Experimental[35]

Materials.—Chlorophylls *a* and *b* were prepared from spinach by the procedure of Strain, *et al.*[4] The methyl chlorophyllides *a* and *b* were prepared from cockleburr by the *in situ* reaction with methanol; details of this procedure will be described elsewhere. Pheophytins *a* and *b* and methyl pheophorbide *b* were prepared from the corresponding chlorophylls following well-described procedures.[36] Methyl pheophorbide *a* was kindly donated by Professor R. B. Woodward. Deuteriochloroform and methanol-d_4 were commercial samples (Volk) and were used without further purification.

N.m.r. Spectra.—The n.m.r. spectra were recorded on Varian spectrometer systems DP 60, DP 40, and A60. Chemical shifts were measured by the conventional side-band techniques using Hewlett–Packard oscillators and frequency counters. Chemical shifts of the spectra determined on the A60 spectrometer were read off the calibrated charts. No particular care was taken to control the temperature of the samples; accordingly, temperatures varied from 28–37°.

Spin decoupling experiments were carried out using wide-line equipment of the DP spectrometers. Field modulation was applied with a Hewlett–Packard 200CD oscillator connected to the Varian V-4250A sweep unit. Phase sensitive detection was carried out with the lock-in amplifier V-4270A. The spectra were recorded as the audiofrequency modulation side bands, while the main radiofrequency field H_1 served as the decoupling field.

(35) The complete numerical chemical shift values of compounds I–IV at the various concentrations studied and the methanol shift values of compounds I and II are available upon request.

(36) H. Fischer and H. Orth, "Die Chemie des Pyrrols," Vol. II$_2$, Akademische Verlagsgerellschaft, Leipzig, 1940.

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

Studies on Polynucleotides. XXIV.[1] The Stepwise Synthesis of Specific Deoxyribopolynucleotides (4).[2] Protected Derivatives of Deoxyribonucleosides and New Syntheses of Deoxyribonucleoside-3′ Phosphates[3]

BY H. SCHALLER, G. WEIMANN, B. LERCH, AND H. G. KHORANA

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The following protected derivatives of the four major deoxyribonucleosides have been prepared: 5′-O-mono-*p*-methoxytrityl- and 5′-O-di-*p*-methoxytritylthymidine from thymidine and the appropriate trityl chloride; N-benzoyl- and N-anisoyldeoxycytidine and from these the corresponding 5′-O-trityl-, 5′-O-mono-*p*-methoxytrityl-, and 5′-O-di-*p*-methoxytrityl- derivatives; N,N,O$^{3'}$,O$^{5'}$-tetrabenzoyldeoxyadenosine and N-benzoyldeoxyadenosine; and from the latter, the corresponding 5′-O-mono-*p*-methoxytrityl- and 5′-O-di-*p*-methoxytrityl-derivatives; 3′,5′-di-O-acetyldeoxyguanosine, N-di-*p*-methoxytrityl-O$^{3'}$,O$^{5'}$-diacetyldeoxyguanosine, N-di-*p*-methoxytritydeoxyguanosine, and N,O$^{5'}$-bis-di-*p*-methoxytritydeoxyguanosine. Phosphorylation of 5′-O-di-*p*-methoxytritylthymidine, of N-benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidine, of N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine, and of N,O$^{5'}$-di-*p*-methoxytrityldeoxyguanosine with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide followed by removal of the protecting groups gave excellent yields of thymidine-3′, deoxycytidine-3′, deoxyadenosine-3′, and deoxyguanosine-3′ phosphates, respectively. A sensitive colorimetric method for the estimation of compounds containing the di-*p*-methoxytrityl group is described.

General Introduction.—Of the two possible approaches to the synthesis of the naturally occurring internucleotidic linkage from two protected nucleoside or nucleotide components, the first is that in which a protected deoxyribonucleoside-5′ phosphate is condensed with the 3′-hydroxyl group of a second suitably protected component.[2a,2b,4,5] In the second approach a protected nucleoside-3′ phosphate is condensed with the 5′-hydroxyl group of a second suitably protected nucleoside, nucleotide, or an oligonucleotide.[2c,4] Both approaches have previously been investigated for the synthesis of deoxyribo-oligonucleotides[2,4] and the first approach was concluded to be superior.[6–8] Therefore this approach has formed the basis of further systematic studies of the synthesis of deoxyribopolynucleotides, which are the subject of the present series of papers.

In the first phase of this work the preparation of suitably protected derivatives of the major deoxyribonucleosides was undertaken. This is the main theme of the present paper. The next phase involved study of the internucleotide bond formation between different nucleosides and nucleotides, since differences in reactivities could have been expected. This phase of the study is reported in the following paper.[9] Two subsequent papers[10,11] report on the synthesis of deoxyribo-oligonucleotides containing different nucleosides in known sequences. Brief reports of portions of this work have already appeared.[12,13]

(1) Paper XXIII: W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2789 (1963).

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

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

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

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

(6) The considerations leading to this conclusion are: (1) It is rather impractical to prepare suitably protected deoxyribonucleoside-3′ phosphates with alkali-labile groups on the amino groups and acid-labile groups such as di-*p*-methoxytrityl group on the 5′-position. For example, as demonstrated in the Experimental section, the di-*p*-methoxytrityl group in N-benzoyl-5′-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate is extremely labile to acid and yet complete removal of the group cannot be carried out selectively prior to removal of the N-benzoyl group since the glycosyl bond in the N-benzoyldeoxyadenosine moiety is also extremely sensitive to acid. (2) The yields of internucleotide bonds using stoichiometric amounts of the two components were lower than usual when bulky substituents are present in the nucleotide component as, for example, 5′-O-tritylthymidine-3′ phosphate (ref. 2c, 8, and 9). (3) A component bearing the free 5′-hydroxyl group and a preformed diester bond is not completely stable in the presence of the carbodiimide reagent; for example, thymidylyl-(3′ → 5′)-3′-O-acetylthymidine reacted in dry pyridine with dicyclohexylcarbodiimide to form small amounts of new products.[2c] Furthermore, triester formation was detected by interaction of an activated diester with the primary hydroxyl group of 3′-O-acetylthymidine.[2c] Side reactions of this type have not been encountered with components which bear the secondary 3′-hydroxyl group. (4) The ready availability of deoxyribonucleoside-5′ phosphates is of practical significance.

(7) It should be emphasized that the analysis described[6] applies to work in the deoxyribonucleotide series. In the stepwise synthesis of ribo-oligonucleotides the favored and, in fact, the only, practical approach has been shown to be that which starts from protected ribonucleoside-3′ phosphates: D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 3112 (1962); D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 1363, 3852 (1963).

(8) G. Weimann and H. G. Khorana, *ibid.*, **84**, 4329 (1962).

(9) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963).

(10) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).

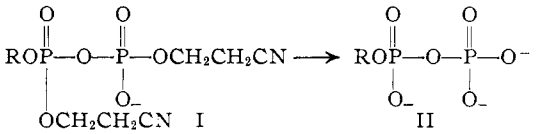
(11) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

Protected Deoxyribonucleosides.—The classical protecting group, triphenylmethyl (trityl), did not prove generally satisfactory for synthesis in the deoxyribonucleotide field[2a,2b] because of the acidic lability of the glycosyl bonds in purine deoxyribonucleosides. Groups like the trityl in their specificity but more labile to acid were therefore sought and the *p*-methoxy-substituted derivatives of the trityl group proved satisfactory.[14] In the present work, 5′-O-monomethoxytrityl-[15] and di-*p*-methoxytrityl-[15] thymidines were prepared by the reaction of the appropriate methoxytrityl chloride with thymidine.

