on so as to show the orientation of the three CH_3 groups with respect to each other. The torsional angles C(4)–C(5)–C(7)–C(10) and C(4)–C(5)–C(7)–C(11) are −94 and +45°, respectively. A comparison with a similar view of the diol (the coordinates for molecule B were used) shows an interesting occurrence; namely, that both CH_3 groups on C(7) are on the *same* side of C(12) as compared to *opposite* sides for the lactone. This means that the barrier to rotation is not as severe as previously supposed. It is possible for the C(11) and C(12) methyl groups to slip past each other. The substantial enlargement of angles C(5)–C(4)–C(12) and C(5)–C(7)–C(11) in both the diol and the lactone over the idealized value of 120° allows enough space for the slippage. The H atoms on C(10) and C(11) are eclipsed with respect to each other. In the diol, the H atoms on C(12) are eclipsed with respect to those on C(11), whereas in the lactone, the C(12) methyl group has rotated so that the H···H distances between CH_3 groups are roughly equidistant. Thus, a rotation about the C(5)–C(7) bond appears to generate a rotation of the C(12) methyl group by gear-like action.

Acknowledgment. We wish to express our appreciation to Dr. Louis A. Cohen of the National Institutes of Health for supplying us with the crystals used in this investigation and for informative discussions. We are grateful for Dr. Bernhard Witkop of the National Institutes of Health for introducing us to the problem.

Polynucleotides. XVI.[1] Oligomers of 8,2′-Anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine 5′-Monophosphate

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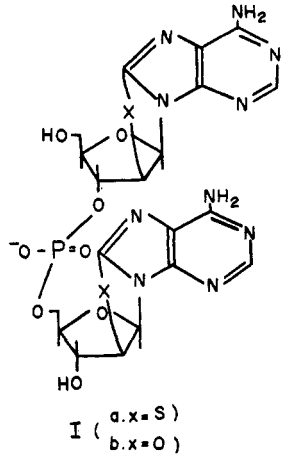
Abstract: N^6-Benzoyl-8,2′-anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine 5′-phosphate (III) was subjected to polymerization using dicyclohexylcarbodiimide in pyridine. The resulting polynucleotides $(pA^s)_n$ were isolated and purified by means of DEAE–cellulose column chromatography, paper chromatography, and paper electrophoresis. Chain lengths were obtained by phosphate analyses of the polymer and its dephosphorylated product formed by alkaline phosphatase digest. All these polynucleotides had CD spectra of similar profile, which had a trough at 277–288 nm and two peaks at around 264 and 222 nm, respectively. Based on the similarity of these spectra with that of A^spA^s previously obtained, the same conformations were assigned to the polymers, *i.e.*, stacked with a left-handed screw axis. The magnitude of Cotton effect [θ] and uv absorbance increased with increasing chain length and reached a plateau at five to six nucleotide units. While [θ] of the pentamer decreased to 62% from 0 to 80°, ε decreased only 15% from 10 to 90°. These phenomena may imply that, owing to a restricted rotation of bases around the glycosidic linkage in each nucleotide unit, the destacking of bases was inhibited to a certain degree.

Recently, we synthesized a dinucleoside monophosphate (Ia) having two 8,2′-anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine (8,2′-*S*-cycloadenosine, A^s) residues linked together with a 3′–5′ phosphodiester linkage.[2,3] We found that compound Ia had a highly stacked conformation with left-handed screw axis by means of its uv, nmr, and CD spectra. In this connection, we also synthesized compound Ib, in which 8,2′-S linkages of Ia were substituted by O linkages.[4] With compound Ib a left-handed stacked conformation was also predicted. In this connection, a versatile method for the specific tosylation of 8-bromoadenosine 5′-monophosphate (BrAMP) and the cyclization to give 8,2′-*S*-cycloadenosine 5′-monophosphate (pA^s) (II) was found.[5] In this paper we report the polymerization of compound II by means of dicyclohexylcarbodiimide (DCC) and properties of the resulting polynucleotides having chain lengths of two to ten nucleotide units.



