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Studies on Polynucleotides. IX.[1] Experiments on the Polymerization of Mononucleotides. Certain Protected Derivatives of Deoxycytidine-5′ Phosphate and the Synthesis of Deoxycytidine Polynucleotides[2]

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3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate (II) has been prepared in quantitative yield by the acid-catalyzed reaction of dihydropyran with deoxycytidine-5′ phosphate. Acetylation of II gave N^6-acetyl-3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate (III) from which N^6-acetyldeoxycytidine-5′ phosphate was obtained by mildly acidic treatment and subsequent chromatography. Benzoylation of II, followed by very brief alkaline treatment, gave N^6-benzoyl-3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate. N^6-Benzoyldeoxycytidine-5′ phosphate was prepared in high over-all yield by benzoylation of deoxycytidine-5′ phosphate with an excess of benzoic anhydride or benzoyl chloride followed by careful alkaline treatment and chromatography of the products. A convenient method for large-scale preparation of N^6-anisyldeoxycytidine-5′ phosphate, the compound used in the polymerization experiments, from deoxycytidine-5′ phosphate is described. The reaction of a molar pyridine solution of a mixture of N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate (25%) and N^6-anisyldeoxycytidine-5′ phosphate (75%) with dicyclohexylcarbodiimide at room temperature for six days gave a polymeric mixture which, after treatment with concentrated ammonia to remove the protecting groups, was separated on a DEAE-cellulose (carbonate) column. Linear deoxycytidine polynucleotides bearing 3′-hydroxyl groups at one end and 5′-phosphomonoester groups at the other end were the major products. Members up to the pentanucleotides were purified by rechromatography on DEAE-cellulose (chloride) columns. Higher members up to the octanucleotide were purified by removal of the phosphomonoester end groups by bacterial phosphatase followed by chromatography on paper sheets. The characterization of all of the linear synthetic polynucleotides as containing specifically the C_3'–C_5' internucleotide bonds is described. The purification and identification of several other products formed during polymerization has been described: the compounds include N-(deoxycytidine-5′ phosphoryl)-N,N′-dicyclohexylurea, deoxycytidine-3′,5′-cyclic phosphate and deoxycytidine cyclic di-, tri- and tetra-nucleotides, which are formed by phosphorylation of the 3′-hydroxyl groups at one end by the 5′-phosphomonoester groups at the other end of the chain.

With the development of a satisfactory method for the polymerization of thymidine mononucleotides and of techniques for the separation and characterization of the resulting polynucleotides,[1,4,5] attention was turned to the polymerization of other deoxyribo-mononucleotides. Two problems were immediately encountered. The first problem, common to most of the mononucleotides, is their insolubility in anhydrous pyridine, which so far is the only medium found satisfactory for polymerization. The second problem, the extent of which varies with the mononucleotide, is the interference from the amino groups in the purine or pyrimidine ring. Thus, in related work[6] on the synthesis of thymidylyl-(5′ → 3′)-deoxycytidine[7] by the reaction of 3′-*O*-acetylthymidine-5′ phosphate with 5′-*O*-trityldeoxycytidine according to the general method previously developed,[8] the major reaction was the phosphorylation of the 6-amino group in deoxycytidine by the activated nucleotide component.[9,10] For the exclusive formation of C_5'–C_3' internucleotide bonds the need clearly was one of preparing deoxyribonucleoside and deoxyribonucleotide derivatives which contained suitably protected amino groups.[9,11] For the specific problems of the polymerization of deoxyribomononucleotides, studies were undertaken on the synthesis of N-acyldeoxyribonucleoside-5′ phosphates. The present paper describes simple and efficient routes to the preparation of N^6-acyldeoxycytidine-5′ phosphate derivatives and the successful polymerization of these pyridine-soluble derivatives to yield homologous deoxycytidine polynucleotides containing C_5'–C_3' internucleotide bonds. Chromatographic methods have been developed for the purification of the individual linear homologs and, in addition, the cyclic-oligonucleotides, analogous to those encountered in the thymidine series,[1,4,5] have been purified and characterized. A brief report of these results has recently been made.[12]

N^6-Acyldeoxycytidine-5′ Phosphate Derivatives.—Pure deoxycytidine-5′ phosphate (I) is available from commercial sources and it was used as the starting material in all the present work. In the first approach, the mononucleotide was converted quantitatively to 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate (II) by acid-catalyzed reaction with dihydropyran.[13,14] Acetylation with

(1) Paper VIII, H. G. Khorana and J. P. Vizsolyi, THIS JOURNAL, **83**, 675 (1961).

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

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

(5) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

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

(7) For the system of nomenclature, see references 8 and 9.

(8) P. T. Gilham and H. G. Khorana, THIS JOURNAL, **80**, 6212 (1958).

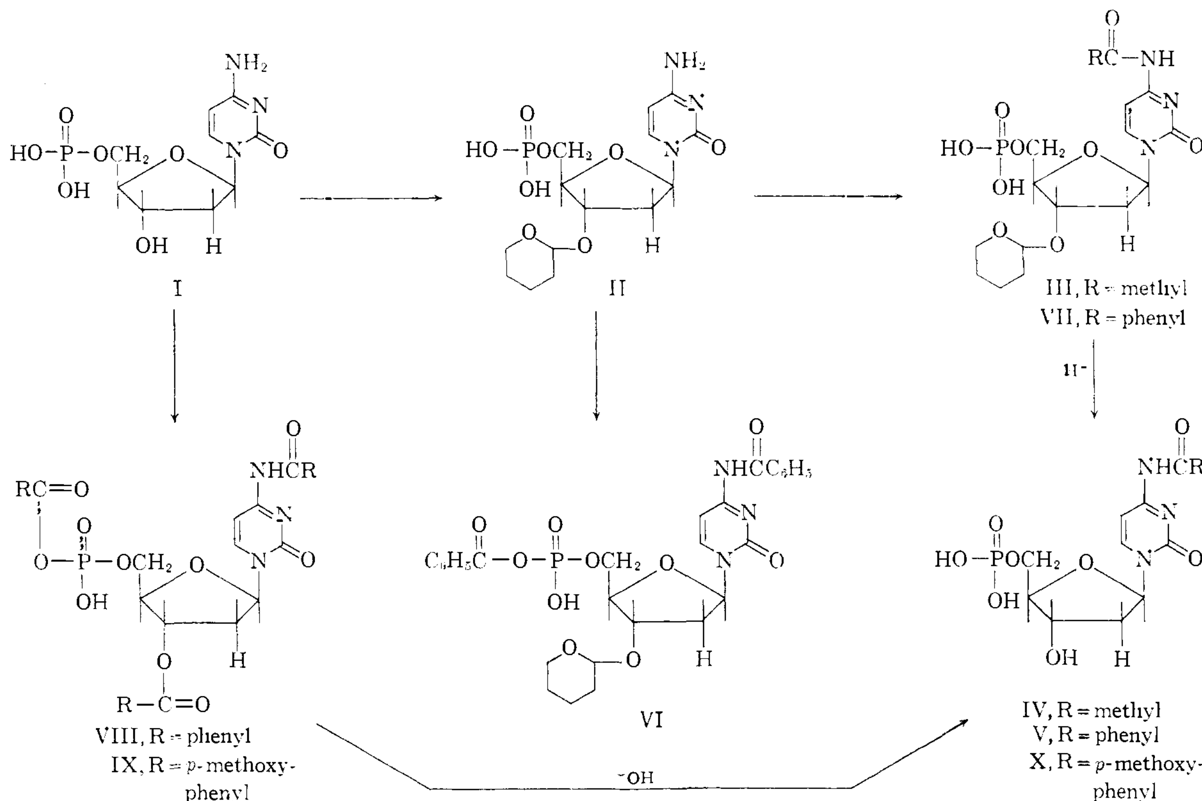
(9) H. G. Khorana, in E. Chargaff and J. N. Davidson, eds., "The Nucleic Acids," Vol. III, Academic Press, Inc., New York, N. Y., 1960, in press.

(10) In the previously recorded phosphorylations of deoxycytidine and derivatives, the phosphorylation of the amino group in the cytosine ring has been noted [A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 34 (1954)]. In the earlier synthesis of cytidine-5′ phosphate [A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2476 (1949)] from 2′,3′-*O*-isopropylidene cytidine by phosphorylation with dibenzylphosphorochloridate, N-phosphorylation must also have occurred, but presumably the phosphoramidate was destroyed during acidic treatment in the work-up. Phosphorylation of 2′,3′-*O*-benzylidene cytidine in this Laboratory with dibenzylphosphorochloridate showed this to be the case (experiment by Dr. Leon A. Heppel). See also G. M. Tener (THIS JOURNAL, in press) for N-phosphorylaion in cytidine by a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide.

(11) *Cf.* P. T. Gilham and H. G. Khorana, THIS JOURNAL, **81**, 4647 (1959).

(12) H. G. Khorana, *Federation Proc.*, in press.

(13) The use of the acid-labile tetrahydropyranyl ethers in polynucleotide work has previously been reported from this Laboratory. [M. Smith and H. G. Khorana, THIS JOURNAL, **81**, 2911 (1959); M. Smith, D. H. Rammler I. Goldberg and H. G. Khorana, forthcoming paper.]



acetic anhydride and pyridine[15] gave III, from which the tetrahydropyranyl group was removed by mild acidic treatment. Partial removal of the N-acetyl group occurred concomitantly, but the desired product, IV, was isolated pure after partition chromatography. The yield of IV was not entirely satisfactory (40–50%, over-all) and moreover the substance did not possess adequate solubility in pyridine. Since the corresponding benzoyl derivative (V) would be expected to be more stable to acidic treatment and to have enhanced solubility, II was benzoylated by prolonged treatment with benzoic anhydride in pyridine. The product obtained was evidently VI, the anhydride linkage in it being very stable to aqueous pyridine, as has been found previously in a related case.[16] Selective alkaline hydrolysis to VII could be achieved by very brief treatment with alkali and this product was obtained pure in high over-all yield after chromatography on a DEAE-cellulose (carbonate form) column. A paper-chromatographic study of the rate of removal of the tetrahydropyranyl group of VII in 50% acetic acid showed that V was formed in essentially quantitative yield, before the N^6-benzoylcytosine residue was appreciably affected. While this route to the synthesis of V has actually not been used in the present work, in view of the simpler and more direct method described below, it should be emphasized that VII is a potentially useful intermediate in the stepwise synthesis of deoxyribopolynucleotides for the purpose of inserting a deoxycytidylyl group into the interior of a chain. Thus, phosphorylation of the 3′-hydroxyl end group of a preformed chain by VII, followed by mild acidic treatment to remove the tetrahydropyranyl group would again selectively expose the 3′-hydroxyl group for a repeat of the phosphorylation reaction.[9,11]

Earlier experiments on the removal of N-acyl groups from different deoxyribonucleosides[8,17] had indicated that they varied considerably in their stability and that in certain cases, *e.g.*, deoxyadenosine, the *O*-acetyl groups were removed more rapidly than the N-acetyl groups under alkaline conditions. Although in the case of N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate the selective removal of the 3′-*O*-acetyl group did not appear to be of preparative value, similar approach using the benzoyl groups seemed more promising for the preparation of the N^6-protected nucleotide.[18] Thus, in test experiments, the benzoyl group in 3′-*O*-benzoylthymidine-5′ phosphate was found to be much more labile to

(14) The preparation of (II) was first carried out by Dr. P. T. Gilham in this Laboratory.

