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Studies on Polynucleotides. XII.[1] Experiments on the Polymerization of Mononucleotides. A Comparison of Different Polymerizing Agents and a General Improvement in the Isolation of Synthetic Polynucleotides[2]

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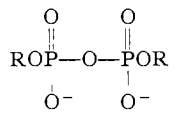
The polymerization of a standard mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate in anhydrous pyridine has been studied with different activating agents. The reagents used were dicyclohexylcarbodiimide (DCC) as previously described, *p*-toluenesulfonyl chloride, 2,5-dimethylbenzenesulfonyl chloride, 2,4,6-trimethylbenzenesulfonyl chloride, diphenylphosphorochloridate and diisopropylcarbodiimide. Of these, dicyclohexylcarbodiimide is most satisfactory. A general improvement in the isolation of synthetic polynucleotides is described which involves the treatment of the total mixture of polymeric products with an excess of acetic anhydride in dry pyridine. This step leads to the cleavage of the frequently encountered side products which consist of oligonucleotides joined together by pyrophosphate linkages between their phosphomonoester groups. With the acetic anhydride-pyridine step, pure thymidine and deoxycytidine oligonucleotides were obtained directly by chromatography of the total polymeric mixtures on DEAE-cellulose (bicarbonate) columns.

In several previous papers of this series[1,4] the polymerizations of a number of deoxyribomononucleotides and the isolation and characterization of the resulting homologous series of polynucleotides have been described. The reagent used in most of the work has been dicyclohexylcarbodiimide (DCC) and the reaction mixtures, at least initially, have been heterogeneous. A further more or less common feature of the polymerization experiments described is that side products are obtained in which some of the oligonucleotides are joined together by means of a pyrophosphate bond between the phosphomonoester groups. The process of internucleotide (phosphodiester) bond formation starting with the mononucleotide is believed to pass through a number of steps, the first one of which is the formation of the symmetrical P^1,P^2-dinucleoside pyrophosphates (I). Of the mononucleotides studied,



I, R = nucleoside

the process of phosphodiester bond formation reached nearest completion in the case of thymidine-5′ phosphate.[4c] In the study of the polymerization of thymidine-3′ phosphate, for example, significant amounts of oligonucleotides containing pyrophosphate bonds were present in the final products.[4b] In continuing our studies on the methods for the polymerization of mononucleotides, we have sought to compare the efficacy of some alternative reagents and the present communication describes the observations we have made on this subject. Further, a procedure involving treatment of the polymeric mixtures with acetic anhydride–pyridine is described which is effective in cleavage of the pyrophosphate bonds present in the polymeric products. Consequently, the isolation of pure homologous polynucleotides from the polymeric mixtures is rendered easier.

Comparative Study of Activating Agents.—In the first study of the polymerization of thymidine-5′ phosphate, *p*-toluenesulfonyl chloride and DCC were used as the reagents.[4a] However, thus far detailed investigations have been reported[4] only of the products obtained using DCC. Since, in contrast with the use of DCC, the reaction mixture using *p*-toluenesulfonyl chloride is homogeneous, a further examination of the latter reagent with a view to increasing the yields of the higher polymers has been undertaken. The polymerization of a mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate was carried out under the conditions recently described for DCC.[4c] The elution pattern obtained after chromatography on a DEAE-cellulose column under the standard conditions[4c] is shown in Fig. 1. A comparison of this elution pattern with that published recently for the analogous experiment with DCC shows that the polymerization did not go as far as it did using DCC. Furthermore, the pattern with the sulfonyl chloride in the range of hexa- and higher polynucleotides (Fig. 1) indicated greater complexity and evidently much lower yields of these higher members were present. In addition there was evidence for the appearance of a new series of compounds with altered ultraviolet absorption characteristics. Thus peaks No. 1, 2, 4 and 7 (Fig. 1) had ultraviolet absorption spectra showing shifts in the λ_{max} from that characteristic of the thymidine chromophore (peak 1, λ_{max} 257 and 263 mμ; peak 2, λ_{max} 252 mμ; peak 4, λ_{max} 252 mμ; peak 7, λ_{max} 260 mμ). It is possible that two of these peaks represent mono- and dinucleotides bearing *p*-toluenesulfonyl groups at the terminal 3′-hydroxyl groups, and that higher homologs of similar compounds also are present in the latter peaks. It should be mentioned that these side products are different from those that contain the pyridinium chromophore at the $C_{3'}$ or $C_{5'}$, carbon of the terminal nucleoside (cf. ref. 4b, 4c and the following paper).

