small volume of water, a small amount of insoluble material removed by centrifugation and the product reprecipitated by the addition of ethyl alcohol and acetone. The voluminous precipitate was collected by centrifugation, then washed successively with acetone and ether. Chromatography in Solvents A and B (R_f's in Table I) showed a single ultraviolet absorbing spot; however, traces of cyanoethyl phosphate and barium carbonate were still present. The process of dissolution in water and precipitation with ethyl alcohol–acetone was repeated again. Pure β-cyanoethyl thymidine-3′ phosphate was obtained finally in a yield of 735 mg. (79.5%).

Anal. Calcd. for $C_{13}H_{18}N_3O_8PBa_{1/2}\cdot H_2O$ (462.01): C, 33.82; H, 4.36; N, 9.09; P, 6.70. Found. C, 34.34; H, 3.99; N, 9.06; P, 6.53.

The molecular weight as determined spectrophotometrically, using a figure of 9700 for $\epsilon_{267\ m\mu}$ of thymidine, was 450.

On paper electrophoresis at *p*H 3.5 and 7.5, the substance was homogeneous, travelling a little faster than thymidylyl-(3′→5′)-thymidine. On treatment with 1 *N* sodium hydroxide at 100° for 10 minutes[18] or with 9 *N* ammonium hydroxide at 60° for 1 hr., the substance was completely converted to thymidine-3′ phosphate. The product thus formed was completely resistant to the 5′-nucleotidase present in crude *Crotalus adamanteus* snake venom.

Reaction of β-Cyanoethyl Thymidine-3′ Phosphate with Dicyclohexylcarbodiimide.—Pyridinium β-cyanoethyl thymidine-3′ phosphate (0.125 mmole) was kept in anhydrous pyridine (0.5 ml.) containing DCC (0.625 mmole) for 4.5 days at room temperature. Water (5 ml.) then was added and the resulting turbid mixture evaporated *in vacuo* at low temperature. The residue was washed with petroleum ether (5 × 20 ml. of 30–80° b.p. fraction) and the gummy residue redissolved in a mixture of pyridine (2.5 ml.) and water (5 ml.). After removal of the insoluble dicyclohexylurea, the solution was kept at room temperature for 10 hr. and then made up to 25 ml. Analysis was performed on aliquots removed from this solution. Thus one aliquot was treated with 1 *N* sodium hydroxide at 100° for 5 minutes and after removal of the sodium ions with pyridinium Dowex-50 resin, the products were chromatographed in Solvent A. Four spots were seen and their concentrations were

(23) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(24) P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

(25) R. J. Hilmoe, *J. Biol. Chem.*, **235**, 2117 (1960).

(26) W. E. Razzell and H. G. Khorana, *ibid.*, **234**, 2105 (1959).

(27) E. Cherbuliez and J. Rabinowitz, *Helv. Chim. Acta*, **39**, 1461 (1956); E. Cherbuliez, G. Cordahi and J. Rabinowitz, *ibid.*, **43**, 863 (1960).

determined by elution with 0.1 *N* hydrochloric acid. Another portion (1/2 of total) after alkaline treatment was applied to the top of a DEAE-cellulose (bicarbonate) column (25 × 2.5 cm. dia.). Elution was carried out with a linear gradient, there being 3 l. of water in the mixing vessel and 3 l. of 0.2 *M* triethylammonium bicarbonate in the reservoir. Seven peaks were obtained; those emerging after the dinucleotide [thymidylyl-(3′→5′)-thymidine-3′ phosphate] were not identified. The mean of the results of this column and those obtained above by paper chromatography are shown in Table II.

TABLE II

PRODUCTS OF THE REACTION OF β-CYANOETHYL THYMIDINE-3′ PHOSPHATE WITH DCC

	Product	Yield, %
1.	Thymidylyl-(3′ → 5′)-thymidine + thymidine-3′,5′ cyclic phosphate[a]	5
2.	Thymidine-3′ phosphate	70
3.	Dithymidine pyrophosphate	1–2
4.	Thymidylyl-(3′ → 5′)-thymidine-3′ phosphate	11
5.	Other products[b]	15

[a] Paper electrophoresis at *p*H 3.5 showed that TpT represented 32% of this mixture and the rest was the 3′,5′ cyclic phosphate. [b] These are the products that emerge from the DEAE-cellulose column after the dinucleotide and on chromatography in Solvent A are at or near the origin (slower than the dinucleotide).

5′-*O*-Tritylthymidine-3′ Phosphate.—5′-*O*-tritylthymidine was prepared by a modification of the procedure described previously[6,28] Thymidine (3.4 g.) and trityl chloride (4 g.; 1.1 molar proportion) were kept at 100° in 50 ml. of anhydrous pyridine for 2 hr. under exclusion of moisture. The solution then was poured into 1 l. of ice-water. After 1 hr. at room temperature, the product was extracted into chloroform (3 x with 150 ml.). The chloroform solution, after drying over Na_2SO_4, was evaporated and the residue crystallized from benzene containing a little acetone. The yield of the product melting at 159–160°[6] (after softening at 115–125°) was 80–85%.

