tracted three times with ether. (Dicyclohexylurea which separated was removed by filtration after the first extraction.) The aqueous solution was evaporated first under reduced pressure and then under suction with an oil-pump. The residue was dissolved in acetone (30 ml.) and a solution of barium iodide (1 mmole) in acetone (5 ml.) was added. The resulting white precipitate was collected by centrifugation and washed four times with 30-ml. portions of acetone. The washed precipitate was dried in a vacuum for one hour at 100° to give barium adenosine-2′,3′ cyclic phosphate (504 mg., 99%)[39] as the hexahydrate. This material was homogeneous as shown by paper chromatography (solvents I–III) and paper electrophoresis at *p*H 7.5. The ratio of phosphorus to adenosine was 1.02.

(39) As estimated spectrophotometrically.

Uridine-2′,3′ cyclic phosphate was prepared by the same procedure in 92% yield as the barium salt. Guanosine-2′,3′ cyclic phosphate was obtained as the mixed ammonium and dicyclohexylguanidinium salts in a yield of 89%, based on ultraviolet absorption of the isolated product. In this case the product was precipitated from the residual formamide upon addition of acetone alone.

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VANCOUVER 8, B. C., CANADA

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Studies on Polynucleotides. I. A New and General Method for the Chemical Synthesis of the C_5'–C_3' Internucleotidic Linkage. Syntheses of Deoxyribo-dinucleotides[1]

BY P. T. GILHAM AND H. G. KHORANA

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A new method has been developed for the specific synthesis of the naturally-occurring (C_5'–C_3') internucleotidic linkage; it involves reaction of a suitably protected deoxynucleotide with a second protected deoxy-nucleoside or -nucleotide in the presence of dicyclohexylcarbodiimide or *p*-toluenesulfonyl chloride. By this approach the three dinucleoside phosphates VIIa, VIIb and VIIc have been prepared in good yield. Procedures are described for the synthesis of deoxyribo-dinucleotides bearing 5′- or 3′-phosphoryl end-groups; these are illustrated by the synthesis of the two isomeric dithymidine dinucleotides (XII and XIV), and a mixed dinucleotide (XVI) containing the nucleosides, deoxyadenosine and thymidine. The results of enzymic and acidic degradative experiments are recorded and these provide additional characterization of the synthetic compounds. Some general observations on the scope and mechanism of this method of "phosphodiester" synthesis also are included.

Recent work in different laboratories has led to the clarification of the nature of the internucleotidic linkage in the nucleic acids.[2] Thus, both ribo- and deoxyribo-nucleic acids may be regarded as consisting of polynucleotide chains in which the individual nucleosides are joined together by C_5'–C_3' phosphodiester bonds. However, relatively little progress has so far been made in the fields of organic synthesis and fine structural (end-group and sequential) analysis of polynucleotides. Recognition of the vital biological functions of the nucleic acids and continuing intensive research in these areas further emphasize the need for a complementary attack on the problems of their macromolecular chemistry. Attention is therefore being devoted in this Laboratory to the various aspects of such problems, and the chemical synthesis of polynucleotides forms, in the initial phase, a major part of our studies. The preparation of synthetic polynucleotides of well-defined structure and covering a wide range of size is most desirable; not only will they provide more information regarding the structures and properties of the nucleic acids, but also their availability will undoubtedly offer new opportunities for chemical, physicochemical and biochemical studies in the polynucleotide field. Thus, for example, by use of these synthetic polynucleotides we are currently investigating chemical and enzymic[3] methods for the structural analysis of the nucleic acids.

The main problems associated with the specific synthesis of the naturally-occurring (C_5'–C_3') internucleotidic linkage are (a) the preparation of suitably protected mononucleosides containing only the desired hydroxyl group (on C_3' or C_5') exposed for a phosphorylation reaction, the protecting groups being such that they can be removed finally under conditions which do not cause disruption of other parts of the molecule, and (b) the creation of the phosphodiester bond, either by stepwise phosphorylation of two protected nucleosides or by adequate activation of the phosphoryl group of a preformed mononucleotide derivative for reaction with the appropriate hydroxyl function of a second nucleoside or nucleotide. In the ribonucleoside (I) series the problems of preparing suitably protected intermediates are, in some ways, complicated by the presence of the *cis*-α-glycol system. Studies on the deoxyribonucleosides II, however, are free from this complication and, although the chemistry of these compounds has been much less explored it is possible to exploit the difference in reactivities

I II

R = purine or pyrimidine

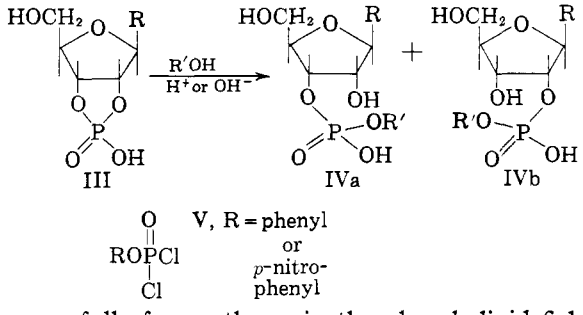
(1) This work has been supported by grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the National Research Council of Canada, Ottawa.

(2) For a recent review see D. M. Brown and A. R. Todd in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, p. 409.

(3) H. G. Khorana, G. M. Tener, W. E. Razzell and R. Markham, *Fed. Proc.*, **17**, 253 (1958); W. E. Razzell and H. G. Khorana, THIS JOURNAL, **80**, 1770 (1958).

of the C_3'– and C_5'–hydroxyl groups. Indeed, the use of this principle is illustrated by the syntheses[4] of a dithymidine phosphate and a dithymidine dinucleotide which are the only two substances containing the C_5'–C_3' linkage synthesized by chemical means so far. The method which was used for the synthesis of the phosphodiester linkage in these compounds included the preparation of a suitably protected nucleoside phosphite, its conversion to the corresponding phosphorochloridate and the use of the latter derivative to phosphorylate the hydroxyl function of a second nucleoside. The difficulty of purification of the intermediates in these syntheses together with the occurrence of side reactions during the final phosphorylation step gave very poor yields of the desired products, and it is unlikely that the method could be extended to the synthesis of higher oligonucleotides.

In this Laboratory attempts first were made to synthesize dinucleoside phosphates of the ribose series by a "transesterification reaction" of the ribonucleoside-2',3' cyclic phosphates (III) · for which satisfactory methods of synthesis are now available.[5] While the synthesis[6] of alkyl ribonucleoside phosphates (IV) by this approach proceeded in high yields when a large excess of the alcohol was used, experiments on dinucleoside phosphate formation from a limited amount of hydroxylic compound (nucleoside) were, in general, unsuccessful. Attention next was turned to the use of a bifunctional reagent such as V for the successive phosphorylation of two protected nucleosides. This type of approach has been used



successfully for syntheses in the phospholipid field by Baer and co-workers.[7] Experiments using the crystalline and easily prepared *p*-nitrophenyl phosphorodichloridate[8] indicated that while C_5'–C_5' linked dinucleoside phosphates, nucleoside-5' alkyl phosphates and related compounds could be prepared in excellent yield,[9] the synthesis of C_5'–C_3' linked dinucleoside phosphates was unsatisfactory. These experiments were, however, preliminary and further work is necessary before the value of the method can be assessed.

In an alternative approach, attention was directed to the activation *in situ* of a monoester of phosphoric acid (*e.g.*, a preformed mononucleotide) and the reaction of the activated derivative with a hydroxylic compound to give the diester directly. The advantages of such an approach are readily apparent since the polynucleotides could be formed stepwise from mononucleotides themselves, these starting materials being well characterized and commercially available. In two preliminary communications[10,11] it already has been reported that the desired activation of monoesters of phosphoric acid may be achieved in solution, by the use of either *p*-toluenesulfonyl chloride or dicyclohexylcarbodiimide (DCC), and that good yields of diesters of phosphoric acid are obtained when a hydroxylic compound also is present in solution. This and the following communication[12] contain a detailed report of the work carried out using this new method of synthesis.

Nomenclature.—In the past a number of methods[13] of shorthand formulation for polynucleotides have been used, in which the nucleosides are designated by one-letter initials and the phosphoryl group by "p." While this abbreviated formulation is sometimes useful from the point of view of conciseness and economy, we have increasingly felt in our present studies the lack of any system of nomenclature for polynucleotides.

Basically, a system similar to that used successfully in the polypeptide field seems most attractive and, analogously, we propose to consider a polynucleotide as a chain in which each *nucleotide* esterifies the hydroxyl function[14a] of the succeeding one rather than as a number of *nucleosides* joined together by phospho-diester bonds.[14b] This type of system requires, of course, further elaboration since, although the naturally occurring polynucleotides probably all have the C_5'–C_3' internucleotidic linkage, synthetic compounds can be made which contain a variety of types of linkage. We intend to use the trivial names, *e.g.*, thymidylic-(5') acid and adenylic-(3') acid to describe the mononucleotides. The number in parentheses refers to the carbon atom involved in the phosphodiester linkage. In order to designate the internucleotidic linkage we shall use a method similar to that already in use for the specification of oligosaccharides. Thus, in the naming of a polynucleotide, the name of each nucleotide radical will be followed by two numbers separated by an arrow, in parentheses, inserted into the name by hyphens. The first and second numbers will refer to the carbon atoms of the preceding and succeeding nucleosides, respectively, which are attached to the phos-

(4) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2632 (1955).

(5) M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6204 (1958).

(6) C. A. Dekker and H. G. Khorana, *ibid.*, **76**, 3522 (1954); G. M. Tener and H. G. Khorana, *ibid.*, **77**, 5349 (1955).

(7) See for example, E. Baer and H. C. Stancer, *ibid.*, **75**, 4510 (1953).

