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Stepwise Synthesis of Certain Deoxyribotrinucleotides

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The chemical synthesis of the deoxyribotrinucleotides 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine and 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxyadenine is described.

The chemical synthesis of oligonucleotides by controlled stepwise condensation of protected fragments has been described.[1] Such structures may serve as models for certain aspects of the properties of nucleic acids; they are useful, for instance, in the determination of enzyme specificity.[2] For our purposes,[3,5,6] it was necessary to synthesize deoxyribotrinucleotides of the type d-pXpYpZ (where X, Y, and Z represent different nucleosides occurring in nucleic acids) or at least the simpler type d-pXpYpY. In the present paper, we describe the chemical synthesis of 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine (VIIIa) and 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxyadenine (VIIIb).

At the time this work was started, a method for preparing the complementary structure d-pXpXpY was known: 5′-O-phosphorylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-deoxycytidine had been synthesized by the polymerization of thymidine-5′ phosphate in the presence of N^6,O-diacetyldeoxycytidine-5′ phosphate.[7] For the desired structures, however, a stepwise approach was indicated; Fig. 1 outlines the procedure employed. N^6-Anisoyl-3′-acetyldeoxycytidine-5′ phosphate (I)[8] was condensed with 5′-tritylthymidine (II) by means of dicyclohexylcarbodiimide. The resulting product III had to be separated from other components of the reaction mixture; established methods for the separation of oligonucleotides were found to be inappropriate since the material was much less polar than the unprotected phosphodiesters commonly encountered. Thus, in a water–ethyl acetate partition, the material distributed almost entirely into the nonaqueous phase. Chromatography on alumina and silica gel was also unsuccessful. Good separation was achieved, however, by chromatography on diethylaminoethylcellulose (DEAE)[10] with very dilute aqueous buffer in absolute methanol. Such a chromatogram is shown in Fig. 2a.

Assay of the individual peaks indicated the presence of derivatives of III in peaks B and D. Peak B could be converted to peak D upon careful alkaline hydrolysis,[9] and the reverse transformation was effected upon treatment of peak D with acetic anhydride in pyridine. Thus, the former was the acetate III of the latter (IV); the 3′-protecting group had presumably been lost adventitiously during manipulation. Since the next step in the synthetic scheme requires removal of the 3′-terminal acetate, anyway, the hydrolysis step was carried out routinely prior to chromatography in subsequent experiments. Examination of a typical column chromatogram shows the disappearance of III (peak B) from the pattern (Fig. 2b).

The material in peak D was assigned structure IV on the basis of: (1) its ultraviolet spectrum, which clearly shows the presence of N^6-anisoylcytidine chromophore; (2) the nature of the blocking groups by their hydrolytic removal and chromatographic identification; and (3) the physical properties and enzymatic susceptibility of the unmasked dinucleoside phosphate. Thus treatment of the latter with purified venom diesterase[11] gave thymidine and deoxycytidine-5′ phosphate, while spleen phosphodiesterase[12] produced deoxycytidine-3′ phosphate and deoxycytidine, as required by the known specificities of these enzymes.

In the next step, IV was condensed once again with the masked mononucleotide I, resulting in derivative Va of the trinucleoside diphosphate. After removal of the base-labile protecting groups, trityl ether VIa was isolated by preparative paper chromatography. Detritylation with acetic acid gave thymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine, a trinucleoside diphosphate recently synthesized[13] by another route. This material was characterized by similar procedures as described for IV.

The last step consisted of the addition of phosphate to the 5′-terminus. This transformation included: (1) complete acylation of VIa, (2) detritylation, and (3) phosphorylation of the resulting VIIIa by Tener's method.[14,15] The desired deoxyribotrinucleotide VIIIa was obtained in an over-all yield of 20%, based on trityl derivative VIa of the trinucleoside diphosphate. This material was abundantly characterized by a variety of physical and enzymatic[3,5,6] methods; by these criteria, it was identical with the product of enzymatic transphosphorylation.

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

(2) In a preliminary communication,[3] the use of 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine (for nomenclature, see ref. 1, pp. 93–96) in studying the mode of action of the exonuclease-I from *E. coli*[4] has been discussed. For a detailed report for the properties of that enzyme,[5] as well as those of the *E. coli* exonuclease-II,[6] see references cited.

(3) G. Scheuerbrandt, A. M. Duffield, and A. L. Nussbaum, *Biochem. Biophys. Res. Comm.*, **11**, 152 (1963).

(4) I. R. Lehman in "Progress in Nucleic Acid Research," J. N. Davidson, and W. E. Cohn, Ed., Vol. II, Academic Press, Inc., New York, N. Y., in press.

(5) I. R. Lehman and A. L. Nussbaum, in preparation.

(6) I. R. Lehman and C. C. Richardson, submitted for publication.

(7) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(8) Obtained by acetylation of the 3′-alcohol.[9] This compound is briefly mentioned by H. Schaller and H. G. Khorana, *Chem. Ind.* (London), 699 (1962), where the wide scope and relative ease of such condensations are discussed.

(9) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961). This material crystallized upor prolonged storage in pyridine.

(10) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).