A mixture of 5′-*O*-tritylthymidine (3 mmole) and pyridinium β-cyanoethyl phosphate (6 mmole) was rendered anhydrous by two evaporations of an anhydrous pyridine solution *in vacuo*. The sirupy mixture was dissolved in 20 ml. of dry pyridine, and DCC (15 mmole) was added to the solution which was kept at room temperature for 4 days. Water (20 ml.) was added, the mixture was extracted with petroleum ether and the aqueous pyridine phase was kept at room temperature overnight. The solution was filtered from dicyclohexylurea and evaporated *in vacuo* after addition of 5 ml. of 1 *M* lithium hydroxide. To the residue a further amount (20 ml.) of 1 *M* lithium hydroxide was added and the solution was kept for 2 hr. at 100°. After cooling, the precipitate of trilithium phosphate was removed by filtration, the precipitate being washed with 10 ml. of 0.01 *M* lithium hydroxide. After two extractions of the aqueous solution with chloroform (15 ml.), ammonium Amberlite 1R-120 resin was added and the solution passed through a column of the same resin in the ammonium form. The total effluent and washings containing the ammonium salt of 5′-*O*-tritylthymidine-3′ phosphate were concentrated *in vacuo* at a bath temperature of below 30°. The concentrated solution was lyophilized, giving 1.69 g. of a fluffy white powder. A small portion (35.94 mg.) was dried for 12 hr. at 100° over potassium hydroxide and phosphorus pentoxide. The recovery (34.4 mg.) indicated a yield of 87.8% of the trityl compound. This product contained a trace of inorganic phosphate, which was removed by dissolving the nucleotide in isopropyl alcohol–concentrated ammonia–water (7–1–2) and filtering from the insoluble precipitate. On the addition of a small amount of acetone to the concentrated solution in the above solvent, the product crystallized at room temperature to give needles.

Anal. Calcd. for $C_{29}H_{32}N_2O_8P.2NH_4$: P, 5.19. Found: P, 4.9.

The above product tends to lose the trityl group very readily, for example, when kept overnight after adding water to the freeze-dried ammonium salt (resulting *p*H ~4). Care is therefore necessary in avoiding acidic *p*H in handling the protected nucleotide. If some detritylation occurs during work-up, the product is purified from the contaminating thymidine-3′ phosphate by partition chromatography on a cellulose column using Solvent A. In one run, 1 mmole of 5′-*O*-tritylthymidine-3′ phosphate containing about 3% of thymidine-3′ phosphate was passed through a column (40 cm. × 3 cm. wide) of cellulose powder which had been preequilibrated with Solvent A. Elution with the same solvent (4 ml. fractions/10 min.) gave the pure trityl compound in fractions 52–87.

Isolation and Properties of the Side Product (IV) in Synthesis of 5′-*O*-Tritylthymidine-3′ Phosphate.—In one experiment, when the reaction mixture was worked up by the addition of 25 ml. of 0.2 *M* lithium hydroxide, a small amount of a second product was obtained. This product, which stayed close to the origin during chromatography in Solvent E, was separated from the main product, 5′-*O*-tritylthymidine-3′ phosphate, by dissolving 300 mg. of the total lyophilized product in Solvent E while warm and adding a few drops of acetone. The product IV crystallized to give long white needles. Contaminating 5′-*O*-tritylthymidine-3′ phosphate was removed by repeating the process. The yield of IV was about 15 mg. after washing with acetone and drying. On treatment with 80% acetic acid at 100°, the products obtained were thymidine-3′ phosphate, another thymidine nucleotide moving slower than thymidine-3′ phosphate (Solvent A) and inorganic phosphate. On heating in 0.1 *N* hydrochloric acid at 100° for 45 minutes, thymidine-3′ phosphate and inorganic phosphate were obtained (no thymine was detectable).

Anal. P, 7.3%; P/Thymidine, 1.49; labile phosphorus to total phosphorus = 1:3.

Rate of Reaction of 5′-*O*-Tritylthymidine-3′ Phosphate with 3′-*O*-Acetylthymidine.—To a solution of 5′-*O*-tritylthymidine-3′ phosphate (0.5 mmole) and 3′-*O*-acetylthymidine (0.5 mmole) in dry pyridine (3 ml.) DCC (2.5 mmole) was added and the mixture was kept at room temperature. At intervals 0.2 ml. aliquots were withdrawn and worked up as follows: To each aliquot was added 2 ml. of water and the aqueous pyridine mixture was kept at room temperature for 5 hr. The *p*H then was brought to about 13 with sodium hydroxide and the alkaline mixture was kept at room temperature for 1 hr. Pyridinium Dowex 50 resin then was added to remove the sodium ions. The resin was removed by filtration and it was washed with water and ethyl alcohol. The total filtrate and washings were evaporated to dryness, 80% acetic acid (10 ml.) was added to the residue, and the solution was heated at 100° for 10 min. Water (20 ml.) was added to the cooled solution and the mixture was evaporated to dryness *in vacuo*. The process of evaporation was repeated and the solid white residue was dissolved in aqueous ethyl alcohol. An analysis of products was carried out by paper chromatography in Solvent A. Four spots were detected corresponding to thymidine-3′ phosphate, P^1,P^2-dithymidine-3′ pyrophosphate, thymidylyl-(3′→5′)-thymidine and thymidine. The spots were eluted with 2 ml. of water and their concentrations determined spectrophotometrically using $\epsilon_{267m\mu}$ = 9700 for thymidine and $\epsilon_{267m\mu}$ = 18500 for the di-nucleoside phosphate. The yields of thymidylyl-(3′→5′)-thymidine obtained as a function of time were: 10 hr. 28%; 20 hr., 32%, 40 hr., 39%, 70 hr., 42%, 170 hr., 45%. No increase in the yield was observed after a further addition of DCC (0.5 mmole) to the solution remaining after 170 hr. and keeping the reaction mixture further for 2 days.