(8) G. R. Cebrian, *Anales. real. acad. espan. fis. y. quim. (Madrid)*, **47B**, 841 (1951); *C.A.* **46**, 11140 (1952).

(9) Unpublished experiments by G. M. Tener, E. H. Pol and H. G. Khorana.

(10) H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chemistry & Industry*, 1523 (1956).

(11) H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957).

(12) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, THIS JOURNAL, **80**, 6223 (1958).

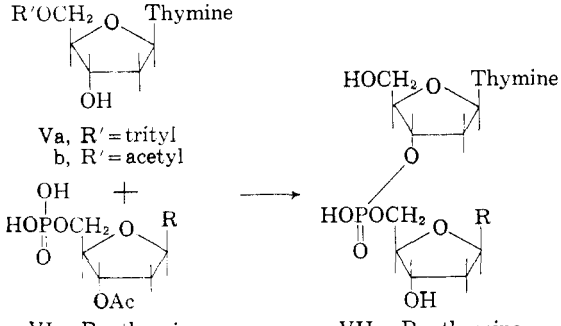
(13) (a) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952); (b) L. A. Heppel, P. R. Whitfeld and R. Markham, *ibid.*, **60**, 8 (1955); (c) H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta*, **26**, 596 (1957).

(14) (a) This approach was first used by R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.*, **197**, 521 (1952), for the naming of certain dinucleotides. (b) This practice has been followed in the naming of dinucleoside phosphates [see *e.g.*, D. T. Elmore and A. R. Todd, *J. Chem. Soc.*, 3681 (1952)], but the method cannot be extended to dinucleotides and higher polymers.

phodiester group. The use of this system is illustrated readily by the names used for the compounds described in this and the following publication[12]: *e.g.*, the dinucleotide XVI becomes deoxyadenylyl-(5′→3′)-thymidylic-(5′) acid. Although this system permits, equally well, the naming of the polynucleotide chain in one or the other direction, we have chosen for the sake of consistency in the present work to name the chain so that the internucleotidic linkage is designated in the order $C_5'\rightarrow C_3'$. However, for maximum simplicity, the choice of naming in one or the other direction may be influenced by the nature of the groups present at the one or the other terminus of the chain.[15]

The Synthesis of Dinucleoside Phosphates.— Deoxyribonucleoside-5′ phosphates are relatively easily accessible and the general synthetic route investigated for the synthesis of C_5'–C_3' linked dinucleoside phosphates consisted of reaction of these 5′-nucleotides with another nucleoside appropriately blocked in the 5′-position. The two derivatives used were 5′-*O*-tritylthymidine (Va) and 5′-*O*-acetylthymidine (Vb). The former was first prepared by Levene and Tipson,[16] who recorded its m.p. as 125°, and subsequently by Michelson and Todd[17] who quoted the m.p. 128° for this product as crystallized from benzene. In our hands, this derivative crystallized from this solvent with one molecule of benzene and, when heated, it softened at 115–125° with effervescence and finally melted at *ca.* 160° (see Experimental). 5′-*O*-Acetylthymidine, which previously has been prepared[4] by the partial deacetylation of 3′,5′-di-*O*-acetylthymidine, was obtained in the present work by the acetylation of thymidine with 1.5 mol. equiv. of acetic anhydride, the product being isolated by chromatography on a silicic acid column.

In the first experiments an anhydrous pyridine solution of thymidylic-(5′) acid was allowed to react with an excess of 5′-*O*-tritylthymidine and 1.5 mol. equiv. of *p*-toluenesulfonyl chloride. After removal of the trityl group by mild acidic hydrolysis, the products were separated by paper chromatography. The desired product, thymidylyl-(5′→3′)-thymidine (VIIa), was present in

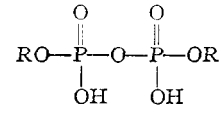


VIa, R = thymine
b, R = *N*-acetyladenine
c, R = *N*-acetylcytosine

VIIa, R = thymine
b, R = adenine
c, R = cytosine

about 50% yield, the other products arising apparently from a polymerization type of reaction which is discussed in the following paper.[12] The identity of the thymidylyl-(5′→3′)-thymidine was confirmed by analysis, ultraviolet absorption characteristics and an electrometric titration which established its equivalent weight and showed the presence of one primary but no secondary phosphate dissociation. The results of enzymic and acidic hydrolyses of the product were consistent with its structure and are mentioned below.

In the above synthesis, an excess of the hydroxylic component 5′-*O*-tritylthymidine had been used in order to minimize the self-condensation of the thymidylic-(5′) acid. A more specific synthesis of the dinucleoside phosphate using stoichiometric amounts of the two components required the protection of the 3′-hydroxyl group of the nucleotide. Subsequent experiments were therefore carried out using 3′-*O*-acetylthymidylic-(5′) acid (VIa) which was prepared either by acetylation of thymidylic-(5′) acid or by the phosphorylation of 3′-*O*-acetylthymidine with dibenzyl phosphorochloridate (see Experimental) followed by hydrogenolysis of the benzyl groups. The condensation of this protected nucleotide with 5′-*O*-acetylthymidine in the presence of DCC was studied carefully so as to ascertain the conditions required for the optimum yield of the diester. The results, which are tabulated in the Experimental section, showed that initially the synthesis was relatively rapid, proceeding to the extent of 50% within the first 18 hours and that the optimum yield (*ca.* 60%) was obtained in about two days. While these experiments were carried out on a small scale, a subsequent preparative experiment gave the thymidylyl-(5′→3′)-thymidine (VIIa) in 66% yield using the reaction (two days) of molar equivalents of 5′-*O*-tritylthymidine (Va) and 3′-*O*-acetylthymidylic-(5′) acid in the presence of an excess of DCC. The other products in this synthesis were the unreacted starting materials, finally obtained as thymidine and thymidylic-(5′) acid, and a small amount of P^1, P^2-di-(thymidine-5′) pyrophosphate (VIII).



VIII, R = thymidine-5′

The above results demonstrated the efficiency of this approach for the specific synthesis of the C_5'–C_3' phosphodiester linkage by the direct use of a mononucleotide. In order to investigate the scope and generality of the method, extension of the synthesis was sought, first of all, by varying the mononucleotide component. To this end, deoxyadenylic-(5′) acid and deoxycytidylic-(5′) acid were acetylated by a standard technique that was developed. The product from the former was presumably the *N*,*O*$^{3'}$-diacetyl derivative VIb, while that from the latter was the *N*,*O*$^{3'}$-diacetyl derivative VIc.[18] Under the conditions used above, the reaction of the diacetyldeoxycytidylic-(5′) acid and 5′-*O*-tritylthymidine (Va) gave the deoxycytidylyl-(5′→3′)-thymidine (VIIc) in 65%

(15) Thus, for example, in ribo-oligonucleotides bearing 2′,3′-cyclic phosphate end-groups, the direction $C_5'\rightarrow C_5'$ was adopted for the abbreviated formulations (ref. 13a, b).

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

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

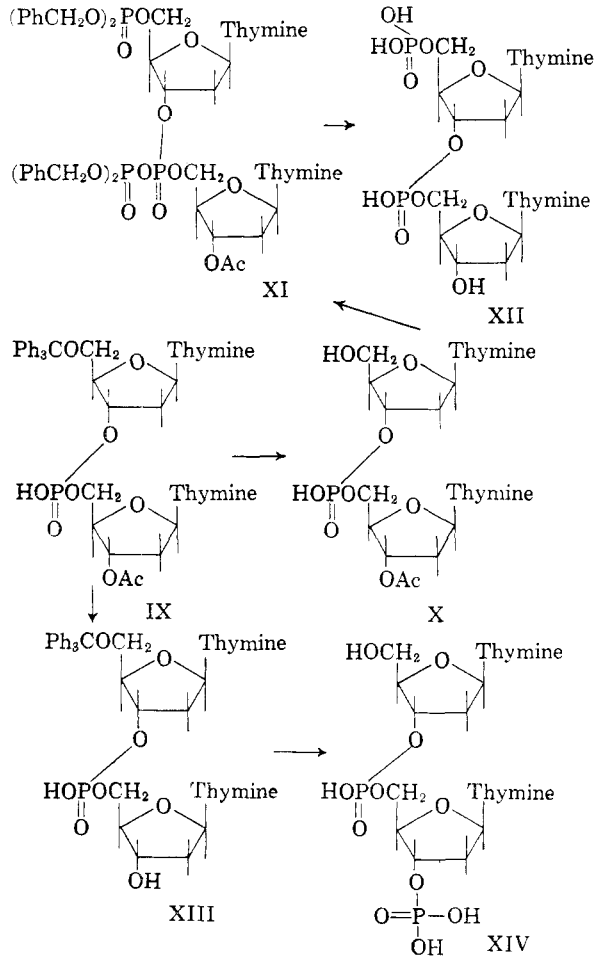
(18) It is probable that the *N*-acetyl group is situated at the N^6-position of the cytosine ring [*cf.* D. M. Brown, A. R. Todd and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956)].

yield. It was necessary, however, to modify the acidic conditions for the removal of the trityl groups since those used above (80% aqueous acetic acid under reflux for ten minutes) caused, in this case, partial hydrolysis of the glycosyl bond in the deoxycytidine fragment of the product. The lability[19] of the bond was confirmed by the observation that, when deoxycytidylic-(5′) acid or its diacetyl derivative was heated in 80% aqueous acetic acid under reflux for one hour a considerable portion of each compound was degraded to cytosine. The conditions ultimately used in the synthesis of VIIc for the removal of the trityl groups without simultaneous hydrolysis of the glycosyl bond included treatment with 80% acetic acid at room temperature for two days.