2,5-Dimethylbenzenesulfonyl chloride and 2,4,6-trimethylbenzenesulfonyl chloride also were tried

(1) Paper XI, R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 2926 (1961).

(2) This work has been supported by grants from the National Cancer Research Institute of the National Institutes of Health and the National Research Council of Canada.

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(4) (a) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6223 (1958); (b) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959); (c) H. G. Khorana and J. P. Vissolyi, *ibid.*, **83**, 675 (1961); (d) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).

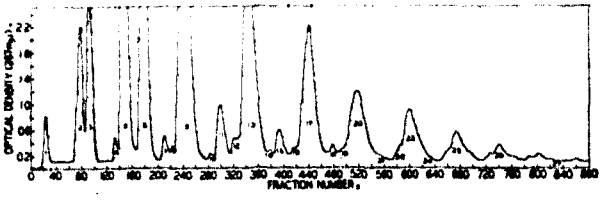
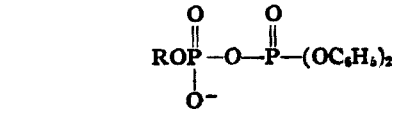


Fig. 1.—Chromatography on a DEAE-cellulose (bicarbonate) column of the polymeric mixture obtained by reaction of a mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate with *p*-toluenesulfonyl chloride. Conditions of chromatography have been described previously (page 684 of ref. 4c).

as polymerizing agents since it might have been expected that these would be more sterically hindered for the esterification of the 3′-hydroxyl group in thymidine-5′ phosphate. The results in regard to the composition of the products and the extent of polymerization were, however, very similar to those obtained with *p*-toluenesulfonyl chloride.

Diphenylphosphorochloridate was selected as another example of a reactive anhydride which could bring about polymerization, perhaps by a simpler mechanism in which the phosphorylating species would be of the type II. The elution pattern obtained after chromatography under standard conditions is shown in Fig. 2. The main features are that polymerization did not go very far and that the reagent was much less efficient in that large amounts of pyrophosphates survived. This was evident from the extra peaks present; for example, peak No. 5 corresponded to P^1,P^2-dithymidine pyrophosphate (I, R = thymidine-5′) and peak 9, following pTpT[5] and preceding the cyclic thymidine trinucleotide, corresponded to a pyrophosphate presumably formed by the joining of pT and pTpT through their phosphomonoester groups. Convincing evidence for the presence of pyrophosphates was obtained by treatment of the polymeric mixture with an excess of acetic anhydride (see the following section) and subsequent chromatography. The elution pattern was simpler in that the extra pyrophosphate peaks were absent. From these results it may be concluded that synthesis using diphenylphosphorochloridate also proceeded *via* exchange reactions involving the formation of complex pyro- and poly- or meta-phosphates, as has been proposed previously (see, *e.g.*, the following paper). The fact that large amounts of pyrophosphates survived is ascribed to the lower reactivity of diphenylphosphorochloridate as compared with *p*-toluenesulfonyl chloride.



II, R = nucleoside

Finally, as a variant of the carbodiimide class of compounds, diisopropyl-carbodiimide was tried with the hope that the smaller carbon content of this reagent as compared with that of DCC might provide more homogeneous reaction mixtures. The reaction mixture using diisopropylcarbodiimide, although insoluble at the start, did become homogeneous on the second day, in contrast with the reaction mixtures obtained using DCC which were heterogeneous for several days. The results of an experiment carried out under conditions identical with those used with DCC showed that the elution pattern was in general similar. However, the polymerization did not proceed as far as when DCC was used. Thus only 10% of the total nucleotide content of the polymeric products was eluted after the hexanucleotide peak. In the previously published experiment with DCC,[4c] approximately 25% of the total mixture appeared after the hexanucleotide peak.