5′-*O*-Tritylthymidylyl-(3′→5′)-thymidine (VIII).—(a) A mixture of pyridinium 5′-*O*-tritylthymidine-3′ phosphate (0.295 mmole), 3′-*O*-acetylthymidine[29] (0.6 mmole) and DCC (1.21 mmole) was kept in anhydrous pyridine (1 ml.) for four days at room temperature. Water (10 ml.) then was added and after 3 hr. at room temperature an aliquot was chromatographed on paper in Solvent A. All the starting mononucleotide had disappeared, there being, in addition to 3′-*O*-acetylthymidine, one strong spot with R_f 0.72 and a very weak spot with R_f 0.88. The mixture was evaporated *in vacuo* at low temperature and water (10 ml.) was readded to the residue. A white gum remained insoluble. DCC was removed by repeated trituration with petroleum ether and the insoluble gum was taken up in chloroform

(28) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **109**, 623 (1935).

(29) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

(25 ml.) by vigorous agitation of the total mixture with this solvent. The chloroform layer was separated after centrifugation to separate the layers, and the extraction with chloroform was repeated once. The chloroform layer now contained more than 90% of the desired product with R_f 0.72 (Solvent A). The chloroform solution was evaporated *in vacuo* and the residue was thoroughly triturated with 0.1 *M* sodium hydroxide (2 × 15 ml.). After filtration to remove dicyclohexylurea, the milky solution was extracted with ether (10 ml.) twice, the ether being backwashed with 0.01 *N* sodium hydroxide. The ether extraction removed the faster-travelling impurity in Solvent A (see above). The aqueous phase of the chloroform extract and the alkaline solution were combined and concentrated *in vacuo* to a small bulk. Ammonium Amberlite 1R-120 resin was added and, after filtration to remove the resin (water wash), the aqueous solution was applied to the top of a DEAE-cellulose column (20 cm. × 2 cm. dia.) in the carbonate form. After a water wash which removed thymidine quantitatively, the desired product (VIII) was recovered by washing the column with a 1:4 mixture of ethyl alcohol and 0.5 *M* triethylammonium bicarbonate. (The 20% concentration of ethyl alcohol in the eluent is safe in that no carbon dioxide is expelled from the column.)

The evaporation of the eluate containing the product was carried out carefully at 20–25° under reduced pressure and repeated addition of some *n*-octyl alcohol during the evaporation was necessary to avoid foaming. The yield of pure 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine as estimated spectrophotometrically was 85%. The residue of the triethylammonium salt was passed through a small column of ammonium Amberlite 1R-120 resin and the effluent and washings lyophilized to give the ammonium salt (191 mg.; 80.5%) as a white powder. A portion of this sample was reprecipitated by adding ether to an ethyl alcoholic solution and the precipitate dried overnight at 100° in a high vacuum.

Anal. Calcd. for $C_{39}H_{42}N_4O_{12}P.NH_4$: P, 3.85. Found: P, 3.64.

Detritylation by the standard acidic treatment gave thymidylyl-(3′→5′)-thymidine, which was homogeneous by paper chromatography and paper electrophoresis. Digestion with spleen phosphodiesterase gave thymidine-3′ phosphate and thymidine in equal amounts.

(b) By Tritylation of Thymidylyl-(3′→5′)-Thymidine.—Anhydrous pyridinium thymidylyl-(3′→5′)-thymidine (0.1 mmole) was reacted in anhydrous pyridine (5 ml.) with tritylchloride (0.5 mmole) at room temperature. The reaction mixture was examined at different intervals by paper chromatography in Solvent A. Spectrophotometric estimation of the eluted spots showed about 50% reaction after 2 days, 64% after 5 days. After the latter period, water (10 ml.) was added under cooling and the mixture extracted with ether. The aqueous solution was concentrated *in vacuo* to a small volume and the total solution applied to a sheet of Whatman 3 MM paper and the chromatogram developed in Solvent A. The band with R_f 0.72 corresponded to the desired product and was eluted with water. Lyophilization followed by drying over potassium hydroxide gave 50.5 mg. (62.5%) of a white powder.

5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylic-(3′) Acid (IX) by Phosphorylation of 5′-*O*-Tritylthymidylyl-(3′→5′)-thymidine.—A mixture of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine (0.1 mmole) and pyridinium β-cyanoethyl phosphate (0.5 mmole) was rendered anhydrous by two evaporations of its solution in 5 ml. of anhydrous pyridine. Anhydrous pyridine (0.5 ml.) followed by DCC (1 mmole) then was added to the sirupy residue. A brown precipitate and the crystalline dicyclohexylurea separated immediately. The sealed reaction mixture was shaken for 2 days at room temperature, DCC (0.5 mmole) and anhydrous pyridine (0.5 ml.) were added and the shaking was continued for another 2 days. Ten ml. of 20% aqueous pyridine was added and the mixture was kept at room temperature for 3 hr. After adding 1 ml. of 1 *M* lithium hydroxide, the mixture was extracted twice with petroleum ether to remove DCC and the aqueous phase was evaporated to dryness. To the residue 5 ml. of 1 *M* lithium hydroxide was added and the solution was heated for 1 hr. at 100°. After cooling, trilithium phosphate was removed by filtration and the clear alkaline solution was passed through ammonium Amberlite 1R-120 resin. The effluent and washings were concentrated under reduced pressure and temperature to a small volume and the concentrate examined directly by paper chromatography in Solvent A. Two spots, with R_f's 0.72 (unreacted starting material) and 0.28 [5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid], were present. Hydrolysis of an aliquot of the reaction mixture with 80% acetic acid at 100° for 10 minutes followed by paper chromatography again showed two spots: one, R_f 0.34, corresponding to thymidylyl-(3′→5′)-thymidylic-(3′) acid. A spectrophotometric determination of the intensities of the two spots showed the yield of the phosphorylation product to be 74%. The separation of the desired dinucleotide was achieved in either of the following two ways. (a) The total mixture from the above was applied to 2 sheets of Whatman 3 MM paper and the chromatograms were developed in Solvent A. The major band with R_f 0.28 was eluted with water and the solution lyophilized. The yield was 57 mg. corresponding to 62%. (b) The preferred procedure involved chromatography on a DEAE-cellulose column (22 cm. × 2 cm. dia.). Elution was carried out using a linear gradient with 0.03 *M* triethylammonium bicarbonate (*p*H 7.5) (2 l.) in the mixing vessel and 0.15 *M* solution (2 l.) of the same salt in the reservoir. Fifteen-ml. fractions at 10 minute intervals were collected. Only two ultraviolet absorbing peaks were again obtained. Fractions 40–85 contained the starting material while fractions 170–256 (peak 2) contained the desired product. The combined peak was evaporated *in vacuo*, the residual triethylammonium salt being converted to ammonium salt by passage through ammonium 1R-120 ion exchange resin. Lyophilization gave the ammonium salt as a white powder. The yield was 62 mg. (67.5%) after drying overnight *in vacuo* at 100°.