The glycosyl bond in purine deoxynucleotides is extremely labile and, in experiments on the synthesis of deoxyadenylyl-(5′→3′)-thymidine by straightforward application of the above procedure, it was noted that in aqueous acetic acid under any conditions the rate of hydrolysis of deoxyadenosine or deoxyadenylic-(5′) acid to adenine was comparable to that of the trityl group. Since further attempts to remove the trityl group from 5′-*O*-tritylthymidine by hydrogenolysis using palladium catalysts in neutral solution were unsuccessful, the less accessible 5′-*O*-acetylthymidine (Vb) was used instead. The reaction of this derivative with *N*,*O*$^{3'}$-diacetyldeoxyadenylic-(5′) acid (VIb) in the presence of DCC gave, after alkaline hydrolysis, the deoxyadenylyl(5′→3′)-thymidine (VIIb) in 50% yield.

The Synthesis of Dinucleotides.—Extension of this work to the synthesis of true dinucleotides (containing a C_5'–C_3' phosphodiester linkage and bearing a monoesterified phosphate end-group) was next undertaken, and two methods capable of general application were devised for this purpose. The first involved the phosphorylation of a suitably protected dinucleoside phosphate to give a dinucleotide with either a 5′- or a 3′-phosphoryl end-group. The second method involved synthesis from two mononucleotide derivatives one of which contained a fully protected phosphoryl group.

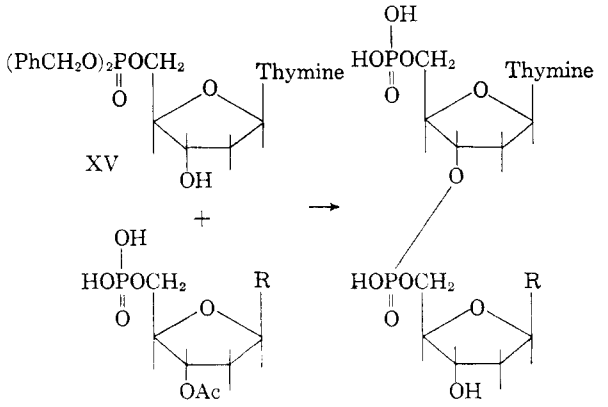
The first of the above methods required the selective unblocking of either the 3′-hydroxyl group at one end or the 5′-hydroxyl group at the other end of 3′-*O*-acetylthymidylyl-(5′→3′)-5′-*O*-tritylthymidine (IX) the intermediate in the synthesis of thymidylyl-(5′→3′)-thymidine described above. Thus, in the preparation of a dithymidine dinucleotide with a 5′-phosphoryl end-group (XII) the crude intermediate IX was treated with acid to remove the trityl group and the product, 3′-*O*-acetylthymidylyl-(5′→3′)-thymidine (X), was purified by chromatography on a cellulose powder column. Phosphorylation of this intermediate with dibenzyl phosphorochloridate gave, presumably, the tetrasubstituted pyrophosphate XI which was treated directly with alkali to hydrolyze (a) the assumed pyrophosphate linkage and (b) the acetyl group. After removal of the benzyl groups by hydrogenolysis the thymidylyl-(5′→3′)-thymidylic-(5′) acid (XII) was purified by paper chromatography and isolated as its crystalline sodium salt in 50% yield based on X. Analytical data for this product were consistent with the structure given and a potentiometric titration of the dinucleotide in its free acid form showed the ratio of primary to secondary phosphate dissociations to be 2. Further characterization based on acidic and enzymic hydrolyses is described below.



For the preparation of the isomeric dinucleotide containing a 3′-phosphoryl end-group, the crude intermediate IX was treated with alkali to give the thymidylyl-(5′→3′)-5′-*O*-tritylthymidine (XIII) which was also purified on a cellulose powder column. The phosphorylation of this compound proved somewhat more difficult than that described above, the lower yields being undoubtedly due to the relative inertness of the secondary (3′) hydroxyl group. The crude phosphorylated product was treated with (a) alkali to cleave the pyrophosphate structure corresponding to XI, (b) acetic acid to hydrolyze the trityl group, and finally was hydrogenated to remove the benzyl groups. Purification by paper chromatography in two solvent systems gave the desired 3′-*O*-phosphorylthymidylyl-(5′→3′)-thymidine (XIV) as the major new product.

(19) It has been stated [A. M. Michelson and A. R. Todd, *ibid.*, 34 (1954)] that *N*-acetylation of "cytidine and deoxycytidine labilizes the glycosidic linkage, giving them a stability similar to that of the corresponding purine nucleosides." In our experiments on the acidic hydrolysis of deoxycytidylic acid and its diacetyl derivative we found that the presence of *N*-acetyl group caused no significant increase in the lability of the glycosyl bond.

The second synthetic method, which is more general in scope, required a mononucleotide containing a protected phosphoryl group. Such a compound, 5′-*O*-dibenzylphosphorylthymidine[4] (XV) was obtained by the alkaline hydrolysis of 3′-*O*-acetyl-5′-*O*-dibenzylphosphorylthymidine. The protected nucleotide was allowed to react with 3′-*O*-acetylthymidylic-(5′) acid (VIa) and DCC under the same conditions as those used for the dinucleoside phosphates except that the reaction was terminated after one day. Removal of the protecting groups and purification of the product by paper chromatography gave the thymidylyl-(5′→3′)-thymidylic-(5′) acid (XII) in 35–40% yield. Even when the reaction was allowed to proceed for two days the yield was considerably lower than that obtained in the preparation of the dinucleoside phosphates. The lower yield is ascribed to the complication introduced by monodebenzylation of the dibenzylphosphoryl group, a reaction that was separately shown to occur (in 5′-*O*-dibenzylphosphorylthymidine) to a significant extent after one day in pyridine solution at room temperature. Since the rate study of the phosphorylation reaction discussed above had shown that a favorable yield could be obtained even after 24 hours, the above synthesis was carried out for this time in order to minimize the side reactions.



VIa, R = thymine
b, R = *N*-acetyladenine

XII, R = thymine
XVI, R = adenine

As a variant, reaction at a lower temperature was used for the synthesis of the mixed dinucleotide XVI to keep the debenzylation at a minimum. Molar equivalents of *N*,*O*$^{3'}$-diacetyldeoxyadenylic-(5′) acid (VIb) and 5′-*O*-dibenzylphosphorylthymidine (XV) were allowed to react with DCC in pyridine at 0–4° for 2 days and after removal of the protecting groups and purification as before the deoxyadenylyl-(5′→3′)-thymidylic-(5′) acid (XVI) was obtained in 40% yield.

Enzymic Hydrolyses.—The present studies have made extensive use of appropriate enzymes as tools for the confirmation of the structures of the synthetic products. Conversely, it is clear that the synthetic substrates may be used fruitfully to determine the specificities and the modes of action of the various nucleases. While detailed studies along these lines will be reported in subsequent communications, it is pertinent to describe briefly here the results of enzymic experiments which provide support for the structures assigned to the various synthetic products.

Crude snake venom (from *Crotalus adamenteus*) degraded, as expected, all the dinucleoside phosphates and the two dinucleotides bearing 5′-phosphoryl groups, thymidylyl-(5′→3′)-thymidylic-(5′) acid (XII) and deoxyadenylyl-(5′→3′)-thymidylic-(5′) acid (XVI), to the corresponding nucleosides and inorganic phosphate. The purified venom diesterase fraction,[20] however, hydrolyzed thymidylyl-(5′→3′)-thymidine (VIIa) to give, as expected, equal amounts of thymidylic-(5′) acid and thymidine. It hydrolyzed the dithymidine dinucleotide with the 5′-phosphoryl end-group to thymidylic-(5′) acid only and the mixed dinucleotide XVI to equal amounts of deoxyadenylic-(5′) acid and thymidylic-(5′) acid. In agreement with the previous findings, the dithymidine dinucleotide XIV with the 3′-phosphoryl end-group was extremely resistant to the action of this diesterase.[21]

A prostate phosphomonoesterase preparation,[22] free from diesterase activity, converted both of the dithymidine dinucleotides (XII and XIV) to thymidylyl-(5′→3′)-thymidine, thus providing further confirmation of their structures.

A phosphodiesterase from calf spleen has recently been highly purified by Hilmoe.[23] In agreement with the previous findings[24] on the use of this preparation in the hydrolysis of ribo-oligonucleotides, it was found that the dinucleotide XII bearing the 5′-phosphoryl end-group was resistant to the enzyme while the isomeric substance XIV with the 3′-phosphoryl end-group was degraded readily to thymidylic-(3′) acid. Thymidylyl-(5′→3′)-thymidine also was hydrolyzed readily, yielding equal amounts of thymidylic-(3′) acid and thymidine.

Acidic Hydrolyses.—Studies of the acidic hydrolysis of deoxyribonucleic acids have been the subject of numerous investigations.[25,26] The general conclusions emerging from these studies are that acidic treatment results, initially, in the cleavage of the labile purine glycosyl bonds. This event, in turn, causes labilization of the phosphate ester linkages attached to the liberated "aldehydo" deoxyribose moieties. Although the pyrimidine oligonucleotides are more resistant to acidic hydrolysis, the initial step in their degradation appears again to be the cleavage of the glycosyl bond.[26] The results of the experiments on the hydrolysis of the compounds described herein are in general agreement with these findings.

(20) J. F. Koerner and R. L. Sinsheimer, *J. Biol. Chem.*, **228**, 1049 (1957). We are grateful to Dr. W. E. Razzell for carrying out some of these experiments.

(21) M. P. de Garilhe, L. Cunningham, U. Laurila and M. Laskowski, *ibid.*, **224**, 751 (1957).

(22) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952).