(11) R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.*, **198**, 293 (1952).

(12) R. J. Hilmoe, *ibid.*, **235**, 2117 (1960).

(13) H. Schaller, G. Weimann, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 355 (1963).

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

(15) Initial difficulties[16] in this sequence, eventually resolved by improvements in procedure,[13] led us to search for alternate methods, and an enzymatic transphosphorylation[17] was used in our first synthesis[3] of 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine (VIIIa).

(16) No such difficulties had been encountered in a control synthesis of 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxycytidine[7] from 5′-tritylthymidylyl-(3′ → 5′)-deoxycytidine by entirely analogous procedures; see Experimental.

(17) The 5′-phosphorylation by a phosphotransferase preparation, as described for simple nucleosides by Brawerman and Chargaff,[18] of a number of deoxyribooligonucleotides has been recorded elsewhere.[19] The method is of particular interest when the preparation of blocked intermediates is impractical.

(18) G. Brawerman and E. Chargaff, *Biochim. Biophys. Acta*, **15**, 549 (1954).

(19) A. L. Nussbaum and A. M. Duffield, *Chem. Ind.* (London), 1729 (1963).

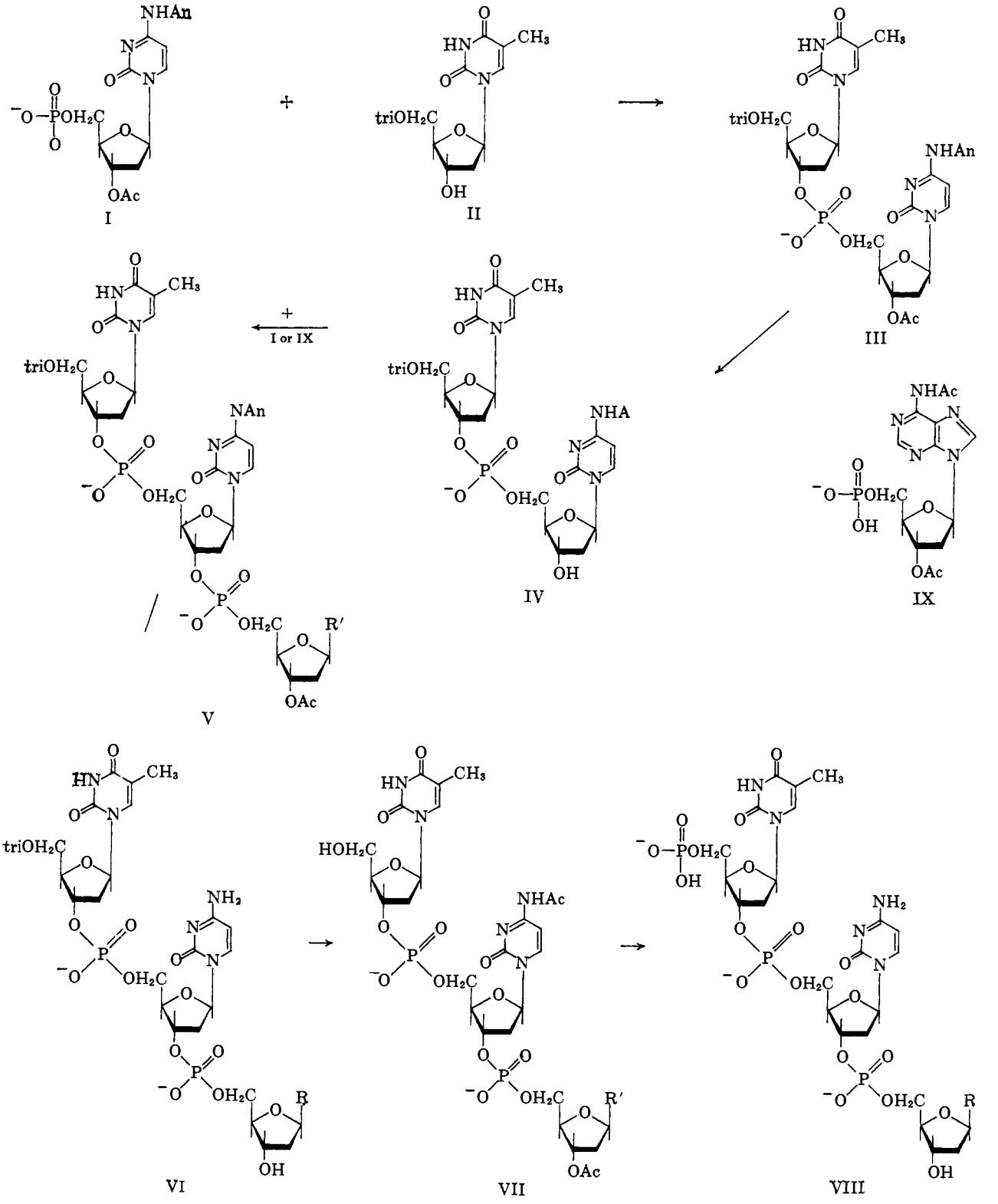

Fig. 1.—An, anisoyl; tri, triphenylmethyl; Ac, acetyl; Va, R′ = N⁶-anisoylcytosine; Vb, R′ = N⁶-acetyladenine; VIa and VIIIa, R = cytosine; VIb and VIIIb, R = adenine; VIIa, R′ = N⁶-acetylcytosine; VIIb, R′ = N⁶-acetyladenine.