Anal. Calcd. for $C_{39}H_{41}N_4O_{15}P_2.3NH_4$ (921.73): P, 6.72. Found: P, 6.6.

Detritylation with acetic acid gave a single product as shown by paper chromatography (Solvents A and B) and paper electrophoresis (*p*H 2.8 and 7.5). Thymidylyl-(3′→5′)-thymidylic-(3′) acid thus obtained was completely degraded by the spleen phosphodiesterase to thymidine-3′ phosphate under appropriate incubation conditions. Incubation with the venom phosphodiesterase preparation failed to affect the dinucleotide under the conditions which completely degraded thymidylyl-(3′→5′)-thymidine to thymidine-5′ phosphate and thymidine.

In the above phosphorylation reaction, when the ratio of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine to β-cyanoethyl phosphate to DCC was 1:5:5, only about 23% of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid was obtained after a four day reaction period.

5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylic-(3′) Acid (IX). Condensation of 5′-*O*-Tritylthymidine-3′ Phosphate with β-Cyanoethyl Thymidine-3′ Phosphate.—(a) 5′-*O*-tritylthymidine-3′ phosphate (0.2 mmole of pyridinium salt) was rendered anhydrous by repeated evaporation of its solution in anhydrous pyridine in a flask with a female joint. To the solution in dry pyridine (1 ml.) DCC (0.5 mmole) was added and the mixture was kept overnight. Formation of a brown color and separation of dicyclohexylurea were observed. Pyridinium β-cyanoethyl thymidine-3′ phosphate (0.1 mmole) was separately rendered anhydrous in a 10 ml. flask with a male joint and DCC (0.5 mmole) was added to its solution in 1 ml. of dry pyridine. The contents of the two flasks were mixed by opening the flasks one into the other under exclusion of moisture and the mixture was kept at room temperature for four days. Water (10 ml.) then was added and the mixture was kept further overnight. After filtration to remove dicyclohexylurea the solution was extracted twice with petroleum ether. After the addition of 10 ml. of 9 *N* ammonia, the aqueous phase was evaporated to a sirup under reduced pressure (addition of some octyl alcohol was necessary during evaporation). Twenty ml. of 9 *N* ammonia were added to the sirup and the mixture was kept at 60° for 1.5 hr. Chromatography in Solvent A showed mainly the presence of 5′-*O*-tritylthymidine-3′ phosphate and 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid. The ammoniacal solution was largely evaporated and the concentrate applied to the top of a DEAE-cellulose column (35 cm. × 3.5 cm. dia.) in the carbonate form. Elution was begun using a linear gradient: the mixing vessel contained 4 l. of water and the reservoir 4 l. of 0.25 *M* triethylammonium bicarbonate. Elution was continued using 4 l. of 0.25 *M* salt in the mixing

vessel and 4 l. of 0.5 *M* salt in the reservoir. Ten minute fractions (13–15 ml.) were collected. The elution pattern is given in Fig. Ic. A very minor peak (probably thymidine-3′,5′ cyclic phosphate) appeared in fractions 60–70°. A peak corresponding to thymidine-3′ phosphate appeared in fractions 145–170. 5′-*O*-tritylthymidine-3′ phosphate (0.094 mmole) appeared in fractions 230–330 and 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid appeared in fractions 390–520. The fractions containing the dinucleotide (IX) were combined and evaporated under reduced pressure at a temperature below 30°. The yield as determined spectrophotometrically was 0.058 mmole (58% based on β-cyanoethyl thymidine-3′ phosphate). The product was homogeneous in Solvents A and D and on paper electrophoresis at *p*H 7.5, being identical with the product prepared above.

In a second run using the above procedure, the acetic acid treatment to remove the trityl group was given before chromatography on a DEAE-cellulose column. The percentages of the different nucleotide materials were the same, except for the change in position of the different peaks.

(b) The above experiment was repeated exactly except that the ratio of 5′-*O*-tritylthymidine-3′ phosphate to β-cyanoethyl thymidine-3′ phosphate used was 1:1. The elution pattern obtained after the DEAE-cellulose chromatography was similar to that described in the above experiment (Fig. 1b). The yield of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine-3′ phosphate was 55%, there being about 16% of thymidine-3′ phosphate and about 20% of 5′-*O*-tritylthymidine-3′ phosphate.