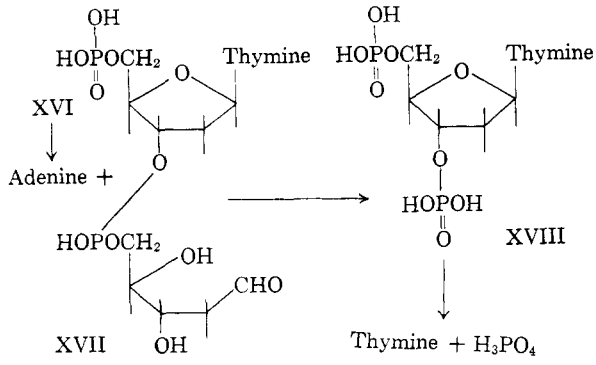
(23) R. J. Hilmoe, National Institutes of Health, Bethesda 14, Md., unpublished work. We are grateful for a gift of this preparation.

(24) L. A. Heppel and R. J. Hilmoe, "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 565; L. A. Heppel, P. J. Ortiz and S. Ochoa, *Science*, **123**, 415 (1956).

(25) (a) P. A. Levene and L. W. Bass, "Nucleic Acids," Am. Chem. Soc. Monograph Series, Chemical Catalog Co., Inc. (Reinhold Publ. Corp.), New York, N. Y., 1931; (b) C. Tamm, M. E. Hodes and E. Chargaff, *J. Biol. Chem.*, **195**, 49 (1952); (c) C. A. Dekker, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 947 (1953); (d) W. E. Cohn and E. Volkin, *Biochim. et Biophys. Acta.*, **24**, 359 (1957).

(26) H. S. Shapiro and E. Chargaff, *ibid.*, **26**, 596, 608 (1957).

During the acid treatment of the two synthetic compounds containing the purine adenine it was possible to discern, by paper chromatography, the various steps in their degradation. In the dinucleotide XVI, treatment with 1 *N* hydrochloric acid at room temperature caused rapid hydrolysis of the purine glycosyl bond giving adenine and, presumably, the compound XVII. Although the latter product remained apparently unchanged at room temperature in the acid solution, it decomposed rapidly when heated at 100°, yielding 3′,5′-di-*O*-phosphorylthymidine (XVIII); some hydrolysis to thymine and inorganic phosphate also occurred under these conditions. Similarly, deoxyadenylyl-(5′→3′)-thymidine (VIIb) gave initially, on treatment with acid, adenine and the presumed "aldehydo" compound (corresponding to XVII) and the latter, when heated, was degraded to thymidylic-(3′) acid (partial hydrolysis of this mononucleotide to thymine and inorganic phosphate also occurred). The synthetic products containing pyrimidine residues were, as expected, more resistant to acid hydrolysis. However, thymidylyl-(5′→3′)-thymidine (VIIa) was completely degraded in 1 *N* hydrochloric acid at 100° after 3 hours to thymine, inorganic phosphate and thymidylic acid (probably a mixture of the 3′- and 5′-isomers). It has already been shown above that the glycosyl bond of deoxycytidylic-(5′) acid was more labile to acid than that of thymidine and this difference in lability was also apparent in the hydrolysis of deoxycytidylyl-(5′→3′)-thymidine (VIIc). Partial degradation of this dinucleoside phosphate in 1 *N* hydrochloric acid at 100° for one hour gave mainly cytosine and thymidylic acid, together with a smaller amount of thymine.



The influence of structure and the position of phosphoryl end-groups on the mode of hydrolysis is illustrated strikingly by the results of the acidic degradation of the two isomeric dithymidine dinucleotides. Thymidylyl-(5′→3′)-thymidylic-(5′) acid required two hours at 100° for complete hydrolysis in 1 *N* hydrochloric acid. At no stage could mononucleotidic material be demonstrated to have been formed as an intermediate and the products were 3′,5′-di-*O*-phosphorylthymidine, thymine and inorganic phosphate. The 3′-*O*-phosphorylthymidylyl-(5′→3′)-thymidine, however, was markedly more stable to acid; about 50% degradation had occurred after 3 hours under the above conditions, the products being thymidylic acid, thymine and inorganic phosphate. Thus, in the "5′-ended" dinucleotide (XII), the thymidine residue that has the more labile glycosidic linkage is that which is esterified in the 5′-position only, whereas in the "3′-ended" dinucleotide (XIV) the more labile thymidine residue is that which is esterified in both the 3′- and 5′-positions.

Ultraviolet Absorption Characteristics.—The ultraviolet spectra of the five compounds VIIa, X, XII, XIII, and XIV described above, which contained two thymidine residues, all had λ_{max} 266–267 mμ. The values of ϵ_{max} (P) (the molar absorptivity maximum based on one gram atom of phosphorus per liter)[27] were determined and the values of ϵ_{max} so obtained all lay within the range 18,300–18,500, being 2.6 to 3.7% lower than the value of 19,000 calculated for two thymidine residues. This decrease in optical density also was observed in the two compounds containing a purine and a pyrimidine residue. Deoxyadenylyl-(5′→3′)-thymidine (VIIb) and deoxyadenylyl-(5′→3′)-thymidylic-(5′) acid (XVI) had λ_{max} 261 mμ and ϵ_{max} values (determined as above) of 20,200 and 19,800, respectively. The value of ϵ_{261}, calculated for a 1:1 mixture of the two nucleosides involved, is 23,700 (using ϵ_{261} 14,800 for deoxyadenosine and ϵ_{261} 8,900 for thymidine). These results are in general accord with the recent findings[26,28] that the molar absorptivities of dinucleotides generally are lower than the values obtained by the summation of the absorptivities of their component mononucleotides. In particular, we confirm the significant observation[28] that the diminution in ϵ_{max} for the purine-containing compounds is considerably higher than that for the compounds containing only pyrimidines.

General Observations.—As mentioned above, the only approach which had been used previously for the synthesis of the C_5'–C_3' internucleotidic linkage involved the reaction of a protected nucleoside benzyl phosphorochloridate with another suitably protected nucleoside. A variant of this approach, where the phosphorochloridate was first converted to a mixed anhydride of diphenylphosphoric acid, gave improved yields in the synthesis of C_5'–C_5' linked dinucleoside phosphates[29] but was ineffective for the synthesis of C_5'–C_3' linked compounds.[4] In contrast with the low yields obtained by the phosphorochloridate method the present approach gave the C_5'–C_3' linked compounds in 50–66% yields. The powerful nature of the present method of phosphorylation becomes further apparent when it is considered that the 3′-hydroxyl function in deoxyribonucleosides is, in general, markedly inert. For example, serious difficulties have been experienced repeatedly in phosphorylating it with reagents such as dibenzyl phosphorochloridate.[30]

It is an important advantage of the present method that *monoesters* of phosphoric acid (mononucleotides) can be used and, further, that the desired products, *diesters*, are obtained directly. The

(27) E. Chargaff and S. Zamenhof, *J. Biol. Chem.*, **173**, 327 (1948).

(28) R. L. Sinsheimer, *ibid.*, **208**, 445 (1954); M. Privat de Garilhe and M. Laskowski, *ibid.*, **223**, 661 (1956).

(29) R. H. Hall, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 3291 (1957).

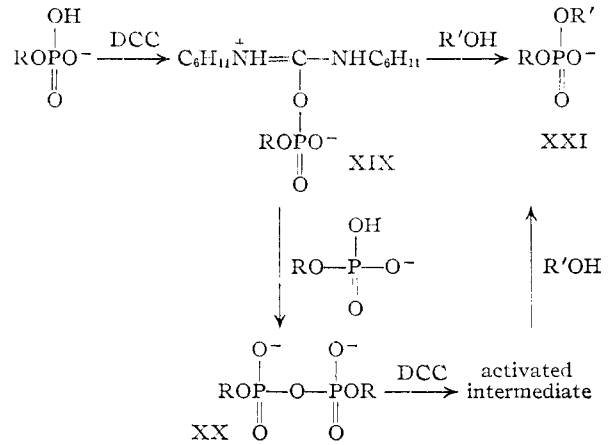
(30) See synthesis of XIV in Experimental section; also *cf.* D. H. Hayes, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 808 (1955).

acidic dissociation in the latter compounds aids their purification. Conventional approaches, involving disubstituted phosphorochloridates as the intermediates, would give, initially, neutral triesters as products which would be more difficult to purify. The purification step at intermediate stages assumes added importance in extending the synthesis to higher oligonucleotides.

Although the present method for the synthesis of C_5'–C_3' internucleotide bonds has been shown, so far, to be quite general, further studies involving various other suitably protected deoxyribo-nucleosides and -nucleotides are required. Another outstanding problem is the investigation of the phosphorylation of suitably protected dinucleotides by mononucleotides. These studies, which are essential for developing the synthesis of mixed higher oligonucleotides, are now in progress.

Of the two reagents dicyclohexylcarbodiimide and *p*-toluenesulfonyl chloride,[31] the former was by far the preferred reagent in the present work since it could be used in excess without the danger of causing side reactions and, furthermore, the only product arising from its use was dicyclohexylurea which readily could be removed from the reaction mixtures.

Although a detailed discussion of the mechanism of the phosphorylation reaction will be presented in a forthcoming publication, it may be noted here that this mechanism can be clearly distinguished from that described in the preceding publication,[5] by hich diesters were formed from the reaction of an alkyl phosphate with DCC in the presence of a large excess of an alcohol. There, it was concluded that the synthesis involved (i) reaction of the alkyl phosphate to form an adduct and (ii) the alcoholysis of this adduct, presumably in its protonated form XIX to yield the diester XXI. On the other hand, in the present experiments using stoichiometric amounts of the hydroxylic compound, the attack on XIX by another alkyl phosphate anion would be the preferred reaction, forming the pyrophosphate XX.