The availability of the protected dinucleoside phosphate IV makes it possible to synthesize deoxyribotrinucleotides containing a third base. This is illustrated by the following sequence: the use of $N^6,O^{3'}$-diacetyldeoxyadenylic acid[20] (IX) in place of I at the appropriate stage gave the corresponding masked trinucleoside diphosphate Vb, which upon complete unblocking gave thymidyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxyadenine, a sequential isomer of thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxycytidine already described.[21] Chemical phosphorylation of the appropriately masked precursor VIIb, followed by hydrolysis, gave 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxylytidylyl-(3′ → 5′)-deoxyadenine (VIIIb).[22]

(20) R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 2932 1961).

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

(22) The yield (12%) was substantially lower than that of VIIIa described above; this was due to loss by depurination during the acidic detritylation step. One would now[23] use the more labile substituted ethers, which can be removed without concomitant depurination.

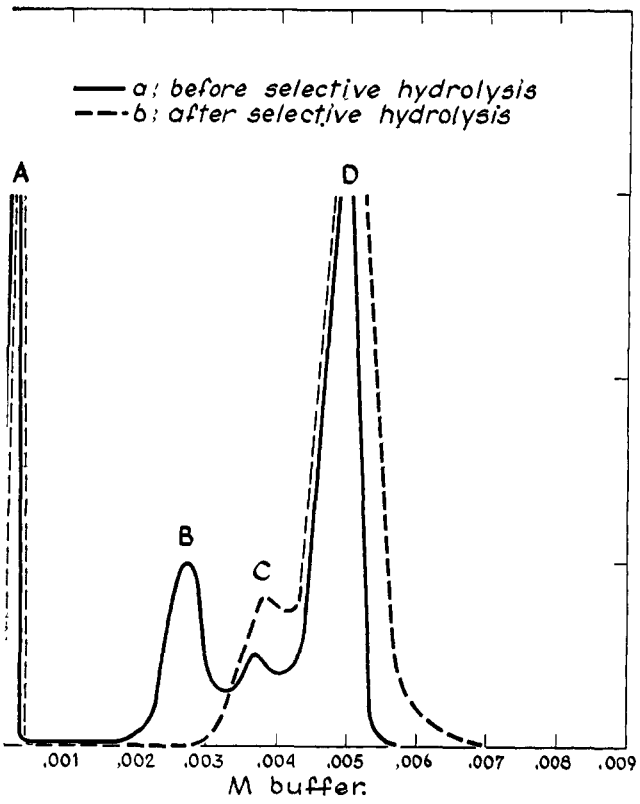

Fig. 2.—DEAE-cellulose column chromatography of mixtures containing "masked" d-TpC's. Elution carried out with triethylammonium bicarbonate in methanol. For details, see Experimental.

A series of experiments was carried out aimed toward the synthesis of a partially blocked ^{32}P-deoxycytidylic acid for use in the condensations here described. These proved abortive since other methods turned out to be more direct; but since the precursor nucleosides were well characterized, their synthesis has been included in the Experimental section.

Experimental[24]

N^6-Anisoyl-3′-acetyldeoxycytidine-5′ Phosphate (I).—N^6-Anisoyl-deoxycytidine-5′ phosphate was prepared according to the literature,[7] Of this, the total crude yield from 5′-deoxycytidylic acid (1.2 g.) was treated with a 1:1 mixture of pyridine–acetic anhydride (10 ml.) for 18 hr. at room temperature. Water (10 ml.) was added and the mixture set aside for 3 hr. Vacuum concentration at 30° left a residual oil which, after titration with ethyl ether, gave a tan powder (980 mg.), m.p. 143–145° dec. This material in paper chromatographic system A traveled as a single spot (R_f 0.78), somewhat faster than the precursor 3′-ol. The material was recrystallized from methanol–ethyl acetate containing a trace of moist pyridine to give an analytical sample, no change in melting point, $\epsilon^{max}_{303\ m\mu}$ 22,500 (pH 5 in water).

Anal. Calcd. for $C_{19}H_{22}O_{10}N_3P \cdot C_5H_5N \cdot H_2O$: C, 49.65; H, 5.03; N, 9.65; P, 5.34. Found: C, 49.34; H, 5.21; N, 9.76; P, 5.88.

Partial Hydrolysis.—The acetate (I, 5 μmoles) was taken up in pyridine (200 μl.), and 2 *N* sodium hydroxide solution (300 μl.) plus water (100 μl.) were added. The two-phase system was agitated for 25 min. at room temperature, passed over a small column of Dowex 50W-X8 (pyridinium cycle), and concentrated to a small volume. Paper chromatography in system A indicated regeneration of the precursor 3′-ol.

Complete Hydrolysis.—The acetate (I, 2 μmoles) in concentrated ammonia (0.5 ml.) was allowed to stand at room temperature for 8 hr. Paper chromatographic assay showed the presence of 5′-deoxycytidylic acid.