I (a. X = S ; b. X = O)

Synthesis of the Polynucleotides. To obtain a suitable substrate for the polymerization reaction, we first attempted to synthesize the N^6-dimethylamino-

(1) Part XV of this series: M. Ikehara and M. Hattori, *Biochim. Biophys. Acta*, in press.
(2) M. Ikehara, S. Uesugi, and M. Yasumoto, *J. Amer. Chem. Soc.*, **92**, 4735 (1970).
(3) S. Uesugi, M. Yasumoto, M. Ikehara, K. N. Fang, and P. O. P. Ts'o, *ibid.*, **94**, 5480 (1972).
(4) M. Ikehara, S. Uesugi, and J. Yano, *Nature (London), New Biol.*, **240**, 16 (1972).
(5) M. Ikehara and S. Uesugi, *Tetrahedron Lett.*, 713 (1970); *Tetrahedron*, **28**, 3687 (1972).

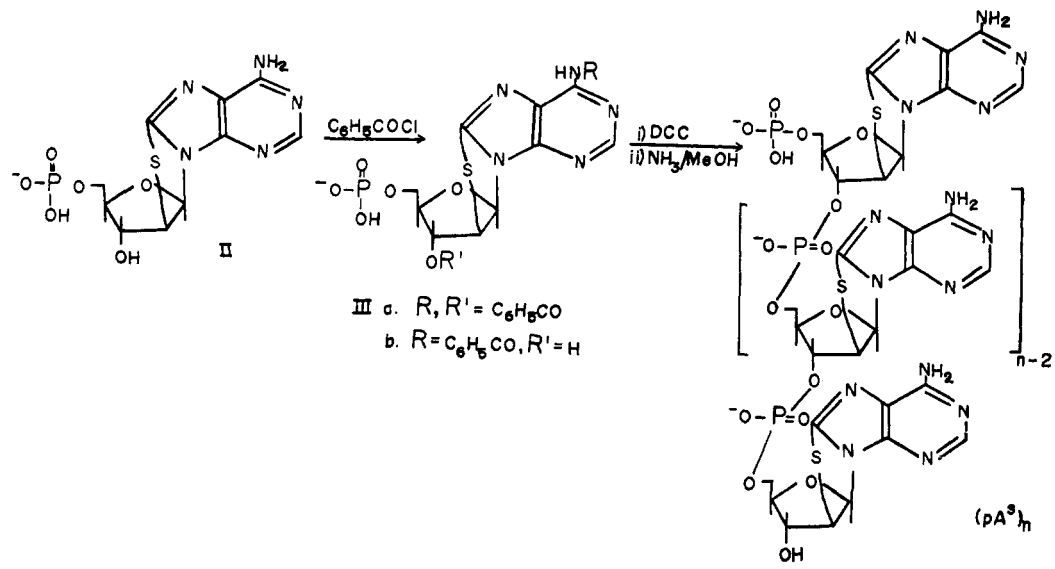


Table I

Peak	Fractions pooled	TOD_{270}[a]	Yield, %	Major component	Paper chromatography[b] $(pA^s)_n$	Paper chromatography[b] $A^s(pA^s)_{n-1}$	Paper electrophoresis[c] $(pA^s)_n$	Paper electrophoresis[c] $A^s(pA^s)_{n-1}$
1	23–42			Nonnucleotidic material				
2	110–136	362	2.6	Unidentified				
3	276–335	1620	12	pA^s	1.00		1.00	0.00
4	405–445	459	3.3	$(pA^s)_2$	0.66	1.10	1.04	0.30
5	461–570	5190	38	Unidentified + $(pA^s)_3$	0.40	0.77	1.01	0.54
6	579–700	1590	12	$(pA)^s_4$	0.48	0.21	0.96	0.70
7	711–795	1190	8.7	$(pA)^s_5$	0.12	0.30	0.95	0.73
8	806–850	665	4.9	$(pA^s)_6$	0.05	0.12	0.94	0.77
9	866–900	505	3.7	$(pA^s)_7$	0.02	0.055	0.87	0.74
10	901–945	510	3.7	$(pA^s)_8$	0.01	0.03	0.08	0.78
11	946–985	451	3.3	$(pA^s)_{\geq 9}$				
12	986–1060	692	5.1					

[a] Total optical density. [b] Performed in solvent A, R_{pA^s} was given. [c] Performed at pH 7.5, R_{pA^s} was given.

