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Studies on Transfer Ribonucleic Acids and Related Compounds. VII.[1] Synthesis and Properties of Cyclic Oligoadenylic Acids

Eiko Ohtsuka, Hisayo Tsuji, and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University[2]

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N,2′-O-Dibenzoyladenosine 3′-phosphate was polymerized using dicyclohexylcarbodiimide as the activating reagent to give linear and cyclic oligoadenylic acids. When 5′-O-monomethoxytrityl-N,2′-O-dibenzoyladenosine 3′-phosphate was added as the chain terminator linear oligoadenylic acids were obtained as major products. Cyclic di- and triadenylic acids were also synthesized by cyclization of the corresponding linear oligonucleotides and were found to be sensitive to RNase M. Their CD spectra were measured in aqueous and methanolic solution.

It has been believed that the loop structure of the anticodon of tRNA's favors the interaction with mRNA by means of hydrogen bonding.[3–5] It is of interest to study properties of cyclic oligonucleotides, although cyclic oligonucleotides which are linked by 3′—5′ phosphodiester linkages are not exact models of the loop structure. Optical studies of cyclic oligothymidylic acids suggested quite different stacking of bases between linear- and cyclic-oligonucleotides.[6] Cyclic oligonucleotides of adenosine[7] (II) (Chart 1) and uridine[8] were characterized as side products of polymerization of mononucleotides. Since properties of cyclic-ribooligonucleotides have not been studied extensively, it was planned to synthesize comparatively larger cyclic adenylic acids.

In this paper two synthetic approaches for cyclic oligoadenylic acids are reported. One approach involved a polymerization of N,2′-O-dibenzoyladenosine 3′-phosphate (Ia) without a chain terminator and the cyclic oligonucleotides were isolated by ion exchange chromatography. The other route was to cyclize linear oligonucleotides of known chain length which had proper protecting groups. For the latter approach N,2′-O-dibenzoyladenosine 3′-phosphate (Ia) and a chain terminator 5′-O-monomethoxytrityl-N,2′-O-dibenzoyladenosine 3′-phosphate (Ib) were polymerized to increase the yield of linear oligoadenylic acids. Partly protected linear oligonucleotides were isolated after detritylation and subjected to cyclization.

Polymerization of N,2′-O-Dibenzoyladenosine (Ia)

N,2′-O-Dibenzoyladenosine 3′-phosphate (Ia) was polymerized using dicyclohexylcarbodiimide (DCC) as the activating reagent in pyridine for 6 days. The reaction mixture was treated with acetic anhydride to cleave pyrophosphate as described in the polymerization of a mixture of N-benzoyl, 2′-O-acetyladenosine 3′-phosphate and N,2′,5′-triacetyladenosine 3′-phosphate.[7] The products having acyl groups were separated by triethyl aminoethyl (TEAE)-cellulose ion exchange chromatography.[9] As summarized in Table I, the result of

1) Part VI: E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Am. Chem. Soc.*, **95**, 4725 (1973).
2) Location: *6-1-1, Toyonaka, Osaka, 560, Japan.*
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4) G. Högenauer, *Eur. J. Biochem.*, **12**, 527 (1970).
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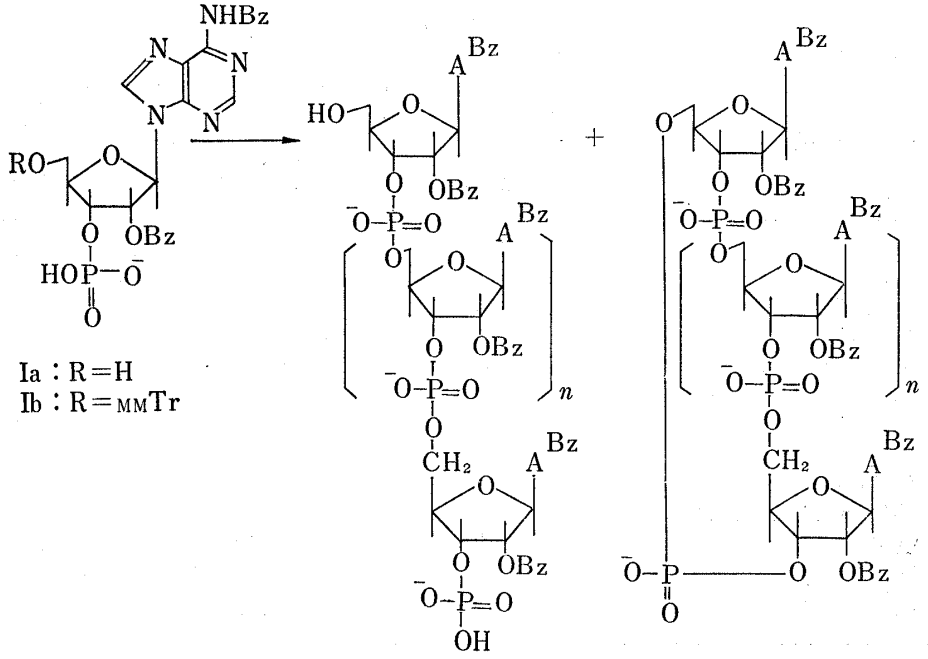