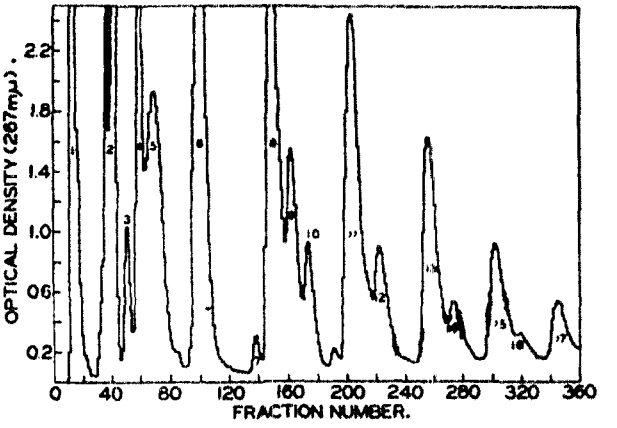


Fig. 2.—Chromatography on a DEAE-cellulose (bicarbonate) column of the polymeric mixture obtained by reaction of a mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate with diphenylphosphorochloridate. For conditions of chromatography see text.

From the comparative studies available, it may be concluded that DCC is the best of the reagents tested so far.[6] Because of the desirability of synthesizing polymers larger than those hitherto available, work on alternative methods of polymerization is being continued.

The Use of Acetic Anhydride in Cleavage of Pyrophosphate Bonds.—An earlier publication[7] has described the reversible formation of symmetrical pyrophosphates of the type I by treatment of monoalkyl phosphates with acetic anhydride in pyridine. Essentially quantitative cleavage of the pyrophosphates to give monoalkyl phosphates occurred when the pyrophosphates (I) were treated with an excess (about twentyfold) of acetic anhydride in dry pyridine followed by hydrolysis with water.[8] The finding promised to offer a very mild method for the hydrolysis of the P^1,P^2-disubstituted pyrophosphates, and its practical application in the cleavage of the pyrophosphate bonds surviving in the polymerization experiments has now been investigated.

The general technique used was to treat the total polymeric mixture in anhydrous pyridine with an

(5) Abbreviations as currently in use in the *J. Biol. Chem.* and as have been defined in earlier papers of this series (ref. 1, 4c, 4d).

(6) It may well be that the highest degree of polymerization obtained with DCC as compared with other reagents with which homogeneous solutions were obtained is due to the fact that diester bond synthesis proceeds well in the insoluble phase, where a higher concentration of the activated nucleotidic material is present.

(7) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

(8) For a discussion of the probable mechanism see ref. 7.

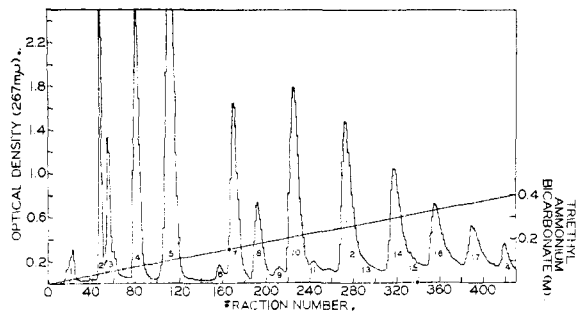
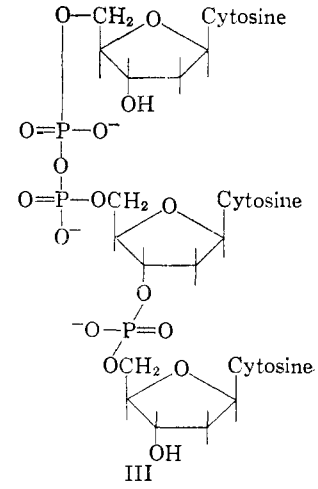