5′-Tritylthymidylyl-(3′ → 5′)-N^6-anisoyldeoxycytidine (IV).—The foregoing acetate (88 mg., 152 μmoles) was dried by several concentrations with pyridine, and dissolved in dry pyridine (0.3 ml.) by warming. After cooling, dicyclohexylcarbodiimide (150 mg.) was added, and the resulting solution was well stoppered and allowed to stand at room temperature for 24 hr. 5′-Tritylthymidine[28] (II, 100 mg.) was added and the mixture allowed to react at 55° for another 48 hr. A second addition of 5′-tritylthymidine (100 mg., total added 435 μmoles or 2.4 equiv.), followed by dicyclohexylcarbodiimide (150 mg.), was succeeded by another 3-day heating at 55°. The reaction was terminated by the addition of water (1 ml.) and the mixture allowed to stand for 4 hr. at room temperature. Partial vacuum concentration at 30° was followed by partitioning the total product between water and ethyl acetate. The organic layer was concentrated to dryness, pyridine (2 ml.), water (1 ml.), and 2 *N* sodium hydroxide (3 ml.) were added, and the resulting two-phase mixture agitated intensively on an Adams Cyclo-Mixer for 25 min. Dowex 50W-X8 (H^+ cycle) was added to discharge the resulting raspberry red color and the whole slurry poured over a column (10 × 1 cm.) of the same resin (pyridinium cycle). The column was washed with a 1:1 pyridine–water mixture (100 ml.) and the combined eluate concentrated to dryness *in vacuo*. The residue was dissolved in methanol (5 ml.) and chromatographed on a DEAE-cellulose (bicarbonate cycle) column (3.5 × 35 cm.) made up in methanol. Continuous addition of methanol (2 l.) containing 2 *N* triethylammonium bicarbonate (14 ml., pH 7.5) to a mixing reservoir initially containing methanol (2 l.) during column irrigation, resulted in the elution pattern shown in Fig. 2b. The fractions of the main peak D were combined, concentrated, and lyophilized. The product (82.5 μmoles, 54% based on starting nucleotide) possessed a maximum in the ultraviolet (methanol) at 273 mμ (ε calculated per P = 27,300).

For characterization, an aliquot of IV (0.8 μmole) in methanol (1.3 ml.) was treated with concentrated ammonia (5 ml.). After 7 hr. at room temperature the solution was concentrated to dryness and streaked on 1 cm. of Whatman filter paper grade 3 MM. It was developed for 18 hr. in system B and the appropriate zones (visualized by examination under an ultraviolet source) were extracted with water (d-trityl TpC) and ethanol (anisoic acid). The extracts were assayed by ultraviolet spectrometry, and in the case of trityl alcohol, by means of a quantitative color test.[29] The results are shown in Table I.

TABLE I

	μmole recovd.	Relative proportion	Theor.
Anisoic acid[a]	0.74	(1.00)	1
d-Trityl TpC[b]	.65	0.88	1
Trityl content[c]	.73	0.99	1

[a] Using $\epsilon_{251.5\ m\mu}$ = 14,100 (ethanol). [b] An extinction coefficient of 22,100 was used. P. T. Gilham and H. G. Khorana record (*J. Am. Chem. Soc.*, **80**, 6212 (1958)) ε = 21,200 for d-TpC. Addition of 900 for the trityl substituent is based on data from E. A. Braude in "Determination of Organic Structures by Physical Methods," E. A. Braude and F. C. Nachod, Ed., Academic Press, Inc., New York, N. Y., 1955, p. 157. [c] Measured on an aliquot of recovered d-tri TpC.[29]

d-Trityl TpC (10 optical density units) in 80% aqueous acetic acid (2 ml.) was allowed to stand at room temperature for 7 hr.

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

(24) Evaporations of aqueous solvents were carried out below 30° at oil pump vacuum. Paper chromatography utilized the solvent mixture isobutyric acid–0.5 *N* ammonia, 10:6 (system A), and isopropyl alcohol–ammonia–water, 7:1:2 (system B). Spots were visualized by quenching of paper fluorescence produced by a Mineralight source (Ultra-Violet Products, Inc., San Gabriel, Calif.), or (destructively) by spraying the paper with a solution specific for phosphorus.[25] Spots were extracted by immersion of the excised quenching area in water for 20 min., three such extractions sufficing in most cases. Ultraviolet spectra were taken in a Zeiss PMQ II spectrophotometer.

Paper electrophoresis was carried out as described by Markham and Smith.[26] Dowex 50W-X8 was washed with concentrated hydrochloric acid–water 1:1, followed by distilled water to pH 5. Diethylaminoethylcellulose (DEAE), type 40, purchased from the Brown Co., Berlin, N. H., was washed with 0.5 *N* sodium hydroxide, then distilled water to neutral, and finally 0.02 *M* buffer, typically ammonium bicarbonate. Phosphorus was determined by a micromethod.[27] Other elemental analyses were performed by E. Meier and J. Consulo, Stanford University Department of Chemistry.