Early experiments on the formation of deoxycytidylyl-(3′ → 5′)-thymidine by reaction of 3′-O-acetylthymidine-5′ phosphate with 5′-O-trityldeoxycytidine showed that the reaction occurred preferentially with the 6-amino group of the cytosine ring.[16] Similarly, interference from the amino group of the adenine ring was also observed in the synthesis of the trinucleotide thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxycytidine.[2b] From these results, the need for protecting the amino groups in the different nucleosides became clear and, therefore, in the polymerization studies suitably protected deoxyribonucleoside-5′ phosphates were used.[17-19] In the present work protected derivatives of deoxycytidine and deoxyadenosine containing only the 3′-hydroxyl group free have been obtained by first preparing the mono-N-benzoyl or anisoyl derivatives and from the latter the 5′-O-di-*p*-methoxytrityl derivatives. Thus deoxycytidine was converted to the N,3′,5′-tribenzoyl or trianisoyl derivative by reaction with benzoyl or anisoyl chloride, respectively. Alkaline treatment of these derivatives under carefully controlled conditions gave the N-benzoyl- and N-anisoyldeoxycytidines in good yield. Subsequent reaction with mono- or di-*p*-methoxytrityl chloride gave the appropriate 5′-O-methoxytrityl derivatives.[20] In the case of deoxyadenosine, benzoylation gave N,N,O$^{3'}$,O$^{5'}$-tetrabenzoyldeoxyadenosine and subsequent alkaline treatment again gave the mono-N-benzoyl derivative in good yield. It is of interest that in the attempted preparation of N-anisoyldeoxyadenosine from the polyanisoyl derivative by alkaline treatment, the major reaction was the cleavage of the glycosyl bond to give N-anisoyladenine.

Acetylation of deoxyguanosine so as to prepare N,3′,5′-triacetyldeoxyguanosine did not proceed satisfactorily,[21] although in the case of deoxyguanosine-5′ phosphate[19] acetylation of the ring occurred readily.[22] The major product from deoxyguanosine was 3′,5′-di-O-acetyldeoxyguanosine[21] in agreement with the finding of the previous workers.[23] The ring-substituted[24] derivatives prepared included N-di-*p*-methoxytrityl-3′,5′-diacetyldeoxyguanosine from 3′,5′-diacetyldeoxyguanosine, and N-di-*p*-methoxytrityldeoxyguanosine by removal of the acetyl groups. N,5′-O-Bis-*p*-methoxytrityldeoxyguanosine was prepared in good yield by direct treatment of deoxyguanosine with di-*p*-methoxytrityl chloride. This last derivative served well for a number of syntheses,[8] including that of deoxyguanosine-3′ phosphate (see below).

Deoxyribonucleoside-3′ Phosphates and Certain Derivatives.—Phosphorylation of 5′-O-di-*p*-methoxytritylthymidine, N-anisoyl-5′-di-*p*-methoxytrityldeoxycytidine, N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine, and N,5′-O-bis-di-*p*-methoxytrityldeoxyguanosine with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide[2c,25] (DCC) gave the corresponding 3′-cyanoethyl phosphates in excellent yields. In all these products the selective removal of the β-cyanoethyl group by mild alkaline treatment was practical and the 3′-phosphates were isolated in high yield. A side product encountered in these phosphorylations had the properties of the corresponding unsymmetrical pyrophosphate II and most probably arose from I during alkaline treatment to remove the β-cyanoethyl group. Compounds of the type II



would be expected to have considerable stability and these must have partly survived during the initial treatment of the anhydrous phosphorylation mixtures with water.

In the case of the phosphorylation product of protected deoxycytidine, it was possible to remove the 5′-O-di-*p*-methoxytrityl group selectively by acidic treatment and N-anisoyldeoxycytidine-3′-β-cyanoethyl phosphate was isolated. However, in deoxyadenosine derivatives, the glycosyl bond was too labile to permit the isolation of N-benzoyldeoxyadenosine-3′ phosphate or its cyanoethyl ester. On the other hand, by selective removal of the N-benzoyl group, the preparation of 5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate was possible.

The four deoxyribonucleoside-3′ phosphates were checked carefully for purity and further characterized by resistance toward the 5′-nucleotidase in *Crotalus adamanteus* venom. The present methods give practical yields of these compounds and of their protected derivatives mentioned above. It should be noted that previously[23] the preparation of the purine deoxyribonucleoside-3′ phosphates, in particular, has been far from satisfactory. It is possible that one reason for the low yields was the lack of protection of the amino groups. Even in the case of 5′-O-trityldeoxycytidine, the yield of the isolated deoxycytidine-3′ phosphate was rather low.[25,26]

(12) H. Schaller and H. G. Khorana, *Chem. Ind.* (London), 699 (1962).

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

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

(15) These are abbreviations, respectively, for *p*methoxyphenyldiphenylmethyl and di-*p*-methoxyphenylphenylmethyl groups.

(16) B. Lerch and H. G. Khorana, unpublished experiments.

(17) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961).

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

(19) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963). This work was partly done concurrently with the present work and some experiments with the di-*p*-methoxytrityl group for protecting the amino group in deoxyguanosine-5′ phosphate are also reported there.

(20) Early in the present work, 5′-O-trityldeoxycytidine was prepared by the method of A. M. Michelson and A. R. Todd [*J. Chem. Soc.*, 34 (1954)] and this was allowed to react with a limited amount of benzoic anhydride. N-Benzoyl-5′-O-trityldeoxycytidine was obtained as the major product (see Experimental).

(21) A part of the cause of failure to accomplish N-acetylation could be due to the insolubility of 3′,5′-di-O-acetylguanosine which separates from the reaction mixture. More recently, using the base tri-*n*-hexylethylammonium hydroxide to solubilize the nucleoside and carrying out the acetylation over prolonged periods, it has proved possible to acetylate the guanine ring in deoxyguanosine.

(22) Comment on this difference in rates has been made elsewhere (ref. 19).

(23) D. H. Hayes, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.*, 808 (1955).

(24) The exact position of the tritylation on the guanine ring is not known with certainty. Elsewhere,[19] comments have been made on the site of acylation in the guanine ring. Alkylations have been proved to occur at the N-7 position in the guanine ring [*e.g.*, P. Brooks and W. D. Lawley, *J. Chem. Soc.*, 3927 (1961); J. A. Haines, C. B. Reese, and A. R. Todd, *ibid.*, 5281 (1962); J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **85**, 193 (1963)]. From the properties of the trityl derivatives it seems doubtful if this group is at N-7 position.

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

(26) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 34 (1954).

During the present work, the methoxytrityl derivatives of nucleosides were detected on paper chromatograms by virtue of the characteristic color formed on spraying the chromatograms with acid (see also ref. 2c). The formation of the color has been made the basis of a sensitive quantitative method for the determination of di-*p*-methoxytrityl derivatives.

The protected nucleosides described in this paper have also proved useful in the synthesis of *p*-nitrophenyl esters of deoxyribonucleoside-3′ phosphates.[27]

Experimental

General Methods.—Paper chromatography was performed by the descending technique using mostly Whatman 40 paper The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water, 7:1:2; solvent B, *n*-butyl alcohol–acetic acid–water, 5:2:3; solvent C, ethyl alcohol–1 *M* ammonium acetate (pH 7.5), 7:3; solvent D, *n*-propyl alcohol–concentrated ammonia–water, 55:10:35. Paper electrophoresis was performed in a high voltage (4000 v.) apparatus in which the paper was immersed in water-cooled high boiling petroleum fraction (Varsol).

The trityl-containing compounds were made visible on paper chromatograms after spraying the chromatograms with the perchloric acid spray and warming in an oven. The compounds containing mono-*p*-methoxytrityl group appeared yellowish orange; those containing di-*p*-methoxytrityl group appeared orange-red.

Tests for resistance toward the 5′-nucleotidase of *Crotalus adamanteus* snake venom were performed as follows: The nucleotide solution (about 1 μmole), water (0.05 ml.), and ammonium carbonate (0.01 ml. of 2 *M*) were mixed to give a total volume of 0.09 ml. A freshly prepared solution (10 mg. of lyophilized venom/ml. of 0.05 *M* TRIS hydrochloride buffer, pH 8, 0.01 ml.) was added and the mixture incubated at 37°. Under these conditions thymidine-5′ phosphate was converted quantitatively to thymidine in under 1 hr., while the deoxyribonucleoside-3′ phosphates described in this paper were totally resistant up to 5-hr. incubation periods.