(c) When in the experiment a above, a ratio of 5′-*O*-tritylthymidine-3′ phosphate to β-cyanoethyl thymidine-3′ phosphate of 1:2 was used, the yield of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid was again about 54% (as based on 5′-*O*-tritylthymidine-3′ phosphate), but the total elution diagram was much more complex (Fig. Ia). Although several of the peaks were practically insignificant in amount of material, there were obtained in all a total of 12 peaks. Five of the peaks contained the trityl group but only two of these exceeded 50 optical density units at 267 mμ. One of these represented the desired dinucleotide and the second emerged after the latter from the anion exchanger column.

5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (XI).—An anhydrous solution of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylic-(3′) acid (IX) (0.056 mmole), 3′-*O*-acetylthymidine (0.2 mmole) and DCC (0.5 mmole) in dry pyridine (1 ml.) was kept at room temperature for 3 days. Water (5 ml.) then was added and after a further period of several hours, the mixture was filtered from urea and then evaporated under reduced pressure. Dilute sodium hydroxide was added to *p*H ~13 and the mixture was extracted with petroleum ether. After 1 hr., the alkaline solution was passed through a column of ammonium Amberlite 1R-120 resin, the effluent and washings being evaporated to a small bulk. Chromatography of aliquots before and after detritylation showed the disappearance of the starting dinucleotide and the presence of three ultraviolet light absorbing spots. The total reaction mixture, without detritylation, was chromatographed on a DEAE-cellulose (carbonate) column (27 cm. × 2 cm. dia.), elution being carried out first with 3 l. of water (mixing vessel) and 3 l. of 0.1 *M* triethylammonium bicarbonate (*p*H 7.5) (reservoir) and then 2 l. of 0.1 *M* triethylammonium bicarbonate (mixing vessel) and 2 l. of 0.2 *M* salt (reservoir). Ten minute fractions (11–12 ml.) were collected. Fractions 8–33 contained thymidine, fractions 90–100 contained a small amount of ultraviolet absorbing material which was not further investigated, fractions 267–355 contained a trityl containing substance (side product XII and XIII, see below) and fractions 424–540 contained 5′-*O*-tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine. The yield of this product, as estimated spectrophotometrically, was 49%. The product obtained as the hygroscopic triethylammonium salt was converted to the ammonium salt by the use of an ion exchange resin and the solution lyophilized (31.5 mg., 47%, after drying at 100° for 12 hr.).

Anal. Calcd. for $C_{49}H_{55}N_8O_{19}P_2.2NH_4$; P, 5.5. Found, P, 5.1.

The product was homogeneous on paper chromatography and identical with a sample synthesized earlier.[7] Ten mg. of the substance was detritylated in the usual way and the product, after removal of acetic acid and triphenylcarbinol by ether extraction, was homogeneous in Solvents A and B and on paper electrophoresis at *p*H 7.5 and 2.8. Incubation with spleen phosphodiesterase gave thymidine-3′ phosphate and thymidine in the ratio 2:1 on complete degradation.

Properties of Side Products XII and XIII.—The material in fractions 267–355 (see above) (triethylammonium bicarbonate concentration about 0.07 *M*) was present in a total amount of about 450 optical density units at 267 mμ. In solvent A, it had the same mobility as the main product (5′-*O*-tritylthymidylyl-thymidylyl-thymidine) described above. The trityl compound was degraded on treatment with 1 *N* sodium hydroxide at 100° for 1 hr. to give the products listed in Table III. The products of hydrolysis were characterized by paper electrophoresis and paper chromatography. Their relative concentrations were determined after elution of the spots with water. Analysis showed the phosphorus content to be 5.1%. The structures XII and XIII for this compound require 4.7% phosphorus. The compound was detritylated on usual treatment with 80% acetic acid (λmax. at acid, neutral and alkaline *p*H of detritylated compound, 265 mμ) but was stable to further treatment at 100° for 6 hr. The detritylated product was resistant to the action of bacterial alkaline phosphomonoesterase[30] and on paper electrophoresis, at *p*H 2.5 and 7.5, moved slower than thymidylyl-(3′→5′)-thymidine. Digestion with spleen and venom phosphodiesterases caused partial degradation to give in both cases a product which was evidently neutral (not thymidine) as judged by its zero mobility on paper electrophoresis. Other products observed were mononucleotides (presumably, thymidine-5′ and -3′ phosphate) and another minor product which was not investigated further.

TABLE III

PRODUCTS OF ALKALINE HYDROLYSIS OF COMPOUND XII AND XIII

Product	Concentration (μmole × 10^{-1})
Thymidine	0.67
5′-*O*-tritylthymidine	.38[a]
TpT[b]	.62
5′-*O*-trityl-TpT	.53
TpTpT[c]	.51
5′-*O*-trityl-TpTpT[32]	.55

[a] The low value as compared with TpTpT; the corresponding degradation product may be due to incomplete elution with water. [b] This, presumably, consisted of a mixture of thymidylyl-(3′→5′)-thymidine and thymidylyl-(5′→5′)-thymidine. [c] This was, presumably, thymidylyl-(5′→5′)-thymidylyl-(3′→5′)-thymidine.

