ANALOGS OF PYRIMIDINE MONO- AND POLYNUCLEOTIDES.

III.* ANALOGS OF OLIGOTHYMIDYLIC ACID

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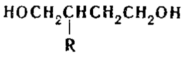
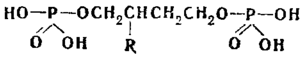
Analogs of oligothymidylic acid were obtained by polycondensation of N_1-(1,4-dihydroxy-2-butyl)thymine with N_1-(1,4-dihydroxy-2-butyl)thymine and also by polycondensation of the appropriate monophosphates in pyridine and dimethylformamide under the influence of dicyclohexylcarbodiimide. Oligomers with ester character are formed in the polycondensation of the monophosphate, whereas oligomers of pyrophosphate character are formed in the polycondensation of the diol and diphosphate.

We were able to show by conformational calculations [2] that the optimum number of methylene links for which a conformation similar to the conformation of nucleic acids is retained is four to five when monosaccharides in the framework of the polymeric chain of nucleic acids are replaced by polymethylenediol residues.

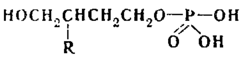
The present paper is devoted to a study of the possibility of polycondensation of the previously synthesized analogs of thymidylic acid based on N_1-(1,4-dihydroxy-2-butyl)thymine (I), its 1',4'-diphosphate (II), and isomeric N_1-(1,4-dihydroxy-2-butyl)thymine monophosphates (IIIa, IIIc) [1, 3] under the influence of dicyclohexylcarbodiimide or triisopropylbenzenesulfonyl chloride.


$HOCH_2\underset{\substack{|\\R}}{C}HCH_2CH_2OH$

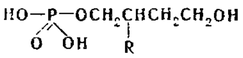
I


$HO-\underset{O\ \ OH}{P}-OCH_2\underset{\substack{|\\R}}{C}HCH_2CH_2O-\underset{O\ \ OH}{P}-OH$

II


$HOCH_2\underset{\substack{|\\R}}{C}HCH_2CH_2O-\underset{O\ \ OH}{P}-OH$

III a


$HO-\underset{O\ \ OH}{P}-OCH_2\underset{\substack{|\\R}}{C}HCH_2CH_2OH$

IIIb

R= thymidyl

In addition to this, we set out to establish the nature of the bonding in the oligomers obtained and to find a satisfactory method for the synthesis of analogs of oligothymidylic acid containing phosphate bonds. An investigation of the possibility of complexing of these oligomers with the complementary chains of homopolynucleotides and a study of their conformational sensitivity to a change in the medium (pH, ionic strength, and temperature) seem of interest. These factors are extremely important for substantiation of the expediency of research dealing with studies of the biological activity of such synthetic models of nucleic acids on a molecular level.

It is known that the most widespread and successful method for the chemical synthesis of oligonucleotides is condensation of the appropriately protected mononucleotides under the influence of dicyclohexylcarbodiimide and aromatic sulfonyl chlorides [4]. Khorana and Vizsolyi have proposed that the polycondensation of thymidine 5'-phosphate be carried

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*See [1] for communication II.

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out in the presence of 3'-O-acetylthymidine in order to increase the molecular weight and reduce the fraction of cyclic products [5].

In order to create analogs of oligothymidylic acid on the basis of the monomers proposed by us, in addition to polycondensation of monophosphates III, it seemed expedient to effect polycondensation of I and II. In the latter case one might have expected the formation of products of higher molecular weight than those obtained as a result of the polycondensation of thymidine 5'-phosphate and 3'-O-acetylthymidine recommended in the literature, inasmuch as 3'-O-acetylthymidine, while reducing the probability of cyclization, simultaneously reduces the molecular weight because of disruptions in the stoichiometry of the functional groups.

In addition to the above-indicated task, we also set out to select an appropriate method for the evaluation of the molecular weights of the oligomers obtained. Gel chromatography on Sephadex seems the most acceptable method for this purpose. It is known that for a number of polymers there is a linear dependence between the distribution coefficients (K_d) and the degree of polymerization (n). In the determination of the molecular weights by this method we used the dependence found for a number of oligothymidylates with n = 1-14 during their gel filtration on Sephadex G-25 [6]. However, this method for the determination of the molecular weights becomes inaccurate for analogs of oligothymidylic acid that contain a considerable number of pyrophosphate groups, inasmuch as the charge of the oligomers obtained changes [7]. It was therefore necessary in this case to limit ourselves to the characteristics of the molecular weight in terms of the distribution coefficients.