(15) The mixed acetyl-phosphate anhydride is also formed but the conditions used for work-up (about 2 hr. at room temperature in aqueous pyridine) are sufficient for its hydrolysis.

(16) G. M. Kellerman, *J. Biol. Chem.*, **231**, 431 (1958).

(17) P. T. Gilham and H. G. Khorana, unpublished work; R. K. Ralph and H. G. Khorana, unpublished work.

(18) In a recent paper, Michelson [*J. Chem. Soc.*, 3655 (1959)] has reported on the selective acetylation of the 6-amino group in cytidine-2′,3′-cyclic phosphate by using 1 mole of acetic anhydride. In this Laboratory, N^6-benzoyl-5′-*O*-trityldeoxycytidine had been prepared previously by treatment of 5′-*O*-trityldeoxycytidine with 1 mole of benzoic anhydride.[6] This approach, using limited amount of the acylating agent, however, is not feasible for the preparation of N^6-acyldeoxycytidine-5′ phosphates from deoxycytidine-5′ phosphate, because the preferred reaction of acetic anhydride or other anhydrides with the mononucleotide would be to form the mixed acyl phosphate type of anhydride, and this would probably undergo the exchange reactions previously described [H. G. Khorana and J. P. Vizsolyi, THIS JOURNAL, **81**, 4660 (1959)]. The preferred approach in the present work has therefore been to use a large excess of the acylating agent and to remove subsequently all acyl groups except that on the N^6-amino group.

alkali than the N[6]-benzoyl group in deoxycytidine derivatives. These observations led to the more direct preparation of N[6]-benzoyldeoxycytidine-5′ phosphate (V) from deoxycytidine-5′ phosphate. Benzoylation with an excess of benzoic anhydride[19] in pyridine gave, presumably, VIII, which when treated with a mixture of pyridine and aqueous sodium hydroxide under carefully controlled conditions gave V as the major product. The latter was purified by column chromatography and characterized on the basis of elementary analysis and characteristic ultraviolet absorption spectrum. In its behavior on paper chromatograms, it was identical with a sample prepared *via* II. The presence of the free 3′-hydroxyl group was further shown by the fact that V was dephosphorylated by the 5′-nucleotidase[20] present in the crude snake venom to form N[6]-benzoyldeoxycytidine.[6]

In the above preparation of N[6]-benzoyldeoxycytidine-5′ phosphate, a trace of deoxycytidine-5′ phosphate was also formed and, therefore, chromatography was necessary for purification. *p*-Methoxybenzoyl (anisyl) esters are more stable to alkali than the parent benzoyl esters[21] and assuming that the same relationship would hold between the N[6]-benzoyl- and N[6]-anisyldeoxycytidine derivatives, deoxycytidine-5′ phosphate was anisylated with an excess of anisyl chloride. The chloroform-soluble product, presumably IX, was obtained in essentially quantitative yield and the anisyl groups in it were indeed more stable to alkali. The alkaline treatment necessary to give the N[6]-anisyldeoxycytidine-5′ phosphate (X) could be controlled very conveniently and no deoxycytidine-5′ phosphate[22] was detected in the product isolated by the work-up procedure described in the Experimental Section. Although for characterization and elemental analysis a sample was chromatographed on a column, the material obtained directly was suitable[22] for polymerization work. The over-all yield of N[6]-anisyldeoxycytidine-5′ phosphate from deoxycytidine-5′ phosphate has been about 90% in the many runs performed on 1–2 mmole scale.

The rates of hydrolysis of the various N-acyldeoxycytidine-5′ phosphate derivatives in sodium hydroxide and 9 *N* ammonia at room temperature are recorded in Table I. As can be seen, ammonia is uniformly more effective than sodium hydroxide[23] and the order of stability of the different derivatives is, as expected: N[6]-anisyl > N[6]-benzoyl > N[6]-acetyl. Since concentrated ammonia was used for removal of the N[6]-anisyl group after the polymerization reaction (see below), it was established that during the removal of the N-acyl groups from N[6]-acyldeoxycytidine-5′ phosphates with ammonia, the product obtained was exclusively deoxycytidine-5′ phosphate, no deamination[25] to uracil derivatives being detected (see Experimental).

TABLE I

RATES OF CLEAVAGE OF N[6]-ACYL GROUPS FROM N[6]-ACYLDEOXYCYTIDINE-5′ PHOSPHATES

Solutions of the different nucleotides (approximately 1×10^{-4} *M*) were made up in 9 *N* ammonia or sodium hydroxide and the loss of the absorption at characteristic wave length was followed at different time intervals

Compound	Time required for 50% degradation: 9 *N* Ammonia	Sodium hydroxide: *p*H 12	*p*H 13	1 *N*
N[6],3,-*O*-diacetyldeoxycytidine-5′ phosphate[a]	1.5 min.	6.3 min.	3.5 min.	2.9 min.
N[6]-benzoyldeoxycytidine-5′ phosphate[b]	16 min.	......	2.5 hr.	.....
N[6]-anisyldeoxycytidine-5′-phosphate[c]	64 min.			>5 hr.

[a] Measured at 298 mμ. [b] Measured at 303 mμ. [c] Measured at 320 mμ.

Partial deamination was observed on treatment of the N[6]-acyldeoxycytidine-5′ phosphate derivatives with 50% acetic acid, the extent varying with the nature of the N-acyl group. N,O-Diacetyldeoxycytidine-5′ phosphate required three to four days at room temperature for complete removal of the N-acetyl group. Although minor products were formed, which evidently arose from the cleavage of N-glycosyl bond, the major reaction was the removal of the acetyl group to form deoxycytidine derivatives. Deamination occurred but only to the extent of 5–10%.[26] N[6]-Benzoyldeoxycytidine-5′ phosphate was more stable to 50% acetic acid: after 8 days at room temperature, more than 50% of the starting material appeared unchanged and the other nucleotidic products were deoxycytidine-5′ phosphate and deoxyuridine-5′ phosphate.[26] The ratio of deoxycytidine to deoxyuridine compound was roughly 2:1. The pattern of degradation of N-anisyl deoxycytidine-5′ phosphate on treatment with hot 50% acetic acid was very complex, deamination and N-glycosyl bond cleavage were apparently prominent reactions.

Deoxycytidine Polynucleotides.—N[6]-Benzoyl- and N[6]-anisyl-deoxycytidine-5′ phosphates were both

(19) The anhydride was used in the early work, but the use of the more reactive benzoyl chloride, as described in the Experimental section, is preferred.

(20) This enzyme requires for its action the presence of an unsubstituted 3′-hydroxyl group in ribo- and deoxyribonucleoside-5′ phosphates. See *e.g.*, R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3747 (1957). Similarly, 3′-*O*-acetylthymidine-5′ phosphate is resistant to this enzyme [G. M. Tener, P. T. Gilham, W. E. Razzell, A. F. Turner and H. G. Khorana, *Ann. N. Y. Acad. Sci.*, **81**, 757 (1959)].

(21) L. P. Hammett, "Physical Organic Chemistry," McGraw–Hill Book Co., Inc., New York, N. Y., 1940, p. 121.

(22) There is usually present a very small amount of the faster-travelling N[6],3′-*O*-dianisyldeoxycytidine-5′ phosphate which need not be removed for the purpose of polymerization experiments, since an additional (25%) amount of the nucleotide bearing 3′-protected hydroxyl group (N,*O*-diacetyldeoxycytidine-5′ phosphate) is incorporated, in any case, before polymerization (see below). When the directly obtained N-anisyldeoxycytidine-5′ phosphate is spotted on paper chromatograms at a very high level (more than 1 μmole in 1.5 cm. diameter spot), very faint traces of one or two very fast-travelling fluorescent contaminants have also been detected. The amounts of these are negligible.

(23) The results seem to be in contrast with those on N[6]-benzoylcytosine,[24] which was found to be stable to half-saturated ammonia at 0° for 15 hr. but was completely converted to cytosine by 0.1 *N* sodium hydroxide at room temperature in 15 hr.

(24) D. M. Brown, A. R. Todd and S. Varadarajan, *J. Chem. Soc.*, 2384 (1956).

(25) During prolonged treatment of N[6]-anisyl- and N[6]-benzoyldeoxycytidine-5′ phosphates with sodium hydroxide (Table I), some deamination might have occurred, but this has not been checked. Deamination in the hot with barium hydroxide is extensive. See below for deoxycytidine-3′,5′-cyclic phosphate hydrolysis.

(26) Deaminations of N-acyl cytosines on treatment with hot aqueous acetic acid were previously found by Brown, *et al.*[24] The deaminations with N-acyl cytosines appear to be much more extensive than those observed in the present series of compounds. Thus N[6]-acetylcytosine gave with 80% acetic acid approximately equal amounts of cytosine and uracil. N[6]-Benzoylcytosine gave under similar conditions uracil and cytosine in the ratio 33:1.[24]

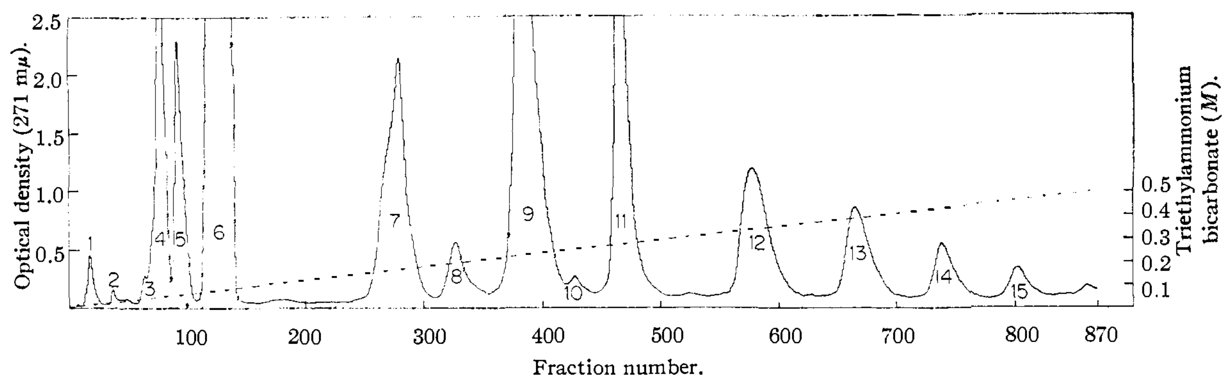
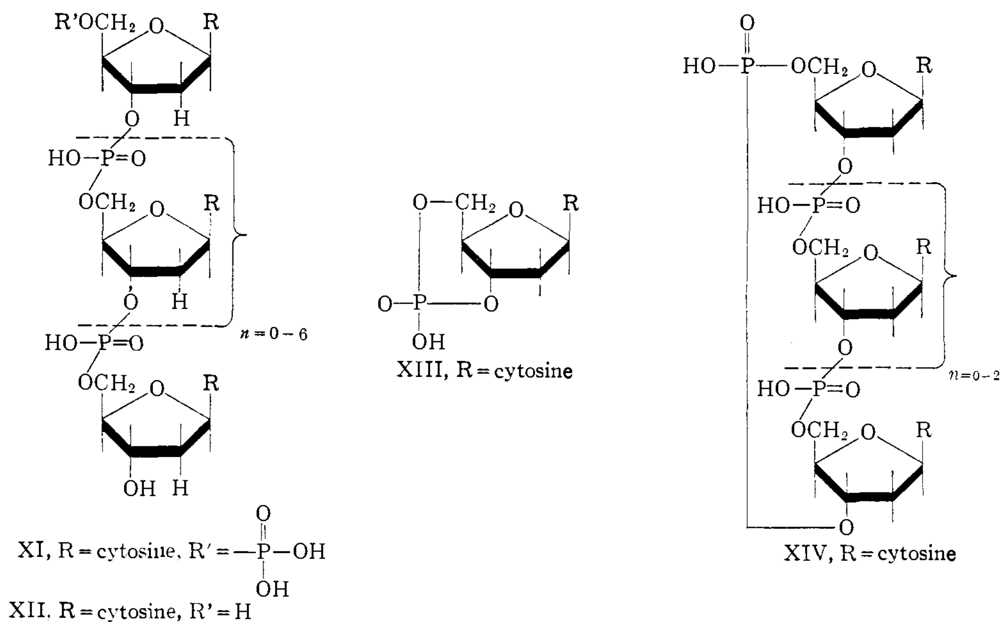


