Anal. Calcd. for $C_{17}H_{17}I_2NO_4$: C, 36.91; H, 3.10; I, 45.80. Calcd. for $C_{17}H_{16}I_3NO_4$: C, 30.07; H, 2.38; I, 56.07. Found: C, 35.28; H, 2.92.

Repetition of the above experiment with addition of iodine extended over 3 hours and a molal ratio of iodine to thyronine of 34:1 resulted in apparent loss of iodine from the starting thyronine.

Anal. Found: C, 37.62; H, 3.40; I, 44.48.

(b) **Iodine Monochloride.**—To 3,5-diiodo-2',5'-dimethyl-DL-thyronine (0.75 g., 1.4 mmoles) dissolved in glacial acetic acid (5 ml.), a solution of freshly redistilled iodine monochloride (0.26 g., 1.6 mmoles) in acetic acid (2.1 ml.) was added. The reaction mixture was stirred for one hour at room temperature, then heated at 60° for 1.5 hours. The product was isolated and purified as described for 3,5,5'-triiodo-2'-methyl-DL-thyronine. Analysis showed only slight increase in iodine content.

Anal. Found: C, 35.86; H, 3.11; I, 48.60.

In a second attempt, iodine monochloride (1.3 mmoles) was added in two portions, one hour apart, to the diiodothyronine (0.56 mmole) and the reaction mixture placed in a 60° water-bath for 3 hours. Isolation and purification was carried out as before. Analysis indicated loss of iodine.

Anal. Found: C, 37.52; H, 2.94; I, 33.58.

Acknowledgment.—This work was supported in part by grant-in-aid funds from Smith, Kline and French Laboratories and from Research Funds of the University of California Academic Senate. We are grateful to Mr. Robert Day for the synthesis of 3,5-dinitro-DL-tyrosine-N-acetyl ethyl ester.

SAN FRANCISCO, CALIF.

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

Studies on Polynucleotides. V.[1] Stepwise Synthesis of Oligonucleotides. Syntheses of Thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine and Deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine[2]

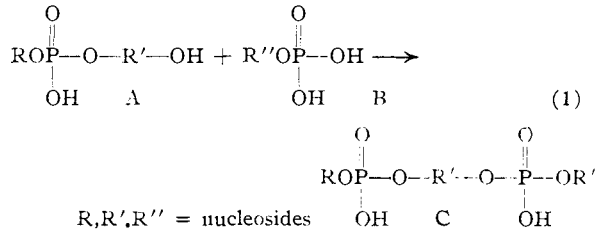
BY P. T. GILHAM AND H. G. KHORANA

RECEIVED MARCH 13, 1959

The reaction of 3′-*O*-acetylthymidylic-(5′) acid (X) with thymidylyl-(5′ → 3′)-5′-*O*-tritylthymidine (VIII) in the presence of dicyclohexylcarbodiimide, followed by mild alkaline and acidic treatments of the product, gave thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine ("trithymidine diphosphate") (XIV) in 68% yield. In the application of this method to the stepwise synthesis of mixed oligonucleotides, *N*,*O*³′-diacetyldeoxycytidylic-(5′) acid (XI) was treated with deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine (IX) and the product afforded, after successive alkaline and acidic treatments, deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine (XV) in 31% over-all yield. The synthetic products were characterized by chemical and enzymic degradations.

In initiating a program of studies in the polynucleotide field we have recently developed a general method for synthesis of the C_5'–C_3' internucleotide linkage.[3,4] This method involves the direct activation of the *phosphomonoester* group of a mononucleotide by dicyclohexylcarbodiimide (DCC) or *p*-toluenesulfonyl chloride in the presence of a suitably protected nucleoside or nucleotide. By this method good yields of a number of dinucleoside phosphates and dinucleotides containing the typical internucleotidic linkage were obtained.[4] The synthesis of larger oligonucleotides would entail the formation of a phosphodiester linkage between a fragment bearing the phosphomonoester group and a second fragment bearing the appropriate hydroxyl group, one or both of these fragments containing preformed diester bonds. The present communication describes our initial experiments to determine whether the above method of diester synthesis can be used to form a second internucleotide bond in the presence of a preformed one. Syntheses of thymidylyl-(5′ → 3′)thymidylyl-(5′ → 3′)-thymidine[5] ("trithymidine diphosphate") (XIV) and deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine[5] (XV) have resulted.[6]