Condensation of 5′-*O*-Tritylthymidylyl-(3′→5′)-thymidine-3′ Phosphate With β-Cyanoethyl Thymidine-3′ Phosphate: Synthesis of Thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine-3′ Phosphate.—Anhydrous pyridinium 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine-3′ phosphate (0.028 mmole) was brought into reaction with DCC (1 mmole) in dry pyridine (0.5 ml.).[31] To the solution then was added a pyridine (0.5 ml.) solution of a mixture of β-cyanoethyl thymidine-3′ phosphate (0.044 mmole) and DCC (0.5 mmole). The sealed mixture was kept at room temperature for 4 days. Water (10 ml.) then was added and the mixture was allowed to stand overnight. After the addition of 10 ml. of 1 *N* sodium hydroxide the solvent was removed *in vacuo*. A further amount of sodium hydroxide (10 ml. of 1 *N*) was added and the insoluble material removed by filtration. The combined filtrate and washings were extracted twice with ether and the alkaline solution heated at 100° for 0.5 hr. An excess of pyridinium Dowex 50 ion exchange resin was added and, after removal of the resin, the solution was concentrated *in vacuo* to a small bulk. (During this evapo-

(30) A. Garen and C. Levinthal, *Biochim. et Biophys. Acta*, **38**, 470 (1960).

(31) An aliquot (0.01 ml.) of the solution was removed and added to 0.1 ml. of water. DCC was removed by extraction with petroleum ether. After 5 hr. at room temperature, the solution was chromatographed in Solvent A. Two spots were visible, one with R_f of the starting material and a weak spot with R_f 0.47, perhaps the symmetrical pyrophosphate derived from the starting material.

ration some detritylation occurred, the *p*H of the solution being around 6.) Detritylation was effected by treatment with 80% acetic acid at 100° for 10 minutes. After removal of acetic acid, an aliquot was chromatographed on a strip of DEAE-cellulose paper, development being carried out with 0.3 *M* triethylammonium bicarbonate. Strong spots with R_f's corresponding to thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine-3′ phosphate and thymidine-3′ phosphate were visible, together with some thymidylyl-(3′→5′)-thymidine-3′ phosphate. The total solution of the reaction products was adjusted to *p*H 7 with ammonia and applied to the top of a DEAE-cellulose column (27 cm. × 2.5 cm. dia.). Elution was carried out using a linear gradient, with 3 l. of water in the mixing vessel and 3 l. of 0.2 *M* triethylammonium bicarbonate (*p*H 7.5) in the reservoir. Elution was continued using up to 1 *M* triethylammonium bicarbonate at a flow rate of 11–12 ml./10 min. Eight ultraviolet absorbing peaks were obtained. The position of their emergence, concentration and their composition are listed in Table IV.

TABLE IV

PRODUCTS OF CONDENSATION OF 5′-*O*-TRITYLTHYMIDYLYL-(3′ → 5′)-THYMIDINE-3′ PHOSPHATE AND β-CYANOETHYL THYMIDINE-3′ PHOSPHATE IN PRESENCE OF DCC

Peak no.	Fractions	Total optical density at 267 mμ	R_f in solvent C	Composition
1	49–61	51.2	0.60	Thymidylyl-(3′ → 5′)-thymidine + thymidine-3′,5′ cyclic phosphate
2	90–120	219	.42	Thymidine-3′ phosphate
3	196–220	89.5	.38	Thymidylyl-(3′ → 5′)-thymidine-3′ phosphate
4	230–250	12	..	Not identified
5	286–304	42	.23	Not identified
6	305–355	345	.30	The trinucleotide (XVI)
7	430–450	27	.18	Complex pyrophosphates (see text)
8	451–470	48	.24	TpTpTpTp[32]

The yield of 345 optical density units (peak 6) corresponded to 44% of theoretical as based on the amount of 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine-3′ phosphate used. The combined peak was concentrated under reduced pressure to a small volume, the concentrate was passed through a short column of ammonium Dowex-50 resin and the effluent and washings were lyophilized to give a white powder (11.6 mg.). The product, which was homogeneous by paper chromatography and paper electrophoresis, was completely degraded by the spleen phosphodiesterase to thymidine-3′ phosphate.

Anal. Calcd. for $C_{30}H_{40}O_{22}N_6P_3 \cdot 4NH_4$: P, 9.2. Found: P, 8.8.

The material in peak No. 7 of Table IV moved faster than thymidine tetranucleotide (TpTpTpTp)[32] on paper electrophoresis at *p*H 7.5 and had lower mobility than the tetranucleotide both in Solvent C and on DEAE-cellulose paper.

An aliquot (10 o.d. units at 267 mμ) of the material was treated with an excess of acetic anhydride–pyridine mixture.[33] After work-up, four products with thymidine absorption spectrum were obtained. The R_f's in Solvent C were 0.03, 0.19, 0.21 and 0.45. (The starting material had R_f 0.18 in Solvent C.) The evidence thus indicated cleavage of pyrophosphate bonds by the acetic anhydride–pyridine mixture.[1]

Thymidylyl-(3′→5′)-3′-*O*-acetylthymidine.[6]—This was prepared by the reaction of 3′-*O*-acetylthymidine-5′ phosphate[6a] (1 mmole) with 5′-*O*-tritylthymidine (2 mmole) in the presence of DCC (7.5 mmole) in 3 ml. of dry pyridine. On working up after 4 days and removal of the trityl group with acetic acid, the product was purified on 3 sheets of Whatman 3 MM paper using Solvent B. The yield of the desired product was 82% and, in addition, the deacetylated product, thymidylyl-(3′→5′)-thymidine was present to the extent of 6%.

(32) Abbreviations as currently adopted by the *J. Biol. Chem.* (See under Instructions to Authors in current issues.)