A complex mixture of products, which was separated by means of anion-exchange chromatography on QAE-Sephadex, was formed as a result of polycondensation of monophosphate III under the influence of dicyclohexylcarbodiimide. Approximately 30% of the material eluted with 0.05 N ammonium carbonate buffer was a substance that was identical to the previously obtained N_1-(1,4-dihydroxy-2-butyl)thymine cyclophosphate [1]. Commencing with 0.5 N buffer, oligomeric products, the yield of which amounts to 28%, are eluted. In order to investigate the character of the bond, the oligomers were treated with acetic anhydride — a reagent that quantitatively destroys the pyrophosphate bonds [8]. Treatment with acetic anhydride leads to a change in the percentage of the separated fractions and reduces the oligomer fraction to 17%. According to the results of gel filtration, oligomeric products with n = 7-8 are eluted commencing with 0.7 N buffer.

The data from chromatographic separation of the products of polycondensation of monophosphates III under the influence of triisopropylbenzenesulfonyl chloride before and after treatment with acetic anhydride are analogous to the results obtained in the preceding case with the aid of dicyclohexylcarbodiimide. The degree of polymerization of the highest-molecular-weight products is five in this case, and the fraction of these substances is about half the fraction obtained when dicyclohexylcarbodiimide is used.

Thus experiments on the polycondensation of monophosphates III leads to results that are in complete agreement with the literature data for thymidine 5'-phosphate both with respect to the formation of phosphate bonds between the monomeric links and with respect to the molecular weights of the products obtained [5].

An investigation of polycondensation of I and II under the influence of dicyclohexylcarbodiimide was carried out in dimethylformamide (DMFA) in connection with the fact that diphosphate II is insoluble in pyridine. The experiments carried out under these conditions showed that I does not react at all, whereas II forms a set of oligomers containing only pyrophosphate bonds. The distribution coefficient of the highest-molecular-weight portion of the polycondensation products on Sephadex G-25 is 0.08. This sort of reaction might be due to decomposition of DMFA during the reaction to give strong bases, which inhibit dicyclohexylcarbodiimide, but repeated experiments on the polycondensation carried out as described in [9] with the addition of Dowex-50 Py^+ resin also did not give the desired result. The low solubility of I in DMFA was noted in supplementary experiments, and this might be the reason for its low reactivity.

Different results were obtained when salt II was used with a tertiary amine in pyridine with triisopropylbenzenesulfonyl chloride as the condensing agent. Fractionation of the mixture of reaction products obtained in this case, which was carried out after separation of the water-insoluble substances by dialysis against water and 2 N sodium chloride

TABLE 1. Results of Fractionation of the Products from the Polycondensation of I and II under the Influence of Triisopropylbenzenesulfonyl

Method of separation	Percentage of the fraction, %	K_d
Ether-soluble	26	—
Water-soluble	74	—
From them by dialysis:		
passed into water	48	—
passed into 2 N NaCl	14,8	0,28
Remained in the dialysis bag	11.2	0,098

solution, made it possible to isolate a number of fractions of oligomers (see Table 1), which, as will be subsequently shown, contain both pyrophosphate and phosphate bonds.

Approximately one fourth of the reaction products that were insoluble in water dissolved in diethyl ether. These substances proved to be products of transformation of starting I under the influence of the sulfonyl chloride, inasmuch as we were also able to obtain such compounds by reaction of I with the sulfonyl chloride in pyridine, and the degree of transformation reached 68% after 6 h. These substances can be partially separated by paper chromatography. Inasmuch as their isolation was not the goal of the present research they were not identified.

It was shown by anion-exchange chromatography that the unchanged diol is not present in the water-soluble fraction of the polycondensation products. Thus ~50% of I underwent polycondensation, i.e., the stoichiometry of the components in the reaction mixture is disrupted, and this apparently also leads to the formation of oligomers containing pyrophosphate bonds as well as phosphate bonds. The pyrophosphate character of the oligomers obtained is confirmed by destruction of a nondialyzed sample with acetic anhydride, during which 55% of diphosphate II is liberated. A portion (18.5%) of the oligomers remains undisrupted, and they consequently contain phosphate bonds. According to the results of gel filtration on Sephadex G-25, the degree of polymerization of these oligomers is 6-7.