Two modifications of the general procedure of diester synthesis were considered advisable in the present work on the formation of a second phosphodiester linkage between a preformed diester (A) and a phosphomonoester (B). (The reaction is represented simply by equation 1, the diester A



carrying the appropriate hydroxylic function on the component R′.) The first was prompted by a consideration of the possible complications due to diester linkage in (A). Although the precise mechanism of the diester synthesis remains to be elucidated, it seems likely that the phosphomonoester (B) is converted by DCC to tri- and higher meta- (I) or polyphosphates (II) and that these reactive products serve as the phosphorylating agents.[7] From previous work it is also known that diesters of phosphoric acid (A) react with DCC in pyridine to

(1) Paper IV, W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959).

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

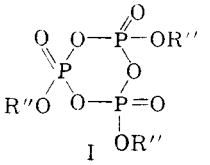
(3) H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chemistry & Industry*, 1523 (1956); H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957).

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

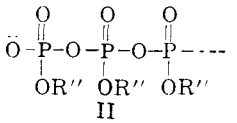
(5) The nomenclature used is that which was proposed in the first paper of this series (ref. 4).

(6) This work has been reported briefly; H. G. Khorana and P. T. Gilham, *Federation Proc.*, **18**, 259 (1959).

(7) Ref. 4 and unpublished results by H. G. Khorana and J. P. Vizsolyi.

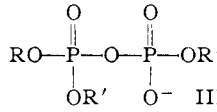


I

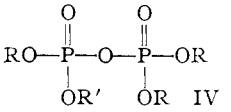


II

form anhydrides.[8] Compounds resulting by anhydride formation between the two reaction components (A and B) would initially be anhydrides of the type III. These and anhydrides derived from their subsequent reactions with DCC might not be efficient phosphorylating agents. To avoid such



III



IV

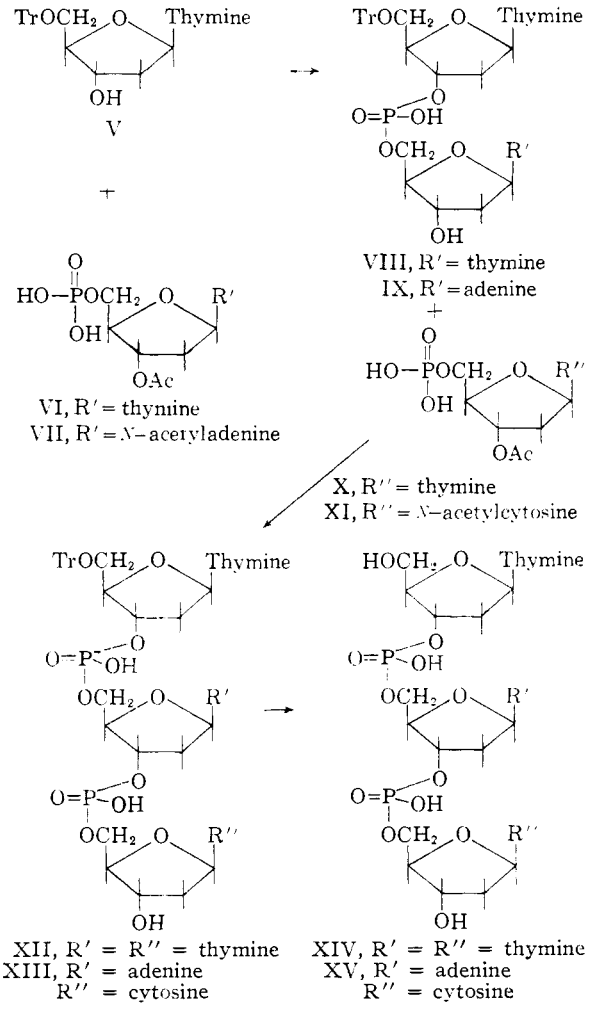
complications, the experimental procedure now used involved the prior reaction of the diester component (A) with an excess of DCC to form the fully substituted pyrophosphate (IV) and the subsequent addition of the phosphomonoester component. (Compounds of the type IV readily are reconverted to the original diesters by mild treatment with alkali.) Although the possibility of anhydride exchange reactions on the subsequent addition of the phosphomonoester cannot be excluded, the reaction between the monoesters and DCC apparently is very rapid and probably would result in the preferential formation of compounds of the type I or II. The second modification introduced was that, instead of using 1:1 molar proportions of the two reactants which in the previous work[4] gave about 60% yields of the desired diesters, two mol. equiv. of the more readily available component (mononucleotide) were used. Higher yield with respect to the diester component then would be expected.