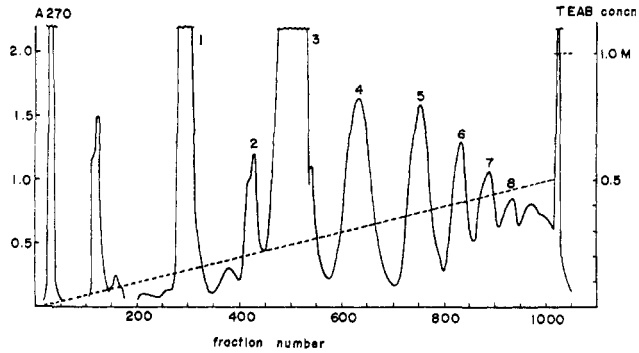


Figure 1.

methylene derivative of II. However, the protected nucleotide had a low solubility in pyridine or DMF and the benzoyl protection on N^6 as used by Khorana and collaborators[6] was employed. The pyridinium salt of pA^s (II) was treated with benzoyl chloride in pyridine to give a perbenzoylated derivative (IIIa). This compound was hydrolyzed with 0.25 *N* sodium hydroxide at 0° to give a N^6-benzoyl compound (IIIb). Structure of this compound was confirmed by a shift of uv λ_{max} from 280 to 309 nm, showing that an acyl group was introduced at the N^6 position. Properties in paper chromatography and electrophoresis also supported this structure. For the final proof, compound IIIb was treated with methanolic ammonia to give the starting material pA^s. The polymerization of compound IIIb was performed in DMF with DCC in the presence of Dowex 50 (pyridinium form) resin to avoid the inhibition of the reaction by amines existing in the reaction mixture. After 20 days at room temperature the reaction was stopped by adding water; the product was treated with acetic anhydride and pyridine to degrade pyrophosphates and with methanolic ammonia to remove protecting groups. The column chromatography of the reaction mixture on DEAE-cellulose (bicarbonate form) was performed by a linear gradient technique using 0–0.5 *M* triethylammonium bicarbonate buffer of pH 7.5. Results are shown in Figure 1 and Table I. Each fraction was purified further by paper chromatography. Peaks no. 11 and 12 were purified by a column chromatography on DEAE-cellulose in the presence of 7 *M* urea.[7] It may be emphasized that these longer polynucleotides could be eluted with 0.4–0.5 *M* NaCl, which was generally used for eluting much longer (20 nucleotide units) polynucleotides. After the elution with 1 *M* triethylammonium bicarbonate buffer,

(6) H. G. Khorana, *Pure Appl. Chem.*, **17**, 349 (1968).

(7) R. V. Tomlinson and G. M. Tener, *Biochemistry*, **2**, 697 (1963).

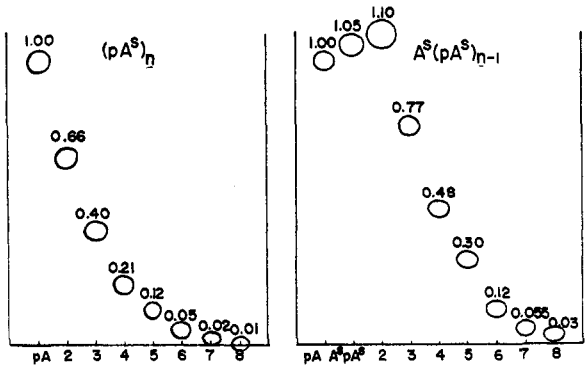


Figure 2.

the column was eluted with the 1 *M* buffer containing 5–7 *M* urea to obtain longer polymers. The yield of different nucleotides is summarized in Table I. The chain length was determined for linear oligomers $(pA^s)_n$ by the removal of terminal phosphates with *E. coli* alkaline phosphatase[8] and comparing $\epsilon(p)$ per residue for each original oligonucleotide $(pA^s)_n$ with that for products $A^s(pA^s)_{n-1}$, respectively. As shown in Table II, $\epsilon(p)$ values of di- to hexanucleotides were

Table II

n	$(pA^s)_n$ $\epsilon(p)$	$A^s(pA^s)_{n-1}$ ϵ_{calcd}
2	1.75×10^4	1.88×10^4
3	1.67	1.73
4	1.62	1.65
5	1.61	1.63
6	1.66	1.64
7	1.61	
8	1.63	

quite consistent with expected values. For hepta- and octanucleotides, comparison of R_f values before and after the phosphatase treatment (Figure 2) and eluting positions from the DEAE column suggested the structure of these polymers. The dinucleoside monophosphate (A^spA^s) obtained from $(pA^s)_2$ by the phosphatase treatment was identical by the criteria of PPC and PEP with that synthesized previously.[2,3]