In the study of the rate of the reaction of 5′-*O*-acetylthymidine with 3′-*O*-acetylthymidylic-(5′) acid and DCC, paper chromatography showed that, in the first few minutes, before any phosphorylation had occurred, the major product (after alkaline hydrolysis of the acetyl groups) was P^1,P^2-di-(thymidine-5′) pyrophosphate. Furthermore, in separate experiments[32] it has been established that pyrophosphates can serve equally well as starting materials in the diester synthesis with DCC or *p*-toluenesulfonyl chloride. Since pyrophosphates of this type are not effective as phosphorylating agents, it must be postulated that the mechanism involves further activation of the pyrophosphate intermediate XX in order to effect phosphorylation of the alcohol. It should be noted that, in the case of DCC, this subsequent activation is inhibited by the presence of water or strong bases such as tri-*n*-butylamine. When strong bases are employed for the reaction of an alkyl phosphate with DCC at room temperature[5] or when partially aqueous media are used, the corresponding pyrophosphates are the ultimate products. Indeed, these procedures have been used extensively for the synthesis of nucleotide coenzymes and related compounds.[33]

Experimental

5′-*O*-Tritylthymidine.—Thymidine (0.456 g.) was treated with trityl chloride (0.536 g.) in pyridine (13 ml.) solution according to Levene and Tipson.[16] After one week at room temperature the mixture was poured dropwise into cold water (100 ml.) with vigorous stirring. The gum which formed solidified after two hours stirring and the product (0.79 g.) was removed by filtration, washed with water and dried *in vacuo*. Recrystallization from benzene gave the 5′-*O*-tritylthymidine as needles containing one molecule of benzene. When heated, the product softened with effervescence from 115 to 125° and on further heating it set to a solid which finally melted at *ca.* 160°. Levene and Tipson[16] record the m.p. as 125° and Michelson and Todd[17] gave the m.p. of their product (recrystallized from benzene) as 128°.

Anal. Calcd. for $C_{29}H_{28}N_2O_5 \cdot C_6H_6$: C, 74.7; H, 6.1; N, 5.0. Found: C, 74.7; H, 6.05; N, 5.2.

A portion of the material was heated *in vacuo* at 118° for 7 hours during which time it became an amorphous solid and the liberated benzene was retained in a Dry Ice trap. The loss in weight was 13.9% (theoretical for one molecule of benzene, 13.9%). The heated compound then had a m.p. 158–160°.

5′-*O*-Acetylthymidine.—Dry thymidine (5 g.) was warmed in anhydrous pyridine (30 ml.) until it had dissolved. The solution was cooled and acetic anhydride (2.9 ml.) added. After 24 hours at room temperature the solution was evaporated to dryness *in vacuo*. The resulting gum was dissolved in water and the solution again evaporated *in vacuo*. The residue was then dissolved in dichloromethane (*ca.* 50 ml.) and, on standing in the cold, the solution deposited crystals of thymidine (0.43 g.) uncontaminated by acetylated products. The dichloromethane-soluble product was chromatographed on a column (4 cm. × 30 cm.) of silicic acid (Mallinckrodt) and the eluates were analyzed by paper chromatography in solvent system II. Continued elution with dichloromethane failed to remove any of the material. Elution with chloroform gave, almost immediately, the crude 3′,5′-di-*O*-acetylthymidine (2.4 g.). Recrystallization from benzene gave the product as prisms (2.25 g., 33%), m.p. 126–127°. Elution with chloroform containing 2% methanol yielded the monoacetylated material (2.27 g.) which, on repeated recrystallization from ethyl acetate, gave needles of 5′-*O*-acetylthymidine (1.45 g., 25%), m.p. 150°. Michelson and Todd[4] report the m.p.'s 125 and 146° for the diacetyl- and the 5′-*O*-acetylthymidine, respectively.

Acetylation of Mononucleotides.—Pyridine solutions of 3′-*O*-acetylthymidylic-(5′) acid, *N*,*O*$^{3'}$-diacetyldeoxycytidylic-(5′) acid and *N*,*O*$^{3'}$-diacetyldeoxyadenylic-(5′) acid were prepared in the following manner. The mononucleo-

(31) There is little doubt that other chlorides or anhydrides of strong acids could function in the synthesis equally well.

(32) A. F. Turner and H. G. Khorana, THIS JOURNAL, not yet published.

(33) See, *e.g.*, M. Smith and H. G. Khorana, *ibid.*, **80**, 1141 (1958), and references therein.

tides were converted to their pyridinium salts by use of a column of Amberlite IR-120 (pyridinium) ion-exchange resin, followed by addition of a few ml. of pyridine to the eluates and evaporation of the solution to dryness *in vacuo*. Each mononucleotide (1 mmole) then was dried *in vacuo* and shaken with a mixture of dry pyridine (5 ml.) and acetic anhydride (5 ml.) until it had dissolved. The solution was allowed to stand for 24 hours and then evaporated *in vacuo* to dryness. The residual gum was dissolved in aqueous pyridine and allowed to stand for a few hours in order to hydrolyze any mixed anhydrides[34] present. The solution next was evaporated *in vacuo* and freeze-dried from water a number of times to remove traces of acetic acid. Finally, the product was dissolved repeatedly in pyridine and the solution evaporated to dryness *in vacuo* to remove water and the material then was made up to 10 ml. with dry pyridine. The ultraviolet spectrum of the acetylthymidylic-(5′) acid was similar to that of the parent nucleotide while the product from the acetylation of deoxyadenylic-(5′) acid had λ_{max} 273 mμ at *p*H 8. *N*,*O*$^{3'}$-Diacetyldeoxycytidylic-(5′) acid had λ_{max} 246, 296 mμ at *p*H 7 and $\epsilon_{280}/\epsilon_{260}$ mμ, 0.775 (*cf.* spectrum of *N*,*O*$^{3'}$-diacetyldeoxycytidine[19]).

Deoxyadenylic-(5′) acid and its diacetyl derivative were unstable in acetic acid–water (4:1) solution at room temperature. Paper chromatography showed that after 48 hours both compounds had undergone considerable decomposition to adenine. Under these conditions the glycosyl bonds of deoxycytidylic-(5′) acid and its diacetyl derivative were stable, but when heated under reflux in acetic acid–water (4:1) for one hour both compounds gave about 50% of cytosine.

The *O*-acetyl groups of the acetylated derivatives could be removed by hydrolysis with sodium hydroxide solution at *p*H 12–13 for 0.5 hour at room temperature. The *N*-acetyl groups were not completely hydrolyzed under these conditions but were removed by treatment with strong ammonia solution for 2 hours at room temperature.

5′-*O*-Dibenzylphosphorylthymidine.—3′-*O*-Acetylthymidine[17] was phosphorylated with dibenzyl phosphorochloridate by a modification of the procedure reported by Michelson and Todd.[17]

Dibenzyl phosphorochloridate was prepared by the reaction of dibenzyl phosphite (6.46 g.) with N-chlorosuccinimide (3.29 g.) dissolved in anhydrous benzene (50 ml.) for two hours at room temperature. The solution was decanted from the precipitated succinimide, concentrated to a small bulk at low temperature and the concentrate added to a frozen solution of 3′-*O*-acetylthymidine (2 g.) in anhydrous pyridine (25 ml.). The mixture was allowed to gradually warm up with shaking to form a clear homogeneous melt and then kept at −20° overnight. The solution, which was practically colorless, was then mixed with sodium bicarbonate (5 g.) and a small volume of water. The mixture was evaporated *in vacuo* at 20° and the residual syrup dissolved in chloroform and water. The chloroform layer was separated, washed with water, 1 *N* hydrochloric acid (twice) and then with dilute sodium bicarbonate solution several times. It was finally dried over anhydrous sodium sulfate and evaporated *in vacuo* to give the 3′-*O*-acetyl-5′-*O*-dibenzylphosphorylthymidine as an oil (3.9 g.). For the removal of the acetyl group a solution of sodium hydroxide (0.56 g.) in water (20 ml.) was added to a dioxane (20 ml.) solution of the phosphorylated product. The clear solution, after being allowed to stand at room temperature for 40 min., was neutralized with acetic acid and evaporated *in vacuo* at low temperature. The residue was taken up in chloroform, the solution washed with water and dilute sodium bicarbonate solution, and then dried over sodium sulfate. Evaporation gave an oil (3.5 g.) which was dissolved in dioxane and made up to 50 ml. This product was chromatographically pure and spectrophotometric analysis of an aliquot showed that the yield of 5′-*O*-dibenzylphosphorylthymidine was 5.2 mmoles (75%).

3′-*O*-Acetylthymidylic-(5′) Acid.—3′-*O*-Acetyl-5′-*O*-dibenzylphosphorylthymidine obtained as above from 3′-*O*-acetylthymidine (1.85 g.) was dissolved in dioxane and hydrogenated in the presence of palladium oxide–barium sulfate catalyst[35] (0.75 g.). When the hydrogen uptake was complete (*ca.* 1 hour), the catalyst was removed by centrifugation and the solution freeze-dried to give the pure 3′-*O*-acetylthymidylic-(5′) acid as a white powder (2.5 g.). This product was identical with that obtained by acetylation of thymidylic-(5′) acid by the general procedure described above. The substance, which was very hygroscopic, was stored as a solution in anhydrous pyridine.