Thymidylyl-(5′ → 3′)-5′-*O*-tritylthimidine (VIII) prepared from 5′-*O*-tritylthymidine (V) and 3′-*O*-acetylthymidylic-(5′) acid (VI) as previously described,[4] was treated in pyridine with an excess of DCC for one hour and then with two mol. equiv. of 3′-*O*-acetylthymidylic-(5′) acid for two days at room temperature. The products were treated with alkali and separated by paper chromatography. The trityl derivative XII was obtained in 68% yield and catalytic hydrogenolysis of this product gave, in excellent yield, thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine (XIV). The identity of this compound was confirmed by the following experiments. It was chromatographically identical with the product obtained by the dephosphorylation by prostatic phosphomonoesterase of thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidylic-(5′) acid ("trithymidylic acid").[9] Degradation (Scheme I, R′ = R″ = thymidine) with a spleen diesterase preparation[10] gave two mol. equiv. of thymidylic-(3′) acid and one mol. equiv. thymidine. This result taken together with the fact that the nucleotide used as the starting material was a 5′-phosphate, served to establish that the synthetic product contained two C_5'–C_3' phosphodiester linkages.

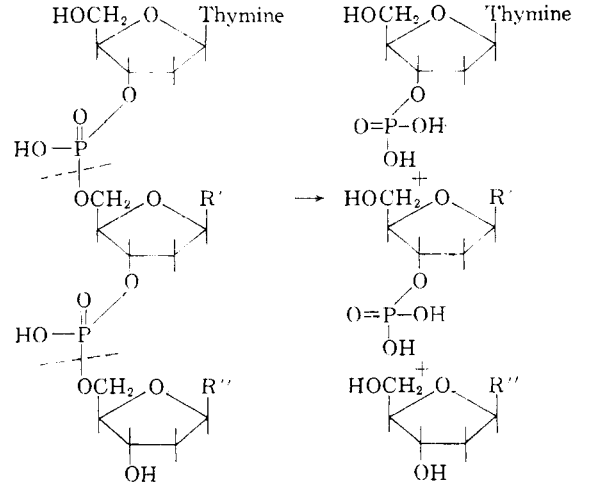
(8) For a recent discussion of carbodiimide reactions see M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 6204 (1958).

(9) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6223 (1958).

(10) Kind gift of Drs. R. J. Hilmoe and L. A. Heppel of the National Institutes of Health, Bethesda, 14, Md.



The method was next applied to the synthesis of a "trinucleoside diphosphate" containing three different deoxyribonucleosides. 5′-*O*-Tritylthymidine (V) and *N*,*O*^{3}-diacetyldeoxyadenylic-(5′) acid (VII) were treated with DCC, then, as in previous experiments,[4] the *O*-acetyl group was removed by treatment with dilute sodium hydroxide and the



SCHEME I.—Degradation of synthetic products by spleen diesterase. Bonds cleaved are shown by dotted lines.