Fig. 1.—Chromatography of total mixture of deoxycytidine polynucleotides from 1 mmole of mononucleotide on a DEAE-cellulose (bicarbonate) column. Conditions of chromatography as in text. For identification of different peaks and distribution of nucleotide material see Table II.

soluble in anhydrous pyridine. For polymerization a mixture of N^6-anisyldeoxycytidine-5′ phosphate (75%) and N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate (25%) was reacted in anhydrous pyridine with dicyclohexylcarbodiimide under conditions described in the preceding paper.[1] The N^6-anisyl group was removed from the resulting products by treatment with concentrated ammonia and subsequent chromatography on a DEAE-cellulose (carbonate form) column gave results shown in Fig. 1. The distribution of the nucleotide material and the composition of the different peaks are listed in Table II. (The system of abbreviations for designation of polynucleotides in the tables and text is that described in the preceding paper.[1])



Linear Polynucleotides.—The di-, tri-, tetra-, penta-, hexa-, hepta- and octa-nucleotides (general structure XI; n = 0–6) were in peaks No. 7, 9, 11, 12, 13, 14 and 15, respectively.

Further processing of the individual peaks was necessary to obtain pure linear polynucleotides. The impurities present were phosphomonosterase-resistant and were probably pyrophosphates (see below.)[27] Rechromatography on DEAE-cellulose columns in the carbonate form, a technique which was very successful in the purification of thymidine polynucleotides,[1] singularly failed to separate the impurities from the desired products.[28] Encouraging results were obtained by rechromatography at pH 5 on DEAE-cellulose (chloride form) columns using a mixture of lithium acetate and lithium chloride as eluents. Typical results obtained with the di-, tri-

(27) While these pyrophosphate types of impurities did not separate from normal polynucleotides (XI) with similar charges, it is interesting that the cyclic oligonucleotides were well resolved from the linear members. Thus, the dinucleotide (XI; n = 0) (peak 7) with three negative charges is well separated from the cyclic trinucleotide (peak 8) also bearing the same net charge. Similarly the linear trinucleotide (XI; n = 1) is well separated from the cyclic tetranucleotide (XIV; n = 2). Evidently the shape of the molecule influences the elution from such columns.

(28) A variety of other conditions for purification of deoxycytidine oligonucleotides by rechromatography was tried without success. The attempts included: (1) chromatography on DEAE-cellulose (chloride form) columns at pH 2–3 using very dilute hydrochloric acid and lithium chloride as eluents, (2) chromatography on columns of Dowex-1 (2% cross-linked) ion exchange resin in the chloride and in the formate form, (3) chromatography on columns of DEAE-cellulose in the formate and in the acetate forms using, respectively, formate buffer (pH 3.5) and acetate buffer (pH 5) as eluents. The oligonucleotides were held too tightly on these columns, no elution being effected with concentrations up to 1 M with respect to formate.

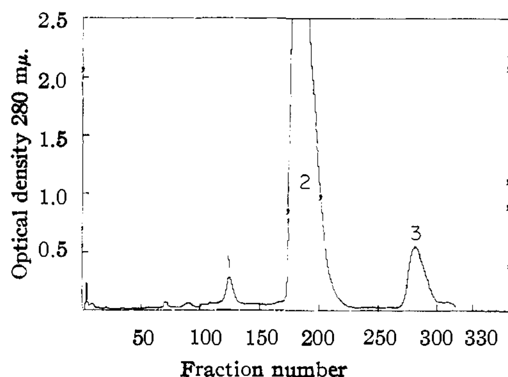


Fig. 2.—Rechromatography of trinucleotide peak (peak 9 of Fig. 1) on a DEAE-cellulose (chloride form) elution with lithium chloride + lithium acetate (pH 5). Conditions as described in text. Peak 2, the major peak, is of pure trinucleotide, pCpCpC

and tetra-nucleotides are illustrated with respect to the trinucleotide (Fig. 2). The main impurity in this and the other oligonucleotides, now carrying higher negative charge emerged after the desired product. Although similar conditions failed to give satisfactory results with the pentanucleotide peak (peak 12 of Fig. 1), purification was achieved by working at somewhat higher *p*H (*p*H 5.8).[29] No success was had in purifying the hexanucleotide peak (peak 13) by this procedure[30] and thus far the major constituents of peaks 13–15 (hexa- to octa-nucleotides) have been purified only as the corresponding members (XII) lacking the terminal phosphomonoester groups, namely, by treatment of the individual peaks with phosphomonoesterase followed by chromatography on paper strips[31] (see Experimental).

The R_f's of the linear polynucleotides bearing 5'-phosphomonoester end groups (XI; n = 0–6) and of the corresponding members (XII) obtained after enzymic removal of the terminal phosphomonoester groups in a number of solvents are listed in Table III. Single spots were obtained with all the compounds, and the pattern of R_f's shows the homologous nature of the polynucleotides. It should be stressed, however, that paper chromatography, according to our experience, has severe limitations with respect to the higher deoxycytidine polynucleotides bearing phosphomonoester end groups. No solvent system has been found which can be used reliably as a criterion of purity for members higher than tetranucleotide. Convincing proof of homogeneity is furnished if a linear polynucleotide, travelling as a single spot in the ammoniacal solvent systems used, gives, after digestion with phosphomonoesterase, again a *single* faster-travelling product (*cf.* ref. 1). The homogeneity of linear members (XI; n = 0–3) obtained after rechromatography as described above was confirmed in this manner. The higher members, hexa- to octa-nucleotides, purified after enzymic removal of the terminal phosphomonoester groups, are also regarded as pure, since the enzymic treatment confers on the compounds markedly higher mobility in the ammoniacal solvents, and there is little chance of overlap of R_f with either other members of the series d-C(pC)$_n$pC or the phosphomonoesterase-resistant contaminants that were present in the original polynucleotide peaks.

Final evidence for the size of the homologous members of the series d-C(pC)$_n$pC was obtained by degradation with venom phosphodiesterase[1] and determination of the ratios of the mononucleotide to the nucleoside formed. The values obtained, which are given in Table IV, are close to those expected for the various homologs.

Since in the present work, a major problem was to ensure that the synthetic polynucleotides contained exclusively the C_5'–C_3' internucleotide linkages and not any P–N linkages, the homologous series of compounds lacking the terminal phosphomonoester groups[32] (XII; n = 0–6) were subjected

TABLE II

CHROMATOGRAPHY OF DEOXYCYTIDINE POLYNUCLEOTIDES. DISTRIBUTION OF NUCLEOTIDE MATERIAL IN DIFFERENT PEAKS OF FIGURE 1

Peak	Fractions pooled	Total nucleotide material[a] in peaks, %	Remarks, composition of peak, etc.
1	12–27	0.67	Too small for investigation
2	34–55	.40	Too small for investigation
3	59–67	.39	N-pyridiniumdeoxycytidine phosphate compound
4	68–86	6.52	Phosphorylurea (XV) + 3',5'-cyclic phosphate (XIII) + non-nucleotide material + one unidentified nucleotide component
5	88–105	3.98	Anisic acid
6	111–144	32.00	Deoxycytidine-5' phosphate + cyclic dinucleotide (XIV; n = 0)
7	241–307	10.8	Mainly d-pCpC (XI; n = 0)
8	312–350	2.44	Mainly cyclic trinucleotide (XIV; n = 1)
9	360–415	15.00	Mainly d-pCpCpC (XI; n = 1)
10	420–440	1.05	Mainly cyclic tetranucleotide (XIV; n = 2)
11	455–490	11.35	Mainly d-pCpCpCpC (XI; n = 2)
12	560–610	8.20	Mainly d-pC(pC)$_3$pC (XI; n = 3)
13	650–695	5.33	Mainly d-pC(pC)$_4$pC (XI; n = 4)
14	725–760	3.14	Mainly d-pC(pC)$_5$pC (XI; n = 5)
15	790–820	1.84	Mainly d-pC(pC)$_6$pC (XI; n = 6)
1 *M* triethylammonium bicarbonate eluate		3.65	Higher polynucleotides

[a] Total recovery of nucleotidic material was 85%, assuming no hypochromicity in the polynucleotides. If, as is probable, there is some hypochromicity, then the recovery was higher.

(29) The aim in this work was to use a *p*H where the secondary phosphoryl dissociation of the terminal phosphomonoester group was suppressed, and yet the *p*H of the eluent was well removed from the pK_a of the amino groups in deoxycytidine residues. It is possible that the lack of success in obtaining discrete peaks with higher polynucleotides was due to inability to realize this situation.

(30) Rechromatography on DEAE-cellulose (chloride) columns under similar conditions has, in contrast, been used successfully for purification of deoxyadenosine polynucleotides up to octanucleotide (R. K. Ralph and H. G. Khorana, unpublished work).

(31) From these results and those on rechromatography on columns, the conclusion is drawn that the amounts of impurities in the major peaks increase with the increasing size of polynucleotides.

(32) Oligonucleotides bearing 5'-phosphomonoester end groups are

TABLE III

R_f'S OF DIFFERENT COMPOUNDS ON PAPER CHROMATOGRAMS

Compound	R_f in solvents							
	A	B		C		D	F	G
Deoxycytidine-5′ phosphate	0.13	0.12	1	0.33	1	0.57	1	1
3′-O-Tetrahydropyranyldeoxycytidine-5′ phosphate	.39							
3′-O-Tetrahydropyranyl-N⁶-acetyldeoxycytidine-5′ phosphate	.56							
3′-O-Tetrahydropyranyl-N⁶-benzoyldeoxycytidine-5′ phosphate-benzoic acid mixed anhydride	.84							
3′-O-Tetrahydropyranyl-N⁶-benzoyldeoxycytidine-5′ phosphate	.58							
N⁶-Acetyldeoxycytidine-5′ phosphate	.31							
N⁶-Benzoyldeoxycytidine-5′ phosphate	.44							
Deoxycytidine		0.55						
Deoxycytidine-3′,5′ cyclic phosphate		.40						
Deoxycytidine-5′ phosphoryl-(N,N′-dicyclohexyl)-urea (XV)		.83						
d-pCpC			0.41[a]		0.82[a]	0.45	0.91[a]	0.45[a]
d-pCpCpC			.16		.63	.16	.75	.19
d-pC(pC)$_2$pC					.47	.04	.26	.07
d-pC(pC)$_3$pC					.37			
d-pC(pC)$_4$pC					.25			
d-pC(pC)$_5$pC					.16			
d-pC(pC)$_6$pC					.095			
d-CpC		.31		0.48				
d-CpCpC		.12		.35				
d-C(pC)$_2$pC			.36	.24				
d-C(pC)$_3$pC			.10	.18				
d-C(pC)$_4$pC			.03	.13				
d-C(pC)$_5$pC				.09				
d-C(pC)$_6$pC				.06				
Cyclic-dinucleotide (XIV; $n = 0$)		.20						
Cyclic-trinucleotide (XIV; $n = 1$)			.42					
Cyclic tetranucleotide (XIV; $n = 2$)			.09					
Side-product (pyrophosphate, XVI)						.235		
Side-product of trinucleotide (last peak of Fig. 2)						.08		

[a] R_f's under these columns are relative to that of deoxycytidine-5′ phosphate.