Fig. 3.—Chromatography on a DEAE-cellulose (bicarbonate) column of thymidine polynucleotides following treatment with acetic anhydride–pyridine mixture. For conditions of chromatography see text.

excess of acetic anhydride for three days at room temperature. If the polymeric mixture was insoluble in anhydrous pyridine, as, for example, that obtained by polymerizing a mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate, the addition of two equivalents of tri-*n*-butylamine caused solubilization. (A separate experiment in which P^1,P^2-dithymidine-5′ pyrophosphate was treated with acetic anhydride in anhydrous pyridine as the tri-*n*-butylammonium salt showed that degradation to thymidine-5′ phosphate went to completion.) After working up as described in the Experimental Section, chromatography on a DEAE-cellulose column in the bicarbonate form gave the elution pattern shown in Fig. 3. In comparison with the elution pattern of the same polymeric mixture reproduced earlier (Fig. 1 of ref. 4c), the present histogram showed the disappearance of ultraviolet absorbing material between the major peaks. The major peaks corresponding to thymidine dinucleotide and higher homologs were examined by the technique used earlier for ascertaining freedom from impurities and, in particular, pyrophosphates containing the same net negative charge as the homologous polynucleotide bearing a 5′-phosphomonoester group. This technique involved treatment with a phosphomonoesterase preparation which was free from phosphodiesterase or pyrophosphatase activity. Complete conversion of the starting material to a faster-travelling product on chromatography showed the absence of any pyrophosphate impurity.[1,4c,4d] By this technique, the di-, tri-, tetra- and pentanucleotide peaks as obtained from the single chromatographic operation (Fig. 3) were found to be pure. It will be recalled that in the previous work rechromatography of the initially obtained peaks was necessary to obtain pure polynucleotides.

In the recently described synthesis of deoxycytidine polynucleotides,[4d] the contamination of the major oligonucleotide peaks by the pyrophosphate type of compounds was more serious. Thus in the dinucleotide (d-pCpC) peak the substance concluded to be III was present in the amount of about 20%.

The removal of such impurities necessitated rechromatography under altered conditions. In the present work, the polymeric mixture obtained by polymerization of a mixture of N,3′-*O*-diacetyldeoxycytidine-5′ phosphate and N-anisyldeoxy-



III

cytidine-5′ phosphate was treated with acetic anhydride–pyridine and, after subsequent work-up and removal of protecting groups, the mixture was separated on a DEAE-cellulose column as previously described.[4d] Direct examination of the major peaks corresponding to di-, tri-, tetra- and pentanucleotide peaks by paper electrophoresis as well as by the technique of dephosphorylation with phosphomonoesterase showed them to be free from the pyrophosphate type of side products.

In the case of the deoxyadenosine polynucleotides an experiment carried out in a manner similar to that described above showed residual contamination of the linear polynucleotides by small amounts of phosphomonoesterase-resistant materials. Although this experiment was not completely successful, the result is not regarded as detracting from the general value of the acetic anhydride treatment for the cleavage of the pyrophosphate bonds. It should be emphasized, however, that scrupulously anhydrous conditions are necessary during the acetic anhydride-pyridine treatment in order to ensure a large excess of acetic anhydride and thus to shift the equilibrium reaction[7] completely over to the cleavage direction. The acetic anhydride–pyridine treatment, in fact, provides a very mild method for the cleavage of pyrophosphate bonds in sensitive molecules.

Experimental

General Methods.—Paper chromatography, paper electrophoresis and other techniques were as described previously.[4c] The experiments with the bacterial alkaline phosphomonoesterase also were carried out as described previously.[4c] A generous gift of another preparation of this enzyme was made by Mr. J. Schwartz of the Rockefeller Institute. A preparation of semen phosphomonoesterase used after standardizing with a dinucleotide was a gift of Dr. Leon A. Heppel of the National Institutes of Health. The preparation as used was free from pyrophosphatase activity or any appreciable activity against deoxyribo-oligonucleotides.