N-acetyl group by hydrolysis with ammonia solution. Deoxyadenylyl-(5′→3′)-5′-*O*-tritylthymidine (IX) was obtained in 56% yield. Hydrogenolysis[11] of the trityl group gave deoxyadenylyl-(5′→3′-thymidine which was identical with the sample prepared earlier from N,O^3-di-acetyldeoxyadenylic(5′) acid and 5′-*O*-acetylthymidine.[4]

The trityl compound IX now was treated first with an excess of DCC in anhydrous pyridine and subsequently with *N*,O^3-diacetyldeoxycytidylic-(5′) acid[4] (XI). The crude product was treated with alkali and ammonia, as before, and the mixture separated by paper chromatography to give deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine (XIII) in 37% yield. The yield of this material is considerably lower than that of the corresponding trithymidine derivative XII and several side products also were formed. These results may be due to reactions involving the unprotected amino group in the adenine moiety of deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine.

Because of the expected acid lability of the glycosyl bond in the deoxyadenosine moiety of XIII, it was hoped that the removal of the trityl group in this product could be effected by hydrogenolysis, as had been done successfully in the case of deoxyadenylyl-(5′→3′)-5′-*O*-tritylthymidine (IX). However, hydrogenolysis of XIII with a palladium oxide–barium sulfate catalyst was extremely slow and was accompanied by hydrogenation of the cytosine ring. The absence of cytosine in the product was shown by its ultraviolet absorption characteristics and by acidic hydrolysis. The removal of the trityl group of XIII therefore was effected by mild acidic hydrolysis and even though there was some concomitant hydrolysis of the adenine–deoxyribose linkage, deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine (XV) was obtained in 83% yield.

As with the trithymidine diphosphate (XIV) the structure of XV was confirmed by enzymic degradation. Incubation with spleen diesterase[9] gave thymidylic-(3′) acid, deoxyadenylic-(3′) acid and deoxycytidine in approximately equimolar amounts (Scheme I, R′ = adenine R″ = cytosine).

Concluding Remarks.—The results reported here show that the basic method of phosphodiester bond synthesis can be used in the synthesis of higher mixed oligonucleotides. The present work has, however, brought into sharp focus some of the outstanding problems which must be solved in order to develop flexible and efficient syntheses of higher polynucleotides. One such problem is the reactivity of the amino groups in adenine and cytosine portions of the nucleosides. More recent work with deoxycytidine derivatives has shown that the interference from the amino group of this nucleoside is the most serious.[12] Specific protecting groups for the amino groups will therefore have to be used. Secondly, the use of the trityl group for protecting the end 5′-hydroxyl group of the proposed polynucleotide chain evidently is not completely satisfactory, if acidic hydrolysis is to be the only means of its removal. Alternative protecting groups for the primary hydroxyl group with the desired characteristics are being sought. Finally, with the increase in size of the polynucleotide chain, the problems of solubility of the intermediates become serious. Thus, it is worthy of note that while the trityl derivatives of nucleosides and dinucleoside phosphates are soluble in organic solvents, the trityl compound XIII is only sparingly soluble in anhydrous pyridine but readily soluble in water.

(11) It may be noted that in earlier experiments hydrogenolysis of the trityl compounds containing adenine was usually either very sluggish or did not proceed at all. Hydrogenolysis proceeded smoothly when the products were purified by paper chromatography.

(12) B. Lerch and H. G. Khorana, unpublished results.

Experimental

Paper chromatography was carried out with several solvent systems: I, isopropyl alcohol–concentrated ammonia–water (7:1:2); II, *n*-butyl alcohol–acetic acid–water (5:2:3); III, 2 *N* hydrochloric acid–*n*-propyl alcohol (1:3); and IV, 80% saturated ammonium sulfate solution + 2% isopropyl alcohol (the *p*H of the mixture adjusted to 6.0 with ammonium hydroxide). The compounds were detected by observation under an ultraviolet lamp and, unless otherwise stated, the R_f values were determined by descending chromatography on Whatman No. 1 chromatographic paper. The ϵ_{max} (P) values given are the molar absorptivities based on one gram atom of phosphorus per liter.

Thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-5′-*O*-tritylthymidine.—Thymidylyl-(5′ → 3′)-5′-*O*-tritylthymidine[4] (pyridinium salt, 0.14 mmole) and DCC (300 mg.) were dissolved in anhydrous pyridine (3 ml.) and the solution allowed to stand for one hour. A solution of 3′-*O*-acetylthymidylic-(5′) acid (0.28 mmole) in anhydrous pyridine (3 ml.) then was added and the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in anhydrous pyridine (3 ml.) and the mixture allowed to stand for two days. The pyridine was removed *in vacuo* and the residue mixed with water and light petroleum. The aqueous layer was kept at *p*H 12–13 for 30 min. by the addition of sodium hydroxide solution. The aqueous solution then was passed through a column of Amberlite IR-120 (pyridinium) ion-exchange resin. The eluate was concentrated to a small bulk *in vacuo* and applied to two 18″ wide sheets of Whatman 3 MM chromatographic paper and the mixture separated by chromatography in solvent system I. The main band (R_f 0.49) was cut out and eluted with water and spectrophotometric analysis of the eluate showed the yield of the desired trinucleoside diphosphate to be 0.095 mmole (68%). For analysis, 30% of the eluate was concentrated and re-chromatographed in solvent system I; the only band was cut out and eluted with water (5 ml.). The eluate was freeze-dried and the resulting powder (24.3 mg.) was dried *in vacuo* at 90° for 7 hours; λ_{max} 267 mμ, ϵ_{max} (P) 12,900; R_f values 0.51 (solvent I), 0.51 (solvent II).

Anal. Calcd. for $C_{49}H_{52}N_6O_{19}P_2 \cdot 2NH_4$: P, 5.5. Found: P, 4.95.

Thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine.—The above trityl derivative (NH_4^+ salt, 0.066 mmole) was dissolved in aqueous alcohol and hydrogenated in the presence of a palladium oxide–barium sulfate catalyst[13] for 3 hours after which time paper chromatography in solvent system I showed that the hydrogenation was complete. The catalyst was removed by centrifugation and the product applied to Whatman 3 MM chromatographic paper and chromatographed with solvent system I. The band, of R_f 0.24, was cut out and eluted with water and spectrophotometric analysis showed that the yield of the trinucleoside diphosphate was 0.055 mmole (83%). The eluate was freeze-dried to a fine powder (48 mg.) a portion of which was dried *in vacuo* at 100° for 5 hours; λ_{max} 266 mμ, ϵ_{max} (P) 12,700; R_f values 0.24 (solvent I), 0.165 (solvent II).

Anal. Calcd. for $C_{30}H_{38}N_6O_{19}P_2 \cdot 2NH_4$: P, 7.0. Found: P, 6.55.

Enzymic Hydrolysis of Thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine.—To a solution of thymidylyl-(5′ → 3′)-thymidylyl-(5′ → 3′)-thymidine (NH_4^+ salt, 1.5 mg.) in 0.25 *M* sodium succinate buffer (*p*H 6.5, 0.05 ml.) was added spleen diesterase solution[10] (0.05 ml.). The mixture was incubated at 37° for 10 hours after the addition of a little toluene. The total mixture then was applied to a sheet of Whatman 3 MM chromatographic paper. De-

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

velopment with solvent system I gave two bands: a nucleoside, thymidine (R_f, 0.63) and a nucleotide, thymidylic-(3′) acid (R_f, 0.14). The bands were eluted with water and spectrophotometric analysis at 267 mμ gave the value 2.0 for the molar ratio of thymidylic-(3′) acid to thymidine. The R_f values and ultraviolet spectra of the nucleotide and nucleoside were compared with those of authentic compounds as in Table I.

TABLE I

	Max, mμ (*p*H 7)	R_f values in solvent systems I	II	III
Nucleotide	267	0.10	0.30	0.82
Thymidylic-(3′) acid	267	.10	.30	.82
Thymidylic-(5′) acid	267	.10	.30	.75
Nucleoside	267	.60	.62	..
Thymidine	267	.60	.62	..