TABLE IV

RESULTS OF DEGRADATION OF DEOXYCYTIDINE POLYNUCLEOTIDES LACKING TERMINAL PHOSPHOMONOESTER GROUPS BY VENOM PHOSPHODIESTERASE

Details of degradation and paper chromatography as in text. The spots of the nucleotide and nucleoside formed were eluted with 0.1 N hydrochloric acid (5 ml.) for 48 hr. and optical densities determined with appropiate blanks at 280 mμ.

Compound	Product of hydrolysis		Ratio, pC/C	
	pC (optical density/ml.)	C (optical density/ml.)	Found	Theor.
d-CpC	1.780	1.770	1.01	1
d-CpCpC	2.750	1.310	2.09	2
d-CpCpCpC	4.600	1.450	3.17	3
d-C(pC)$_3$pC	6.960	1.625	4.28	4
d-C(pC)$_4$pC	1.615	0.340	4.75	5
d-C(pC)$_5$pC	7.425	1.020	7.28	6
d-C(pC)$_6$pC	3.800	0.500	7.6	7

to the action of the spleen phosphodiesterase.[33-35] Complete degradation occurred in each case and the products formed were deoxycytidine-3′ phosphate[36] and deoxycytidine.

Cyclic Oligonucleotides.—The monomeric member of this series, deoxycytidine-3′,5′ cyclic phosphate (XIII) was present in a small amount (about 0.5%) as one of the four components of peak 4, while the cyclic dinucleotide (XIV; $n = 0$) was one of the two major components of peak 6 (the second component was deoxycytidine-5′ phosphate). Cyclic tri- and tetra-nucleotides were the major components of peaks 8 and 10, respectively. Cyclic di- and tri-nucleotides could be purified by chromatography in isopropyl alcohol–ammonia–water solvent but the purification of the tetranucleotide (XIV; $n = 2$) by this technique was not complete. A sample was purified by treatment of the material obtained after paper chromatography with phosphomonoesterase and rechromatography in the ammoniacal solvent systems. The portion which was resistant was the cyclic tetranucleotide. XIII and the cyclic oligonucleotides were all resistant to phosphomonoesterase. The rate of degradation of the cyclic oligonucleotides by the venom phosphodiesterase[37] was much slower than that of

resistant; see L. A. Heppel and R. J. Hilmoe in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 565.

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

(34) The kinetics and stepwise action of the spleen enzyme on these substrates has been described elsewhere [W. E. Razzell and H. G. Khorana, *ibid.*, in press (1960).]

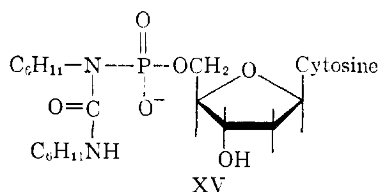
(35) The spleen phosphodiesterase at present provides the only reliable and specific tool for degradation of deoxyribopolynucleotides to deoxyribonucleoside-3′ phosphates and thus to establish the presence of internucleotide bonds involving C$_3$′-hydroxyl groups.

(36) The sole nucleotide formed was deoxycytidine-3′ phosphate (see Experimental section). This result assures that no deamination to uracil compounds occurred during the alkaline and ammoniacal treatments used to remove the N-anisyl groups from the linear polynucleotides.

linear oligonucleotides (XI). This result and the general pattern of elution of these compounds are both consistent with the structures assigned to them.[38] The R_f's of the cyclic oligonucleotides are listed in Table III.

The behavior of deoxycytidine-3',5'-cyclic phosphate (XIII) on treatment with barium hydroxide in the hot was analogous to that of cytidine-3',5'-cyclic phosphate,[39] three products being formed with deoxycytidine spectrum, and deamination to form deoxyuridine phosphates being a prominent reaction. When the products were chromatographed on a Dowex-1-formate column, two of the peaks with deoxycytidine spectrum corresponded to deoxycytidine-5' and deoxycytidine-3' phosphate, the latter being the more abundant of the two. The third product with deoxycytidine spectrum, which has not been identified,[40] emerged between the two above-mentioned products.

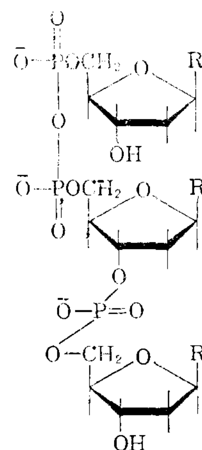
Other Minor Products.—A very minor product (0.2–0.4%) which is the fastest component of peak 4 on paper chromatograms, has been identified tentatively as the phosphorylurea (XV). The substance moves on paper electrophoresis at pH 7.5



much more slowly than deoxycytidine-5' phosphate and even more slowly than the cyclic phosphate (XIII). On being kept in 1 N hydrochloric acid at room temperature, it yields deoxycytidine-5' phosphate as the only ultraviolet absorbing product and the conversion is complete in a few hours. A water-insoluble product, evidently dicyclohexylurea, is concomitantly formed. The substance is stable to 1 N sodium hydroxide at room temperature for at least 4 hr. All these properties are consistent with the formulation XV. The formation of the substance, although not of any practical significance in the polymerization reaction, is of interest for study of the reactions of carbodiimides with phosphomonoesters.[41]

Another extremely minor product, which was present in peak 3, travelled on paper chromatograms run in the isopropyl alcohol–ammonia–water solvent more slowly than deoxycytidine-5' phosphate. Paper electrophoresis at pH 7.5 showed the substance to have net negative charge, the mobility, however, being much less than that of deoxycytidine-5' phosphate. The ultraviolet spectrum of the substance was a composite of deoxycytidine and N-alkylpyridinium cation. The presence of a phosphomonoester group was shown by its susceptibility to phosphomonoesterase, the resulting ultraviolet absorbing product behaving as a cation at pH 7.5. In all these properties the substance[42] corresponds to the minor product appearing at similar position in the elution diagram of thymidine polynucleotides described in the preceding paper.[1] The probable structure for this type of compound has already been discussed there.

A general class of side-products encountered consists of those that emerge together with the desired polynucleotides bearing 5'-phosphomonoester end groups on initial chromatography of the total polymeric mixtures. The side-products, which evidently have the same negative charge at pH 7.5 as the linear homologs bearing the phosphomonoester end groups, have had to be separated by rechromatography at pH 5–5.5 of the individual peaks. The general elution pattern then obtained is that most of the side-products follow the polynucleotide bearing the phosphomonoester groups. This general result and the further specific evidence presented below on the side-product from the dinucleotide (XI; n = 0) leads to the conclusion that the side-products are pyrophosphates containing linear oligonucleotides joined up through their phosphomonoester groups. Thus the side-product present in the dinucleotide (XI; n = 0) is concluded to be the pyrophosphate XVI. On paper electrophoresis at pH 7.5, the substance



XVI, R = cytosine

(37) *Cf.* Cyclic thymidine oligonucleotides, W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 and 2114 (1959).

(38) The alternative possible structures for these would be those of pyrophosphates formed by joining up of linear oligonucleotides. This class of compounds is, however, very rapidly attacked by venom phosphodiesterase. Moreover, the general elution pattern of deoxycytidine cyclic oligonucleotides is practically identical with that of thymidine cyclic oligonucleotides,[1] which have previously been thoroughly characterized.[4] Furthermore, the amounts of the different cyclic oligonucleotides in the two series correspond very closely.

(39) M. Smith, G. Drummond and H. G. Khorana, THIS JOURNAL, **83**, 698 (1961).

(40) While detailed structural work on this product remains to be carried out, it seems reasonable to postulate that it is 2'-deoxyxylofuranosylcytosine-5' phosphate, having arisen by the participation from the 2-keto group of cytosine ring during ring-opening of the cyclic diester and intermediate formation of a cyclonucleoside-5' phosphate [see *e.g.*, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955), for cyclonucleoside formation from 3'-*O*-substituted thymidine and related compounds].

(41) Phosphorylureas have previously been found only as a result of the reaction of the strained five-membered cyclic diesters of phosphoric acid with dicyclohexylcarbodiimide. [H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, THIS JOURNAL, **79**, 430 (1957).] The phosphorylureas described previously are much more labile than XV by virtue of the adjacent hydroxyl group present in them.

(42) The amount of the substance is negligible, as is that of the analogous compound in the thymidine series. It is our impression that the use of concentrated nucleotide solutions as in the present procedures reduces the amount of these products. The amounts encountered in earlier work[4,5] seemed to be much higher.

moves more slowly than the dinucleotide (XI; n = 0), although the two compounds are eluted together from ion exchange columns at the same pH. The result indicates a larger size for XVI. In accordance with the earlier observations on the degradation of pyrophosphates on treatment with an excess of acetic anhydride and pyridine,[43] the substance on similar treatment gave products which, judging from their chromatographic behavior, were deoxycytidine-5′ phosphate and the dinucleotide (XI; n = 0).

General Remarks.—The simple method developed for the selective protection of the amino group in deoxycytidine-5′ phosphate has also been applied to the similar problem in deoxyadenosine-5′ phosphate[30] and should be generally useful in synthetic work in the polynucleotide field.

The presence of some pyrophosphate bonds linking up different oligonucleotides in the final polymerization mixtures has been found in the present work. Similar compounds were noted in the earlier work with thymidine-3′ phosphate.[5] In the polymerization of thymidine-5′ phosphate, similar compounds were also probably present, but in very small amounts, and moreover they could be readily removed. The total results on polymerization can be accommodated on the tentative mechanism of the phosphodiester bond synthesis previously suggested.[4,8,44] The initial steps in the over-all mechanism are the conversion of the mononucleotide to pyro- and then poly- or meta-phosphates. It appears that the insoluble products that invariably appear almost instantaneously on the addition of dicyclohexylcarbodiimide to the anhydrous pyridine solutions of the starting nucleotides are these activated intermediates of the poly- or metaphosphate type. The subsequent phosphorylation of hydroxyl groups to form the phosphodiester bonds is a slower process and the extent of its completion is reflected in the composition of the final products. The solubilities of the intermediate compounds probably influence the rate of phosphodiester bond formation. So far, this process of phosphodiester bond formation at the expense of pyrophosphate bonds has been brought nearest to completion in the case of thymidine-5′ phosphate polymerization. For those nucleotides where the pyrophosphate bonds survive to a serious extent, work is necessary on modification of polymerization conditions and/or the investigation of alternative polymerizing agents. Another possibility is to devise a procedure for the subsequent cleavage of the pyrophosphate bonds that survive. This, in effect, was demonstrated in the present work in the degradation of XVI on treatment with acetic anhydride. Experiments on the treatment of the total polymerization mixture in this manner and on analysis of the products, as well as on studies of efficiency of different polymerizing agents will be reported subsequently.