The preparations of 3′-*O*-acetylthymidine-5′ phosphate, N^6-anisyldeoxycytidine-5′ phosphate, N,3′-*O*-diacetyldeoxycytidine-5′ phosphate and of a mixture of N-benzoyldeoxyadenosine-5′ phosphate and N,3′-*O*-dibenzoyldeoxyadenosine-5′ phosphate have all been described previously.[1,4c,4d]

Polymerization of a Mixture of 3′-*O*-Acetylthymidine-5′ Phosphate and Thymidine-5′ Phosphate. (a) Using *p*-Toluenesulfonyl Chloride.—A mixture of pyridinium 3′-*O*-acetylthymidine-5′ phosphate (0.5 mmole) and pyridinium thymidine-5′ phosphate (1.5 mmole) was rendered

anhydrous by the standard technique involving evaporation of a pyridine solution. The residual sirup was taken up in 1 ml. of dry pyridine and to the solution was added rapidly a solution of *p*-toluenesulfonyl chloride (600 mg.; about 3 mmole) in 1 ml. of anhydrous pyridine. The rapidly mixed solution was kept sealed at 4° in the dark for 2 days. Water (25 ml.) then was added. After a further 18 hr. at room temperature, 3 ml. of 2 *N* sodium hydroxide was added and the orange-yellow solution was allowed to stand for 2 hr. After evaporation to about 2 ml., the concentrate was passed through a column of Dowex-50 resin (ammonium form) (total bed volume, around 20 ml.). The column subsequently was washed thoroughly with water (5 × 20 ml.) and the total effluent evaporated to a small volume. One half of the polymeric mixture then was chromatographed on a DEAE-cellulose column under the standard conditions described previously[4c] for a 1 mmole scale separation. The elution pattern is shown in Fig. 1. Selected fractions corresponding to different peaks were examined for their ultraviolet absorption spectra and composition.

(b) **Using Diphenylphosphorochloridate.**—To a solution of a standard mixture (1 mmole) of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate in dry pyridine (0.5 ml.) was added a solution of 0.325 ml. (1.5 mmole) of diphenylphosphorochloridate in 0.5 ml. of dry pyridine with exclusion of moisture. The clear light yellow solution was kept sealed in the dark at room temperature for 4½ days. Water (10 ml.) was added and the resulting solution kept at room temperature for 12 hr. To one half of the total solution, corresponding to 0.5 mmole of total nucleotide, was added 1.5 ml. of 2 *N* sodium hydroxide, and the alkaline mixture was allowed to stand for about 2.5 hr. at room temperature. Amberlite IR-120 H^+ resin was carefully added to bring the *p*H to neutrality. The resin then was removed and thoroughly washed with water. The total aqueous solution and washings were concentrated to a few ml. and the concentrate adjusted to *p*H around 8. The total was applied to the top of a DEAE-cellulose (bicarbonate) column (approximately 300 ml. total bed volume). After a water wash, elution was carried out with a linear gradient, the mixing vessel containing 4 l. of water and the reservoir 4 l. of 0.4 *M* triethylammonium bicarbonate[9] (*p*H 7.5). About 18 ml. fractions were collected at a flow rate of 1.6 ml./min. The elution pattern obtained is shown in Fig. 2.

The remaining half of the polymeric mixture in aqueous pyridine was evaporated at low temperature *in vacuo* and the process of evaporation after addition of dry pyridine was repeated three times. Finally the mixture was taken up in 5 ml. of dry pyridine. Tri-*n*-butylamine (0.5 ml.; 2 mmole) and acetic anhydride (2 ml.; 20 mmole) were added and the clear solution kept at room temperature for 4 days. After a work-up as described below for similar experiments, the polymeric mixture again was chromatographed on the DEAE-cellulose column used above for similar separation.