Thus, by way of correlating the above data, it can be asserted that the polycondensation of monophosphates III leads mainly to products, the monomeric links in which are bonded by phosphate bonds. On the other hand, oligomers containing a considerable number of pyrophosphate bonds are formed in the polycondensation of I and II. In addition, definite differences in the polycondensation of III and the polycondensation of I and II are also observed. Thus the polycondensation of I and II in the presence of triisopropylbenzenesulfonyl chloride leads to the formation of a precipitate a few minutes after mixing of the reagents, whereas the addition of the sulfonyl chloride to monophosphates III does not disrupt the homogeneity of the system during the entire reaction. However, if the sulfonyl chloride is added to an anhydrous pyridine solution of diphosphate II, a precipitate into which all of the "diphosphate" material is transferred forms. According to the results of anion-exchange chromatography and gel filtration on Sephadex G-25 the precipitate consists of a set of oligomeric products. The phenomenon that we observed is apparently due to the bifunctional nature of diphosphate II. One of the necessary steps in the polycondensation is activation of the monophosphate groups [10], but branched polymers that are insoluble in the solvents used are probably formed in this case because of the peculiarities of the chemical structure of diphosphate II. Partial destruction of the polymer occurs during treatment of the polymeric products with aqueous pyridine and bases, and this is ultimately responsible for the formation of the relatively low-molecular-weight oligomers. Activation of II evidently occurs as in the case of polycondensation of only II in the polycondensation of diphosphate II with I. When the reaction is carried out in pyridine with the sulfonyl chloride, I undergoes two competitive reactions in this case: first, reactions with the activated phosphate groups of diphosphate II, which is evidently hindered in connection with the heterogeneous character of the reaction, and, second, a side reaction involving sulfonation of the hydroxyl groups by the sulfonyl chloride.

Proceeding from the above, it might be assumed that oligomers with a large number of diphosphate bonds and higher molecular weights can be formed as the concentration of I in the reaction mixture increases. The next communication in this series of papers is devoted to verification of the correctness of this assertion.

EXPERIMENTAL

Absolute pyridine was prepared in accordance with the method described in [1]. Triisopropylbenzenesulfonyl chloride was recrystallized three times from pentane and had mp 95°. Analytical grade tri-n-octylamine was used without additional purification. The acetic anhydride was vacuum distilled immediately prior to use. The DMFA was purified by azeotropic distillation with benzene and water, dried with KOH, and vacuum distilled twice immediately prior to use.

Dry Dowex 50 W·4 Py^+ anion-exchange resin was prepared by five evaporations of Dowex 50 W·4 H^+ resin with absolute pyridine. The preparation of the resins for column chromatography was described in [1].

Anion-exchange chromatography was carried out with the aid of a stepwise ammonium carbonate buffer. The products applied to the column containing the anion-exchange resin were diluted in such a way that the ionic strength of the solution corresponded to the ionic strength of a 0.01 N ammonium carbonate buffer; in this case, the uncharged particles were eluted immediately from the column, whereas the anionic particles were retained. Gel filtration was carried out with 1 N ammonium carbonate buffer. A column (74.5 cm^3 with a height of 65 cm) filled with Sephadex G-25 was used for analytical purposes. The distribution coefficients (K_d) during gel filtration were calculated from the formula

$$K_d = \frac{V_e - V_o}{V_i},$$

where V_e is the elution volume of the substance, V_i is the inner volume, and V_o is the outer volume of the volume. The V_o term was taken to be equal to the elution volume of high-molecular-weight polyuridylic acid, and V_i was calculated as the difference in the elution volume of I and the outer volume. The degree of polymerization was determined from the graphical dependence [6] of K_d on n.

Column chromatography was monitored as shown in [1]. Quantitative determination of the substances that absorb in the UV region was accomplished with an SF-16 spectrophotometer.

Polycondensation of Monophosphates III. A) A solution of 0.068 mmole of monophosphates III in the acid form in aqueous pyridine was evaporated to 0.5 ml, and the residue was evaporated to dryness four times with absolute pyridine (7-ml portions). The residue was dissolved in dry pyridine (2.2 ml) containing 84 mg (0.408 mmole) of dicyclohexylcarbodiimide. After 5 days, 5 ml of water was added to the reaction mixture, and it was allowed to stand for 12 h. The precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness twice with water and once with 0.5 N ammonium hydroxide. The residue was dissolved in water, the pH was brought up to 7.5, and the solution was applied to a column filled with QAE-Sephadex; the compounds were eluted with ammonium carbonate buffer while gradually raising the ionic strength of the solution. The substance eluted by 0.05 N buffer was chromatographically homogeneous, had R_f 0.48 (system A) and 0.6 (system B),* and its electrophoretic mobility relative to 5'-UMP was 0.9 at pH 7.5 and 1 at pH 2.8. The distribution coefficient of the substances eluted with 0.5, 0.7, and 1 N buffer that absorbed in the UV region (the substances were combined and the overall yield was 28%) was 0.9 according to the results of gel filtration with a column filled with Sephadex G-25, and this constitutes evidence for their oligomeric nature.