Thymidylyl-(5′→3′)-thymidine.—5′-*O*-Tritylthymidine (145 mg., 0.3 mmole) was mixed with an anhydrous pyridine solution (3 ml.) of 3′-*O*-acetylthymidylic-(5′) acid (0.3 mmole) and the mixture evaporated *in vacuo*. The residual glass was dissolved in anhydrous pyridine (3 ml.) and DCC (310 mg., 1.5 mmoles) added with shaking. After 48 hours at room temperature the reaction mixture was evaporated *in vacuo* until practically all the pyridine was removed. The residue was mixed with 80% aqueous acetic acid (10 ml.) and heated under reflux for 10 min. When cool, the mixture was diluted with water (20 ml.) and filtered. The filtrate was evaporated to dryness *in vacuo* and the residue dissolved in a small amount of water and kept at *p*H 13 for 30 min. by addition of sodium hydroxide solution. The solution then was passed through an Amberlite IR-120(H^+) ion-exchange column and the eluate was evaporated to a small bulk. The crude product was chromatographed on two 18-in. sheets of Whatman 3 MM paper in solvent system I. The R_f values of the bands obtained were 0.68 (thymidine), 0.45 (thymidylylthymidine), 0.25 (P^1,P^2-di-(thymidine-5′) pyrophosphate) and 0.16 (thymidylic-(5′) acid). The main band (R_f 0.45) was cut out and eluted with water. The eluate was freeze-dried, yielding a white powder (122 mg.). A portion of the product (79.15 mg.) was heated at 110° *in vacuo* overnight to yield the dried material (72.35 mg.) indicating an over-all yield of 66%; λ_{max} 267 mμ, ϵ_{max} (P) 18,500.

Anal. Calcd. for $C_{20}H_{26}N_4O_{12}P\cdot NH_4$: P, 5.5. Found: P,[36] 5.1.

A portion of the product was converted to the free acid by passing its aqueous solution through a column of Dowex 50 (H^+) ion-exchange resin. The eluate was evaporated *in vacuo* and the residue dissolved in a small volume of absolute ethyl alcohol. The product was precipitated as a white powder by the addition of excess of ether and collected by centrifugation. It was washed with ether and dried. Titration of this acid with sodium hydroxide showed the absence of any secondary phosphoryl dissociation and the equiv. wt. was found to be 578; calcd. for $C_{20}H_{27}N_4O_{12}P$, 546.

Incubation of the material in tris-(hydroxymethyl)-aminomethane buffer (*p*H 8.8) with crude snake venom showed quantitative degradation to thymidine and inorganic phosphate, while the diesterase fraction[20] released equivalent amounts of thymidine and thymidylic-(5′)-acid, as ascertained by paper chromatography. Paper chromatography showed that when the dinucleoside phosphate was heated at 100° in 1 *N* hydrochloric acid for three hours, it was completely degraded to thymine, mononucleotidic material and some inorganic phosphate.

In the earlier experiment, in which *p*-toluenesulfony chloride was used in place of DCC, a mixture of thymidylic-(5′) acid (0.5 mmole) and 5′-*O*-tritylthymidine (2.5 mmoles) was dissolved repeatedly in anhydrous pyridine and the solution evaporated to dryness *in vacuo* to completely remove all traces of water. The residue finally was dissolved in anhydrous pyridine (3 ml.) and *p*-toluenesulfonyl chloride (150 mg., 0.78 mmole) was added with shaking. The solution was allowed to stand overnight at 0–2° with the exclusion of moisture, then diluted with water (1 ml.) and evaporated *in vacuo* to a sirup. Residual pyridine was removed by solution of the material in dioxane and evaporation of the dissolution a few times and the residue finally was heated under reflux with 80% aqueous acetic acid (10 ml.) for 15 minutes. The solution was cooled and the crystalline precipitate removed by filtration. The filtrate was concentrated *in vacuo* and a small portion of the product was analyzed by paper chromatography in solvent system I. The products were thymidine, thymidylyl-(5′→3′)-thymidine, a cyclic dithymidine dinucleotide[12] and smaller amounts of polymeric materials[12] situated close to the origin. For the calculation of the yield of the desired product, the various phosphorus-containing spots were eluted and the optical densities of their solutions determined at 267 mμ. The yield of thymidylyl-(5′→3′)-thymidine was about 50% of the total phosphorus-containing material. The separa-

(34) *Cf.* A. W. D. Avison, *J. Chem. Soc.*, 732 (1955).

(35) R. Kuhn and H. J. Haas, *Angew. Chem.*, 67, 785 (1955).

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

tion of the bulk of the reaction products was carried out as in the foregoing synthesis.

Rate of Formation of Thymidylyl-(5′→3′)-thymidine.—5′-*O*-Acetylthymidine (28 mg., 0.1 mmole) and DCC (103 mg., 0.5 mmole) were added to an anhydrous pyridine solution (1 ml.) of 3′-*O*-acetylthymidylic-(5′) acid (0.1 mmole) and, at various times, aliquots of 0.1 ml. were withdrawn. Each aliquot was diluted with water and kept at *p*H 13 for 30 min. with sodium hydroxide solution. The solution was then passed through a column of Amberlite IR-120 (H^+) ion-exchange resin and the eluate was neutralized with dilute ammonia. The solution was then evaporated *in vacuo* to dryness and dissolved in water (0.1 ml.). A portion of this solution was applied to a sheet of Whatman No. 1 chromatographic paper and the separation effected by use of solvent system I. The intense spots had R_f values 0.65 (thymidine), 0.41 (thymidylyl-(5′→3′)-thymidine) and 0.11 (thymidylic-(5′)acid), and the weak spots had R_f values 0.84 (unidentified), 0.31 (unidentified) and 0.21 (P^1,P^2-di-(thymidine-5′) pyrophosphate). For each aliquot the spots with R_f values 0.65 and 0.41 were cut out and soaked in water (4 ml.) for 1 hour and the optical densities at 267 mμ of the solutions were determined. The amount of material in each spot was calculated using ϵ_{267} 9,500 for thymidine and ϵ_{267} 18,500 for thymidylyl-(5′→3′)-thymidine. In Table I the yield of thymidylyl-(5′→3′)-thymidine is expressed as a percentage based on the unchanged thymidine obtained in each aliquot.

TABLE I

Time, hours	Thymidine O.D. 267	Thymidine μmole	Thymidylyl-(5′→3′)-thymidine O.D. 267	Thymidylyl-(5′→3′)-thymidine μmole	Yield, %
4	0.497	0.209	0.500	0.108	34
8	.317	.133	.471	.102	43
18	.367	.155	.718	.155	50
23	.431	.182	.934	.202	53
28.5	.162	.068	.395	.085	56
42	.350	.147	.898	.194	57
52.5	.536	.226	1.590	.344	60
73	.320	.135	0.999	.216	61
116.5	.386	.163	1.000	.217	59

In a similar experiment aliquots were withdrawn after 1, 2 and 3 minutes and investigated in the same way. Paper chromatography showed that the products obtained from the 3-minute aliquot were exclusively thymidine, P^1,P^2-di-(thymidine-5′) pyrophosphate and a small amount of thymidylic-(5′) acid.

Deoxycytidylyl-(5′→3′)-thymidine.—5′-*O*-Tritylthymidine (145 mg., 0.3 mmole) was added to an anhydrous pyridine solution (3 ml.) of *N*,*O*³′-diacetyldeoxycytidylic-(5′) acid (0.3 mmole) and the mixture evaporated *in vacuo*. The residue was dissolved in anhydrous pyridine (3 ml.) and DCC (310 mg., 1.5 mmoles) was added. The mixture was shaken until the small amount of gum, which had formed, had redissolved. After a total of 48 hours at room temperature the pyridine was evaporated off *in vacuo* and the residue repeatedly dissolved in aqueous dioxane and the mixture evaporated until no smell of pyridine was detectable. The residue then was dissolved in 80% aqueous acetic acid (10 ml.) and allowed to stand for 2 days. Excess of water was added next and the mixture filtered. The filtrate was evaporated to dryness *in vacuo* at room temperature and the residue dissolved in a small amount of water. The solution was kept at *p*H 13 for 30 min. by the addition of sodium hydroxide solution passed through a column of Amberlite IR-120 (NH_4^+) ion-exchange resin, and the eluate evaporated to a small bulk. The product was applied to 2 sheets of Whatman 3 MM chromatographic paper and chromatographed with solvent system I. The bands produced had these R_f values: 0.70 (thymidine), 0.39 (the dinucleoside phosphate), 0.18 (presumably P^1,P^2-di-(deoxycytidine-5′) pyrophosphate) and 0.12 (deoxycytidylic-(5′) acid). The main band (R_f 0.39) was eluted with water and the solution was freeze-dried yielding the desired product as a fine powder (120 mg.). A portion of this material (80.5 mg.) was heated *in vacuo* at 100° overnight to give 72.0 mg. of the dried product, indicating an over-all yield of 65%; λ_{max} (*p*H 2) 273 mμ, ϵ_{max} (P) 21,200.

Anal. Calcd for $C_{19}H_{25}N_5O_{11}P\cdot NH_4$: P, 5.6. Found: P, 5.2.

A portion of the product was heated with 1 *N* hydrochloric acid at 100° for one hour. Paper chromatography showed that it had partly degraded to cytosine and thymidylic acid together with smaller amounts of thymine and inorganic phosphate. When heated for 3 hours under these conditions the material was degraded completely to cytosine, thymine and inorganic phosphate together with a small amount of thymidylic acid. Identity of the hydrolysis products was confirmed by their ultraviolet spectra: cytosine, λ_{max} 268 mμ (*p*H 7); thymine, λ_{max} 265 mμ (*p*H 7), 290 mμ (*p*H 13).