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

(26) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

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

(28) Prepared according to J. P. Hurwitz, J. A. Urbanski, and J. Chua, *J. Org. Chem.*, **27**, 3033 (1962). We found it useful to follow the course of tritylation, as well as the purity of the product, by thin layer chromatography. Silica Gel G (Merck Darmstadt) was irrigated with chloroform–ethyl acetate (1:1). Under these conditions the product travels 2.5/10 cm.

(29) Trityl alcohol and its ethers reacts with a solution of concentrated sulfuric acid in methanol (1:1, v./v.) to give a stable yellow color at 406 mμ which exhibits Beer's law proportionality in the region examined. Details are to be published elsewhere.

The solution was concentrated to dryness and d-TpC[1] isolated by preparative paper chromatography in system B.

Enzymatic Characterizations of d-TpC. (i) **Spleen Phosphodiesterase.**—d-TpC (2 optical density units) in distilled water (30 μl.) 0.1 *M* succinate buffer (50 μl., pH 6.1), and a spleen diesterase preparation[30] (20 μl.), assayed at 4,8 units/ml., were incubated at 37° for 4 hr. The entire solution was then spotted on Whatman filter paper grade 3 MM and developed in system B against Tp and d-C. Matching spots of comparable intensity were observed.

(ii) **Purified Venom Phosphodiesterase.**—d-TpC (3 optical density units) in water (50 μl.), 1 *M* glycine (20 μl., pH 9.2), 0.1 *M* magnesium chloride (10 μl.), and a preparation of purified venom diesterase[31] (20 μl.), assayed at 800 units/ml.,[11] were treated as above. Paper chromatography revealed the presence of equimolar amounts of d-pC and T.

(iii) **Crude Venom Phosphodiesterase.**—Repetition of the above procedure with a crude preparation from the venom of the rattlesnake gave rise to the appearance of d-C and T.

5'-Tritylthymidylyl-(3' → 5')-N⁶-anisoyl-3'-acetyldeoxycytidine (III).—A reaction as described for the corresponding deacetylated material IV was carried out on one-half the scale (76 μmoles) described above up to just prior to the hydrolysis with sodium hydroxide. Chromatography of the total mixture on DEAE-cellulose is illustrated in Fig. 2a. The desired peak fractions B were pooled, concentrated, and lyophilized. The material thus obtained measured 296 optical density units in methanol (9.8 μmoles, 13%) at its maximum (273 mμ); its spectrum was virtually superimposable on that of its parent alcohol IV. Combination, concentration, and lyophilization of fractions D gave 755 optical density units in methanol at 273 mμ (26.7 μmoles, 35%), of IV); thus, 72% of the combined TpC derivatives had hydrolyzed adventitiously. Structure of the acetate III follows from its polarity, its spectrum ($\epsilon^{MeOH}_{273\ m\mu}$ 27,000), its conversion to d-TpC by a two-stage hydrolytic unmasking as described above, and finally its conversion to the known[7] d-pTpC (*vide infra*).

Deoxycytidylyl-(3' → 5')-thymidine-5' Phosphate.—5'-Tritylthymidylyl-(3' → 5')-[N⁶-anisoyl-3'-acetyl]deoxycytidine (640 optical density units, 23.1 μmoles) was dried overnight under high vacuum at room temperature and dissolved in 80% aqueous acetic acid (5 ml.). After standing for 44 hr. (paper chromatography revealed that total trityl splitting had occurred after 24 hr.), the solution was concentrated to dryness and the residue extracted with *n*-hexane. The remaining gum was dissolved in 20% aqueous pyridine (40 ml.) and passed over a column of Dowex 50W-X8 (pyridinium cycle). The latter was washed with the same solvent (30 ml.) and the combined eluates were concentrated *in vacuo*. A 1 *M* solution of pyridinium β-cyanoethyl phosphate in pyridine (0.115 ml.) was added and the mixture rendered anhydrous by concentrating it several times with dry pyridine. Dicyclohexylcarbodiimide (206 mg., 1 mmole) was added, and the volume of the mixture made up to 1 ml. with anhydrous pyridine. The reaction was allowed to proceed at room temperature for 72 hr. when water (2 ml.) was added and the mixture left to stand overnight. It was then centrifuged and the supernatant removed. The residual solid was extracted several times with water, the suspension being centrifuged each time and the liquid phase combined with the original supernatant. The combined aqueous solution was concentrated to dryness and treated with concentrated ammonia (1 ml.) for 45 min. at 56°. A further quantity of ammonia (1 ml.) was added and heating continued for an additional hour. After cooling, the solution was concentrated, suspended in water (10 ml.), and filtered. The filtrate was adjusted to pH 9, applied to a column of DEAE-cellulose (15 × 2.5 cm., bicarbonate cycle), and eluted by means of a linear gradient of 0.3 *M* triethylammonium bicarbonate (1500 ml., pH 7.5), mixing with water (1500 ml.). A peak emerging at 0.15 *M* buffer was combined, concentrated, and lyophilized. It had λ_{max} at 272 mμ (pH 2), 280/260 = 1.0 and traveled with the R_f expected for pTpC[7]; yield from the tritylated dinucleoside phosphate, 5.42 μmoles (23.4%). An additional 4.16 μmoles of TpC (18%) was eluted earlier with buffer strength 0.035 *M*.