Ia : R=H
Ib : R=MMTr

Bz=benzoyl

IIa : n=0
IIb : n=1
IIc : n=2
IId : n=3

IIIa : n=0
IIIb : n=1

Chart 1

TABLE I

Peak	Fraction pooled	TOD	A_{280} %[a]	Identification
1	39— 68	2247	25	A^{Bz}(OBz)-p cyclic
2	76— 80	70	1.4	A^{Bz}(OBz)-p
3	92—107	637	13	cyclic dinucleotide (IIIa)
4	113—135	227	5	linear dinucleotide (IIa)
5	151—170	171	3.4	mixture of linear and cyclic triadenylic acids (IIb and IIIb)
6	171—195	235	4.7	
7	196—235	423	8.5	
8	1M fraction	805	16	unidentified

a) Percentages are calculated from TOD (4950 A_{280}) eluted from the column.

this polymerization showed the formation of relatively short chain of linear and cyclic oligoadenylic acids, although fractions eluted with 1M buffer was obtained substantially. The unprotected linear di- and tri-nucleotides were identified by paper chromatography, paper electrophoresis (Table II) and phosphatase[10] treatment followed by RNase M[11] digestion. Cyclic triadenylic acid was insensitive to bacterial alkaline phosphatase[10] and distinguished from the trinucleotide with 2′,3′-terminal cyclic phosphate by acidic treatment (pH 2). Cyclic di- and tri-adenylic acids were found to be sensitive to RNase M hydrolysis to give adenosine 3′-phosphate.

Polymerization of a Mixture of N,2′-O-Dibenzoyladenosine 3′-phosphate (Ia) and the 5′-O-Monomethoxytrityl Derivative (Ib)

Four to one (mole/mole) mixture of N,2′-O-dibenzoyladenosine 3′-phosphate (Ia) and 5′-O-monomethoxytrityl N,2′-O-dibenzoyladenosine 3′-phosphate (Ib) was polymerized

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11) M. Irie, *J. Biochem.* (Tokyo), **62**, 509 (1967).

Table II.

Compounds	Paper chromatography Relative mobility to Ap		Paper electrophoresis Relative mobility to Ap pH 7.5
	Solvent A	Solvent B	
Ap	1.0	1.0	1.0
ApAp		0.76	1.05
ApApAp	0.16	0.58	1.11
ApApApAp		0.32	1.14
ApApApApAp		0.19	1.15
ApA		1.2	
ApApA		0.91	
ApApApA		0.57	
ApApApApA		0.37	
5′-C-Pyridinium Ap		0.77	0.51
(Ap)	2.8	1.46	0.52
(ApAp)	1.0	1.14	0.72
(ApApAp)	0.76	0.97	0.83

Table III

Peak	Fraction pooled	TOD A_{280}	%[a]	Identification
1	38— 50	1050	8	5′-C-pyridinium A^{Bz}(OBz)-p
2	64— 74	834	6.4	A^{Bz}(OBz)-p cyclic
3	85— 91	377	2.9	A^{Bz}(OBz)-p
4	111—129	637	4.9	cyclic dinucleotide (IIIa)
5	166—192	1063	8.1	linear dinucleotide (IIa)
6	254—310	1615	12.3	IIb contaminated with IIIb
7	350—400	775	5.9	mainly linear trinucleotide (IIb)
8	430—463	847	6.5	mainly linear tetranucleotide (IIc)
9	518—540	946	7.2	mainly linear pentanucleotide (IId)

a) Percentages are calculated from TOD (13100 A_{280}) eluted from the column.