5′-O-Dimethoxytritylthymidine.—To a suspension of thymidine (2.43 g. 10 mmoles) in dry pyridine (10 ml.), di-*p*-methoxytrityl chloride (3.50 g. 10.35 mmoles) was added and the mixture shaken. Clear solution resulted within 5 min., followed by the separation of some crystals, presumably, of pyridine hydrochloride. The mixture was kept for 4 hr. at room temperature and to it was then added 1 ml. of methyl alcohol. The solution was concentrated to a gum at low temperature and chloroform (20 ml.) and water (10 ml.) were added. The chloroform layer was washed with water and then dried over sodium sulfate. Evaporation of the solvent gave a gum which was dissolved in 200 ml. of hot benzene. Cyclohexane was added carefully until a faint turbidity appeared. On storage at room temperature, 5′-O-di-*p*-methoxytritylthymidine was obtained in a crystalline form. It was collected by filtration and washed with a mixture of cyclohexane and benzene (1:1). The yield was 5.07 g. (93%), m.p. 116–118. After recrystallization from the same solvent mixture, the yield was 4.65 g. (85%), m.p. 123–124°. The ultraviolet absorption characteristics in methyl alcohol were: λ_{max} 269 and 234 mμ, λ_{min} 254 mμ, ϵ_{269} 11,600, ϵ_{234} 23,000, ϵ_{254} 8400.

Anal. Calcd. for $C_{31}H_{32}O_7N_2$ (544.61): C, 68.50; H, 5.93; N, 5.15. Found in a sample dried at 100° under high vacuum for 12 hr. (wt. loss, 0.7%): C, 68.62; H, 5.93; N, 4.87.

The rate of hydrolysis of the di-*p*-methoxytrityl group in the above compound in 80% acetic acid at room temp. was determined as follows: A solution of the substance (4.32 mg.) in 1 ml. of 80% acetic acid was kept at room temperature Aliquots were removed after 1, 2, 3, and 10 min., and diluted with a mixture of water and chloroform. The chloroform layer was removed and the aqueous layer made up to a known volume for spectrophotometric determination of the thymidine present. Hydrolysis was essentially complete in 15 min., the half-life being 1.9 min. at 26°.

5′-O-Mono-*p*-methoxytritylthymidine.—Thymidine (2.42 g., 10 mmoles) was treated in dry pyridine (20 ml.) with mono-*p*-methoxytrityl chloride (3.11 g., 10.1 mmoles) for 15 hr. at room temperature. Some methyl alcohol was then added and the total solution concentrated to a small volume. A mixture of chloroform (100 ml.) and water (100 ml.) was added and the chloroform layer separated, washed with water, and dried over sodium sulfate. The aqueous layer was found to contain 0.305 mmole of unreacted thymidine as determined spectrophotometrically. The dry chloroform solution was evaporated and the residue re-evaporated a few times from chloroform to remove residual pyridine. The residue was redissolved in a small volume of chloroform and the solution applied to a column of aluminum oxide (100 g., deactivated with 10 ml. of water). The column was eluted with chloroform (100 ml.) and then with chloroform (250 ml.) containing 5% methyl alcohol. Fractions of 10-ml. volume were collected. Fractions 5–7 contained *p*-methoxytritanol or its methyl ether; fractions 10–28 contained ultraviolet-absorbing material and after evaporation the desired product was precipitated from a concentrated methyl alcohol solution by the addition first of benzene (100 ml.) and then an excess of ether (800 ml.). The precipitate was collected and washed with ether. The filtrate was concentrated and the concentrate rediluted with ether to give more precipitate. The total yield of the two precipitates was 4.22 g. (82%) m.p. 103–105°. This product was free from thymidine and was homogeneous by paper chromatography in solvents A and C. A further amount (about 500 mg.) of the same product was present in the mother liquor from the above precipitation (this amount being estimated spectrophotometrically).

Anal. Calcd. for $C_{30}H_{30}N_2O_6$ (514.48): C, 69.95; H, 5.88; N, 5.45. Found: C, 70.27; H, 6.36; N, 5.38.

The rate of hydrolysis in 80% acetic acid at 27° was determined. The half-life was 8.5 min.; complete hydrolysis occurred in 1.5 hr.

Phosphorylation of 5′-O-Dimethoxytritylthymidine. Isolation of Thymidine-3′ Phosphate.—A mixture of pyridinium β-cyanoethyl phosphate (0.2 mmole) and 5′-O-dimethoxytritylthymidine (45 mg.) was rendered anhydrous by repeated evaporation of pyridine solution. The dry mixture was dissolved in dry pyridine (1 ml.) and DCC (206 mg., 1 mmole) was added. The sealed mixture was kept at room temperature for 3 days. Water (0.5 ml.) was then added and the mixture evaporated repeatedly after additions of water. The residue was heated in 80% acetic acid for 15 min. The sole product as determined by chromatography in solvent A was thymidine-3′ β-cyanoethyl phosphate. The latter on treatment with sodium hydroxide was converted quantitatively to thymidine-3′ phosphate. The latter product was homogeneous and was resistant to the action of the 5′-mononucleotide as tested according to the procedure described above.

N-Benzoyldeoxycytidine.—Deoxycytidine (915 mg., 4 mmoles) was benzoylated in dry pyridine (20 ml.) with 2 ml. of benzoyl chloride for 1 hr. at room temperature. The total solution containing the crystalline pyridine hydrochloride was poured into 700 ml. of ice water. The gummy precipitate which formed was extracted with ethyl acetate (200 ml.). The ethyl acetate extract was dried over sodium sulfate and then evaporated to an oil. The product, presumably N^6,$O^{3'}$,$O^{5'}$-tribenzoyldeoxycytidine, was precipitated by the addition of 100 ml. of ether, collected by filtration, and washed with ether. The yield was 1.96 g. (92% as calculated for N^6,$O^{3'}$,$O^{5'}$-tribenzoyldeoxycytidine).

The bulk (1.86 g., about 3.6 mmoles) of the above product was dissolved in a mixture of tetrahydrofuran (50 ml.), methyl alcohol (40 ml.), and water (10 ml.), and the solution cooled to 0°; 10 ml. of 2 *N* sodium hydroxide was added and the mixture stirred at 0° for 10 min. Dowex-50 (pyridinium form, 10 ml.) ion exchange resin was added and the neutral solution, which was obtained within 2 min., was filtered and the resin washed thoroughly with water. The total filtrate and washings were concentrated to 100 ml. and then extracted with ether to remove benzoic acid. Some of the desired product began to separate at this stage and the aqueous solution was heated to redissolve the precipitate. The solution was filtered from a little insoluble material and the clear filtrate cooled. N-Benzoyldeoxycytidine separated as a crystalline precipitate and some ether was added to complete the crystallization. The total yield in three crops (concentration after each crop) was 943 mg. (79%), m.p. softening at 174–175°, and on being heated further melted at 194°. The ultraviolet absorption spectrum in water showed λ_{max} at 260 mμ (ε 21,800) and at 302 mμ (ε 11,800); at pH 2, λ_{max} at 260 mμ (ε 20,900) and at 305 mμ (ε 12,900). In 95% ethyl alcohol, the characteristics were: λ_{max} 305 mμ (ε 9500), 260 mμ (ε 20,850), λ_{min} 285 mμ (ε 7000), 230 mμ (ε 9000).

Anal. Calcd. for $C_{16}H_{17}N_3O_5$: C, 58.00; H, 5.17; N, 12.69. Found: C, 57.37; H, 5.46; N, 12.89.

N-Benzoyl-5′-O-trityldeoxycytidine.—5′-O-Trityldeoxycytidine[20] (600 mg., 1.28 mmoles) was treated in anhydrous pyridine (5 ml.) with benzoic anhydride (348 mg., 1.54 mmoles) at room temperature for 7 days. The solution was then evaporated, the residue dissolved in dichloromethane (10 ml.), and the solution applied to a silicic acid column (16 × 1.2 cm.). The column was eluted with 100 ml. of dichloromethane, then a mixture of this solvent and chloroform (1:1), and finally with chloroform; 10-ml. fractions were collected. Fractions 18–28 contained ultraviolet-absorbing material and after evaporation yielded 508 mg. (69.5%) of the desired product. This was taken up in acetone, the solution filtered from a little insoluble material, and the clear filtrate evaporated. The residue was crystallized from acetonitrile to give 362 mg. (49.5%) of the product. On

(27) W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2781 (1963).

heating, the crystals showed sintering at 200° and darkened at 230° without displaying a melting point. The R_f in both solvents A and B was 0.85. The ultraviolet absorption characteristics of the product were: λ_{max} 305 mμ (ε 9600), 261 mμ (ε 20,500); λ_{min} 285 mμ (ε 6900), 241 mμ (ε 13,100).