Physical Properties of the Polynucleotides. Previously we investigated[2,3] properties of the dinucleoside monophosphate A^spA^s and found that this compound had a structure with a strong stacking of bases along a left-handed helical axis. It has been shown that A^spA^s has a pair of CD bands with the opposite sign in B region compared with those of ApA. As shown in Figure 3, a series of polynucleotides $(pA^s)_n$ also had CD curves of the same profile. For the dimer $(pA^s)_2$ a trough appeared at 288 nm and two peaks at 266 and 222 nm. The magnitudes of these bands are around −4000, +2000, and +5000, respectively. Trinucleotide $(pA^s)_3$ showed a trough at 282 nm and peaks at 264 and 222 nm. Magnitudes almost doubled for each band. For polynucleotides having the chain length more than 7, a similar pattern was obtained for the CD spectra. The trough appeared at 277 nm and peaks appeared at 262 and 223 nm. The magnitude of bands increased further to −11,000, +9000, and +15,000,

(8) H. C. Neu and L. A. Heppel, *Biochem. Biophys. Res. Commun.*, **17**, 215 (1964).

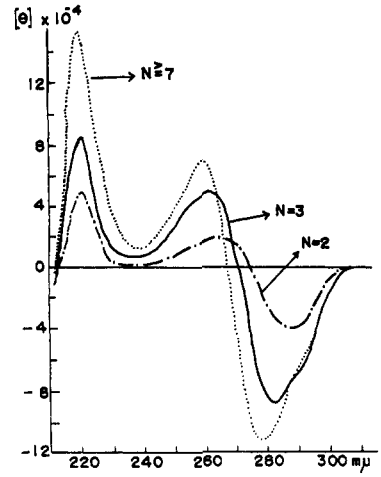


Figure 3.

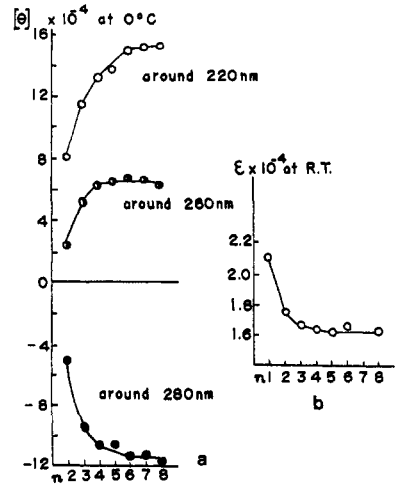


Figure 4.

respectively. There appeared a hypsochromic shift of the position of troughs and a bathochromic shift of the first peak with increase in chain lengths of polymers. Crossover points shifted hypsochromically from the short to long-chain-length oligomers. However, the total profile of the CD spectra was quite similar. This fact suggested that these polymers possessed the same geometrical arrangement. Since a stacked left-handed helical conformation was suggested for A^spA^s from the criteria of CD as well as nmr spectra,[2,3] polynucleotides $(pA^s)_n$ should have the same conformation. An increase in the magnitude of rotational strengths may suggest that the degree of stacking increases with the chain length, until the heptamer is reached.

When $[\theta]$ taken at the extremes was plotted against chain length, we obtained curves as shown in Figure 4a. All the curves reached at a plateau when the chain length became five to six nucleotide units. A similar type of curve was obtained by plotting ϵ against the chain length of the polymer (Figure 4b). In this case also the curve reached a plateau with $n = 4$–5. These facts suggest that $(pA^s)_n$ would form a stable helical conformation of the constant pitch at around chain length of pentamer. This number is somewhat smaller than that observed in the $(pA)_n$ series.[9] If we assume that

(9) A. M. Michelson, T. L. V. Ulbricht, T. R. Emerson, and R. J. Swan, *Nature (London)*, **209**, 873 (1966).

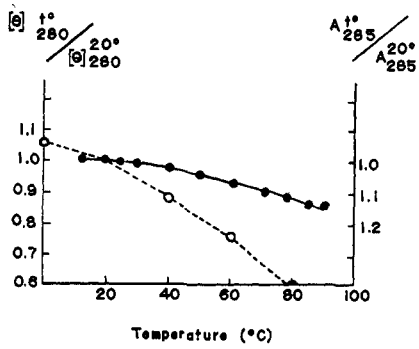


Figure 5.