Experimental

3′-O-Tetrahydropyranyldeoxycytidine-5′ Phosphate.—Deoxycytidine-5′ phosphate[45] (0.306 g., 0.96 mmole) was added to a mixture of freshly distilled dihydropyran (3.0 ml.) and dimethyl sulfoxide (3.0 ml.) and to the mixture was then added a solution (0.25 ml. of 5.5 N) of hydrogen chloride in dry dioxane and the sealed reaction mixture shaken until a clear solution resulted (2–5 min.). After a further period of 50 min., ammonium hydroxide (sp. gr. 0.9) was added in excess and the mixture evaporated to a gum. Dilute ammonium hydroxide was added to the residue and the solution extracted with ether. The aqueous layer was filtered and lithium hydroxide (1.5 ml. of 2 N) was added to the clear filtrate. Ammonia was removed by evaporation and the concentrated alkaline solution neutralized to pH 8 by careful addition of dilute hydrochloric acid. The solution was evaporated under reduced pressure and the resulting gum was taken up in the minimum volume of methyl alcohol. The nucleotide was selectively precipitated by dilution with four volumes of acetone and the precipitate collected by centrifugation. The white solid was washed successively with a methyl alcohol–acetone (1–4) mixture and acetone and after drying was re-precipitated and washed in the same manner. The yield of the acetone-dried powder, which was homogeneous by paper chromatography (see Table III for R_f) was 377 mg., corresponding to a yield of 97% as determined spectrophotometrically.

For analysis, some of the above product was converted to the ammonium salt by treatment with ammonium Amberlite IR-120 ion exchange resin and lyophilized.

Anal. Calcd. for $C_{14}H_{25}N_4O_8P$ (monoammonium salt): C, 41.20; H, 6.19; N, 13.66; P, 7.58. Found: C, 40.99; H, 6.58; N, 13.17; P, 7.1.[46]

For the study of the acidic removal of the tetrahydropyranyl group in the above compound, 7.1 mg. of the lithium salt was dissolved in 0.35 ml. of 10% aqueous acetic acid and the solution kept at room temperature. Aliquots were applied on paper chromatograms, which were developed in Solvent System B. Spectrophotometric estimation of the intensities of the eluted spots showed the following rate for hydrolysis to deoxycytidine-5′ phosphate: 2 hr., 17%; 4 hr., 32%; 8 hr., 54%; 26 hr., 100%.

N⁶-Acetyldeoxycytidine-5′ Phosphate *via* 3′-*O*-Tetrahydropyranyldeoxycytidine-5′ Phosphate.—An aqueous solution of the pyridinium salt of 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate (prepared by treatment of 0.86 mmole of the lithium salt with pyridinium IR-120 Amberlite ion exchange resin) was evaporated under reduced pressure and low temperature with added pyridine.[47] The residual gum was rendered dry by successive re-evaporation with 25 ml. portions of pyridine. The resulting partly crystalline residue was treated with 25 ml. of anhydrous pyridine and then 5 ml. of acetic anhydride added. The mixture was shaken and when a clear solution resulted (5–10 min.), the flask was set aside in the dark for 12 hr. Water (25 ml.) was then added to the almost clear solution, and after being kept at 4° for 3 hr. the solution was evaporated under reduced pressure. To the concentrated solution,[48] aqueous pyridine (100 ml. of 5%) was added and the solution re-concentrated. The process was repeated twice and final y an aqueous pyridine solution was lyophilized[49] to ensure complete removal of pyridinium acetate. Crude N-acetyl 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate was thus obtained as a powder.

The above powder was dissolved in water (24 ml.) containing a drop of pyridine and acetic acid (2.7 ml.) was added. After 3 hr.[50] at room temperature, an excess of pyri-

(43) H. G. Khorana and J. P. Vizsolyi, This Journal, **81**, 4660 (1959).

(44) M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

(45) The mononucleotide used in all the work described here was a commercial product. It was checked carefully for purity as described in the preceding paper.[1]

(46) The phosphorus analysis was carried out on another sample which had been prepared in an identical manner.

(47) The addition of an amount of pyridine at least equal to the volume of the aqueous solution is necessary during evaporation in order to avoid fall of the pH below 6, when the protecting group becomes noticeably labile.

(48) The process of evaporation was not allowed to continue to dryness, since some decomposition of the product was then detected.

(49) During this operation the contents of the flask were never allowed to collapse to a gum. If there was any tendency in this direction, the lyophilization was interrupted while there was still ice present and the contents re-lyophilized after being dissolved in 5% aqueous pyridine.

(50) It appears that the acidic removal of the tetrahydropyranyl group occurs faster with N-acetyl and N-benzoyl (see below) deriva-

dine was added and then pyridinium acetate was removed by repeated evaporation and lyophilization from aqueous pyridine as described above. The resulting powder was dissolved in 2 ml. of 1 *M* ammonium acetate (*p*H 6) and to the solution 5 ml. of isopropyl alcohol was added under shaking. The mixture was applied to the top of a cellulose column (30 cm. × 4.2 cm. diameter) which had been packed in the same solvent mixture (Solvent A, see below under "Paper Chromatography"). Elution was started with the same solvent with a flow rate of 1.3 ml./min. and 15 ml. fractions were collected. Determination of the optical density of the various fractions at 298 mμ, which is the λ_{max} of the desired compound gave these results: peak 1, which emerged in fractions 21–25, contained a total of 1,680 optical density units. (This peak has not been identified.) Peak 2, which appeared in fractions 28–34, contained a total of 830 optical density units and was unchanged N^6-acetyl-3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate. Peaks 3 and 4, which emerged in fractions 39–44 and 48–52, were minor and these were followed by Peak 5, which contained N^6-acetyldeoxycytidine-5′ phosphate. This peak appeared in fractions 55–71[51] and contained 4,400 optical density units. It was evaporated to small bulk with added pyridine and then re-evaporated with aqueous pyridine. The concentrated aqueous solution was passed through an excess of pyridinium Amberlite IR-120 resin (200 ml. total bed volume). The total effluent and washings were freed of pyridinium acetate by re-evaporation and re-lyophilization from aqueous pyridine, as described above, and afforded the pyridinium salt of N^6-acetyldeoxycytidine-5′ phosphate as a hygroscopic white powder.[52] The yield as based on phosphorus analysis[53] was 59%. (In a later run, the yield of the pure product was around 40%.)

The spectral characteristics at *p*H 7 of the ammonium salt isolated by chromatography in solvent A were: λ_{max}, 298 mμ, λ_{min}, 269 mμ; λ_{max}, 245 mμ, λ_{min}, 226 mμ; ϵ_{max} at 298 mμ/P was 8,660.

Anal. Calcd. for $C_{16}H_{21}N_4O_8P \cdot 3H_2O$: C, 39.82; H, 5.64; P, 6.42. Found[52]: C, 40.10; H, 5.53; P, 6.94.[54]

N^6-Benzoyl-3′-O-tetrahydropyranyldeoxycytidine-5′ Phosphate.—An aqueous solution of the pyridinium salt of 3′-*O*-tetrahydropyranyl-deoxycytidine-5′ phosphate (0.80 mmole) was evaporated with added pyridine. The resulting gum was evaporated with five successive portions (10 ml. each) of anhydrous pyridine and the resulting partly crystalline gum was covered with 20 ml. of anhydrous pyridine. Benzoic anhydride (3.6 g., *ca.* 16 mmole) was added and the reaction flask shaken in the dark until the nucleotide had dissolved (2–3 hr.). The clear solution was then kept for a further 24 hr. One half (10 ml.) portion was then withdrawn and added slowly with stirring to 20 ml. of 50% aqueous pyridine. The solution was carefully evaporated to small bulk, adding pyridine from time to time and then water added in excess. The mixture was extracted with ether, the ether extracts washed with aqueous pyridine and the combined aqueous pyridine layer evaporated with pyridine and the volume adjusted to 10 ml. The following operations were performed on aliquots from this stock solution:

(a) A small sample was mixed with an equal volume of concd. ammonia and, after 12 hr. at room temperature, the solution was examined by paper chromatography in Solvent B. 3′-*O*-Tetrahydropyranyldeoxycytidine-5′ phosphate was practically the sole product, a trace of deoxycytidine-5′ phosphate being also present.

(b) A 2-ml. sample was mixed with an equal volume of 2 *N* sodium hydroxide and after 1 minute[55] at room temperature an excess of pyridinium Amberlite IR-120 resin was added. The resin was removed, washed thoroughly with water and the combined solution applied to the top of a DEAE column (14 cm. × 2 cm. diameter) which had been prepared in the bicarbonate form. Elution was carried out by using a linear gradient technique, the two vessels containing 500 ml. each of 0.005 *M* triethylammonium bicarbonate (*p*H 7.4) and 0.125 *M* solution of the same buffer. The flow rate was 1 ml./min. and 5 ml. fractions were collected. The first peak (20–40 fractions) contained benzoic acid. The fractions at the start of the second major peak were all examined for their ultraviolet absorption spectra and were apparently contaminated with substance(s) having characteristic deoxycytidine spectrum. The bulk of the peak containing characteristic N-benzoyideoxycytidine spectrum contained 690 optical density units at 303 mμ and was twice lyophilized to dryness to give a powder which was very hygroscopic. The spectral characteristics were as described below for N^6-benzoyldeoxycytidine-5′ phosphate. A sample which when weighed had obviously absorbed moisture showed a phosphorus content of 3.7%. The yield based on the phosphorus analysis was 71%. The ϵ_{max}/P at 303 mμ was ascertained to be 12,000.

Paper chromatographic examination in solvent A showed a strong spot of the desired compound and a barely visible slower spot which could correspond to either N^6-benzoyldeoxycytidine-5′ phosphate or 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate.

For a study of the rate of removal of the tetrahydropyranyl group, the above product was kept in an excess of 50% aqueous acetic acid at room temperature and aliquots applied on paper chromatograms at different time intervals. The chromatograms were developed in solvent E (see below). After 2 hr., N^6-benzoyldeoxycytidine-5′ phosphate was practically the only product, traces of the unchanged compound and a slower travelling product (debenzoylated product) being present. The rate of removal of the tetrahydropyranyl group, thus, was similar to that observed above for N^6-acetyl 3′-*O*-tetrahydropyranyldeoxycytidine-5′ phosphate.

N-Benzoyldeoxycytidine-5′ Phosphate.—(a) An aqueous solution of pyridinium deoxycytidine-5′ phosphate (0.84 mmole, as determined spectrophotometrically) was evaporated and the residue rendered anhydrous by repeated evaporations from added pyridine. Pyridine (10 ml.) was then added, followed by benzoic anhydride (2.2 g.). The flask was shaken in the dark at room temperature until a clear solution resulted (2–3 days). After a further period of 12 hr., water was added in excess, the mixture was set aside for 3 hr. and the solvent removed under reduced pressure. Water was added and unreacted benzoic anhydride removed by ether extractions. The aqueous layer was concentrated and the oil which separated was dissolved in water (28 ml.) containing a little pyridine. Sodium hydroxide (28 ml. of 2 *N*) was added and after 40 min. at room temperature an excess of pyridinium Amberlite IR-120 resin was stirred in. The aqueous phase was passed over more resin in a column to ensure complete removal of the sodium ions and the total solution was evaporated to a gum with a little pyridine. Two ml. of 1 *M* ammonium acetate (*p*H 6.0) was added, followed by 5 ml. of isopropyl alcohol. The solution was applied to a column of cellulose (32 cm. long × 4.2 cm. diameter) which had been packed and equilibrated in the same solvent mixture (solvent A). Elution with the same solvent gave several peaks (as determined by their absorption at 270 mμ) which were identified by paper chromatography. The results are given.