N-Benzoyl-5′-O-monomethoxytrityldeoxycytidine.—N-Benzoyldeoxycytidine (0.243 g., 0.743 mmole) was treated in dry pyridine (2 ml.) with freshly prepared mono-*p*-methoxytrityl chloride (0.272 g., 0.881 mmole) for 18 hr. Ethyl alcohol was then added to destroy any of the unreacted reagent and the solution evaporated to a gum. This was dissolved in 2 ml. of chloroform and the solution applied to a silicic acid (11 g.) column. The column was eluted with 50 ml. of chloroform and then with chloroform containing 2.5% ethyl alcohol, 10-ml. fractions being collected. The fractions 9–16 contained the desired product (0.415 g., 96.5%) which was recovered by evaporation of the solvent. The product could not be obtained in a crystalline form.

A solution of the product (855 mg.) in 5 ml. of benzene was chromatographed on a column (bed volume 50 ml.) of alumina (10% deactivated with water). Elution was carried out with benzene (100 ml.), benzene + 5% methanol (200 ml.), benzene + 10% methanol (200 ml.); 13-ml. fractions were collected at a flow rate of *ca.* 1 ml./min. After a little of monomethoxytrityl-containing material in fractions 3 and 4, benzoyl-5′-O-monomethoxydeoxycytidine was the only product and it was present in fractions 14–19. By evaporation and drying under high vacuum, 818 mg. of the pure product was recovered. The ultraviolet absorption characteristics of the product were: λ_{max} 305.5 mμ (ε 9600), 260 mμ (ε 21,600), 233 mμ (ε 23,500); λ_{min} 289 mμ (ε 7700), 226 mμ (ε 22,500).

Anal. Calcd. for $C_{36}H_{33}N_3O_5$ (603.65): C, 71.70; H, 5.53; N, 6.96. Found: C, 70.33; H, 5.84; N, 6.73.

N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidine and N-Benzoyl-3′,5′-bis-O-di-*p*-methoxytrityldeoxycytidine.—N-Benzoyldeoxycytidine (660 mg., 2 mmoles) and di-*p*-methoxytrityl chloride (990 mg., 2.92 mmoles) in dry pyridine (2.5 ml.) were shaken at room temperature. The clear solution which resulted in 10 min. was kept for 1.5 hr. (Pyridine hydrochloride separated after 30 min.) Methyl alcohol was then added and the solution evaporated to a gum at low temperature. This was dissolved in a mixture of water (10 ml.) and ethyl acetate (10 ml.). The organic layer was washed twice with 10 ml. of water and then dried over sodium sulfate. The solution was then evaporated, the gum was dissolved in 20 ml. of benzene, and the solution re-evaporated. The residue was redissolved in a small volume (2 ml.) of benzene and the solution applied to the top of an alumina (100 g., deactivated with 15 g. of water) column (30 × 2 cm. diam.). Elution was carried out with 200 ml. of benzene and then with ether; 15-ml. fractions were collected. Fractions 4–6 contained a dimethoxytrityl derivative which was nonnucleosidic. Fractions 16–19 (ether front) contained some N-benzoyl-bis-di-*p*-methoxytrityldeoxycytidine (peak A). Fractions 45–54 contained N-benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidine (peak B). The combined fractions containing peak A were evaporated to an oil, which was dissolved in ethyl acetate (1 ml.) and ether (10 ml.). Cyclohexane (50 ml.) was then added and the solution concentrated at about 0° *in vacuo* to about 10 ml. when the product precipitated. The precipitate was collected by filtration, washed with cyclohexane, and dried. The yield of this product was 160 mg. (7.5%), m.p. 124°. The ultraviolet absorption characteristics were: λ_{max} 306 mμ (ε 9600), a broad shoulder at 242–255 mμ, $\epsilon_{260\ m\mu}$ 24,400, $\epsilon_{236\ m\mu}$ 45,700; λ_{min} 293 (8100) and 223 mμ (ε 38,400).

Anal. Calcd. for $C_{58}H_{53}N_3O$ (936.08): C, 74.45; H, 5.72; N, 4.49. Found: C, 73.39; H, 5.92; N, 4.45.

The material in peak B (N-benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidine, 1.12 g., 1.78 mmoles, 89%) was also precipitated from a little acetone, ether, and cyclohexane. The total yield was 890 mg. (70%), m.p. 119°. Some more material (100 mg.) was recovered from the mother liquor and fractions 40–45 and 55–65 from the column chromatography described above. The ultraviolet absorption characteristics were: λ_{max} 306 mμ (ε 9700), 260 mμ (ε 22,250), 236 mμ (ε 29,250); λ_{min} 292 mμ (ε 7900), 251 mμ (ε 21,200), 223 mμ (ε 25,750).

Anal. Calcd. for $C_{37}H_{35}N_3O_7$ (633.71): C, 70.11; H, 5.76; N, 6.63. Found: C, 69.46; H, 5.68; N, 6.16.

N^6,$O^{3'}$,$O^{5'}$-Trianisoyldeoxycytidine.—A mixture of deoxycytidine (1.14 g., 5 mmoles), and anisoyl chloride (3 ml., 21 mmoles) in dry pyridine (25 ml.) was shaken at room temperature. A clear solution resulted after 5 min., followed by the precipitation of pyridine hydrochloride. After 2 hr. the solution was poured into ice water (300 ml.) containing ammonium hydrogen carbonate (20 g.). A further amount of water (200 ml.) and ethyl alcohol (100 ml.) were added. The total suspension was heated to 70–80° and then cooled to 0°. The pH was adjusted carefully with sodium hydroxide (1 ml. of 2 *N*) to 8 and the precipitate collected by filtration immediately and washed with water and then with ether. The yield of the crude product was 3.18 g. (100%). A portion (0.5 g.) was crystallized with methyl alcohol to give needles (0.42 g., 84%), m.p., 185–186°.

Anal. Calcd. for $C_{33}H_{39}O_{10}N_3$ (629.70): C, 63.0; H, 4.97; N, 6.67. Found: C, 62.07; H, 5.09; N, 6.56.

In a repeat of the above preparation, deoxycytidine hydrochloride and an equivalent amount of triethylamine was used as the starting material for anisoylation. The yield was similar.

N^6-Anisoyldeoxycytidine.—N^6,$O^{3'}$,$O^{5'}$-Trianisoyldeoxycytidine (690 mg.)[28] was dissolved in 5 ml. of pyridine and to the solution was added 15 ml. of 1.5 *M* aqueous sodium hydroxide under cooling. An oil which resulted was brought into solution by the further gradual addition of water (total 6 ml. added). This solution was kept at room temperature for 20 min. and was then treated with an excess of pyridinium Dowex-50 ion exchange resin. The solution was filtered from the resin and the resin washed first with water (formation of a precipitate in the aqueous filtrate at this stage) and then with tetrahydrofuran, the latter wash being collected separately.[29] The aqueous layer was filtered from the precipitate and concentrated to about 10 ml. Anisic acid and the desired product crystallized at this stage. The total mixture was washed several times with ether to remove anisic acid and the crystalline precipitate (needles)[30] of the desired product collected by filtration.[31] The yield at this stage was 300 mg. (75% as based on the assumption that the starting material was pure trianisoyldeoxycytidine). The ultraviolet absorption spectral characteristics in water (*cf.* N-anisoyldeoxycytidine-5′ phosphate) were: λ_{max} 303 mμ (ϵ_{max} 24,500); λ_{min} 238 mμ with a shoulder at 250–260 mμ. In ethyl alcohol, λ_{max} was shifted to 288 mμ.

Anal. Calcd. for $C_{17}H_{19}O_6N_3$ (361.36): C, 56.50; H, 5.30; N, 11.63. Found: C, 56.35; H, 5.33; N, 11.45.