(c) **Using Diisopropylcarbodiimide.**—The procedure followed on a 1 mmole scale was exactly as described previously[4c] for DCC except that 0.315 ml. (2 mmole) of diisopropylcarbodiimide was used. The work-up and separation on a DEAE-cellulose column were as previously described.[4c]

Degradation of P^1,P^2-Dithymidine-5′ Pyrophosphate on Treatment with Acetic Anhydride in the Presence of Tri-*n*-Butylamine.—Dithymidine pyrophosphate was prepared essentially by the method of Smith, Moffatt and Khorana: thus pyridinium thymidine-5′ phosphate (0.2 mmole) was rendered anhydrous by repeated evaporation of its pyridine solution and finally the nucleotide was taken up in 2 ml. of this solvent. Tri-*n*-butylamine (0.1 ml.; 0.41 mmole) and DCC (206 mg.; 1 mmole) were added and the reaction mixture kept for about 24 hr. Water (5 ml.) was added and the mixture extracted with ether three times. After filtration to remove dicyclohexylurea, the aqueous layer which, as judged by chromatography in Solvent A,[4c] contained mostly the pyrophosphate, was evaporated to dryness and the residue re-evaporated three times from anhydrous pyridine (5 ml. each time). The anhydrous residue was taken up in 2 ml. of dry pyridine and tri-*n*-butylamine (0.1 ml.) and acetic anhydride (0.5 ml.; 5 mmole) were added. The reaction mixture was kept sealed in the dark for about three days, after which water (5 ml.) was added. After several hours at room temperature, the solution was evaporated, the sirupy residue redissolved in water and the solution re-evaporated *in vacuo*. The process was repeated several times to ensure complete removal of the pyridinium acetate. Sodium hydroxide (1 ml. of 1 *N*) was added and the alkaline solution, after being kept for 1 hr. at room temperature, was passed through an ammonium Dowex-50 ion exchange resin column. The ammoniacal effluent was examined by paper chromatography in Solvent A. Thymidine-5′ phosphate was the sole product.

Treatment of the Polymeric Mixture from Thymidine-5′ Phosphate with Acetic Anhydride and Isolation of Polynucleotides.—The polymerization of a mixture of 3′-*O*-acetylthymidine-5′ phosphate (1 mmole) and thymidine-5′ phosphate (3 mmole), the work-up of the resulting polymeric mixture and chromatography on a DEAE-cellulose (bicarbonate) column have been described previously.[4c] An aliquot of the same solution of the polymeric mixture, corresponding to 0.4 mmole of the starting mononucleotide, was passed through a pyridinium Dowex-50 ion exchange resin column (2 cm. long × 1 cm. dia.) and the total effluent and water washings evaporated to dryness under reduced pressure. Pyridine was added to the residue and the solvent re-evaporated. The residue, which was largely insoluble in dry pyridine, gave a clear solution in 5 ml. of dry pyridine containing 0.2 ml. (about 0.85 mmole) of tri-*n*-butylamine. Acetic anhydride (1.5 ml.) was added and the sealed mixture kept in the dark at room temperature for about four days. Water (10 ml.) was added rapidly with agitation and after a further 1 hr. most of the pyridinium acetate was removed by repeated evaporation after redissolution in water. To the gummy residue then was added 2 ml. of 1 *N* sodium hydroxide, and the milky solution (separation of tri-*n*-butylamine) was kept at room temperature for 2 hr. The total mixture was passed through a column of ammonium Dowex-50 ion exchange resin and the column washed thoroughly with water. The total aqueous effluent was reduced to a small volume, the *p*H was adjusted to about 8 and the solution applied on top of a column of DEAE cellulose (bicarbonate) (35 cm. long × 3 cm. dia.). The column was washed with water and then eluted using a linear gradient of triethylammonium bicarbonate (*p*H 7.5). The mixing vessel contained 4 l. of water and the salt vessel 4 l. of 0.4 *M* triethylammonium bicarbonate. A flow rate of 1.5 ml./min. was maintained, 16–18 ml. fractions being collected. The elution pattern is shown in Fig. 3. The elution was continued as far as the octanucleotide peak. The peak tubes containing the different polynucleotides were combined and processed as described previously.[4c]

The tri-, tetra- and penta-nucleotides thus obtained were ascertained to be free from any pyrophosphate contaminants by their complete dephosphorylation with the semen phosphomonoesterase preparation (Chromatography in Solvent E[4c].)