Treatment of the dinucleotide with purified venom diesterase[11] (*vide supra*) gave rise to equal quantities , pT and pC, as adjudged by paper chromatography.

5'-Tritylthymidylyl-(3' → 5')-deoxycytidylyl-(3' → 5')-deoxycytidine (VIa).—N⁶-Anisoyl-3'-acetyldeoxycytidine-5' phosphate (60 mg., 120 μmoles) was treated overnight in dry pyridine (0.7 ml.) with dicyclohexylcarbodiimide (150 mg.). 5'-Tritylthymidylyl-(3' → 5')-N⁶-anisoyldeoxycytidine (2,400 optical density units (72 μmoles), previously dried by coconcentration with pyridine, was dissolved in dry pyridine (1 ml.) and added to the activated masked mononucleotide. After 7 days at 55° the reaction was terminated by the addition of water (1.5 ml.) and allowed to stand at room temperature overnight. The suspension was partitioned between ethyl acetate and water and the organic layer discarded. The aqueous phase was concentrated to dryness and dissolved in methanol–concentrated ammonia (1:1, 2 ml.) and allowed to stand for 7 hr. at room temperature. The solution was concentrated to dryness, dissolved in water, and streaked onto 75 cm. of Whatman filter paper grade 3 MM. Development of the chromatogram in system B gave rise to seven absorption bands. The material traveling from 10 to 16 cm. (solvent from 43 cm.) was excised and extracted exhaustively with water, yielding 690 optical density units at 274 mμ. Chromatography on a DEAE-cellulose column (bicarbonate cycle) gave a main peak emerging with 0.125 *M* triethylammonium bicarbonate (pH 7.5). This material (VIa), after concentration and lyophilization of the appropriate peak fractions, yielded 490 optical density units at 274 mμ (14.5 μmoles, 20% based on protected dinucleoside phosphate). The 5'-tritylnucleoside diphosphonate (VIa) had λ_{max} 274 mμ (pH 2), 280/260 = 1.23, 250/260 = 0.52, ε/2P = 32,000. A trityl determination[29] indicated the presence of 1.0 mole per 2.0 moles of P.

Thymidylyl-(3' → 5')-deoxycytidylyl-(3' → 5')-deoxycytidine.—The trityl derivative VIa (275 optical density units, 8.2 μmoles) was treated with 80% aqueous acetic acid (2 ml.) during 40 hr. at room temperature. The solvent was removed *in vacuo*, water (10 ml.) was added, and the solution again concentrated. The residue was then partitioned between water and *n*-hexane to remove trityl alcohol, and the aqueous phase concentrated. The residue was dissolved in water (10 ml.) and the pH of the solution adjusted to 8.5. It was then applied to a column of DEAE-cellulose (12 × 3 cm., bicarbonate cycle) and eluted in a linear gradient of 0.3 *M* triethylammonium bicarbonate (1500 ml., pH 7.5) mixing with water (1500 ml.). A main peak emerged at buffer strength 0.1 *M*. Pertinent fractions were combined, concentrated, and lyophilized yielding VIIa (261 optical density units at its maximum of 275 mμ (pH 2), 7.7 μmoles). The ultraviolet spectrum had 280/260 = 1.49, 250/260 = 0.55 (pH 2). Treatment of VIIa with calf spleen phosphodiesterase gave rise to three spots upon paper chromatography in system A, traveling as Tp, Cp, and C in the (weighted) ratios of 1.0:1.1:1.0 (theoretical 1:1:1). At its maximum this material had ε/2P = 31,500 (pH 2).