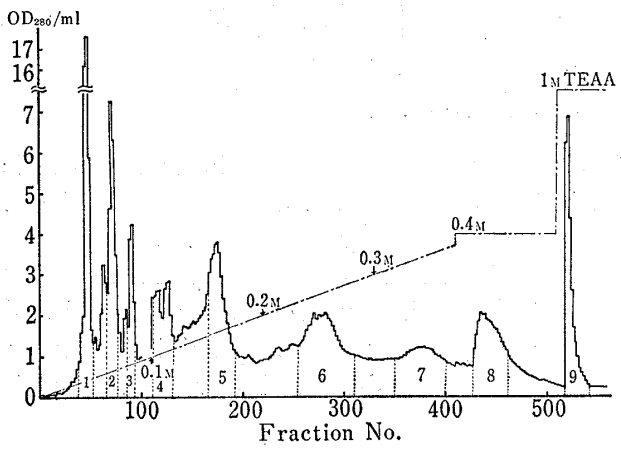

Fig. 1. Chromatography of the Product obtained in the Polymerization of N,2′-O-Dibenzoyladenosine 3′-phosphate (Ia) and 5′-O-Monomethoxytrityl Derivative (Ib) on a Column (2.5 × 50 cm) of TEAE-Cellulose (acetate) in 80% Ethanol

The gradient was 0 to 0.4M and the total volume was 8 liters. Fractions of 17 ml was collected every 20 min.

using DCC. The products were separated by TEAE-cellulose chromatography after the detritylation. The acetic anhydride treatment was omitted because the 5′-hydroxyl group of linear oligonucleotides had to be free for the cyclization. The elution pattern of the chromatography is shown in Fig. 1 and the identification of some of the peaks are given in Table III. The yield of cyclic di- and tri-adenylic acids was less than that in the first polymerization (Table I). Linear oligoadenylic acids, however, were obtained up to the pentanucleotide. The result of chain length determination of linear adenylic acids is summarized in Table IV. *Rf* values and the mobility of difrent compounds are shown in Table II.

Table IV

	Adenosine A_{260}	Ap A_{260}	Ratio
ApA	0.91	0.99	1.0 : 1.08
ApApA	0.37	0.83	1.0 : 2.20
ApApApA	0.27	0.94	1.0 : 3.48

Oligonucleotides were hydrolyzed with RNase M and subjected in paper chromatography in solvent A. Spots were eluted with water (3 ml).

Cyclization of the Di- and Tri-nucleotides

N,2′-O-Benzoylated di- and tri-adenylic acids (IIa, b) were tried to be cyclized with DCC in dilute pyridine solution. Deprotected products were first separated by paper chromatography in solvent C. Cyclic oligonucleotides thus isolated were contaminated with oligonucleotides with terminal 2′,3′-cyclic phosphate (V), which were derived from pyrophosphates of oligonucleotides (IV) by ammonia treatment (Chart 2). Unprotected cyclic di- and tri-adenylic

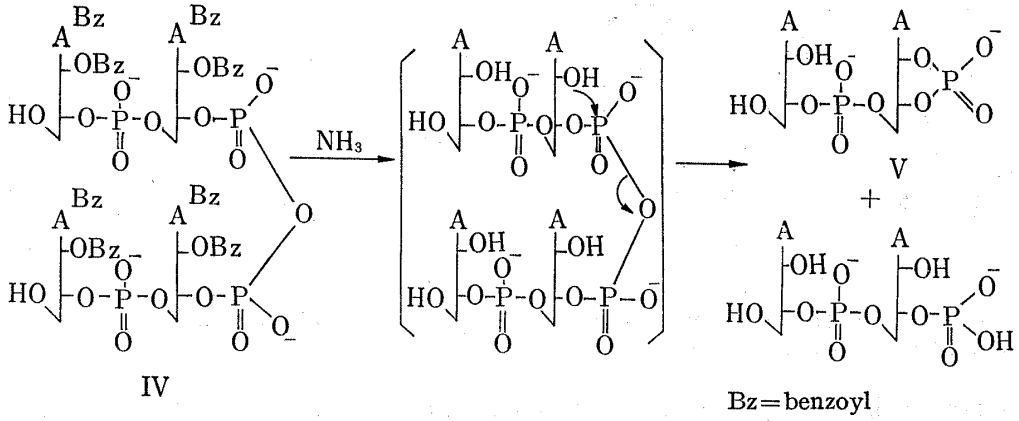

Chart 2

acids were purified by paper electrophoresis (pH 7.5) after acid treatment to cleave the 2′,3′-cyclic phosphate. *Rf* values and relative mobilities in paper electrophoresis are shown in Table II. Ultraviolet (UV) absorption spectra of these cyclic oligonucleotides are summarized in Table V together with those of linear oligomers. ε values of the cyclic di- and tri-adenylic acids at different pH are not too far from those of the mononucleotide. The linear dimer ApAp shows an appreciable increase in absorption at acidic pH. Acidic denaturation does not occur in the case of cyclic di- and tri-adenylic acids. The yields of the cyclic di- and tri-