Peak 1 appeared in fractions 45–53 and consisted of benzoic acid. Peak 2, which was not well resolved from the preceding and succeeding peaks, was relatively minor and was not identified. Its spectrum resembled that of N^6-benzoyldeoxycytidine. Peak 3 was the major peak and appeared in fractions 60–80. It consisted of N-benzoyldeoxycytidine-5′ phosphate and contained 6,400 optical density units at 270 mμ. Peak 4, which consisted of deoxycytidine-5′ phosphate (700 optical density units at 270 mμ) appeared much later (fractions 170–200). Peak 3 was pooled so as to avoid contamination with material of Peak 2 and evaporated to small bulk with pyridine and re-evaporated once with aqueous pyridine to remove isopropyl alcohol. The concentrate was passed over a column of pyridinium Amberlite IR-120 resin (total resin volume, 100 ml.). The total effluent and washings were concentrated to small bulk in a rotary evaporator using low pressure and ice-cooling in the condenser vessel. The process was repeated four times with dilute aqueous pyridine to remove most of the pyri-

tives than with the parent tetrahydropyranyldeoxycytidine-5′ phosphate.

(51) Continued elution after the desired peak gave a small peak containing deoxycytidine-5′ phosphate.

(52) The analyst reported a weight loss (0.234 mg. out of 3.882 mg.) on drying over P_2O_5 for 24 hr. at room temperature. The analyses given are based on the weight of the dried sample.

(53) By the method of E. J. King, *Biochem. J.*, **26**, 292 (1932).

(54) The phosphorus analysis was carried out on a sample weighed soon after lyophilization.

(55) The brief treatment suffices to cleave the mixed anhydride (benzoyl phosphate) bond.

dinium acetate. The concentrate was then diluted with more aqueous pyridine and the solution lyophilized. The product from the first lyophilization was re-dissolved in aqueous pyridine and re-lyophilized. Monopyridinium N-benzoyldeoxycytidine-5′ phosphate was obtained in this way as a white powder which did not absorb any water when left on the balance for 5 min. The yield as based on phosphorus analysis was 49%. The ultraviolet absorption characteristics of the substance as ammonium salt at *p*H 7.5 were: λ_{max}, 304 and 259 mμ; λ_{min}, 283 and 233 mμ. The ϵ_{max}/P at 304 mμ was 12,100. (In 1 *N* NaOH λ_{max} shifted from 304 to 315 mμ.)

Anal. Calcd. for $C_{16}H_{18}N_3O_8P \cdot 1C_5H_5N \cdot 1.5\ H_2O$ (mono-, pyridinium salt): C, 48.7; H, 5.03; N, 10.8; P, 5.99. Found: C, 48.4; H, 5.08; N, 10.7; P, 6.22.

(b) The substance has been prepared by benzoylation of deoxycytidine-5′ phosphate with an excess of benzoyl chloride, followed by treatment with 1 *N* sodium hydroxide and then chromatographed using DEAE-cellulose (acetate form) and triethylammonium acetate as eluent. Except for the fact that shorter time (5 min.) was given for the alkaline treatment, the procedure is exactly as described below for N^6-anisyldeoxycytidine-5′ phosphate under (a).

N^6-Anisyldeoxycytidine-5′ Phosphate.—(a) Pyridine (3 ml.) was added to a finely divided sample of 0.1 mmole of deoxycytidine-5′ phosphate (prepared by lyophilization of a dilute aqueous pyridine solution) followed by 0.3 ml. of distilled anisyl chloride. The clear solution which soon resulted on shaking was kept in the dark at room temperature for 1 hr. Water (10 ml.) was added in an ice-bath and the mixture rapidly extracted three times with 15 ml. portions of chloroform and the total chloroform extracts were washed twice with 5 ml. portions of water. The chloroform solution was then evaporated under reduced pressure and the gum was taken up in 2 ml. of pyridine. To the solution was added 1 ml. of water and after slight cooling, sodium hydroxide (3 ml. of 2 *N*) was added. The clear solution which resulted on shaking within the first minute was kept at room temperature for 25 min. An excess of Amberlite IR-120 (H^+) was added (neutralization to *p*H about 8 is marked by a color change—reddish to pale yellow) and then the aqueous layer was passed slowly through a column (8 cm. × 1.5 cm.) of pyridinium Amberlite IR-120 (20–50 mesh) to ensure complete removal of sodium ions. The total effluent and washings (*ca.* 50 ml.) were evaporated in a rotary evaporator under reduced pressure. (Ice-bath was used to cool the condenser vessel and the temperature of the bath outside the evaporation flask was not allowed to rise above 20°.) When the volume reduced to about 20 ml., anisic acid separated and the *p*H of the solution fell to about 4.6. Three extractions were carried out with 50 ml. portions of ether. Paper chromatographic examination in solvent E showed that N-anisyldeoxycytidine-5′ phosphate (fluorescent spot under ultraviolet light) was practically the sole product. Only traces of faster-travelling fluorescent spots and anisic acid were present. The *p*H of the aqueous solution was around 4. An equal volume of pyridine was added and the solution was carefully evaporated to a small volume. (At least equal volume of pyridine must be present in order to avoid fall of *p*H.) The concentrated aqueous pyridine solution (*p*H about 7) was applied to the top of a DEAE-cellulose (acetate form) column (15 cm. × 2 cm.). After a water wash (*ca.* 50 ml.) elution was begun using a linear salt gradient with the mixing vessel containing 1 liter of water and the second vessel 1 liter of 0.2 *M* solution of triethylammonium acetate (*p*H 5) buffer. A flow rate of 1 to 1.2 ml./min. was maintained and 7–8 ml. fractions were collected. After two very minute peaks between fractions 60–70 and 80–90, N^6-anisyldeoxycytidine-5′ phosphate emerged in fractions 100 to 140. The combined fractions (290 ml. in volume), which accounted practically for all of the ultraviolet absorbing material put on the column, were evaporated under reduced pressure. The residual syrup was taken up in water and the solution again evaporated under reduced pressure. In all, six repetitions of this process were carried out to remove most of the triethylammonium acetate. Finally, the residue was dissolved in water and the solution passed through a column (10 cm. × 1.5 cm.) of pyridinium Dowex-50 ion exchange resin, the column being washed with several bed volumes of water. The total effluent and washings were lyophilized.[56] Monopyridinium N^6-anisyldeoxycytidine-5′ phosphate was obtained as a fluffy white powder.

Anal. Calcd. for $C_{22}H_{25}N_4O_9P \cdot 1H_2O$[57] (monopyridinium salt): C, 49.06; H, 5.08; N, 10.45; P, 5.74. Found: C, 49.45; H, 5.49; N, 10.48; P, 5.53 and 5.87.

The substance had the following spectral characteristics as ammonium salt in *p*H 7.5 phosphate buffer: λ_{max}, 302 mμ, and an inflection at 255 mμ; λ_{min}, 235–236 mμ. The ϵ_{max}/P at 302 mμ was 22,430.

(b) The preparation has been carried out many times, starting with 1 mmole of deoxycytidine-5′ phosphate and the product obtained without column chromatography has been used directly in polymerization experiments. The following points may be noted. The anisylation is carried out on tenfold scale and worked up exactly as above up to the point of alkaline treatment. The syrupy residue obtained on evaporation of the chloroform solution is dissolved in 20 ml. of pyridine. Water (10 ml.) is quickly added under agitation, when spontaneous crystallization may occur. Thirty milliliters of 2 *N* sodium hydroxide is added immediately after and the flask cooled momentarily in an ice-bath. The initial two-phase mixture turns to a clear light orange solution within one minute. The solution is kept for 18 min.[58] at room temperature. An excess of IR-120 (H^+) ion exchange resin (60–70 ml.) is then added and the total aqueous pyridine solution and washings are passed slowly through a pyridinium IR-120 resin column (3 cm. diameter × 20 cm.) to ensure the complete removal of all sodium ions. The total effluent and washings (with 5% pyridine) are concentrated to about 40 ml. Anisic acid, which separates, is removed by filtration and the solid washed with water. The total filtrate is concentrated again at low temperature to about 40 ml. and any anisic acid which separates is again removed. The *p*H of the final solution is 3.8–4 and is safe provided temperature is kept below 20° during evaporation. The aqueous solution is extracted three times with 50 ml. portions of ether and then diluted with a little more than equal volume of pyridine before evaporation. The final residue is dissolved in pyridine to a standard volume. The yield is estimated as follows:

A known aliquot (about 0.1 ml.) is kept in 1 ml. of concentrated ammonia solution in a sealed flask at room temperature for 24 hr. to remove the anisyl group and the solution is evaporated to a solid residue. The latter is dissolved in 1 ml. of 50% aqueous pyridine and 0.1 ml. aliquots are applied in duplicate on paper chromatograms which are developed in Solvent B. Deoxycytidine-5′ phosphate bands are eluted and estimated spectrophotometrically. The overall yield of N-anisyldeoxycytidine-5′ phosphate in different runs has consistently been 86–92%. The standardized pyridine solutions prepared as above have been used within one week. On being kept in refrigerator for 2–4-month periods, they showed, on chromatography in Solvent E, traces of anisic acid and deoxycytidine-5′ phosphate.

Polymerization of a Mixture of N^6,3′-O-Diacetyldeoxycytidine-5′ Phosphate and N^6-Anisyldeoxycytidine-5′ Phosphate.—A pyridine solution of N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate (prepared from 0.25 mmole of deoxycytidine-5′ phosphate as described in the preceding paper[1]) was mixed with a pyridine solution of 0.75 mmole of N^6-anisyldeoxycytidine-5′ phosphate and the total solution evaporated under reduced pressure, using Dry-Ice trap. Ten ml. of fresh pyridine was added to the gum and the solution re-evaporated to a gum on an oil pump. The process was repeated three times subsequently, using anhydrous pyridine each time and admitting dry air into the system after evaporations *in vacuo*. The frothy residue was finally dissolved in 0.5 ml. of anhydrous pyridine under exclusion of moisture, and to it was added rapidly under agitation a solution of 412 mg. (2 mmole) of dicyclohexylcarbodiimide in 0.5 ml. of

(56) As mentioned earlier,[49] lyophilization should proceed throughout from a solid surface, so that a completely solid powder is obtained. The formation of gum causes decomposition because the *p*H of the final product (monopyridinium salt) when tested by dissolving in water is between 2 and 3. For storage as solution, the *p*H should immediately be brought up to neutrality.

(57) The analyst reported a weight loss of 0.182 mg. from 3.160 mg. sample on drying for two days over P_2O_5 at room temperature. The analyses reported are on the residual sample.

(58) The somewhat shorter time reduces further the possibility of the liberation of any deoxycytidine-5′ phosphate. On the other hand, a small percentage of N^6,3′-*O*-dianisyldeoxycytidine-5′ phosphate still remains.[22]

pyridine. The whole heterogeneous mixture was agitated vigorously for about ten minutes, during which time the insoluble layer hardened to a gum. The contents were shaken mechanically in the dark for six days at room temperature. A dilute sodium hydroxide solution (3 ml. of 1 N sodium hydroxide + 5 ml. of water) was added under shaking and the mixture left for about 40 min. at room temperature. During this time, the mixture was extracted twice with ether and the insoluble dicyclohexylurea removed by filtration. The total alkaline solution was then neutralized to *p*H 9 by careful addition of Amberlite IR-120 (H^+) resin and the resin removed by filtration and thoroughly washed with water. The total aqueous solution was evaporated to a syrup and to it was added 25 ml. of concd. ammonia. The stoppered solution was left at room temperature for at least 48 hr. before evaporation. The insoluble anisamide was removed by ether extraction and the aqueous solution adjusted to *p*H 9 with alkali for column chromatography.