(33) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, 81, 4660 (1959).

5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine.—Pyridinium 5′-*O*-tritylthymidylyl-(3′→5′)-thymidine-3′ phosphate (0.048 mmole) was rendered anhydrous by repeated evaporation of its solution in dry pyridine. The residue was finally taken up in 0.5 ml. of dry pyridine and to the solution was added DCC (1 mmole). The sealed mixture was kept at room temperature for 8 hr. A solution of 3′-*O*-acetylthymidylyl-(3′→5′)-thymidine (0.072 mmole) and DCC (0.5 mmole) in pyridine (0.5 ml.) then was added to the first solution and the sealed reaction mixture was kept at room temperature for 4 days. On subsequent addition of water (10 ml.) a brown gum separated which was solubilized by the addition of some pyridine (3 ml.) and shaking. After 2 hr. at room temperature dicyclohexylurea was removed by filtration and the filtrate was evaporated under reduced pressure after the addition of 1 ml. of 9 *N* ammonium hydroxide. The residue was taken up in 5 ml. of 1 *N* ammonia and the milky solution was extracted with ether. The aqueous layer was concentrated to a sirup which was kept in 5 ml. of concentrated ammonia at room temperature for 15 min. After evaporation of ammonia, the solution was made up to 10 ml. Nine-tenths of this solution was applied to the top of a DEAE-cellulose (bicarbonate) column (27 cm. × 2.5 cm.). Elution was performed with a linear gradient, 4 l. of water in the mixing vessel and 4 l. of 0.2 *M* triethylammonium bicarbonate (*p*H 7.5) in the reservoir. The total peaks eluted are listed in Table V. Continued elution with triethylammonium bicarbonate up to 0.5 *M* did not give any more ultraviolet absorbing material.

TABLE V

REACTION PRODUCTS OBTAINED DURING CONDENSATION OF 5′-*O*-TRITYLTHYMIDYLYL-(3′ → 5′)-THYMIDINE-3′ PHOSPHATE AND THYMIDYLYL-(3′ → 5′)-3′-*O*-ACETYLTHYMIDINE

Peak no.	Fractions no.	Total optical density units	R_f (Solvent A)	Identification remarks
1	5–13	..	0.21	Pyridine + ultraviolet absorbing compound with λmax. 261 mμ
2	48–65	525	.42	Thymidylyl-(3′ → 5′)-thymidine
3	65–77	24	.26	Absence of trityl group
4	121–165	53	.73	5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidine
5	181–203	71	.20	Unidentified
6	220–240	65	.33	Unidentified
7	250–355	230	(a) 0.65 (b) .49 (c) .06	(a) and (b) contain trityl groups[a]
8	484–520	60	0.27	5′-*O*-tritylthymidylyl-(3′ → 5′) thymidine-3′ phosphate
9	525–600	363	0.31	5′-*O*-tritylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine

[a] Detritylation of combined (a) and (b) gave on chromatography in Solvent A a total of six spots with R_f's: 0.10, 0.21, 0.29, 0.31, 0.43 and 0.53.

Peak 9 was concentrated to a small volume under reduced pressure at low temperature, the solution was passed through a column of ammonium Dowex-50 resin and the effluent and washings were lyophilized. The yield of this desired product was 23%, as determined spectrophotometrically. The product was homogeneous on paper chromatography and after detritylation was again homogeneous. It was identical with the product obtained by treatment of the previously synthesized thymidine tetranucleotide (pTpTpTpT)[5] with phosphomonoesterase. Incubation of a sample (8 optical density units) with snake venom phosphodiesterase gave thymidine-5′ phosphate and thymidine in the ratio of 3.06:1; theory 3:1.

Anal. Calcd. for $C_{58}H_{69}O_{26}N_8P_3 \cdot 3NH_4$; P, 6.45. Found: P, 5.95.

The Reaction of Thymidylyl-(3′→5′)-3′-*O*-acetylthymidine with Dicyclohexylcarbodiimide.—Thymidylyl-(3′→5′)-3′-*O*-acetylthymidine was kept in 0.5 ml. of anhydrous pyridine in the presence of DCC (0.25 mmole) for 7 days at room temperature. After the addition of water, the aqueous

pyridine mixture was kept for several hours, DCC removed by extraction with ether and the acetyl group removed by keeping the mixture in concentrated ammonia at room temperature for 15 min. The total products were separated by preparative paper chromatography in Solvent A. Three bands were obtained: the main product was thymidylyl-(3′→5′)-thymidine (R_f 0.42); there was a weak band with R_f 0.35, the major new product having R_f 0.21. Examination of the last mentioned material on DEAE-cellulose paper (development with 0.2 *M* triethylammonium bicarbonate) gave three spots: one with R_f 0.79, which was identical with the material (λmax. 261 mμ) in peak 1 (Table V) of the preceding experiment; a weak spot with R_f 0.34, which was identical with the unidentified peak 5 (Table V) of the preceding experiment and another weak spot with R_f 0.18. The total material of R_f 0.21 from paper chromatography was applied to a DEAE-cellulose (carbonate) column (12 cm. × 1 cm. dia.). The water wash contained the above-mentioned material which had R_f 0.79 on DEAE-cellulose (carbonate) paper. This substance (designated XX) showed λmax. at 261–262 mμ and a shoulder at 265 mμ in acid, neutral and alkaline *p*H. Treatment of the solution in the quartz cell with sodium borohydride gave an ultraviolet spectrum typical of thymidylyl-(3′→5′)-thymidine. Five optical density units of the above substance were incubated with snake venom phosphodiesterase. The products were thymidine-5′ phosphate and a second product with R_f 0.33 in Solvent A. The latter product moved towards the cathode on paper electrophoresis at *p*H 7.5 and showed an ultraviolet absorption composite of that of thymidine and N-methylpyridinium cation.