Table V. UV Spectral Properties of Linear- and Cyclic-oligoadenylic Acids

	pH 7			pH 1			pH 13		
	λ_{max}	λ_{min}	$\varepsilon\times10^{-4}$ at 260 nm	λ_{max}	λ_{min}	$\varepsilon\times10^{-4}$ at 260 nm	λ_{max}	λ_{min}	$\varepsilon\times10^{-4}$ at 260 nm
ApAp	258	229	1.39[a]	257	230	1.52	258	230.5	1.45
ApApAp	258	231	1.26[a]	257.5	231	1.46	258	232	1.30
ApApApAp	258	232	1.23[a]	257.5	231.5	1.47	258	235	1.26
(ApAp)	259.5	232.5	1.44	257.5	231	1.48	259.5	235	1.47
(ApApAp)	259.5	236	1.45	257.5	232	1.49	259.5	237.5	1.43

a) Reported values.[15] Values at pH 1 and pH 13 were estimated from those observed at neutral pH and different pH. For ε's of the cyclic oligonucleotides, see Experimental.

oligonucleotides were 19% and 29%, respectively. Oligonucleotides degraded during the reaction and/or work-up procedure were found more than the unchanged starting materials.

Circular Dichroic Spectra

Differences in base stacking in linear and cyclic deoxyoligothymidylic acids were shown by circular dichroism, ultraviolet absorption and nuclear magnetic resonance.[6] Circular dichroic (CD) spectra of cyclic di- and tri-adenylic acids together with the linear oligonucleotides were measured in aqueous solution as shown in Figure 2. The CD spectrum of 3′,5′-cyclic adenylic acid was also measured in aqueous solution and agreed with the one calculated from the optical rotatory dispersion (ORD) spectrum.[12] This spectrum is the same as that of adenosine 5′-phosphate.[13] Probably electronic transitions in these monomers may be similar each other. The spectrum of the cyclic dinucleotide (ApAp)[14] is remarkably different from that of the linear dimer which gives almost same extrema (Table VI) reported previously.[12,15]

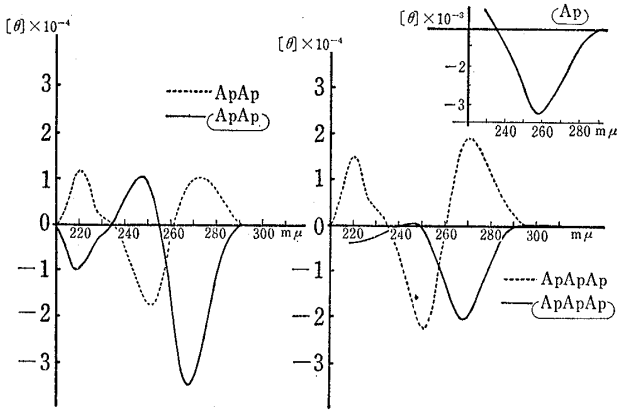

Fig. 2. CD Spectra in 0.01M Potassium Phosphate (pH 7.0) and 0.1M Potassium Fluoride