Deoxyadenylyl-(5′→3′)-thymidine.—5′-*O*-Acetylthymidine (84 mg., 0.3 mmole) and a pyridine solution (3 ml.) of *N*,*O*³′-diacetyldeoxyadenylic-(5′) acid (0.3 mmole) were mixed and evaporated *in vacuo*. The residue was dissolved in anhydrous pyridine (3 ml.) and DCC (310 mg., 1.5 mmoles) was added with shaking. After 48 hours the mixture was evaporated to dryness *in vacuo* and the residue mixed with water (20 ml.). The solution was kept at *p*H 13 for 30 minutes by addition of sodium hydroxide solution and then brought to *p*H 8 by addition of Amberlite IR-120 (H^+) ion-exchange resin. The solution then was passed through a column of Amberlite IR-120 (NH_4^+) ion-exchange resin. The eluate was evaporated *in vacuo* to a small bulk and the product applied to two sheets of Whatman 3 MM chromatographic paper. Chromatography in solvent system I gave bands with these R_f values: 0.87 (unidentified), 0.71 (thymidine), 0.44 (dinucleoside phosphate), 0.19 (presumably P^1,P^2-di-(deoxyadenosine-5′) pyrophosphate) and 0.14 (deoxyadenylic-(5′) acid). The main band (R_f 0.44) was cut out and eluted with water. Freeze-drying of the eluate gave the product as a fine powder (100 mg.). A portion of this material (49.85 mg.), when dried *in vacuo* at 110° overnight gave 44.02 mg. of the dried product, indicating an over-all yield of 50%; λ_{max} 261 mμ, ϵ_{max} (P) 20,200.

Anal. Calcd. for $C_{20}H_{25}N_7O_{10}P\cdot NH_4$: P, 5.4. Found: P, 4.8.

A portion of the product was dissolved in 1 *N* hydrochloric acid and paper chromatography in solvent system I showed that after 0.5 hour at room temperature most of the material had decomposed to adenine (R_f 0.46, λ_{max} 260 mμ at *p*H 7) and a new compound (R_f 0.35). The latter product was stable to acid at room temperature but was completely decomposed when heated in 1 *N* hydrochloric acid at 100° for one hour, yielding thymine (R_f 0.54, λ_{max} 265 mμ at *p*H 7, 290 mμ at *p*H 13) inorganic phosphate and thymidylic acid.

3′-*O*-Acetylthymidylyl-(5′→3′)-thymidine.—5′-*O*-Tritylthymidine (484 mg., 1 mmole) and DCC (1.03 g.) were added with shaking to an anhydrous pyridine solution (10 ml.) of 3′-*O*-acetylthymidylic-(5′) acid (1 mmole). After standing for 2 days the mixture was evaporated to dryness *in vacuo* and the residue mixed with 80% aqueous acetic acid and the solution again evaporated to dryness *in vacuo*. The residue finally was mixed with 25 ml. of 80% aqueous acetic acid and heated under reflux for 10 minutes. When cool, the solution was diluted with water and the crystalline precipitate removed by filtration. The filtrate was evaporated *in vacuo* to dryness and the residue was dissolved in a small amount of *n*-butyl alcohol–water (86:14) mixture and applied to the top of a column (volume 250 ml., length 25 cm.) of cellulose powder (Whatman standard grade) which had been packed wet by use of this solvent mixture. The separation was carried out with this butanol–water solvent and fractions of 5 ml. were collected every 20 minutes. Each fraction was analyzed by paper chromatography in solvent system II. Fractions 50–60 contained unreacted thymidine and fractions 60–74 gave a small amount of unidentified material. Fractions 91–133 were combined and evaporated *in vacuo* to dryness, the residue was extracted with water and the solution filtered. Freeze-drying of the filtrate gave the desired product as a white powder (302 mg.). A portion of this pyridinium salt (100 mg.) was dissolved in water and passed through a column of Amberlite IR-120 (H^+) ion-exchange resin. The product, which was obtained by freeze-drying the eluate, was heated at 85° *in vacuo* for 12 hours to yield the anhydrous 3′-*O*-acetylthymidylyl-(5′→3′)-thymidine (96 mg., representing a yield of 50%); λ_{max} 266 mμ, ϵ_{max} (P) 18,500.

Anal. Calcd. for $C_{22}H_{29}N_4O_{13}P$: P, 5.25. Found: P, 5.5.

Thymidylyl-(5′→3′)-5′-*O*-tritylthymidine.—5′-*O*-Tritylthymidine (484 mg., 1 mmole) and DCC (1.03 g.) were

added with shaking to an anhydrous pyridine solution (10 ml.) of 3′-*O*-acetylthymidylic-(5′) acid (1 mmole). After 2 days the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in dioxane–water (1:1) mixture and kept at *p*H 13 for 0.5 hour by the addition of sodium hydroxide solution. The solution was then brought to *p*H 8 by the addition of Amberlite 1R-120 (H^+) ion-exchange resin and then passed through a column of Amberlite IR-120 (NH_4^+) resin. The eluate was evaporated to dryness *in vacuo* and the residue extracted with water. The aqueous extract was concentrated *in vacuo*, dissolved in a small amount of *n*-butyl alcohol–water (86:14) mixture and applied to the top of a column (volume 250 ml., length 25 cm.) of cellulose powder (Whatman Standard Grade) which had been packed wet by use of this solvent mixture. The separation was effected with the butanol–water solvent; fractions of 4 ml. were collected every 20 minutes and analyzed by paper chromatography in solvent system I. Fractions 53–127 were combined and evaporated to dryness *in vacuo* and the residue extracted with water. The filtered aqueous extract was freeze-dried to give the ammonium salt of the desired product as a white powder (358 mg., 44%). For analysis, a portion of this material was dissolved in a small volume of ethanol and reprecipitated by the addition of excess ether. The product was then dried *in vacuo* at 100° for 12 hours; λ_{max} 267 mμ, ϵ_{max} (P) 18,400.

Anal. Calcd. for $C_{39}H_{40}N_4O_{12}P\cdot NH_4$: P, 3.85. Found: P, 3.45.

Phosphorylation of 3′-*O*-Acetylthymidylyl-(5′→3′)-thymidine.—Dibenzyl phosphorochloridate was prepared by the reaction of dibenzyl phosphate (262 mg., 1 mmole) with N-chlorosuccinimide (134 mg., 1 mmole) in anhydrous benzene (5 ml.) for 2 hours. The benzene solution then was decanted from the precipitated succinimide, concentrated *in vacuo* to a small bulk and added to a frozen (−80°) anhydrous pyridine solution (1 ml.) of the pyridinium salt of 3′-*O*-acetylthymidylyl-(5′→3′)-thymidine (0.1 mmole). The mixture was allowed to warm up with shaking until it was homogeneous and then kept at −20° for 12 hours. The mixture was diluted with water, evaporated to dryness *in vacuo* and the residue dissolved in dioxane–water (1:1) mixture. The solution was kept at *p*H 13 for 0.5 hour by the addition of sodium hydroxide solution and then passed through a column of Amberlite IR-120 (H^+) ion-exchange resin. The eluate was neutralized with dilute ammonia solution and hydrogenated in the presence of the palladium oxide–barium sulfate catalyst.[35] The catalyst then was removed by centrifugation, the solution evaporated to dryness *in vacuo* and the residue dissolved in water (4 ml.). The solution was brought to *p*H 12 by the addition of lithium hydroxide solution and the precipitated lithium phosphate removed by centrifugation. The solution was brought to *p*H 8 by the addition of Amberlite IR-120 (H^+) and then evaporated to dryness *in vacuo*. In order to remove lithium chloride the residue was dissolved in aqueous methanol (2 ml.) and the nucleotidic material precipitated by the addition of excess acetone. The product was applied to two 18-inch sheets of Whatman 3 MM chromatographic paper and separation effected with solvent system II. The main band was cut out, eluted and the eluate passed through a column of Amberlite IR-120 (H^+) ion-exchange resin. The solution then was neutralized with sodium hydroxide solution, evaporated to dryness *in vacuo* and the residue recrystallized from aqueous ethanol to yield plates of the hydrated sodium salt of thymidylyl-(5′→3′)-thymidylic-(5′) acid (46 mg., 50%); λ_{max} 267 mμ, ϵ_{max} (P) 9,150, $[\alpha]^{20}D$ +10.9° (*c* 1.5 water) (Michelson and Todd[4] report $[\alpha]^{20}D$ +7.0° for the hydrated calcium salt).

Anal. Calcd. for $C_{20}H_{25}O_{15}N_4P_2Na_3\cdot 13H_2O$: P, 6.7. Found: P, 6.65.

A portion of this material was heated *in vacuo* at 100° overnight to yield the anhydrous product.

Anal. Calcd. for $C_{20}H_{25}O_{15}N_4P_2Na_3$: P, 8.95. Found: P, 8.8.

The sodium salt was converted to the free acid form by use of Dowex-50 (H^+) ion-exchange resin and electrometric titration of this material showed that the ratio of primary (*p*H range 1–3.5) to secondary (*p*H range 5.5–8) phosphoryl dissociations was 2.

The products of the various enzymic hydrolyses of the dinucleotide were determined by paper chromatography in both solvent systems. Incubation of the product in tris-(hydroxymethyl)-aminomethane buffer (*p*H 8.8) with the unfractionated snake venom (*Crotalus adamenteus*) gave thymidine and inorganic phosphate whereas a purified phosphodiesterase fraction[20] from snake venom degraded the dinucleotide to thymidylic-(5′) acid only. The prostate phosphomonoesterase[22] hydrolyzed the dinucleotide to thymidylyl-(5′→3′)-thymidine and inorganic phosphate. The dinucleotide was resistant to the action of the calf spleen phosphodiesterase.[23]

Paper chromatography in solvent system I showed that the dinucleotide was degraded completely in 1 *N* hydrochloric acid at 100° in 2 hours, the products being 3′,5′-di-*O*-phosphorylthymidine, thymine and inorganic phosphate.