The identity of the weak band with R_f = 0.35 remains unknown.

Phosphorylation of 5′-*O*-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine with a Mixture of β-Cyanoethyl Phosphate and DCC.—Pyridinium 5′-*O*-trityl-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-thymidine (3.3 μmole) was kept in 0.5 ml. of anhydrous pyridine with a mixture of pyridinium β-cyanoethyl phosphate (from 16 mg. of the barium salt) and DCC (42 mg.). After 2 days at room temperature, water (5 ml.) was added and the reaction mixture left overnight. It was then evaporated and to the residue were added 3 ml. of 1 *N* sodium hydroxide and the mixture kept at 100° for 30 min. The trityl group was removed by passing the mixture through a Dowex 50 (H^+) column and keeping the acidic solution for 8 hr. at room temperature. The solution then was neutralized with ammonia and applied to a 9 inch wide strip of Whatman 40 paper. After chromatography in Solvent C, the slowest band corresponded to the desired thymidine tetranucleotide and was eluted. The yield as estimated spectrophotometrically was 43%. Rechromatography on DEAE-cellulose paper showed a small amount (about 5%) of impurity (R_f 0.49) in the main product (R_f 0.12) which had the same mobility as the tetranucleotide previously characterized.[5a] The impurity was removed by applying the total material on a DEAE-cellulose column (15 cm. long × 1 cm. dia.) and eluting with a linear salt gradient, 2 l. of water in the mixing vessel and 2 l. of 0.25 *M* triethylammonium bicarbonate in the reservoir. About 5-ml. fractions were collected at 10 min. intervals. Pure tetranucleotide was eluted in fractions 398–452.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN, AND THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, BRITISH COLUMBIA]

Studies on Polynucleotides. XIV.[1] Specific Synthesis of the $C_{3'}$–$C_{5'}$ Interribonucleotide Linkage. Syntheses of Uridylyl-(3′ → 5′)-Uridine and Uridylyl-(3′ → 5′)-Adenosine[2]

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Uridine-3′,5′ cyclic phosphate (I) on reaction, under its own catalysis, with 2,3-dihydropyran gives quantitatively 2′-*O*-tetrahydropyranyluridine-3′,5′ cyclic phosphate (II). Treatment of II with barium hydroxide affords a mixture of 2′-*O*-tetrahydropyranyluridine-3′ phosphate (III) and the isomeric -5′ phosphate (IV) in the ratio 5:1. On treatment of the mixture of III and IV with trityl chloride in pyridine only IV reacts and the resulting 5′-*O*-trityl ether (V; R = R′ = R″ = H) is separated from III by anion exchange chromatography on a DEAE-cellulose column. 5′-*O*-*p*-anisyldiphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = R′ = H; R″ = OCH_3) and 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (V; R = H; R′ = R″ = OCH_3) were prepared by careful treatment of the mixture of III and IV with, respectively, *p*-anisyldiphenylmethyl chloride and di-*p*-anisylphenylmethyl chloride followed by chromatography. The preparation of 2′,3′-*O*-*p*-anisylideneuridine, 5′-*O*-*p*-anisyldiphenylmethyluridine, 5′-*O*-di-*p*-anisylphenylmethyluridine, 5′-*O*-tri-*p*-anisylmethyluridine and 5′-*O*-tri-*p*-anisylmethyladenosine is described. The rates of hydrolysis in acid of these new protected derivatives of uridine and uridine-3′ and -5′ phosphates were determined. The introduction of each *p*-methoxy group in the trityl group increased the rate of hydrolysis approximately by a factor of ten. Careful treatment of 5′-*O*-di-*p*-anisylphenylmethyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate with cold acetic acid gave quantitatively III, a suitable starting material for polymerization to form $C_{3'}$–$C_{5'}$ linked uridine polynucleotides. Condensation of compounds of the type V with 2′,3′-di-*O*-acetyluridine in the presence of dicyclohexylcarbodiimide followed by ammoniacal and then acetic acid treatment (4 hr. at room temperature) gave pure uridylyl-(3′ → 5′)-uridine (50%) which was fully characterized by analytical and enzymic methods. Prolonged treatment with acetic acid during work-up of the above condensation product increased the yield of uridylyl-uridine to 70%, but a trace (1%) contamination by the rearranged product, uridylyl-(2′ → 5′)-uridine, was then detected. N,N,2′,3′-tetrabenzoyladenosine (XII) was prepared in excellent yield by benzoylation of 5′-*O*-trityladenosine or 5′-*O*-tri-*p*-anisylmethyladenosine with an excess of benzoyl chloride followed by removal of the 5′-*O*-protecting group. Condensation of derivatives of the type V with XII followed by removal of the protecting groups gave 50–70% yield of uridylyl-(3′ → 5′)-adenosine, the results being analogous to those obtained in the synthesis of uridylyl-(3′ → 5′)-uridine.

In the deoxyribopolynucleotide field, progress has been made in recent years both in the area of the stepwise synthesis of $C_{3'}$–$C_{5'}$ linked oligonucleotides as well as on the polymerization of suitably protected deoxyribomononucleotides.[1,5–7] In

(1) Paper XIII, G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962) (preceding paper).

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(6) H. G. Khorana, *Federation Proc.*, **19**, 931 (1960).

(7) 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.