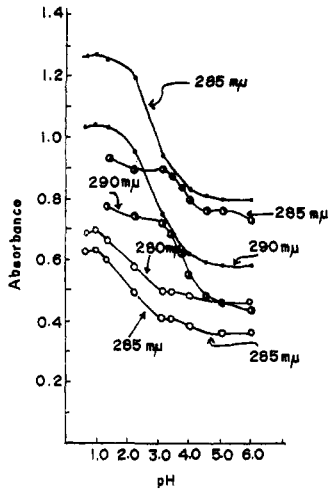


Figure 6.

the main force for stabilizing the conformation of single chain polymer in the neutral solution is the stacking of neighboring bases, the stacking in $(pA^s)_n$ must be stronger than in $(pA)_n$, presumably because of restricting the rotation of bases by the anhydro linkage. The same conclusion was reached in the case of A^spA^s.[2,3]

When the ratio of the uv absorbance of $A^s(pA^s)_4$ at different temperatures ($A^{t°}$) and at 20° ($A^{20°}$) was plotted against the temperature, we obtained the curves in Figure 5. From the curves we can see that $A^s(pA^s)_4$ showed a relatively slow melting process and a hyperchromicity of only 15% from 10 to 90°. Assuming a hypochromicity of 60% after complete digestion of $A^s(pA^s)_4$, we can predict a melting temperature higher than 90° for this nucleotide. In contrast to the slow melting, the ratio of rotational strengths $[\theta]^{t°}/[\theta]^{20°}$ of $A^s(pA^s)_4$ decreased rather steeply to an extent of 62% from 0 to 80°. A simple two-state model[10] may not suffice to interpret these thermal transition data. If we consider that the hypochromicity mainly depends on the vertical stacking of neighboring bases and the rotational strength on the coupling of transition moments of these bases, the first melting process of $A^s(pA^s)_4$ may be interpreted by assuming a lateral movement of overlapped bases without greatly changing the distance between them. Although this phenomenon was suggested earlier[10] in the case of ApA, it occurred more clearly in A^spA^s because of the fixation of the base to the carbohydrate moiety. In the region of the

(10) D. Glaubiger, D. A. Lloyd, and I. Tinoco, Jr., *Biopolymers*, **6**, 409 (1968).

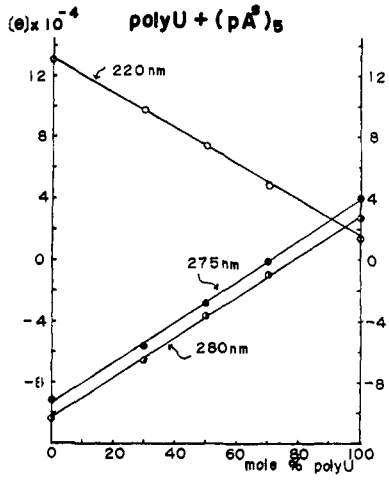


Figure 7.

relatively higher temperature, the destacking process occurs gradually.

pK values of the pA^s monomer and oligomers were obtained either by uv absorption or by CD. As shown in Figure 6, uv absorption of pA^s, A^spA^s, and $(pA^s)_5$ showed a transient decrease at pH 3.8, 2.8, and 2.2, respectively. This indicated that the pK_a of pA^s decreased with oligonucleotide formation. By CD measurement at 280 and 285 nm, $[\theta]$ of $(pA^s)_5$ also rapidly increased at around pH 2.2. These facts are consistent with those observed in the case of poly A.[11] These experiments indicated that strong stacking of bases in $(pA^s)_n$ oligomers occurs at least in the acidic condition.

Finally, we have studied the complex formation of $(pA^s)_n$ with poly U in neutral solution. As shown in Figure 7, the mixing study of $(pA^s)_5$ and poly U at 0° in the presence of 0.01 M Mg^{2+} did not reveal any indication of complex formation even after 24 hr of incubation. This had also been observed in the case of A^spA^s with poly U.[3] From these experiments we can deduce that, because of the left-handedness of the helix of $(pA^s)_n$, complex formation with poly U, which has right-handed helicity, is impossible. Furthermore, the rigid fixation of bases at $\varphi_{CN} = -108°$ in each nucleotide unit of poly A^s may not be suitable for hydrogen bonding with uracil, even if the former incompatibility did not exist.