Preparation and Isolation of Deoxycytidine Polynucleotides.—The reaction of a mixture of N,3′-*O*-diacetyldeoxycytidine-5′ phosphate (0.25 mmole) and N-anisyldeoxycytidine-5′ phosphate (0.75 mmole) with DCC (2 mmole) in 1 ml. of anhydrous pyridine was carried out exactly as has been described recently.[4d] Water (10 ml.) was added after about six days and the mixture kept at room temperature for two days. The dark-colored solution was filtered from dicyclohexylurea, the latter being washed with 20% aqueous pyridine. The total volume of the filtrate and washings was 23 ml. Pyridine (25 ml.) was added before evaporation under reduced pressure. The residue was taken up in 10 ml. of pyridine and re-evaporated. Finally, the residue which was now partly insoluble in anhydrous pyridine was rendered completely anhydrous by readdition of dry pyridine and evaporation of the solvent. Pyridine (10 ml.) and acetic anhydride (2.5 ml.) were added and the sealed reaction mixture shaken in the dark at room temperature. Except for a small oily deposit on the walls, a clear solution resulted after a few hours.[10] After a total of three days when the solution was practically clear, cold water (30 ml.) was quickly added and the solution kept at room temperature overnight. After addition of 10 ml. of pyridine, the solution was evaporated under reduced pressure and at a bath

(9) For preparation of triethylammonium bicarbonate solution see M. Smith, D. H. Rammler, I. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962).

(10) The experiment was carried out before the use of trialkylamine to aid solubilization was introduced. The treatment with acetic anhydride would now be carried out in the presence of an amine such as tri-*n*-butylamine.

temperature of below 25°. The process of redissolution in dilute aqueous pyridine and evaporation was repeated four times to remove most of the pyridinium acetate without allowing the *p*H to drop below 4 (because of lability of N-acetyl groups at acidic *p*H and to avoid any deamination). Concentrated ammonia (50 ml.) then was added and the solution kept at room temperature for three days. Most of the ammonia then was evaporated and the aqueous solution extracted three times with ether. The aqueous layer was brought to *p*H 8.5 with ammonia and applied to a column (33 cm. long × 4 cm. dia.) of DEAE-cellulose (bicarbonate form). The column was washed with about 200 ml. of water and then eluted with a linear gradient of triethylammonium bicarbonate (*p*H 7.5). At first the mixing vessel and the salt vessel contained, respectively, 4 l. of water and 4 l. of 0.25 *M* triethylammonium bicarbonate (*p*H 7.5) and then the same volume of, respectively, 0.25 *M* and 0.5 *M* triethylammonium bicarbonate. A flow rate of 1.5 ml./min. was maintained and 15–17 ml. fractions were collected. The ultraviolet absorption of the fractions was read at 271 mμ. The following is a description of the elution pattern obtained, which in appearance resembled that given previously (Fig. 1 of ref. 4d).

Peak 1, as judged from the spectrum, consisted of non-nucleotidic material. Peak 2 consisted of the material with λmax at 295 mμ, which arises from the reaction of acetic anhydride and pyridine. Peak 3 was anisic acid. Peaks 4 and 5 were, respectively, the mononucleotide and the cyclic dinucleotide. Peak 8 was linear dinucleotide (d-pCpC) and constituted 11.8% of the total nucleotidic material eluted. Peak 10, by analogy with the elution pattern described in the previous paper,[4d] was cyclic trinucleotide. Peaks 12 (9.3%), 15 (12.4%) and 17 (5.7%) consisted of, respectively, tri-, tetra- and penta-nucleotides. Further tests of purity were carried out with aliquots of these major peaks.