Chromatography of Polymeric Mixture on a DEAE-Cellulose (Carbonate) Column.—The aqueous solution as obtained above was applied on top of a DEAE-cellulose (carbonate) column (33 cm. × 4 cm. diameter) and washed in with three 20-ml. portions of water. The column was further washed with 700 ml. of water to remove any unabsorbed (anisamide, etc.) material and then elution was begun using a linear gradient technique with 4 l. of 0.002 M triethylammonium bicarbonate (*p*H 7.5) in the mixing vessel and an equal volume of 0.25 M triethylammonium bicarbonate (*p*H 7.5) in the reservoir. When this eluent had passed through, the elution was continued with 0.25 and 0.5 M triethylammonium bicarbonate, respectively, in the mixing vessel and the reservoir (4 liters of each, again). Approximately 20 ml. fractions were collected at a flow rate of 3 ml./min. The elution pattern is shown in Fig. 1. The distribution of the nucleotidic material and identification of the different peaks are shown in Table I. Each of the pooled peaks was evaporated under reduced pressure to a syrup, which was re-dissolved in water and the solution re-evaporated. Several repetitions of this process were usually necessary to ensure complete removal of triethylammonium bicarbonate. The final residues were dissolved in water and processed further as desired. Those that were subjected to purification by paper chromatography were passed through small beds (2–3 cm. × 1 cm.) of ammonium Dowex-50 ion exchange resin to convert to the ammonium salt form. Others were adjusted to *p*H 8–9 with ammonia before rechromatography.

Rechromatography of Linear Polynucleotides.—Examination of the peaks corresponding to di- and tri-nucleotides on paper electrophoresis at *p*H 7.5, paper chromatography in solvent D and the lack of complete disappearance of the original 'spots' after treatment with phosphomonoesterase all indicated non-homogeneity of the peaks.

The procedure for purification by rechromatography is illustrated with respect to peak 9 (trinucleotide). The total material was applied as a solution in 0.05 M lithium acetate buffer (*p*H 5) on top of a DEAE-cellulose (chloride form) column (15 cm. × 2.2 cm. dia.) pre-equilibrated with the same buffer. (In the subsequent experiments the columns were not pre-equilibrated and the solutions were applied at *p*H 8–9.) No difference in results was noted. Elution was carried out using a linear gradient technique, the mixing vessel containing 0.05 M lithium acetate buffer (*p*H 5) and the reservoir containing 0.05 M lithium acetate buffer + 0.1 M lithium chloride. The volume of liquid in both vessels was 2 l. each. A flow rate of 1.2 ml./min. was maintained and 12 ml. fractions were collected. The elution pattern obtained is shown in Fig. 2. The main central peak corresponded to the pure trinucleotide, isolated by the procedures described below.

For rechromatography of peak 7 (dinucleotide) by the above technique, the mixing vessel contained 2 l. of 0.05 M lithium acetate buffer (*p*H 5) and the reservoir contained 2 l. of 0.05 M lithium acetate + 0.05 M lithium chloride. The major peak of pure dinucleotide appeared first, followed much later by the minor peak (14%) corresponding to the pyrophosphate (XVI).

For rechromatography of peak 11 (tetranucleotide) the column size and general conditions were as above, except that the elution was carried out using 4 l. of 0.05 M lithium acetate in the mixing vessel and 4 l. of 0.05 M lithium acetate + 0.2 M lithium chloride in the reservoir. The major peak appeared at lithium chloride concentration between 0.115 and 0.125 M.

Repeated attempts at rechromatography of the pentanucleotide (peak 12) under above conditions failed to give good sharp peaks; the material emerged slowly and continuously over a wide area after the chloride concentration was over 0.1 M. With the pentanucleotide satisfactory results were obtained when the *p*H of the buffer was raised to 5.8. The major central peak, although close to the preceding and succeeding peaks of impurities, was of pure pentanucleotide. Attempts to purify the hexanucleotide (peak 13) by this technique have failed so far.

Recovery of Polynucleotides after Rechromatography.—The pooled peaks of pure polynucleotides and of side-products (*e.g.*, XVI from the original dinucleotide peak), as obtained above, were all neutralized to *p*H 9 with ammonia and diluted with water so as to reduce the concentration of the chloride ions to below 0.03–0.04 M. The total solutions were passed slowly (about 1.5 ml./min.) through small columns (1.2 cm. × 4–5 cm. long) of Dowex-1 (2% cross-linked) (chloride form). The column was washed subsequently with water and then with 0.05 N HCl in the case of di- and tri-nucleotides and with 0.1 N HCl in the case of higher polynucleotides. The effluent was collected in fractions which were examined for ultraviolet absorbing material. The polynucleotides were all eluted rapidly in a total of 40–50 ml. volume. The combined acidic solution of the polynucleotide was neutralized with lithium hydroxide and evaporated to dryness, first using a water aspirator and then an oil pump. To the residue were added 2 ml. of methyl alcohol and the resulting solution or suspension was transferred to a centrifuge tube. Inclusive of washings, the total volume of methyl alcohol used was 4–5 ml. Acetone (about 20 ml.) was added, followed by 5 ml. of ether and the precipitated polynucleotide collected by centrifugation. The centrifugate was washed by adding 2 ml. of methyl alcohol, followed by an excess of acetone. The process was repeated once and then the material was washed finally with acetone and dried at room temperature. The lithium salt thus obtained was converted to the ammonium salt by passing through an ammonium Dowex-50 ion exchange resin bed, the latter being washed thoroughly with water brought to *p*H 8–9 with a trace of ammonia. Sometimes the lithium salts of polynucleotides were passed through pyridinium Dowex-50 ion exchange resin beds in which case 2–5% aqueous pyridine was used to wash the bed thoroughly to ensure complete elution of the polynucleotide. (The final concentrated solutions of ammonium or pyridinium polynucleotides tended to have acidic *p*H by virtue of the evaporation of the volatile bases and the *p*H was adjusted routinely to about 8 with ammonia.)

Degradation of Polynucleotides Using Venom Phosphodiesterase.—The preparation of venom phosphodiesterase used throughout the present work and the general conditions of its use are as described in the preceding paper.[1] Thus the degradations of compounds of the type XII (results in Table IV) were performed by lyophilizing solutions of the compounds containing appropriate amounts in small test tubes and adding to the residue 0.01 ml. of 2 M ammonium carbonate buffer (*p*H 9) and 0.04 ml. of the phosphodiesterase preparation. The amounts of the different compounds used were: about 3 optical density units (at 280 mμ in 0.1 N hydrochloric acid) of CpC; about 4–5 optical density units of CpCpC and proportionately increasing optical density units of the higher members. On the average a sufficient amount of each compound was used so as to liberate 1–1.5 optical density units of the terminal deoxycytidine after complete degradation. The total time of incubation was about 6 hr. at 37°. This period of time and the amount of the enzyme used are in several fold excess of the minimal conditions that were found to be necessary for complete degradation of pCpCpC described below.

The following experiments with the diesterase showed that the rate of degradation of cyclic oligonucleotides (XIV) was much lower than that of linear polynucleotides: the linear trinucleotide pCpCpC (0.02 ml., 7.5 optical density units) was treated with 0.02 ml. of phosphodiesterase + 0.01 ml. of ammonium carbonate buffer at 37°. Degradation to the mononucleotide (pC) was complete in the first aliquot examined after 1 hr. by paper chromatography in solvent C. Lyophilized samples of cyclic dinucleotide (about 20 optical density units), cyclic trinucleotide (about 10 optical density units) and cyclic tetranucleotide (about 4 optical density

units) were each incubated under the standard conditions but with larger amounts (0.05 ml.) of the phosphodiesterase preparation. Aliquots were removed after 4 and 8 hr. and examined by paper chromatography in solvent B. Cyclic dinucleotide was about 60% degraded after 8 hr., cyclic trinucleotide was degraded about 60% in 4 hr. and almost completely in 8 hr. The cyclic tetranucleotide was completely degraded in 8 hr.

Enzymic Removal of Terminal Phosphomonoester Groups.—Bacterial alkaline phosphatase was used in all the preparations of compounds of the type XII and for testing purity of the different oligonucleotides (XI). The preparation and the manner of its use have been described in the preceding paper.[1]

Dephosphorylation of N^6-Benzoyl- and N-Anisyl-deoxycytidine-5′ Phosphate.—Solutions of the nucleotides (about 1 μmole in 0.03 ml. of water) as ammonium salts were incubated with a solution (0.02 ml.) of crude rattlesnake venom[59] in 0.1 *M* trihydroxymethylaminomethane buffer (*p*H 8) and an additional 0.01 ml. of 1 *M* solution of the same buffer. Dephosphorylation of the nucleotides to the N^6-acylnucleosides was complete in the first aliquot examined after 45 min. The aliquot was applied on a paper chromatogram which was developed in solvent E; the original nucleotide spots were replaced by spots travelling faster (close to the front) on the chromatogram. N^6-Benzoyldeoxycytidine thus formed was identical in its mobility in Solvent H with a crystalline sample of the substance.[6]

Degradation of the Series $C(pC)_npC$ by Spleen Phosphodiesterase and Identification of the Resulting Mononucleotide as Deoxycytidine-3′ Phosphate.—The preparation of spleen phosphodiesterase used was given by Dr. W. E. Razzell. It had been prepared by the method of Hilmoe.[33] The activity of preparation was determined against TpT and CpC.

In the first experiment 1 μmole of TpT (in 0.02 ml. volume) was incubated in 0.02 ml. of 1 *M* ammonium acetate buffer (*p*H 6.5) with 0.02 ml. of the enzyme preparation. Aliquots were removed and examined on paper chromatograms in solvent B. Hydrolysis to thymidine-3′ phosphate and thymidine was complete in about 1 hr. In another experiment with CpC, 0.2 μmole of the substrate (0.04 ml. volume) was incubated with the amounts given above of the enzyme and buffer. Hydrolysis was complete within the time (1 hr.) the first aliquot was examined.