In conclusion, polynucleotides derived from cyclonucleotide pA^s, in which the base moiety has restricted rotation around the nucleoside linkage due to an anhydro bond, form a left-handed polynucleotide helix with a fairly strong stacking between neighboring bases. Complex formation of these polynucleotides with other polynucleotides having the same fixed base moiety is now under investigation.

Experimental Section[12]

Paper chromatography (PPC) was performed on Toyo filter paper No. 51A in the following solvents: (A) 1-propanol–concentrated ammonia–H_2O (55:10:35); (B) ethanol–1 M ammonium acetate

(11) A. Rich, D. R. Davies, F. H. C. Crick, and J. D. Watson, *J. Mol. Biol.*, **10**, 28 (1961).

(12) Uv absorption spectra were taken with a Hitachi EPS-3T spectrophotometer, and CD spectra were taken with a JASCO ORD/UV-5 spectropolarimeter in a path length of 10 mm at a concentration of 1–2 OD_{max}/ml. Calibrations were made with *d*-10-camphorsulfonic acid.

(7:3). Paper electrophoresis (PEP) was performed with a Toyo electrophoretic apparatus at 35 V/cm in a buffer, 0.05 *M* triethylammonium bicarbonate (pH 7.5). Migration ratios were represented as R_{pA}, which stands for relative migrating ratio to 5′-AMP, or R_{pA-A}, which stands for that assuming 5′-AMP = 1.0 and adenosine = 0.0.

N^6-Benzoyl-8,2′-anhydro-8-mercapto-9-(β-D-arabinofuranosyl)-adenine 5′-Monophosphate (III). 8,2′-*S*-Cycloadenosine 5′-phosphate (II, free acid, 410 mg, 1 mmol) was dissolved in pyridine and evaporated twice with added pyridine. The residue was dissolved in 20 ml of pyridine, and benzoyl chloride (2.5 ml) was added. By a brief shaking all solid materials were dissolved and white precipitates came out. After keeping the reaction mixture for 1 hr at room temperature, $CHCl_3$ (50 ml) and water (50 ml) were added. Further extraction with chloroform (2 × 25 ml) and evaporation of the chloroform layer gave a glass having a single spot at R_f 0.76 (starting material 0.15) on paper chromatogram in solvent B. The residue was dissolved in 50% pyridine (200 ml) and cooled in an ice bath. A solution of 0.5 *N* NaOH (200 ml), which was cooled previously in an ice bath, was added, and the solution was kept at 0° for 20 min. The reaction mixture was neutralized by adding Dowex 50-X8 (pyridinium form) and resin was removed by filtration. The filtrate was passed through a column (2 × 17 cm) of the same resin, which was washed with 10% pyridine (250 ml) and water (150 ml). Eluents were evaporated to give a residue, which had R_f 0.40 (starting material 0.13) and R_f 0.58 (dibenzoyl derivative, 6%) on PPC in solvent B: uv $\lambda_{max}^{H^+}$ 237, 309 nm; $\lambda_{max}^{H_2O}$ 237.5, 302 nm; $\lambda_{max}^{OH^-}$ 244 (sh), 304 nm; PPC, R_f (B) 0.40; PEP, R_{pA-A} 0.93.

Polymerization of N^6-Benzoyl-8,2′-*S*-cycloadenosine 5′-Phosphate (III). The pyridinium N^6-benzoyl pA[8] (*ca.* 1 mmol) was dissolved in pyridine (20 ml) and evaporated. The residual solid was dissolved in a mixture of DMF (2 ml) and pyridine (2 ml) and evaporated to dryness. This treatment was repeated three times. During this treatment all solvents were added in a drybox, and evaporation was done by an oil pump under the strict exclusion of the moisture. The residue, obtained above, was dissolved in DMF (7 ml), and anhydrous Dowex 50-X8 (1 g, pyridinium form) was added. The solvent was removed by evaporation. The residue was dissolved in pyridine (1 ml) and evaporated again. This was repeated twice for complete drying. Finally, the residue was dissolved in DMF (1.5 ml), and DCC (550 mg) was added. The reaction mixture was concentrated for 0.5 ml and kept at room temperature for 25 days. After addition of 50% pyridine (10 ml), the mixture was kept at room temperature for 1 hr and precipitates were filtered. The precipitate was washed with 50% pyridine; the filtrate and washings were combined and extracted twice with petroleum ether. The water layer was evaporated to dryness. To the residue were added pyridine (10 ml), triethylamine (0.14 ml), and acetic anhydride (2.5 ml). The reaction mixture was kept at room temperature for 2 days in the dark. The reaction was stopped by adding water (25 ml), and after 3 hr the solvent was removed by evaporation with added 1-propanol. The residue was taken up in concentrated ammonia (40 ml) and kept at 50° for 3 hr. The solution was evaporated with added 1-propanol and adjusted to 100 ml with water. Uv absorption of this solution was $\lambda_{max}^{H_2O}$ 271 nm. The total optical density at 260 nm was 16,800 units.