5′-O-Di-*p*-methoxytrityldeoxycytidine-3′ Phosphate, N-Anisoyldeoxycytidine-3′ β-Cyanoethyl Phosphate and Deoxycytidine-3′ Phosphate.—An anhydrous solution of N-anisoyl-5′-O-di-*p*-methoxytrityldeoxycytidine (330 mg., 0.5 mmole), pyridinium β-cyanoethyl phosphate (3 mmoles), and DCC (1.25 g., 6.06 mmoles) in dry pyridine (2.5 ml.) was kept at room temperature for 2 days. Water (about 1.5 ml.) was then added and the mixture extracted with petroleum ether. The extracts were washed back with water and the water wash combined with the aqueous pyridine solution. The combined solution was diluted with pyridine to a total volume of 25 ml. Aliquots from this stock solution were worked up as follows: (a) A 5-ml. portion was withdrawn, treated with a little ammonia, and the ammoniacal solution concentrated to a small volume. The residue was treated with 5 ml. of concentrated ammonium hydroxide for 2.5 days at room temperature. The solution was filtered from the precipitate, which had deposited, and concentrated to an oil at reduced temperature. Paper chromatography in solvent A showed one main spot corresponding to 5′-O-di-*p*-methoxytrityldeoxycytidine-3′ phosphate and two faint spots, one of which was at the origin and the second corresponded to deoxycytidine-3′ phosphate. The concentrated aqueous solution was treated with 6 ml. of 80% acetic acid for 20 min. The acidic solution was then evaporated and the residue taken up in water and the solution extracted with ether to remove di-*p*-methoxytritanol. Paper chromatography in solvents A and C of the aqueous solution showed the major product to be deoxycytidine-3′ phosphate (R_f in solvent A, 0.08; R_f in solvent C, 0.41). The yield of the desired nucleotide was estimated spectrophotometrically to be 85%. This product was resistant to the action of crude snake venom, *Crotalus adamanteus*, under the conditions described above. A minor product (6.4%) accompanying deoxycytidine-3′ phosphate and having deoxycytidine-like spectrum had R_f (in solvent A) 0.03 and (solvent C) 0.15. On paper electrophoresis at pH 7.1 it had mobility 1.4 relative to deoxycytidine-3′ phosphate.

N-Anisoyldeoxycytidine-3′ β-Cyanoethyl Phosphate.—Another 5-ml. aliquot from the above stock solution was evaporated to dryness. A small amount (0.01 ml.) of concentrated ammonium

(28) The sample used was obtained by anisoylation of deoxycytidine hydrochloride in pyridine and triethylamine. The yield of the product before crystallization was 5% higher than theoretical for the trianisoyl derivative. Presumably, this result was caused by the formation of some (25–30%) of the N,N-$O^{3'}$,$O^{5'}$-tetraanisoyl derivative in the presence of the more powerful catalyst triethylamine. *Cf.* the formation of N^1,N^6,$O^{2'}$,$O^{3'}$,$O^{5'}$-pentabenzoylcytidine on benzoylation of cytidine in the hot: D. M. Brown; A. R. Todd, and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).

(29) This precipitate and some more recovered from the tetrahydrofuran wash by evaporation and subsequent treatment with aqueous ethyl alcohol (total 53 mg.) corresponded to unreacted starting material and, presumably, N,O-dianisoyldeoxycytidine.

(30) This product can be recrystallized (needles) from hot water.

(31) It is interesting that the aqueous filtrate from this concentrated solution was found to contain in addition to a trace (0.2% of total) of N-anisoyldeoxycytidine, some (about 10% as based on the starting material) deoxycytidine. Under the alkaline conditions used in the above experiment, the half-life for the cleavage of N-anisoyl group was 2.5 hr. at 25°.

hydroxide was added and the solution obtained after addition of water was again evaporated. The residue was treated with 5 ml. of 80% acetic acid for 20 min. and the solution then evaporated. The residue was taken up in water and extracted with ether. Paper chromatography of the aqueous solution showed a single spot in solvents A (R_f 0.69) and C (0.83) corresponding to N-anisoyldeoxycytidine-3′ β-cyanoethyl phosphate. The yield as determined spectrophotometrically was 88%.

N,N,O³′,O⁵′-Tetrabenzoyldeoxyadenosine and N-Benzoyldeoxyadenosine.—A suspension of dry deoxyadenosine (1.25 g., 5 mmoles) in anhydrous pyridine (15 ml.) was treated with 2.5 ml. of benzoyl chloride for 2 hr. at room temperature. The resulting solution was then poured into ice water and the insoluble product extracted with chloroform (3 × 100 ml.). The chloroform extract was washed with water and then evaporated to a gum. Crystallization was effected from aqueous ethanol, the yield of N,N,O³′,O⁵′-deoxyadenosine being quantitative. The melting point of the crystalline product was 173–174°.

Anal. Calcd. for $C_{37}H_{40}N_5O_7$: C, 68.30; H, 4.60; N, 10.70. Found: C, 68.50; H, 4.31; N, 10.12.

For preparation of N-benzoyldeoxyadenosine, the gum obtained above was directly dissolved in a mixture of ethyl alcohol (15 ml.) and pyridine (10 ml.) and the solution was treated with a mixture of 20 ml. of 2 *N* sodium hydroxide and 20 ml. of ethyl alcohol at room temperature for 5 min. An excess of pyridinium Dowex-50 ion exchange resin was then added to remove sodium ions, the resin was then removed by filtration, and the filtrate and washings concentrated *in vacuo* to a small volume. Water (25 ml.) was added and the mixture extracted with ether (3 × 50 ml.). The aqueous suspension was heated and the resulting solution cooled slowly. N-Benzoyladenine separated first and it was removed by filtration. The filtrate after concentration and storage yielded N-benzoyldeoxyadenosine (1.15 g., 65%) as colorless needles, m.p. 113–115°. The ultraviolet absorption characteristics of the product were: λ_{max} (water), 280 mμ, ϵ_{max} 20,700; λ_{max} (pH 2) 285 mμ, ϵ_{max} 22,500.

Anal. Calcd. for $C_{17}H_{17}O_4N_5$: C, 57.4; H, 4.82; N, 19.75. Found: C, 56.6; H, 4.89; N, 19.55.

When in the above preparation, aqueous alcoholic sodium hydroxide was replaced by 2.5 *M* sodium methoxide solution after 5 min. at 0° the main product (70%) formed was N-benzoyladenine.

Anal. Calcd. for $C_{12}H_9ON_5$: C, 60.2; H, 3.75; N, 29.3. Found: C, 59.94; H, 3.93; N, 28.23.

Attempted Synthesis of N-Anisoyldeoxyadenosine.—Dry deoxyadenosine (2.51 g., 10 mmoles) was treated with an excess of anisoyl chloride (8.53 g., 50 mmoles) in pyridine (20 ml.) for 2.5 hr. The solution was then poured into ice water and the product was extracted with chloroform. The extract was evaporated *in vacuo* and the gum dissolved in a mixture of ethyl alcohol (30 ml.) and tetrahydrofuran (20 ml.). The solution was treated with a mixture of 40 ml. of 2 *N* sodium hydroxide and 40 ml. of ethyl alcohol for 5 min. at room temperature and 40 min. at 0°. The alkali was neutralized by addition of an excess of pyridinium Dowex-50 ion exchange resin. The resin was removed and the filtrate and washings were concentrated. The concentrate was diluted with water and the anisic acid extracted with ether. The residual solution contained as the major product N-anisoyladenine (1.8 g., 67%), m.p. 236–238°.

Anal. Calcd. for $C_{13}H_{11}O_2N_5$: C, 58.0; H, 4.09; N, 26.0. Found: C, 57.89; H, 4.15; N, 25.81.

N-Benzoyl-5′-O-monomethoxytrityldeoxyadenosine.—N-Benzoyldeoxyadenosine (0.390 mg., 1.1 mmoles) was dissolved in anhydrous pyridine (2 ml.) and to the solution was added monomethoxytrityl chloride (0.410 g., 1.33 mmoles). The sealed mixture was kept in the dark at room temperature overnight. Some ethyl alcohol was then added and the solution evaporated to a gum *in vacuo*. The residue was dissolved in chloroform (1 ml.) and the solution applied to the top of a silicic acid (11 g.) column. The column was eluted with chloroform (50 ml.) and then with chloroform containing 1.25% of ethyl alcohol (total volume 150 ml.); 10-ml. fractions were collected. Fractions 8–17 contained the protected nucleoside which was recovered by evaporation (0.253 g., 76.5%). A portion (48.9 mg.) of this product was dissolved in 2 ml. of benzene and the solution further chromatographed on an alumina (15% deactivated with water) column (20 × 1 cm.). Elution was carried out with benzene (100 ml.) and then with ether (100 ml.). Pure N-benzoyl-5′-O-monomethoxytrityldeoxyadenosine was present in fractions 12–14, it being preceded by some other methoxytrityl-containing product. The fractions containing the desired product were evaporated to dryness and the residue taken up in a mixture of acetone (10 ml.) and cyclohexane (10 ml.). Upon concentration, the product separated as a white precipitate (42.5 mg., 87% recovery), m.p. 104–107°. The ultraviolet absorption characteristics in ethyl alcohol were: λ_{max} 280 and 232 mμ, λ_{min} 255 and 224 mμ, ϵ_{280} 20,000, ϵ_{255} 12,500, ϵ_{232} 26,000, ϵ_{224} 25,000.