5'-O-Phosphorylthymidylyl-(3' → 5')-deoxycytidylyl-(3' → 5')-deoxycytidine (VIIIa).—5'-Tritylthymidylyl-(3' → 5')-deoxycytidylyldeoxycytidine (234 optical density units, 7.0 μmoles) was dried by repeated concentration with dry pyridine. Acetic anhydride–pyridine (1:1, 10 ml.) was added and the solution allowed to stand at room temperature for 24 hr. It was then cooled to 0°, water (5 ml.) added, and the solution allowed to stand for 1 hr. at room temperature. The reaction was then concentrated and coconcentrated three times with water (2 ml.); 80% aqueous acetic acid (5 ml.) was added and the solution allowed to stand at room temperature for 44 hr. It was concentrated and coconcentrated with water until all the acetic acid had been removed. The dry residue was leached with *n*-hexane (3 × 10 ml.), dissolved in 50% aqueous pyridine (10 ml.), and passed over a column of Dowex 50W-X8 (1 × 10 cm., pyridinium cycle). The column was washed with the same solvent (150 ml.) and the combined eluates concentrated to dryness. Two further concentrations with dry pyridine (5 ml.) left a relatively anhydrous residue. Dowex 50W-X8 resin (pyridinium cycle, 0.2 ml.),[13] pyridinium β-cyanoethyl phosphate[14] (40 μmoles), and dimethylformamide (0.25 ml.) were added and the resulting suspension concentrated several times with anhydrous pyridine. Dry pyridine (0.25 ml.) and dicyclohexylcarbodiimide (120 mg.) were added and the condensation allowed to proceed at room temperature in the dark for 2 days. Water (1 ml.) was added and after overnight standing most of the liquid phase was removed by concentrating under vacuum. Concentrated ammonia (1 ml.) was added and the suspension heated at 55° for 1 hr.; a further addition of concentrated ammonia (1 ml.) was followed by another 1.5-hr. heating period. The reaction was diluted with water (10 ml.), filtered, and the precipitate washed well with water. The combined filtrate was concentrated almost to dryness, diluted with water (25 ml.), and the pH adjusted to 8.5. It was then applied to a DEAE-cellulose column (15 × 2.5 cm., bicarbonate cycle) and eluted using a gradient of 0.3 *M* triethylammonium bicarbonate (1500 ml., pH 7.5), mixing with water (1500 ml.). A main peak, emerging with 0.18 *M* buffer, was concentrated and lyophilized yielding 62.4 optical density units at 275 mμ, which were not completely homogeneous as shown by paper chromatography in system A. Preparative separation on Whatman filter paper grade 3 MM removed a more polar impurity and gave VIIIa (48.4 optical density units at 276 mμ, 1.4 μmoles, yield 20% based on VIa); pTpCpC had the properties: ultraviolet maximum at 276 mμ, 280/260 = 1.54, 250/260 = 0.52 (pH 2), ε/3P = 31,800. Treatment with purified venom diesterase gave rise to pT and d-pC in the ratio of 1.0:2.0 (theoretical 1:2). Treatment with Exonuclease I (Lehman's phosphodiesterase from *E. coli*)[4] gave d-pC and d-pTpC in the ratio 1.1:1.0 (theoretical 1:1). Further treatment of the dinucleotide (obtained from the latter digest by preparative paper

(30) Purchased from the Worthington Biochemical Corp.

(31) *Crotalus adamenteus* venom was obtained from the Ross Allen Reptile Farm, Silver Springs, Fla.

chromatography) with purified venom diesterase gave similar amounts of pT and d-pC, as adjudged by paper chromatography.

5'-O-Phosphorylthymidyl-(3' → 5')-deoxycytidylyl-(3' → 5')-deoxyadenosine (VIIIb).—5'-Tritylthymidylyl-(3' → 5')-N⁶-anisoyldeoxycytidine (IV) (4300 optical density units, 154 μmoles) in aqueous pyridine (1:1, 10 ml.) was passed over a column of Dowex 50W-X8 (10 × 1 cm., pyridinium cycle) and the column washed with 50% aqueous pyridine (120 ml.). The combined eluates were concentrated to dryness and coconcentrated several times with dry pyridine. N⁶,O³'-Diacetyldeoxyadenylic acid[20] (200 μmoles, pyridinium salt) and Dowex 50W-X8 resin (200 μl., pyridinium cycle) were added, and the reaction mixture rendered anhydrous as before. Dry pyridine (2 ml.) and dicyclohexylcarbodiimide (412 mg.) were added and the reaction heated at 55° for 5 days. After the addition of water (2 ml,), the mixture was agitated and left to stand overnight at room temperature. It was then concentrated to a small volume and partitioned between ethyl acetate and water; the aqueous phase was concentrated to dryness and the residue treated with concentrated ammonia (10 ml.) in methanol (5 ml.). After 7 hr. at room temperature, the solvent was removed and the residue streaked on 6 × 14 cm. of Whatman filter paper grade 3 MM and developed in system B. The most prominent zone had traveled from 10 to 18 cm. (solvent front at 40 cm.), and it was extracted exhaustively with water yielding 715 optical density units at 266 mμ. Of this, 543 optical density units were further purified by chromatography on DEAE-cellulose (15 × 4 cm., bicarbonate cycle) using a linear gradient of water (1500 ml.) being continuously diluted with a solution of 0.3 *M* triethylammonium bicarbonate (1500 ml., pH 7.5). The desired d-trityl TpCpA (VIb) (250 optical density units at its maximum 265 mμ, 280/260 = 0.72, 250/260 = 0.70 (all at pH 2)) was eluted with 0.16 *M* buffer. The appropriate tubes of this, the main peak, were combined, concentrated, and lyophilized.

An aliquot of the material obtained prior to column chromatography was treated with 80% aqueous acetic acid (5 ml.) for a period of 16 hr. at room temperature. Electrophoresis (pH 7.5) gave three regions: adenine (corresponding to an estimated 30% of depurination), d-TpCpA (VIb), and a most rapidly migrating zone, presumably the other fragment of depurination, d-TpCp, not further investigated. The nature of the trinucleoside diphosphate was verified by the following data: ultraviolet spectrum, λ_{max} 265 mμ, 280/260 = 0.77, 250/260 = 0.73 (all at pH 2); R_f in system A 0.52; products of digestion with purified venom diesterase: T, pC, and pA in the ratio 1.0:0.95:1.0 (theoretical 1:1:1).