Paper chromatography in solvent system I also showed that the nucleotide was not degraded by incubation with snake venom (*Crotalus adamanteus*), in 0.25 *M* tris buffer (*p*H 8) under the same conditions that thymidylic-(5′) acid was degraded to thymidine.

Deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine.—5′-*O*-Tritylthymidine[4] (0.5 mmole) and DCC (0.52 g.) were dissolved in an anhydrous pyridine solution (5 ml.) of $N,O^{3'}$-diacetyldeoxyadenylic-(5′) acid (0.5 mmole). After two days the pyridine was evaporated *in vacuo* and the residue was mixed with light petroleum and water. The aqueous layer was kept at *p*H 12–13 for 0.5 hour by the addition of sodium hydroxide solution. Sodium ions then were removed by the addition of Amberlite 1R-120 (pyridinium) ion-exchange resin. The solution was filtered and the filtrate mixed with an equal volume of concentrated ammonia solution. After two hours the solution was concentrated to a small bulk and the product dissolved in a little aqueous alcohol and applied to two Whatman 3 MM chromatographic paper sheets (18″ wide). The mixture was separated with solvent system I and the main band (R_f 0.69) cut out and eluted with aqueous alcohol. Spectrophotometric analysis of this eluate showed that the yield of the dinucleoside phosphate was 0.28 mmole (56%). A portion of the product was evaporated to dryness *in vacuo*, dissolved in a little water and freeze-dried. The white powder, so obtained, then was dried *in vacuo* at 90° for 8 hours; λ_{max} 261 mμ, ϵ_{max} (P) 20,900 (in water); λ_{max} 260 mμ, ϵ_{max} (P) 20,800 (in 0.01 *N* HCl); R_f values 0.64 (solvent I), 0.70 (solvent II).

Anal. Calcd. for $C_{39}H_{39}N_7O_{10}P\cdot NH_4$: P, 3.8. Found: P, 3.7.

A portion of the product was dissolved in aqueous alcohol and hydrogenated in the presence of the palladium oxide–barium sulfate catalyst[10] for 4 hours. The product (R_f values 0.32 (solvent I), 0.35 (solvent II)) was chromatographically identical with deoxyadenylyl-(5′ → 3′)-thymidine synthesized previously.[4]

Deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine.—An aqueous alcohol solution of deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine (NH_4^+ salt, 0.433 mmole) was passed through a column of Amberlite 1R-120 (pyridinium) ion-exchange resin, the eluate was mixed with some pyridine and concentrated *in vacuo* to a small bulk. The residue was dried by repeated evaporation *in vacuo* of its solution in dry pyridine. The residue finally was dissolved in dry pyridine (5 ml.) and DCC (1 g.) added with shaking. After one hour a solution of $N,O^{3'}$-diacetyldeoxycytidylic-(5′) acid[4] (pyridinium salt, 0.8 mmole) in dry pyridine (8 ml.) was added and the total volume of the reaction mixture was reduced to about 5 ml. by evaporation *in vacuo*. After 2 days the mixture was evaporated to dryness *in vacuo* and the residue mixed with light petroleum and water. The aqueous layer was separated and kept at *p*H 12–13 for 0.5 hour by the addition of sodium hydroxide solution. The solution then was passed through a column of Amberlite 1R-120 (pyridinium) ion-exchange resin, the eluate was mixed with an equal volume of concentrated ammonia solution and allowed to stand for 2 hours. The solution then was concentrated *in vacuo* to a small bulk and applied to Whatman 3 MM chromatographic paper (three 18 in. wide sheets) and the mixture separated with solvent system I. The band with R_f 0.44 was cut out and eluted with water. Spectrophotometric analysis of the eluate showed that the yield of the product was 0.16 mmole (37%). For analysis, a portion of the product was freeze-dried and the remaining powder dried *in vacuo* at 100° for 10 hours; λ_{max} 263 mμ, ϵ_{max} (P) 14,000 (in water); λ_{max} 267, ϵ_{max} (P) 14,900 (in 0.01 *N* HCl); R_f values 0.44 (solvent (I), 0.52 (solvent II).