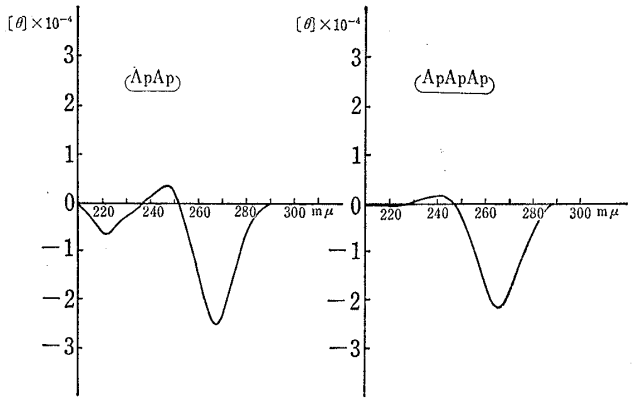

Fig. 3. CD Spectra in Methanol

The opposite sign of the Cotton effect at long wave length can be interpreted as a result of a change of the mode of exciton splitting of B_{2u} transition of adenine, due to a different ordered conformation of the bases in (ApAp) from that in ApAp. The possibility of distorsion of adenine bases for this abnormal CD spectra may be excluded by the fact that ultraviolet absorption spectra of (ApAp) and ApAp are essentially same (Table V). The CD spectrum of (ApApAp) showed a smaller negative Cotton effect at long wave length compared to (ApAp). The shape resembled that of the cyclic monomer but slightly shifted to the blue. Circular dichroic spectra of (ApAp) and (ApApAp) in methanol are shown in Figure 3. As summarized in Table VI there are no dramatic differences in shape of the spectra in aqueous and methanolic

TABLE VI. Extrema of CD Spectra shown in Fig. 2 and Fig. 3

Sample	Solvent	λ_{peak}	$[\theta]_{peak} \times 10^{-4}$	$\lambda_{crossover}$	λ_{trough}	$[\theta]_{trough} \times 10^{-4}$
(ApAp)	aqueous buffer	248	1.08	255	268	−3.69
(ApApAp)	aqueous buffer	245	0.07	248	268	−2.14
(ApAp)	methanol	248	0.352	252	268	−2.53
(ApApAp)	methanol	243	0.183	248	268	−2.20

12) C.A. Bush and H.A. Scheraga, *Biopolymers*, **7**, 395 (1965).
13) M. Ikehara, S. Uesugi, and K. Yoshida, *Biochem*, **11**, 836 (1972).
14) (ApAp) refers to cyclic diadenylic acid. Other abbreviations are as suggested by IUPAC-IUB combined commission, *J. Biol. Chem.*, **241**, 531 (1966). For protected oligonucleotides see ref. 1 and 9.
15) J. Brahms, A.M. Michelson, and K.E. Van Holde, *J. Mol. Biol.*, **15**, 467 (1966).

media. This could mean that conformation of these cyclic oligoadenylic acids is determined mainly by the backbone structure rather than the hydrophobic interaction of bases. $\overline{(ApApAp)}$ even showed smaller θ value in aqueous solution. Magnitude of the long-wave length Cotton effect, however, decreased in methanol in the case of $\overline{(ApAp)}$. This may imply that in the water solution two adenine bases of $\overline{(ApAp)}$ could interact probably by the stacking forces. It may be interesting to observe CD spectra of macrocyclic oligonucleotides.

Experimental

General Methods——Paper chromatography was performed by the descending techniques in the following solvents: A, Isopropanol–concentrated ammonia–water (7:1:2, v/v); B, *n*-propanol–concentrated ammonia–water (55:10:35, v/v). Paper electrophoresis was performed at 900 V/40 cm using 0.05 M triethylammonium bicarbonate, pH 7.5. Other general methods are mostly as described previously.[1,9]

Optical Measurements——Circular dichroism measurements were performed at room temperature (20—23°) using a JASCO ORD/UV-5 spectropolarimeter with attachment for CD measurement. The buffer used was 0.01 M potassium phosphate (pH 7.0) containing 0.1 M potassium fluoride. Ultraviolet spectra were taken by a Hitachi EPS-3T spectrophotometer. All results are expressed as residue extinction or residue ellipticity. The samples were measured in 1 cm path length cells at the concentration of 0.5—1.0×10^{-4}M.

Extinction coefficients of the cyclic oligonucleotides were calculated from an increase of absorption after enzymatic hydrolysis. RNase M (25 μg) were used for the oligonucleotide (2—3 A_{260} units) in 0.1 M ammonium acetate (pH 5.0, 100 λ) at 37° for 4 hr. The incubation mixture was diluted with 0.01 M potassium phosphate (pH 7.0) and 0.1 M potassium fluoride (3 ml). The same amount of the oligonucleotide were treated under the same condition without the enzyme. An extinction coefficient of 1.54×10^4 at 260 nm was used for Ap.