Anal. Calcd. for $C_{37}H_{33}O_5N_5$: C, 70.06; H, 5.26; N, 11.15. Found: C, 69.73; H, 5.48; N, 11.40.

On treatment with 80% acetic acid at 25° for 10 min. the substance partially decomposed. The products on chromatography in solvent C were: unchanged starting material (50%), N-benzoyldeoxyadenosine (29%), and N-benzoyladenine (21%).

N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine.—Dry N-benzoyldeoxyadenosine (710 mg., about 2 mmoles) was treated with di-*p*-methoxytrityl chloride (750 mg., 2.2 mmoles) in pyridine (5 ml.) for 3 hr. at room temperature. The mixture was then poured into ice water and the product extracted several times with ethyl acetate (spectrophotometric determination indicated less than 5% of starting material in aqueous phase). The organic layer was dried over sodium sulfate and the dried solution evaporated *in vacuo* to a gum, which was dissolved in 10 ml. of benzene. The solution was applied to an alumina (Woelm, neutral alumina, 15% deactivated; 70 g. dry weight) column. Elution was carried out with 150 ml. of benzene and then continued with 250 ml. of ether. The flow rate was 12–13 ml. in 15 min. Fractions 5–7 were deeply yellow and contained, presumably, bis-di-*p*-methoxytrityl-N-benzoyldeoxyadenosine.

Anal. Calcd. for $C_{69}H_{53}N_6O_8 \cdot 2C_6H_6$ (1115): N, 6.25; OCH_3, 11.1. Found: N, 5.98; OCH_3, 11.01.

Fractions 15–37 contained the desired product and after combining were evaporated *in vacuo*. The residue was redissolved in acetone–cyclohexane and the solution re-evaporated. A white powder (1.07 g., 81%) was obtained after drying for 5 hr. at 80° under high vacuum. The ultraviolet absorption characteristics in ethyl alcohol were: λ_{max} 277–282 and 234 mμ, λ_{min} 260 and 224 mμ, ϵ_{277} 20,500, ϵ_{234} 31,500.

Anal. Calcd. for $C_{35}H_{35}N_5O_6$ (657.7): C, 69.4; H, 5.35; N, 10.6; OCH_3, 9.4. Found: C, 69.55; H, 5.73; N, 10.06; OCH_3, 8.98.

N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ Phosphate.—N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine (660 mg.) was treated in anhydrous pyridine (7 ml.) with a mixture of pyridinium β-cyanoethyl phosphate (from 960 mg. of the crystalline barium salt) and DCC (3 g.) for 4 days at room temperature. A mixture of pyridine (4 ml.) and water (8 ml.) was then added and the diluted reaction mixture was kept overnight at room temperature; DCC was then extracted with petroleum ether and the aqueous pyridine solution of the product made up to 50 ml. A portion of this stock solution was processed further to prepare 5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate (see the following preparation). For the preparation of N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate, 25 ml. of the stock solution (corresponding to 0.44 mmole of the nucleotide) was evaporated to a gum. Sodium hydroxide (5 ml. of 1 *N*) was added and the mixture kept at 0° for 18 min. Pyridinium Dowex-50 was added until the alkalinity disappeared. The resulting solution was filtered from the resin and the resin washed thoroughly with aqueous ethyl alcohol. The combined filtrate was concentrated very carefully at low temperature using a Dry Ice–acetone bath as a trap and the concentrate applied to the top of a DEAE–cellulose (bicarbonate form) column (30 × 3.5 cm. diam.). The column was washed first with 200 ml. of water containing 20% ethyl alcohol and then with 1 l. of water. Elution was begun with a linear gradient of triethylammonium bicarbonate, with 2 l. of water in the mixing vessel and an equal volume of 0.2 *M* triethylammonium bicarbonate (pH 7.5) in the reservoir. Elution was then continued with 2 l. of 0.2 *M* salt in the mixing vessel and an equal volume of 0.3 *M* salt in the reservoir. A flow rate of 1.5–1.8 ml./min. was maintained, fractions being collected at 10-min. intervals. The water wash contained some di-*p*-methoxytritanol and another unidentified ultraviolet-absorbing material (R_f, 0.8 in solvent C). Two minor succeeding peaks present in fractions 50–75 were discarded. The next peak contained a small amount of deoxyadenosine-3′ phosphate and the next peak contained N-benzoyldeoxyadenosine-3′ phosphate (R_f in solvent C, 0.47). The main peak appeared in fractions 180–350. It was cut into three portions. The first portion, fractions 180–225, contained 0.021[32] mmole of product; the second portion, fractions 226–275, contained 0.1 mmole of the product; while the third portion, fractions 276–350, also contained 0.1 mmole of the product. Portions 1 and 2 contained pure N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate while the last portion contained, in addition, a small amount of a faster traveling (solvent C) material (di-*p*-methoxytrityl containing). The identity of this side product has as yet not been established. It traveled, after detritylation, slower (R_f 0.73, solvent C) than N-benzoyldeoxyadenosine-3′ phosphate (R_f 0.79). On paper electrophoresis at pH 7.5, its mobility was one-third of that of deoxyadenosine-3′ phosphate.

The pure N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate was recovered by concentrating the pooled fractions under a high vacuum (oil pump and Dry Ice–methyl alcohol

(32) Assuming an ϵ_{max} of 15,900 at 260 mμ.

trap), treating the concentrate with an excess of pyridinium Dowex-50 ion exchange resin and storing the product as its solution in pyridine. The ultraviolet absorption characteristics of the pure product (after elution of the ammonium salt from a paper chromatogram) were: λ_{max} 282 mμ, λ_{min} 260 mμ with inflection at 230 mμ, $\epsilon_{280}/\epsilon_{260\ m\mu}$ 1.5; $\epsilon_{230}/\epsilon_{260\ m\mu}$ 2.20.

5'-O-Di-*p*-methoxytrityldeoxyadenosine-3' Phosphate. (a).—Twenty milliliters of the stock solution described in the preceding preparation was treated with 2 ml. of concentrated ammonium hydroxide and the solution evaporated to dryness. The residue was taken up in 30 ml. of concentrated ammonium hydroxide and the sealed mixture kept at room temperature for 48 hr. Paper chromatography in solvent A showed a single major nucleotidic product with R_f 0.50 (R_f of starting material 0.85), there being traces of deoxyadenosine-3' phosphate and of another ultraviolet-absorbing material close to the origin. The total solution after concentration was applied to two sheets of Whatman 3 MM paper and the chromatograms developed in solvent A. The strong band of the product was eluted with dilute ammonia and the eluate was passed through a short column of ammonium Dowex-50 ion exchange resin. The total effluent including washings was lyophilized to give a white powder (510 mg.). The weight after drying over phosphorous pentoxide *in vacuo* was 485 mg. (72% of theory). During the lyophilization and manipulation some 10% of the product decomposed to deoxyadenosine-3' phosphate as indicated by paper chromatography.