DEAE-Cellulose Column Chromatography of the Polymerization Products. The solution containing polynucleotides thus obtained was applied to a column (4 × 38 cm) of DEAE-cellulose (bicarbonate form). After the column was washed with water, it was eluted with 0–0.25 *M* triethylammonium bicarbonate (pH 7.5, total 10 l.) in a linear gradient. One fraction was 15 ml and the flow rate was 90 ml/hr. The column was eluted further with 0.25–0.5 *M* buffer in a linear gradient and finally with 1 *M* buffer. The elution pattern is shown in Figure 1 and the total optical density of each fraction is recorded in Table I. The column was eluted further with 1 *M* buffer containing 5 *M* and 7 *M* urea. Higher oligonucleotides (total OD_{260}[130]) were obtained. Each fraction was pooled and evaporated to dryness, and the residue was evaporated several times with added water to remove triethylammonium bicarbonate completely. The residue was dissolved in water and purified further by PPC and/or column chromatography as follows.

Purification of Polynucleotides. (a) Oligonucleotides having nucleotide units under 8 were purified by preparative PPC in solvent A. For longer oligomers PPC was performed for a few days by the descending technique.

(b) Fraction no. 345–426 were evaporated to a residue and applied to a column of DEAE-cellulose (1 × 50 cm, Cl^- form). Elution was carried out with 0.3–0.5 *M* NaCl (total 0.8 l.) in a linear gradient. One fraction was 5 ml and the flow rate was 20 ml/hr. The major peak was obtained at 0.4 *M* NaCl concentration. Each fraction was diluted with water to three volumes and applied to a small column of DEAE-cellulose (bicarbonate form). Nucleotides were eluted with 1 *M* ammonium bicarbonate and desalted by several evaporations with added water.

(c) Fraction no. 966–982 were obtained by evaporation of the buffer and the residue was dissolved in water (50 ml). The solution was applied to a column (1 × 10 cm) of DEAE-cellulose (Cl^- form), which was washed with 7 *M* urea containing 0.02 *M* Tris-HCl (pH 7.5). After the washing the column was eluted with 0.05–0.3 *M* NaCl (total 1.4 l.) containing 7 *M* urea by a linear gradient technique.

(d) Fractions after no. 986 were evaporated to give a residue, which was dissolved in water (50 ml) and applied to a column (1 × 51 cm) of DEAE-cellulose. After the water-wash, the column was eluted with 0.1–0.5 *M* NaCl containing 7 *M* urea and 0.05 *N* HCl (total 2 l.) by a linear gradient. One fraction was 5 ml and the flow rate was 20 ml/hr. The major peak appeared at 0.45 *M* NaCl concentration.

Phosphate Analysis[13] of the Linear Oligonucleotides and Their Dephosphorylated Compounds. Linear oligonucleotides obtained by extracting with water from the paper chromatogram in solvent A were adjusted to 5 OD_{260}/ml; 1-ml portions of this solution were taken into three Kjehldahl tubes and 6% $HClO_4$ (0.5 ml) was added. After heating for 4 hr, the amidol (0.5 ml) and the molybdate solutions (0.25 ml) were added to each tube, and the volume was adjusted to 5 ml. Absorbance at 675 nm was measured after 15 min.

Linear oligonucleotides (30 OD units) were incubated with *E. coli* alkaline phosphatase[8] (0.4 mg/ml, 60 μl) in a buffer (total volume 600 μl) of 1 *N* NH_4HCO_3 (60 μl) at 37° for 4 hr. The entire volume was applied to filter paper and developed in solvent A, and the dephosphorylated oligomers were extracted with water; 5 OD/ml of this solution was analyzed as before. Results are summarized in Table II.

(13) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).