Polymerization of N,2′-O-Dibenzoyladenosine 3′-phosphate (Ia)——Pyrimidinium N,2′-O-dibenzoyladenosine 3′-phosphate (0.36 mmole) was dissolved in 50% aqueous pyridine and passed through a small column of pyridinium Dowex 50×2. The effluent and washings were evaporated with added pyridine. The nucleotide was precipitated from its anhydrous pyridine solution with ether–pentane (3:2) and the precipitate was coevaporated with pyridine 3 times. The dried residue was dissolved in pyridine (2 ml) and DCC (1.45 mmoles) was added. The solution was concentrated to *ca.* 1.5 ml and kept for 6 days at 30°. Aqueous pyridine (50%, 20 ml) was added and DCC was removed with pentane (10 ml) 3 portions. After 6 hr the filtered solution was evaporated with pyridine. The dried residue was treated with acetic anhydride (1 ml) in pyridine (2.5 ml) for 2 days. The volatile materials were evaporated and the residue was treated with 50% pyridine for overnight. The reaction mixture was dissolved in 80% ethanol (40 ml) and applied to a column (2.5×50 cm) of TEAE-cellulose (acetate). Elution was carried out using a linear gradient of triethylammonium acetate in 80% ethanol. Salt concentration was 0 to 0.4 M and the total volume was 8 liters. Fractions of 15 ml were collected every 20 min. The result of the chromatography was summarized in Table I.

Polymerization of a Mixture of N,2′-O-Dibenzoyladenosine 3′-phosphate (Ia) and 5′-O-Monomethoxytrityl-N,2′-O-dibenzoyladenosine 3′-phosphate (Ib)——Pyridinium salt of Ia (0.94 mmole) and pyridinium salt of Ib (0.25 mmole) were passed through a column of pyridinium Dowex 50 as described above. The dry mixture was treated with DCC (4.8 mmoles) in pyridine (3 ml). The homogeneous solution was concentrated to a sirup and kept for 5 days at 30°. Aqueous pyridine treatment was carried out as above and the mixture was precipitated in ether–pentane (3:2) from their anhydrous pyridine solution. The precipitate was treated with 80% acetic acid (30 ml) for 2 hr and acetic acid was evaporated with water. Ninety percent of the reaction mixture was applied to a column of TEAE-cellulose (acetate). The elution pattern and conditions are shown in Fig. 1 and the identification of peaks are shown in Table III.

Cyclization of A^{Bz}(OBz)-p-A^{Bz}(OBz)-p (IIa)——The triethylammonium salt of the protected dinucleotide (IIa) (300 A_{280}, 8.3 μmoles) were passed through a column (*ca.* 1 ml) of pyridinium Dowex 50. The effluent and washings were evaporated to dryness. The residue was dissolved in pyridine (0.5 ml) and precipitated in ether–pentane (3:2) mixture. The precipitate was coevaporated with dry pyridine 3 times and treated with DCC (0.18 mmole) in pyridine (1.5 ml) for 6 days at 30°. Aqueous pyridine (50%, 2 ml) was added and DCC was removed with pentane. After 4 hr the filtered solution was made anhydrous with pyridine and precipitated in ether–pentane (3:2). The precipitate was treated with acetic anhydride (0.5 ml) in pyridine (1 ml) for 2 days at room temperature. The volatile materials were removed and the residue was treated with aqueous pyridine as described for the reaction mixture of the polymerization of A^{Bz}(OBz)-p. The mixture was treated with 15 N methanolic ammonia and the deprotected nucleotides were subjected to paper chromatography in solvent B. The starting material was recovered 13% and 29% of the UV absorbing material showed the same mobility as $\overline{(ApAp)}$ obtained by polymerization. The material showing the same *Rf* as $\overline{(ApAp)}$ was eluted from paper chromatogram and adjusted with hydrochloric acid to pH 2. The solution

was evaporated *in vacuo* and applied to paper electrophoresis. Adenosine 2′,3′-cyclic phosphate was hydrolyzed with this treatment and adenosine 3′,5′-cyclic phosphate was not changed under the same condition. The electrophoresis resolved cyclic diadenylic acid and the linear dinucleotide which was probably derived from the dinucleotide having the terminal cyclic phosphate (V). The yield after this treatment was 19%. *Rf* values are shown in Table II. The cyclic dinucleotide (ApAp) was subjected to RNase M digestion and found to be hydrolyzed completely under the condition described in general methods.

Cyclization of A^{Bz}(OBz)-p-A^{Bz}(OBz)-p-A^{Bz}(OBz)-p (IIb)——The trinucleotide (450 A_{280}, 8.3 μmoles) was treated with DCC (0.1 mmole) as described for the cyclization of the dinucleotide. The deprotected product was applied to paper chromatography in solvent B and the material which travelled in *Rf* 0.97 (40% of the mixture) was transfered to paper electrophoresis after pH 2 treatment. The overall yield was 29%.