B) A solution of tri-n-octylamine (0.28 mmole) in pyridine was added to a 50% aqueous pyridine solution of III (0.14 mmole), and the mixture was evaporated. The residue was evaporated three times with dry pyridine, after which a pyridine solution of triisopropylbenzenesulfonyl chloride (0.58 mmole) was added, and the mixture was concentrated to 0.5 ml and allowed to stand for 24 h. The reaction was terminated by the addition of 2 ml of concentrated ammonium hydroxide in 6 ml of ethanol. The mixture was evaporated twice in ammonium hydroxide. The residue was dissolved in 20 ml of water, and the pH of the solution was brought up to 7.5; the solution was applied to a column filled with QAE-Sephadex and eluted with ammonium carbonate buffer. The distribution coefficient of the highest-molecular-weight portion of the reaction products (10% of the sum of the separated substances) was 0.3, according to the results of gel filtration on Sephadex G-25.

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*See [1] for the composition of the systems.

Treatment of the Products of Polycondensation of Monophosphates III with Acetic Anhydride. A 200 oe sample* of an aqueous solution of the products of polycondensation of III was dried repeatedly by the action of dicyclohexylcarbodiimide by evaporation with dry pryidine. A 1-ml sample of freshly distilled acetic anhydride was added to the anhydrous pyridine solution (1 ml), and the mixture was allowed to stand in the dark without access to moisture for 3 days. Water (1 ml) was then added to the reaction mixture with cooling, and the solution was evaporated to dryness after 30 min. The residue was dissolved in water, and the solution was passed through a column filled with QAE-Sephadex (V_k = 2 ml); the products were eluted with the stepwise gradient of an ammonium carbonate buffer. The distribution coefficient of the highest-molecular-weight portion of the reaction products was 0.25, which corresponds to an average degree of polymerization of 7 to 8 [6].

The products of polycondensation of III were similarly worked up under the influence of triisopropylbenzenesulfonyl chloride. The highest-molecular-weight fraction of the hydrolysis products had a distribution coefficient of 0.43, which corresponds to an average degree of polymerization of 5.

Polycondensation of I and II in DMFA under the Influence of Dicyclohexylcarbodiimide. An aqueous pyridine solution containing 0.149 mmole each of I and II was evaporated to dryness, the residue was dissolved in dry pyridine, and the solution was again evaporated (this operation was repeated four times). The dried mixture of products was dissolved in freshly distilled DMFA, and Dowex-50 Py^+ resin (0.5 ml) and a solution of dicyclohexylcarbodiimide (14.9 mmole) in DMFA (the total volume of solvent was 7 ml) were added. After 5 days, 3 ml of water was added to the reaction mixture, and it was allowed to stand overnight. The dicyclohexylurea was removed by filtration and washed with water. The filtrate was evaporated to dryness, and the residue was dissolved in water and passed through a column filled with Dowex-50 H^+ resin. The eluate (40 ml) was neutralized to pH 7.5 with ammonia and applied to a column filled with QAE-Sephadex; the products were eluted by the standard method. Half of the substance that absorbs in the UV region was not retained in the column but was eluted with the starting 0.01 N buffer. According to the results of paper chromatography, this product is starting I: R_f 0.73 (system A), 0.78 (system B), and 0.63 (system C) [1].

Polycondensation of I and II in Pyridine under the Influence of Triisopropylbenzenesulfonyl Chloride. A 9.09-g (19.5 mmole) sample of diphosphate II was passed through a column filled with Dowex-50 H^+ resin (250 ml), the solution was evaporated to 50 ml, diluted with 50 ml of pyridine, and combined with a pyridine solution of tri-n-octylamine (78 ml). The final mixture was evaporated to a viscous mass, and the residue was dissolved in dry pyridine and combined with a solution of 4.19 g (19.5 mmole) of diol I in pyridine. The resulting mixture was dried thoroughly by repeated evaporation with dry pyridine. The residue was dissolved in 60 ml of dry pyridine, and a solution of 31 g (92.7 mmole) of triisopropylbenzenesulfonyl chloride in 45 ml of pyridine was added. The mixture was held at 10° for 21 h, after which 200 ml of water was added, and the mixture was allowed to stand for 12 h. The resulting precipitate was removed by filtration and washed with water. The precipitate consisted of triisopropylbenzenesulfonic acid, which is soluble in alcohol, and a mixture of side products from I, which is soluble in ether.