The degradation of CpCpC and higher homologs up to $C(pC)_6pC$ was carried out using sufficient amounts of the homologs so as to release about 1–1.5 (0.08–0.11 μmole) optical density units of terminal deoxycytidine. All the incubations were carried out using the substrates in 0.05 ml. water and adding 0.02 ml. of 1 *M* ammonium acetate buffer (*p*H 6.5) and 0.03–0.04 ml. of the enzyme preparation standardized above. Incubations were carried out for a period of 5 hr. at 37°. Subsequent examination on paper chromatograms (solvent B) showed that degradation to deoxycytidine-3′ phosphate and deoxycytidine was complete in every case, except in the case of $C(pC)_5pC$ and $C(pC)_6pC$ where a trace of ultraviolet absorbing material was seen under the ultraviolet lamp at the origin. (The amounts were too small to ascertain if the material was nucleotidic. In all cases the degradations were concluded to be over 95% complete.) The nucleotide bands from all the degradations were eluted and combined and rerun on a paper chromatogram and re-eluted. The total material (about 2 μmole) was characterized as deoxycytidine-3′ phosphate as follows: (a) About 0.25 μmole of the material was dephosphorylated by digestion with the alkaline phosphomonoesterase and the resulting deoxyribonucleoside examined on a paper chromatogram developed in solvent H, using deoxycytidine and deoxyuridine as markers. (The solvent is particularly suited to the separation of the two nucleosides.) There was only one strong spot present, corresponding in mobility with deoxycytidine. The result rules out the possibility of any deamination during the alkaline treatments given in the work-up of the polymeric mixtures. (It may be noted that in earlier experiments N^6-benzoyl- and N^6-anisyl-deoxycytidine-5′ phosphates had also been treated with concentrated ammonia to remove the acyl groups and the nucleotides formed shown to be free from deoxyuridine-5′ phosphate by paper electrophoresis at *p*H 3.5 and also by dephosphorylation with phosphomonoesterase and subsequent chromatography in solvent H.) (b) An artificial mixture of one μmole of synthetic deoxycytidine-3′ phosphate and deoxycytidine-5′ phosphate (2μ mole) was made and its separation effected on a column (8 cm. × 1 cm. dia.) of Dowex-1 (8% cross-linked) (formate form) ion exchange resin. The elution was carried out with 0.01 *M* formic acid.[60] The elution positions of the two substances, which were well separated from each other, were thus determined. Deoxycytidine-3′ phosphate obtained as above was then chromatographed under identical conditions. It emerged as a single peak in the expected position and, furthermore, all of the ultraviolet absorbing material applied was accounted for by the peak.

Acidic Degradation of Deoxycytidine-5′ Phosphoryl-(N, N′-dicyclohexyl)-urea (XV).—About 5 optical density units of the substance was treated with 0.2 ml. in 1 *N* hydrochloric acid at room temperature. Aliquots were removed at intervals and examined by paper chromatography in Solvent B. The small amount of the substance did not permit an accurate determination of the rate of hydrolysis, but more than 50% apeared to be converted to deoxycytidine-5′ phosphate within 2 hr. In another experiment the total amount of the phosphorylurea available from two polymerization runs on 1 mmole scale was hydrolyzed with 1 *N* hydrochloric acid. A highly water-insoluble product was formed which could be crystallized from aqueous ethyl alcohol. The amount was too small for detailed characterization but from its physical appearance was evidently dicyclohexylurea.

Degradation of the Pyrophosphate, XVI, by Acetic Anhydride.—XVI was prepared either by separation of the original peak 7 (Fig. 1) on paper chromatograms developed in solvent D or preferably by rechromatography on a DEAE-cellulose (chloride form) column as described above for linear polynucleotides. XVI emerged after the major linear dinucleotide. About 10 optical density units (at 280 mμ in acid) of the substance were passed through a small bed of Dowex-50 (pyridinium form) ion exchange resin and the bed washed thoroughly with 5% aqueous pyridine. The residue was evaporated to dryness under reduced pressure and rendered anhydrous by re-evaporation of added pyridine. One ml. of pyridine and 0.25 ml. of acetic anhydride were added and the sealed flask shaken mechanically in the dark for 18 hr. Water was added in excess and after 2 hr. at room temperature, most of the pyridinium acetate was removed by repeated evaporation under reduced pressure. The residue was then kept in concd. ammonia for 4 hr. at room temperature. Ammonia was evaporated and the residue chromatographed in solvent D. Three bands, corresponding in R_f's to the starting material, the dinucleotide (XI; n = 0) and deoxycytidine-5′ phosphate were present. In addition, a heavy ultraviolet absorbing material, but non-nucleotidic in character, was present. The product, as mentioned above, arises from the reaction of acetic anhydride with pyridine.

Products of Acidic Treatment of N^6-Acyldeoxycytidine-5′ Phosphates: (a) N^6-Benzoyldeoxycytidine-5′ Phosphate.—The nucleotide was kept at room temperature in an excess of 50% aqueous acetic acid. Aliquots were removed and examined by paper chromatography in solvent A. Even after 8 days, more than 50% of the starting material appeared to be present and the nucleotidic products of hydrolysis were deoxyuridine-5′ phosphate and deoxycytidine phosphate, the latter being the slower-travelling product in the solvent. There were two fast-travelling ultraviolet absorbing products visible, which were presumably benzoic acid and benzamide. The ratio of deoxycytidine-5′ phosphate to deoxyuridine-5′ phosphate was estimated spectrophotometrically to be roughly 2:1. In another experiment in which the starting nucleotide was heated in 50% acetic acid at 100°, both deoxyuridine-5′ phosphate and deoxycytidine-5′ phosphate were again formed, but there was also observed another fluorescent product travelling with the R_f of the starting material, and the results were therefore not clear-cut.

The pattern seen on chromatograms afer heating N-anisyldeoxycytidine-5′ phosphate in 50% acetic acid was very complex and there were at least seven products noted. It is probable that both deamination to uracil derivatives and N-glycosyl bond cleavage occurred during the above treatment.

(59) *Crotalus adamanteus*, purchased from Ross Allen's Reptile Farm, Florida.

(60) W. E. Cohn in "Biochemical Preparations," Vol. V, D. Shemin, editor, J. Wiley and Sons, Inc., New York, N. Y., 1957, p. 40.

(b) **N^6,3'-O-Diacetyldeoxycytidine-5' Phosphate.**—The lyophilized material prepared as described previously was kept at room temperature in 50% acetic acid. Most of the N-acetyl group had been removed in 3–4 days. After a total of 7 days, the total products were streaked on a strip of paper which was developed in solvent G. Although there were three other trace bands present, most of the nucleotide material was contained in two bands. Of these, the faster travelling band (band 1) was very much stronger than the slower band (band 2). Bands 1 and 2 were eluted and treated with concentrated ammonia, to remove *O*-acetyl group, and the resulting products were dephosphorylated by incubation with alkaline phosphomonoesterase. The nucleosides obtained were chromatographed in solvent H. Band 1 was found to give mostly deoxycytidine but also some deoxyuridine (the amount of the latter was estimated to be 12%). Band 2 also gave deoxycytidine and a barely visible trace of deoxyuridine. From these results, it is concluded that band 1 originally contained mostly 3'-*O*-acetyldeoxycytidine-5' phosphate and some deoxyuridine-5' phosphate, while band 2 contained mostly deoxycytidine-5' phosphate, some deacetylation of the 3'-*O*-acetyl group having occurred. The extent of deamination to uracil nucleotide was 10%.

Paper Chromatography and Paper Electrophoresis.—Paper chromatography was performed using double-acid washed papers (Whatman No. 40 or 44) and the descending technique. The solvent systems used were: solvent A, isopropyl alcohol–1 *M* ammonium acetate (*p*H 6) (5–2, v./v.); solvent B, isopropyl alcohol–concd. ammonia–water (7–1–2); solvent C, *n*-propyl alcohol–concd. ammonia–water (5.5–1–3.5); solvent D, ethyl alcohol–0.5 *M* ammonium acetate (*p*H 3.8), (5–2); solvent E, ethyl alcohol–1 *M* ammonium acetate (*p*H 7.5) (5–2); solvent F, isobutyric acid (100 ml.)–1 *N* ammonia (60 ml.)–0.1 ethylenediaminetetraacetate-disodium (1.6 ml.); solvent G, *n*-butyl alcohol–acetic acid–water (5–2–3); solvent H, *n*-butyl alcohol–water (86–14). The R_f's of different compounds in the different solvent systems are listed in Table III.

Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith,[61] using 0.05 *M* triethylammonium bicarbonate (*p*H 7.5) buffer and 0.05 *M* ammonium acetate (*p*H 3.5) buffer or simply 1 *M* acetic acid for acidic *p*H.

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

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL AND THE DEPARTMENT OF PHARMACOLOGY OF THE UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER 8, B. C., CANADA]

Cyclic Phosphates. IV.[1] Ribonucleoside-3',5' Cyclic Phosphates. A General Method of Synthesis and Some Properties

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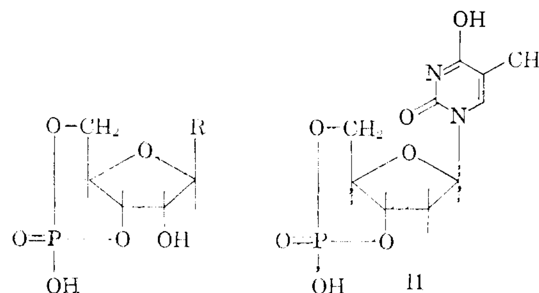
A general method for the synthesis of ribonucleoside-3',5' cyclic phosphates is described. It involves the reaction of a 4-morpholine-N,N'-dicyclohexylcarboxamidinium (III) salt of a ribonucleoside-5' phosphate with dicyclohexylcarbodiimide under dilute conditions. Using this method, adenosine-, guanosine-, cytidine- and uridine-3',5' cyclic phosphates have been prepared in yields of 60–85%. The preparation of N^6-benzoyl cytidine-5' and N-benzoylguanosine-5' phosphates, the starting materials in two of the above syntheses, is described. Acidic, alkaline and enzymic degradations of the cyclic phosphates have been studied. The results show that the six-membered phosphate ring exerts a profound and characteristic influence on the stabilities of the glycosyl bonds in the nucleotides. Purine glycosyl bonds are rendered remarkably stable to acid while the glycosyl bond in uridine-3',5' cyclic phosphate is strikingly labilized. Barium ions catalyze the alkaline hydrolysis of the six-membered phosphate ring, the products being the nucleoside-3' and nucleoside-5' phosphates in the ratio of 5:1. Some observations on the action of different phosphodiesterases on the cyclic phosphates are recorded.

An adenine nucleotide was recognized by Sutherland and co-workers[5,6] as a factor stimulating the conversion of inactive glycogen phosphorylase to the active form in liver preparations. The substance,[6] which proved to be identical with a product arising from the barium hydroxide-catalyzed degradation of adenosine-5' triphosphate,[7a] was shown by Lipkin and co-workers[7b] to be adenosine-3',5' cyclic phosphate (I, R = adenine).

A minor product formed during the polymerization of thymidine-5' phosphate was independently identified as thymidine-3',5' cyclic phosphate[8] (II)



I, R = purine or pyridine

and at the same time several methods for its synthesis were described.[8] In further work, uridine-3',5' cyclic phosphate (I, R = uracil) was synthesized in good yield from uridine-5' phosphate and was used as the key intermediate in the specific synthesis of the naturally occurring $C_{3'}$–$C_{5'}$ inter-ribonucleotidic linkage.[9]

The recognition of the biological importance of adenosine-3',5' cyclic phosphate and of the potentialities of this class of compounds as intermediates in the synthesis of ribopolynucleotides made it

(1) Paper III, H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, THIS JOURNAL, **79**, 430 (1957).

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

(3) The Institute for Enzyme Research, University of Wisconsin, Madison 5, Wis.

(4) Department of Pharmacology, University of British Columbia. The work in this department has been supported by a grant from The Life Insurance Medical Research Fund, New York.

(5) T. W. Rall, E. W. Sutherland and J. Berthet, *J. Biol. Chem.*, **224**, 463 (1957); T. W. Rall and E. W. Sutherland, *ibid.*, **232**, 1065 (1958).

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

(9) M. Smith and H. G. Khorana, *ibid.*, **81**, 2911 (1959).