(b).—The following alternative procedure eliminated the partial decomposition encountered above and, therefore, gave a pure sample of 5'-O-di-*p*-methoxytrityldeoxyadenosine-3' phosphate. A batch (900 mg.) of N-benzoyl-5'-O-di-*p*-methoxytrityldeoxyadenosine was treated with a mixture of pyridinium β-cyanoethyl phosphate (from 1.3 g. of the barium salt) and DCC in dry pyridine (5 ml.) for 4 days at room temperature. Water (10 ml.) was added and the reaction mixture kept overnight at room temperature. Dicyclohexylurea was removed by filtration and washed thoroughly with 1:1 aqueous pyridine. The total aqueous pyridine solution was evaporated at a temperature below 15°. Pyridine was added frequently during the evaporation. The residue was dissolved in 10 ml. of pyridine and the solution treated with 30 ml. of 1 *N* lithium hydroxide for 15 min. at 0°. The small amount of precipitate present was removed by centrifugation and the clear supernatant was treated with pyridinium Dowex-50 resin to remove alkali. The resin was removed by filtration and washed thoroughly with pyridine. The total aqueous pyridine solution was concentrated to a gum at a low temperature, frequent additions of pyridine being made. The gum was dissolved in a mixture of concentrated ammonium hydroxide (50 ml.) and tetrahydrofuran (25 ml.) and the solution kept at room temperature for a week. Subsequent chromatography in solvent C showed the main product to be 5'-O-di-*p*-methoxytrityldeoxyadenosine-3' phosphate, but some di-*p*-methoxytritanol and another fluorescent product (R_f 0.86) containing the di-*p*-methoxytrityl group were present. There was also a small amount of deoxyadenosine-3' phosphate (R_f 0.21) and a trace of another very slow traveling ultraviolet absorbing product. The solution was evaporated *in vacuo* and the residue dissolved in the solvent system isopropyl alcohol–concentrated ammonia–water (7:1:2, chromatographic solvent A). Some material which remained undissolved was filtered off and the solution applied to the top of a cellulose powder column (105 × 4.5 cm. diam.) packed in the same solvent. Fractions of 1.5–2 ml. were collected at 10-min. intervals. Di-*p*-methoxytritanol and some other product appeared in fractions 341–391. Fractions 391–402 contained the fluorescent (under ultraviolet light) material and there was some overlap in the succeeding fractions (402–412) of this product with the desired 5'-O-di-*p*-methoxytrityldeoxyadenosine-3' phosphate. Fractions 413–441 contained the latter product in pure state, whereas in fractions 442–517 the same product was contaminated with another slower traveling (solvent C) compound. Fractions 413–441 were pooled and evaporated *in vacuo* and the residue converted to the pyridinium salt by passage through a pyridinium Dowex-50 column at 0°. The eluate was concentrated in the presence of an excess of pyridine under a vacuum obtained with an oil pump and using Dry Ice–methyl alcohol as the condensing agent in the trap. The loss of any of the 5'-O-di-*p*-methoxytrityl group was thus circumvented. The yield of pure 5'-O-di-*p*-methoxytrityldeoxyadenosine-3' phosphate was 35%. An additional 10% was recovered by reprocessing the fractions preceding and succeeding the pure compound. Treatment of a portion (10 optical density units) of the lyophilized material with 80% acetic acid for 5 min. at 0° followed by rapid application on a chromatogram (solvent A) showed essentially complete hydrolysis to deoxyadenosine-3' phosphate, only a trace of the starting material being present (no adenine could be detected).

3',5'-Di-O-acetyldeoxyguanosine.—It was prepared by acetylation of deoxyguanosine with an excess of acetic anhydride (5 ml.) either in pyridine (50 ml.) alone or in a mixture (1:1) of pyridine and dimethylformamide for 3 days at room temperature. The product did not melt up to 270°; it turned somewhat brown. Hayes, *et al.*, record decomposition point of 222°.

When acetylation was carried out with acetyl chloride (5 molar equivalents), the product after 3 days was again 3',5'-di-O-acetylguanosine, no N-acetylation being detected[33] (ultraviolet absorption spectrum characteristic of deoxyguanosine and not of N-acetyldeoxyguanosine-5' phosphate[13]). The lack of N-acetylation was probably due to the insolubility of 3',5'-di-O-acetylguanosine in the solvent.

N-Di-*p*-methoxytrityl-O$^{3'}$,O$^{5'}$-diacetyldeoxyguanosine.—3',5'-Di-O-acetyldeoxyguanosine (360 mg., 1 mmole) was treated in dry pyridine (10 ml.) with di-*p*-methoxytrityl chloride (400 mg., 1.18 mmoles) at room temperature. The clear solution which resulted in 15 min. was kept at room temperature. The reaction was complete after 9.5 hr. as ascertained by paper chromatography of an aliquot in solvent C. After a total of 12.75 hr., water (1 ml.) was added and the reaction mixture then partitioned between ethyl acetate (100 ml.) and water (100 ml.). The organic layer was dried over sodium sulfate and then concentrated to an oil, which was dissolved in benzene (5 ml.). Cyclohexane (10 ml.) was added to this solution slowly and the precipitation which ensued was completed by the addition of ether (50 ml.). The product (370 mg.) was collected and washed with ether; m.p. 135–138°. The filtrate on concentration and reprecipitation with an excess of ether yielded 210 mg. of the same desired product. The total yield (580 mg.) was 89%. The ultraviolet absorption spectrum showed the characteristics expected of monotritylation on the ring: λ_{max} 277μ, 262μ, and 233 mμ; λ_{min} 272 and 250 mμ; ϵ_{277} 17,000, ϵ_{262} 18,400, ϵ_{233} 25,800, ϵ_{272} 16,400, ϵ_{250} 17,300.

Anal. Calcd. for $C_{35}H_{35}N_5O_8$ (653.71): C, 64.5; H, 5.37; N, 10.79. Found: C, 64.75; H, 5.38; N, 11.66.

N-Di-*p*-methoxytrityldeoxyguanosine.—N-Di-*p*-methoxytrityl-3',5'-di-O-acetylguanosine (180 mg., 0.28 mmole) was dissolved in a mixture of methyl alcohol (10 ml.) and water (3 ml.) and the solution was treated at 0° with 0.6 ml. of 2 *N* sodium hydroxide for 5 min. Pyridinium Dowex-50 (3 ml.) was then added to neutralize the alkali. The resin was removed by filtration and washed with methyl alcohol and pyridine. The total filtrate was evaporated and the evaporation repeated after addition of some benzene. The residue was dissolved in a little pyridine and methyl alcohol. On the addition of benzene a precipitate appeared which increased on further dilution of the mixture with ether. The precipitate was collected and washed with ether. The yield of the product was 135 mg., m.p. 162–167°. It was crystallized from benzene containing a small amount of methyl alcohol; m.p. 165–168°.

Anal. Calcd. for $C_{31}H_{31}O_6N_5$ (569.63): C, 65.6; H, 5.47; N, 12.18. Calcd. for $C_{31}H_{31}O_6N_5 + C_6H_6$: C, 66.6; H, 5.55; N, 10.5. Found: C, 64.90; H, 5.53; N, 10.95.

The ultraviolet absorption spectrum taken in ethyl alcohol showed: λ_{max} 277 mμ (ϵ 17,100), λ_{max} 262 mμ (ϵ 18,100), λ_{max} 234 mμ (ϵ 25,200); λ_{min} 271 mμ (ϵ 16,500), λ_{min} 251 mμ (ϵ 17,100).

N,O$^{5'}$-Bis-di-*p*-methoxytrityldeoxyguanosine. (a) **From Deoxyguanosine.**—Deoxyguanosine (0.54 g., 2 mmoles) was obtained as a finely divided powder by lyophilization of its solution in dilute ammonium hydroxide. The nucleoside was treated in pyridine (30 ml.) with 800 mg. (2.3 mmoles) of di-*p*-methoxytrityl chloride at room temperature for 1.5 hr. after which time a further amount (200 mg., 0.6 mmole) of the reagent was added. After 6 hr. the nucleoside had still not gone into solution and another addition of the reagent (600 mg., 1.95 mmoles) was made. After a further 2-hr. period, a clear solution had resulted. (Crystalline precipitate of pyridine hydrochloride had formed.) Methyl alcohol was added and the solution concentrated. The concentrate (2.5 ml.) was partitioned between chloroform (100 ml.) and water (100 ml.). The organic layer was separated and the solution after drying was concentrated. The concentrated solution was applied to the top of an alumina (20% deactivated) column (35 × 2.5 cm. diam.). The column was washed with chloroform (200 ml.), 6-ml. fractions at 20-min. intervals being collected. Elution was then continued with chloroform containing 6% ethyl alcohol. Fractions 3–17 contained di-*p*-methoxytrityl methyl ether and possibly di-*p*-methoxytritanol. Fractions 45–70 contained bis-di-*p*-methoxytrityldeoxyguanosine. These fractions were combined and concentrated. To the concentrate, ether (about 300 ml.) was added. The precipitate (1.15 g.) was collected, the mother liquor giving a further amount (0.25 g.) of the same product. The total yield therefore was 1.40 g. (1.61 mmoles, 80%).