Peak 8, corresponding to the dinucleotide, gave a single band on paper electrophoresis at *p*H 7.5. The corresponding peak in the previously described histogram (Fig. 1 of ref. 4d) contained 15–25% of a second slower-travelling band corresponding to the pyrophosphate III. Furthermore, as expected, the material of peak 8 in the present run was completely converted to a faster-travelling band (d-CpC) on digestion with phosphomonoesterase.

Peak 12, consisting of linear trinucleotide, also was pure: Digestion with phosphomonoesterase gave a single faster-travelling band, no ultraviolet absorption with R_f corresponding to that of the starting material surviving.

The peaks 15 and 17, (tetra- and penta- nucleotides, respectively) on treatment with the phosphomonoesterase, gave only faint traces of ultraviolet absorbing materials with R_f's of the starting materials. The unreacted materials could be cyclic penta- and hexa- nucleotides or traces of undegraded pyrophosphate compounds.

Treatment of Deoxyadenosine Polynucleotides with Acetic Anhydride and Pyridine.—A mixture of 993 mg. of N-benzoyldeoxyadenosine-5′ phosphate and N,3,*O*-dibenzoyldeoxyadenosine-5′ phosphate was prepared from deoxyadenosine-5′ phosphate (2 mmole) by the method described previously.

This material was evaporated from dry pyridine several times to remove residual traces of water and finally concentrated in 1 ml. of dry pyridine. Dicyclohexylcarbodiimide (824 mg.) then was added rapidly to the solution, with stirring. The solution became clear, then slowly deposited a viscous gum. The mixture was shaken for 24 hr. at 20° in the dark, when a clear brownish solution resulted. A further 412 mg. of dicyclohexylcarbodiimide and 1 ml. of pyridine were added and the resulting gummy precipitate was shaken for 24 hr., when a clear solution again resulted. After shaking for a total of 5 days, water (10 ml.) was added to the reaction and the flask set aside for 4 hr. The solution then was extracted with ether and filtered to remove dicyclohexylurea. The urea was washed with 20% aqueous pyridine (15 ml.), and the filtrate and washings were combined, pyridine (30 ml.) was added and the whole evaporated *in vacuo*, then several times from dry pyridine. The residue was dissolved in dry pyridine (10 ml.). Acetic anhydride (1.5 ml.) was added to an aliquot of this solution (2.5 ml.) in a total volume of 10 ml. of dry pyridine. After 3 days at 20° the clear brown solution was cooled and ice-water (30 ml.) was rapidly added. After 24 hr. the solution was evaporated repeatedly from aqueous pyridine to remove some of the pyridinium acetate.

The residual gum was dissolved in conc. ammonium hydroxide (50 ml.) in a sealed flask for 48 hr. After removal of the ammonia *in vacuo* the solution was extracted with ether, adjusted to *p*H 8 and applied to the top of a DEAE-cellulose (bicarbonate) column (35 cm. × 3.5 cm. dia.). The column was washed with water and eluted with a linear gradient of triethylammonium bicarbonate (*p*H 7.5) as described for similar separations.[1]

Fractions containing individual peaks were combined and evaporated *in vacuo* (<20°). The products were stored in water (5 ml.) at −10°, prior to identification by paper chromatographic comparison with polymers characterized earlier.

The homologous series of peaks were essentially homogeneous when chromatographed in Solvent E, apart from a faint trace of a faster-moving homologous series of compounds (perhaps the dephosphorylated polymers).[1]

After treatment of the linear polynucleotides with semen phosphomonoesterase for 5 hr. at 37° and rechromatography in Solvent E, the compounds were almost completely dephosphorylated to give materials of higher R_f. Traces of material remaining were readily degraded to deoxyadenosine-5′ phosphate by venom phosphodiesterase, suggesting that these were not cyclic polynucleotides.