The solution was concentrated by evaporation, the resulting precipitate was removed by filtration, and 50 ml of the solution was dialyzed in a cellophane bag against 2 liter of water four times (6 h each time). Dialysis against 2 N NaCl was repeated four times (8 h each time). The products that passed into 2 N NaCl were freed from salt by evaporation and decantation of the solution and then by exhaustive dialysis against water. The results of the dialysis are presented in Table 1.

The UV spectrum of the substance that was soluble in alcohol and other data were in agreement with the corresponding data for diisopropylbenzenesulfonic acid R_f 0.87 (system A), λ_{max} 280 nm (ε 500). The chromatographic and spectral characteristics of the products that were soluble in ether were in agreement with the corresponding data for the products obtained by treatment of 101.2 mg (0.47 mmole) of I and 660 mg (2.35 mmole) of triisopropylbenzenesulfonyl chloride in 5.2 ml of pyridine for 6 h. In the latter case, reaction

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*The symbol "oe" corresponds to the extinction of the "nucleotide" substance in 1 ml of a neutral solution in a 1-cm thick quartz cuvette.

products with R_f 0.65 and λ_{max} 255 nm and R_f 0.51 and λ_{max} 270 nm, as well as indistinctly separated products with higher mobilities, were detected along with triisopropylbenzenesulfonic acid (R_f 0.87) and diol I (R_f 0.72) by paper chromatography (system A).

Treatment of the Nondialyzable Sample of the Analog of Polythymidylic Acid with Acetic Anhydride. A solution of 0.2 ml of tri-n-octylamine in 2 ml of pyridine was added to 200 oe of the analog of polythymidylic acid in 60 ml of 50% aqueous pyridine, and the mixture was dehydrated by repeated evaporation with dry pyridine. The residue was dissolved in 3 ml of pyridine, and 2 ml of acetic anhydride was added, after which the mixture was allowed to stand for 3 h. It was then evaporated to dryness with 1 N ammonium hydroxide three times. The residue was dissolved in water, and the solution was extracted with diethyl ether. The aqueous layer was collected and evaporated to dryness, and the residue was dissolved in water and passed through a column filled with Dowex-50 H^+ resin. The pH of the eluate (20 ml) was brought up to 7.5, and the eluate was applied to a column filled with QAE-Sephadex; the products were eluted with an ammonium carbonate buffer. Paper chromatography in three systems showed that the product eluted by the 0.3 N buffer was identical to diphosphate II: R_f 0.03 (A), 0.07 (B), and 0.16 (C) [1].

Polycondensation of Diphosphate II under the Influence of Triisopropylbenzenesulfonyl Chloride. Water (4 ml) and a solution of 0.25 ml of tri-n-octylamine in 3 ml of pyridine were added to a solution of 0.126 mmole of II in 4 ml of pyridine, after which the mixture was evaporated to 1 ml. Dry pyridine (8 ml) was added to the residue, and the mixture was again evaporated (the operation was repeated four times). The residue was dissolved in 5 ml of dry pyridine, and a solution of 400 mg of triisopropylbenzenesulfonyl chloride in 5 ml of dry pyridine was added. The mixture was then allowed to stand for 24 h, after which the precipitate was removed by filtration and washed thoroughly with dry pyridine. All of the filtrates were combined and evaporated, and the residue was dissolved in alcohol. The UV spectrum of an alcohol solution of the product was identical to the spectrum of a solution of triisopropylbenzenesulfonic acid.

The slightly soluble precipitate was suspended in aqueous acetone solution and made alkaline with concentrated ammonia (100 ml). The suspension was evaporated several times to dryness with 2 N ammonium hydroxide, after which it was dissolved in 30 ml of water (the solution was turbid). A 5-ml sample of the solution was diluted to 30 ml with water and chromatographed with a column filled with QAE-Sephadex. According to the UV spectra, the diphosphate II introduced into the reaction had been completely precipitated.

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