The purified d-trityl TpCpA (VIb, 246 optical density units) was coconcentrated with dry pyridine (3 × 3 ml.) and the residue acetylated with pyridine (5 ml.) containing acetic anhydride (5 ml.). After 2 days at room temperature the solution was cooled to 0°, water (5 ml.) was added, and after 2 hr. at room temperature the solution was concentrated to dryness and coconcentrated with water (2 × 10 ml.). The residue was treated with 80% aqueous acetic acid (5 ml.) for 16 hr. at room temperature when the solvent was removed and the product coconcentrated with water (2 × 5 ml.). The dried material was leached with *n*-hexane (2 × 10 ml.) and the insoluble material passed over a column of Dowex 50W-X8 (10 × 1 cm., pyridinium cycle) in 50% aqueous pyridine. The column was washed with the same solvent (100 ml.) and the combined eluates concentrated to dryness. Dowex 50W-X8 resin (200 μl., pyridinium cycle) and pyridinium β-cyanoethyl phosphate (70 μmoles) were added, and the mixture dried by the usual procedure. Anhydrous pyridine (0.25 ml.), dry dimethylformamide (0.25 ml.), and dicyclohexylcarbodiimide (130 mg.) were added and the reaction allowed to proceed during 48 hr. at room temperature. Concentrated ammonia (1 ml.) was added and the suspension heated at 55°. After 1 hr. and 1.5 hr., concentrated ammonia (1 ml.) was added and heating continued for a total of 2 hr. The suspension was filtered, the precipitate washed exhaustively with water, and the combined filtrate concentrated. The residue was diluted with water (25 ml.) and the pH adjusted to 8.5. It was then applied to a DEAE-cellulose column (16 × 2.5 cm., bicarbonate cycle) and eluted with a linear gradient of 0.3 *M* triethylammonium bicarbonate (1500 ml., pH 7.5), mixing with water (1500 ml.). A peak emerging at 0.18 *M* buffer contained material having the same paper chromatographic migration characteristics as the product from the enzymatic transphosphorylation of d-TpCpA.[19] The appropriate fractions were combined, concentrated, and lyophilized, yielding the desired trinucleotide VIIIb (29.5 optical density units at its maximum 265 mμ, 12% based on VIb. The trinucleotide had the characteristics: 280/260 = 0.75, 250/260 = 0.75, ε/3P = 33,000 (all at pH 2); R_f in system A 0.43; purified venom diesterase gave pT:dpC:dpA in the ratio 1.16:1.00:1.00 (theoretical 1:1:1).

N⁶,3',5'-Trianisoyldeoxycytidine.[32]—Deoxycytidine hydrochloride (263.7 mg., 1 mmole) was suspended in pyridine (30 ml.), anisoyl chloride (4 ml.) was added dropwise, and the mixture stirred at room temperature for 1 hr. The reaction was terminated by pouring it into ice-cold 1 *M* ammonium bicarbonate (300 ml.). The slurry was allowed to come to room temperature when it was heated on the steam bath for 0.5 hr., cooled, and extracted with ethyl acetate. The organic extract was dried over sodium sulfate and concentrated to dryness. The residue was suspended in benzene (10 ml.) and applied to an alumina column (50 g., Merck) and eluted with benzene (3 × 50 ml.), ether (3 × 50 ml.), and chloroform (3 × 50 ml.). Concentration of the latter left a crystalline residue which upon crystallization from ethyl acetate gave the triacyl derivative (258 mg., 41%). This material had a m.p. 166–168° and traveled as a single spot in thin layer chromatography (1.8 to 2.3/10 cm., silica gel G, chloroform–ethyl acetate 1:1).[33] The infrared spectrum (Nujol) lacked hydroxyl absorption but had strong ester bands (5.85 and 8.04 μ) and amide bands (6.00 and 6.03 μ).

Anal. Calcd. for $C_{33}H_{31}O_{10}N_3$: C, 62.95; H, 4.96; N, 6.68. Found: C, 62.84; H, 5.12; N, 6.76.

N⁶-Anisoyldeoxycytidine.—The foregoing trianisoyl derivative (200 mg.) in pyridine (6 ml.) was diluted with water (3 ml.) and 2 *N* sodium hydroxide (9 ml.). The resulting two-phase system was agitated vigorously at room temperature for 25 min. when the upper layer was separated and neutralized with glacial acetic acid; 1 *M* ammonium bicarbonate (10 ml.) was added and the solution extracted with ethyl acetate. The organic layer was concentrated to a thick oil, which crystallized upon trituration with chloroform, Crystallization from methanol gave the monoanisoate, 56.9 mg., 49%, m.p. 176–177°, resolidifying and final decomposing at 230–240°. The material was homogeneous in thin layer chromatography (0.8 to 1.3 cm./10 cm., silica gel G, 3% methanol in ethyl acetate). The infrared spectrum indicated associated hydroxyl (2.98 μ), no ester bands, and amide bands (6.00 and 6.08 μ).

Anal. Calcd. for $C_{17}H_{19}O_6N_3 \cdot 0.5CH_3OH$: C, 55.69; H, 5.61; N, 11.13, Found: C, 55.45; H, 5.69; N, 11.18.

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(32) These reactions were patterned after the corresponding transformations for the nucleotides; see ref. 7.

(33) Visualized by spraying the plate with a 1:1 solution of concentrated sulfuric acid in methanol and heating for 10 min. at 120°.