Anal. Calcd. for $C_{48}H_{60}O_{15}P_2\cdot 2NH_4$: P, 5.5. Found: P, 5.1.

Deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine.—The removal of the trityl group from the above compound by hydrogenolysis in the presence of the palladium oxide–barium sulfate catalyst was very slow and the product (R_f 0.18, solvent I), isolated by paper chromatography did not contain the base cytosine. The product had λ_{max} 260 mμ (in water) and 258 mμ (in acid) (*cf.* spectra of deoxyadenylyl-(5′ → 3′)-5′-*O*-tritylthymidine above). Hydrolysis of the product with dilute hydrochloric acid at 100° produced adenine and thymine as the only ultraviolet-absorbing materials, these products being identified by their R_f values and ultraviolet spectra.

Detritylation was effected by acidic hydrolysis when the deoxycytidylyl-(5′ → 3′)-deoxyadenyl-(5′ → 3′)-5′-*O*-tritylthymidine (NH_4^+ salt, 7.3 μmoles) was dissolved in 80% aqueous acetic acid (0.5 ml.) and allowed to stand for 26 hours. The solution was applied to Whatman 3 MM chromatographic paper and chromatography with solvent system I gave the deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine as the main band (R_f 0.18) together with weaker bands of adenine (R_f 0.51) and unchanged material (R_f 0.41). Elution of the desired product with water and spectrophotometric analysis of the eluate showed the yield to be 6.1 μmoles (83%). Freeze-drying of the eluate gave the trinucleoside diphosphate as a white powder which could not be obtained anhydrous; λ_{max} 263 mμ, ϵ_{max} (P) 14,000 (in water); λ_{max} 267 mμ, ϵ_{max} (P) 14,800 (in 0.01 *N* HCl). The product moved as a single band on electrophoresis in *p*H 3.5 buffer and had R_f values 0.13 (solvent I) and 0.11 (solvent II).

TABLE II

	λ_{max} (mμ) *p*H 7	*p*H 2	R_f values in solvent systems I	II	III	IV
Nucleoside	271	279	0.54	0.49	..	..
Deoxycytidine	271	280	.53	.49	..	..
Nucleotide A	267	..	.10	.30	0.86	..
Thymidylic-(3′) acid	267	..	.10	.30	.87	..
Thymidylic-(5′) acid	267	..	.10	.30	.80	..
Nucleotide B	260	257	.10	.27	..	0.15
Deoxyadenylic-(3′) acid	259	257	.10	.27	..	.15
Deoxyadenylic-(5′) acid	259	257	.10	.27	..	.21

Enzymic Hydrolysis of Deoxycytidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-thymidine.—The above trinucleoside diphosphate (NH_4^+ salt, 1.1 mg.), 0.25 *M* sodium succinate buffer (*p*H 6.5, 0.05 ml.), spleen diesterase solution[10] (0.05 ml.) and a trace of toluene were kept at 37° for 11 hours. The total mixture, then applied to Whatman 3 MM chromatographic paper and developed in solvent system I, gave a nucleoside band (R_f 0.54) and a nucleotide band (R_f 0.27 and 0.30). On extended chromatography in solvent system I for a period of several days the nucleotide band separated into two bands, A the faster traveling and B the slower traveling band. By comparison of their ultraviolet spectra and R_f values with those of authentic compounds (Table II), the nucleoside and nucleotides A and B were identified as deoxycytidine, thymidylic-(3′) acid[14] and deoxyadenylic-(3′) acid[15] and their relative molar concentrations as determined by spectrophotometric analysis were 1:1.2:0.9, respectively.

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(14) A. F. Turner and H. G. Khorana, THIS JOURNAL, **81**, 4651 (1959).

(15) We are grateful to Dr. L. Cunningham of Marquette University, Milwaukee, Wis., for a sample of this material which he prepared by enzymatic degradation of deoxyribonucleic acid (L. Cunningham, *ibid.*, **80**, 2546 (1958)).