Phosphorylation of Thymidylyl-(5′→3′)-5′-*O*-tritylthymidine.—A solution of the ammonium salt of thymidylyl-(5′→3′)-5′-*O*-tritylthymidine (81 mg., 0.1 mmole) in water was passed through a column of Amberlite IR-120 (pyridinium) ion-exchange resin and the eluate evaporated to dryness *in vacuo*. The residue was rendered anhydrous by repeatedly dissolving it in anhydrous pyridine, evaporating the solution to dryness *in vacuo* and finally dissolving in anhydrous pyridine (1 ml.). The solution was frozen (−80°) and mixed with dibenzyl phosphorochloridate prepared from dibenzyl phosphite (524 mg.) and N-chlorosuccinimide (268 mg.) as in the foregoing experiment. The mixture was allowed to warm up until it formed a homogeneous solution and then kept at −20° for 60 hours. The solution was then evaporated *in vacuo*, the residue dissolved in dioxane–water (1:1) mixture and kept at *p*H 13 for 0.5 hour by the addition of sodium hydroxide solution. The solution now was passed through a column of Amberlite IR-120 (H^+) and the eluate which had *p*H 1–2 was allowed to stand at room temperature for 48 hours. The solution was neutralized with lithium hydroxide solution and hydrogenated in the presence of the palladium oxide–barium sulfate catalyst.[35] After removal of the catalyst the solution was concentrated *in vacuo* and dissolved in a small volume of water. The solution was brought to *p*H 12 with lithium hydroxide solution and the precipitated lithium phosphate removed by centrifugation. The solution was brought to *p*H 8 by addition of Amberlite IR-120 (H^+) and then evaporated to dryness *in vacuo*. To remove lithium chloride the residue was dissolved in a small volume of aqueous methanol and the product precipitated by addition of excess of acetone. Chromatographic analysis of this product in solvent system II showed that the desired dinucleotide was contaminated by a number of minor products with low R_f values. The crude material was applied to two 18-inch sheets of Whatman 3 MM chromatographic paper and separation effected by prolonged chromatography in solvent system II. The dinucleotide band was cut out and eluted and the eluate passed through a column of Amberlite IR-120 (H^+) ion-exchange resin and the solution then neutralized with ammonia solution. The solution was concentrated and applied to two 18-inch sheets of Whatman 3 MM paper and chromatographed in solvent system I. The main band was cut out and eluted and the eluate freeze-dried to yield the 3′-*O*-phosphorylthymidylyl-(5′→3′)-thymidine as a white powder (12.5 mg., 19%), λ_{max} 266 mμ, ϵ_{max} (P) 9,200.

On electrophoresis in 0.02 *M* citric acid buffer the product moved as one band, having the same mobility as that of its isomer, thymidylyl-(5′→3′)-thymidylic-(5′) acid.

The dinucleotide was degraded on incubation with the prostate monoesterase[22] and the products were shown by paper chromatography to be thymidylyl-(5′→3′)-thymidine and inorganic phosphate. Incubation with calf spleen diesterase[23] completely degraded the dinucleotide to thymidylic-(3′) acid only. The identity of the mononucleotide formed was shown by extended chromatography in solvent system I, under which conditions thymidylic-(3′) acid separates from its 5′-isomer. Also, the mononucleotide was resistant to the action of crude snake venom under the conditions which completely hydrolyzed thymidylic-(5′) acid to thymidine and inorganic phosphate. The dinucleotide itself also was very resistant to the action of snake venom.

The dinucleotide was heated in 1 *N* hydrochloric acid at 100° for 3 hours and paper chromatography showed that it was 50% degraded to a mononucleotide, thymine and inorganic phosphate.

Thymidylyl-(5′→3′)-thymidylic-(5′) Acid.—A solution of 5′-*O*-dibenzylphosphorylthymidine (0.1 mmole) in di-

oxane (1 ml.) was mixed with an anhydrous pyridine solution (1 ml.) of 3′-*O*-acetylthymidylic-(5′) acid (0.1 mmole). The mixture was evaporated at *ca.* 0° to dryness *in vacuo*, the residue was dissolved in anhydrous pyridine (1 ml.) and DCC (103 mg.) was added with shaking. The mixture was allowed to stand for 24 hours and then evaporated to dryness *in vacuo*. The residue was dissolved in dioxane–water (1:1) mixture and the solution kept at *p*H 13 for 0.5 hour by addition of sodium hydroxide solution. The solution was passed through a column of Amberlite IR-120 (H^+) ion-exchange resin, the eluate was concentrated *in vacuo* to a small volume and allowed to stand for 24 hours. It was then neutralized with ammonia solution and hydrogenated in the presence of palladium oxide–barium sulfate catalyst.[35] After removal of the catalyst the solution was applied to an 18-inch sheet of Whatman 3 MM chromatographic paper and the products separated by prolonged chromatography in solvent system I. The dinucleotide band was cut out, eluted and the eluate passed through a column of Amberlite IR-120 (H^+) resin. The eluate was neutralized with sodium hydroxide solution, evaporated to dryness *in vacuo* and the residue recrystallized from aqueous alcohol to yield plates (32–36 mg., 35–40%) of the product identical with the thymidylyl-(5′→3′)-thymidylic-(5′) acid described above.

Deoxyadenylyl-(5′→3′)-thymidylic-(5′) Acid.—A solution of 5′-*O*-dibenzylphosphorylthymidine (0.3 mmole) in dioxane (3 ml.) was mixed with an anhydrous pyridine solution (3 ml.) of *N*,*O*³′-diacetyldeoxyadenylic-(5′) acid (0.3 mmole). The mixture was evaporated at *ca.* 0° to dryness *in vacuo*, the residue was dissolved in anhydrous pyridine (3 ml.), and DCC (310 mg.) added with shaking. The solution was kept at 0–2° for 48 hours and then evaporated to dryness *in vacuo*. The residue was dissolved in dioxane–water (1:1) mixture and kept at *p*H 13 for 0.5 hour with sodium hydroxide solution. The solution was brought to *p*H 6 by addition of acetic acid and then filtered. The filtrate was hydrogenated in the presence of palladium oxide–barium sulfate catalyst[35] and after removal of the catalyst the solution was passed through a column of Amberlite IR-120 (NH_4^+) ion-exchange resin. The eluate was concentrated *in vacuo* to a small bulk, applied to two 18-inch sheets of Whatman 3 MM chromatographic paper and developed in solvent system I until separation was effected. The dinucleotide band was cut out, eluted and the eluate freeze-dried to yield the product as a white powder (90 mg.). A portion (60.72 mg.) of this product was heated *in vacuo* at 100° for 12 hours to yield the anhydrous material (53.52 mg.). Thus the over-all yield for a di-ammonium salt was 40%; λ_{max} 261 mμ, ϵ_{max} (P) 9,900.

Anal. Calcd. for $C_{20}H_{25}N_7O_{13}P_2\cdot(NH_4)_2$: P, 9.25. Found: P, 8.85.

The product was shown to be homogeneous by paper chromatography in the two solvent systems and by electrophoresis in 0.02 *M* citric acid buffer.

Incubation of the product with the unfractionated snake venom gave thymidine and deoxyadenosine identified by paper chromatography in solvent system I. Hydrolysis of the dinucleotide by the phosphodiesterase[20] from snake venom gave thymidylic-(5′) acid and deoxyadenylic-(5′) acid separated and identified by electrophoresis in 0.02 *M* citric acid buffer.

The dinucleotide was dissolved in 1 *N* hydrochloric acid and paper chromatography in solvent system I showed that after 15 minutes most of the adenine had been liberated with formation of a new compound (with the same R_f value as the dinucleotide itself). This product was not degraded further when left in the acid solution for 18 hours. When the dinucleotide was heated in 1 *N* hydrochloric acid at 100° for one hour the products, which were identified by paper chromatography and ultraviolet spectra, were adenine (λ_{max} 260 mμ, *p*H 7), thymine (λ_{max} 265 mμ, *p*H 7; 290 mμ, *p*H 13), 3′,5′-di-*O*-phosphorylthymidine, inorganic phosphate and small amount of thymidylic acid.

	I	II
Thymine	0.54	0.65
Cytosine	.46	.47
Adenine	.46	.59
Thymidine	.60	.62
Deoxycytidine	.53	.49
Deoxyadenosine	.56	.61
Thymidylic-(5′) acid	.105	.30
Deoxycytidylic-(5′) acid	.07	.26
Deoxyadenylic-(5′) acid	.095	.27
3′-*O*-Acetylthymidylic-(5′) acid	.18	.47
N,*O*³′-Diacetyldeoxycytidylic-(5′) acid	.12	.48
N,*O*³′-Diacetyldeoxyadenylic-(5′) acid	.16	.47
5′-*O*-Acetylthymidine	.65	.73
3′,5′-Di-*O*-acetylthymidine	.73	.84
5′-*O*-Tritylthymidine	.85	.90
5′-*O*-Dibenzylphosphorylthymidine	.83	.91
3′,5′-Di-*O*-phosphorylthymidine	.015	.14
P¹,P²-Di-(thymidine-5′) pyrophosphate	.19	.17
Thymidylyl-(5′→3′)-thymidine	.34	.32
Deoxycytidylyl-(5′→3′)-thymidine	.30	.36
Deoxyadenylyl-(5′→3′)-thymidine	.32	.35
3′-*O*-Acetylthymidylyl-(5′→3′)-thymidine	.53	.45
Thymidylyl-(5′→3′)-5′-*O*-tritylthymidine	.72	.76
Thymidylyl-(5′→3′)-thymidylic-(5′) acid	.04	.18
3′-*O*-Phosphorylthymidylyl-(5′ → 3′)-thymidine	.05	.16
Deoxyadenylyl-(5′→3′)-thymidylic-(5′) acid	.04	.14

Paper Chromatography.—The R_f values listed above were measured by descending chromatography on Whatman No. 1 chromatographic paper in two solvent systems: I, isopropyl alcohol–concentrated ammonia–water (7:1:2); II, butanol–acetic acid–water (5:2:3). Those compounds containing acid functions were chromatographed as their ammonium salts. The spots were located by observation under an ultraviolet lamp and phosphorus-containing spots also were detected by use of a spray.[37]

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(37) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).