Anal. Calcd. for $C_{52}H_{49}N_5O_8$ (871.95): C, 71.62; H, 5.66; N, 8.08. Found: C, 71.98; H, 6.03; N, 7.91 (see also the analysis as based on the di-*p*-methoxytrityl group determination).

(33) In a parallel experiment benzoylation with benzoyl chloride did cause N-benzoylation as clearly shown by the ultraviolet absorption spectrum. *Cf* the spectrum of N-benzoylguanosine-5' phosphate: M. Smith, G. I. Drummond, and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 693 (1961).

Ultraviolet absorption spectrum was: λ_{max} 276 mμ (ε 19,000), λ_{min} 273 mμ (ε 18,800), shoulder 262 mμ (ε 20,800), λ_{max} 234 mμ (ε 43,000), λ_{min} 224 mμ (ε 39,000).

(b) **From N-Di-*p*-methoxytrityldeoxyguanosine.**—N-Di-*p*-methoxytrityldeoxyguanosine (3.74 g., 6.4 mmoles) was treated in dry pyridine with 2.60 g. (7.4 mmoles) of di-*p*-methoxytrityl chloride at room temperature. After 5 hr. a clear solution resulted. Methyl alcohol was then added and after concentration the mixture was partitioned between chloroform and water. The concentrated chloroform solution was applied to an alumina (20% deactivated) column (90 × 4 cm. diam.). The column was eluted with 300 ml. of chloroform and then with chloroform (700 ml.) containing 2% ethyl alcohol. The effluent with the latter eluent was collected in 10-ml. fractions. The elution was continued with chloroform (800 ml.) containing 5% ethyl alcohol, the size of each fraction being now 15 ml. Peak A obtained in fractions 11–20 contained di-*p*-methoxytrityl compounds (nonnucleosidic material). Fractions 46–90 contained the desired bis-N,O$^{5'}$-di-*p*-methoxytrityldeoxyguanosine. When 150 fractions had been collected, the column was eluted with methyl alcohol. Some of the starting material (N-di-*p*-methoxytrityldeoxyguanosine) was thus recovered (0.8 g., 1.4 mmoles, 22%). The fractions containing the desired product were concentrated and the product precipitated with ether. In all, 3.5 g. (4.0 mmoles, 63%) of the product was thus recovered.

Deoxyguanosine-3′ Phosphate.—A solution of N,O$^{5'}$-bis-di-*p*-methoxytrityldeoxyguanosine (870 mg., 1 mmole), pyridinium β-cyanoethyl phosphate (6 mmoles), and DCC (2.5 g.) in pyridine (10 ml.) was kept at room temperature for 2 days. Water (3 ml.) was then added and the mixture extracted with cyclohexane.[34] The aqueous pyridine layer was made up to a total volume of 25 ml. with pyridine (stock solution). The yield of the phosphorylation product (N,O$^{5'}$-bis-di-*p*-methoxytrityldeoxyguanosine-3′ β-cyanoethyl phosphate, R_f 0.88 in solvent A) was 97%, using the ϵ_{max} value of $\epsilon_{276\ m\mu}$ 19,000 determined previously for N,O$^{3'}$-bis-di-*p*-methoxytrityldeoxyguanosine-5′ phosphate.[19]

A 5-ml. portion of the above solution was evaporated with added water to remove pyridine. The residue was then dissolved in 5 ml. of 80% acetic acid and the solution kept at room temperature. As judged by paper chromatography in solvent A, the removal of the 5′-O-di-*p*-methoxytrityl group was complete in 10 min. However, the complete removal of the N-di-*p*-methoxytrityl group necessitated prolonging the acidic treatment to 4 hr. After 4 hr. acetic acid was removed by evaporation and the residue shaken with a mixture of water and ether. The aqueous layer on paper chromatography in solvent C showed the major product (90%) with R_f 29 to be deoxyguanosine-3′ β-cyanoethyl phosphate, there being in addition a minor spot (3%, R_f 0.03) corresponding to deoxyguanosine-3′ phosphate and another spot (7%) with R_f 0.20 which has not been identified.[35]

Paper electrophoresis at pH 7.1 showed only two products, the major one corresponding to deoxyguanosine-3′ β-cyanoethyl phosphate and the other one, faster traveling, corresponding to the unidentified product.[35]

A portion of the above aqueous solution was treated with an equal volume of 2 *N* sodium hydroxide for 20 min. at room temperature. The alkali was removed by treatment with an excess of pyridinium Dowex-50 (pyridinium form) resin. Paper chromatography in solvent A of the resulting aqueous pyridine solution showed mostly deoxyguanosine-3′ phosphate, there being a minor product traveling slower (close to the origin), corresponding, presumably, to deoxyguanosine-3′ pyrophosphate.[35] The final yield of the pure nucleotide as based on the protected nucleoside was 89%. This product was resistant to the action of the crude snake venom under conditions which caused complete dephosphorylation of thymidine-5′ phosphate.

Quantitative Determination of Di-*p*-methoxytrityl-Containing Compounds.—The method developed was based on the fact that the di-*p*-methoxytrityl perchlorate has an absorption maximum at 500 mμ. For example, the compounds are readily detected on paper chromatograms by virtue of their bright orange color when the chromatograms are sprayed with a perchloric acid solution. The quantitative assay developed utilized a 1:1 solution of ethanol–perchloric acid (70%). A standard curve was prepared by dissolving 66.9 mg. of di-*p*-methoxytrityl chloride in 1 l. of ethyl alcohol (concentration/ml. thus was 0.000198 mmole). The optical density was read at 500 mμ by taking aliquots of the standard solution and diluting to 10 ml. with the 1:1 ethyl alcohol–perchloric acid mixture. The optical density was proportional to the concentration. Thus 0.1 ml. gave an optical density of 0.145, 0.2 ml. of 0.291, 0.4 ml. of 0.58, 1.0 ml. of 1.42, and 2.0 ml. of 2.85. Using the straight line curve thus obtained, the method was used with di-*p*-methoxytrityl containing compounds as: (a) **5′-O-di-*p*-methoxytritylthymidine.** A solution of 6 mg. in 10 ml. of ethyl alcohol was prepared; 1 ml. was diluted to 10 ml.; 1 ml. of this diluted solution was treated with 3 ml. of 70% perchloric acid and the total made up to 10 ml. with ethyl alcohol. The optical density of this solution at 500 mμ was 0.78. From this, the concentration of di-*p*-methoxytrityl group in the original solution was calculated to be 0.33 mg./ml. or 3.3 mg. in the total solution. The molecular weight of di-*p*-methoxytritylthymidine was thus found to be 550 (theoretical 544.6).

(b) **N-Benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine.**—A stock solution of 21.27 mg. of the substance in 25.3 ml. of ethyl alcohol was prepared. 1 ml. was diluted to 25 ml. with ethyl alcohol; 1 ml. of this solution on dilution with perchloric acid and ethyl alcohol to 10 ml., as above, gave an optical density of 0.38, while 2 ml. gave 0.76. The dimethoxytrityl group concentration and then the molecular weight of the starting material was thus determined to be 635 (theoretical 657).

(c) **N,O$^{5'}$-Bis-di-*p*-methoxytrityldeoxyguanosine.**—A stock solution containing 9.7 mg. in 10 ml. of ethyl alcohol was used; 1 ml. was diluted to 10 ml.; 0.2 ml. of this solution when made up to 10 ml. with the reagent perchloric acid and ethyl alcohol to 10 ml. gave ϵ_{500} 0.325. The molecular weight was thus calculated to be 875 (theoretical 871).

(34) During these extractions an oily layer, presumably of the bulky phosphorylation product, separated. During subsequent addition of more pyridine a clear solution again resulted.

(35) This is very likely a mixed pyrophosphate of deoxyguanosine-3′ phosphate and β-cyanoethyl phosphate.