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Polynucleotides. XLVIII.[1] Synthesis and Template-Directed Polymerization of Tri-(adenylic Acid) having a 2′-O-(*o*-nitrobenzyl) Group at the 3′-End

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Polymerization of tri(adenylic acid) bearing a 2′-O-(*o*-nitrobenzyl) group and a 3′-phosphate group at the 3′-end($A-A-A_p^{NB}$) was attempted in the presence of poly(uridylic acid), poly(U), using water-soluble carbodiimide. The nitrobenzyl group can be removed photochemically under mild conditions. A reaction containing the trimer and poly(U) in 1 : 2 ratio at 0° for 15 days gave polymerized products (up to the 9-mer) in 14% yield and was proved to be dependent on the template. Raising the reaction temperature to 15° accelerated the polymerization rate. Reaction with a doubled trimer concentration at 0° did not improve the polymerization yield. Although the newly formed phosphodiester linkage was exclusively 3′—5′, some isomerization (7%) to 2′—5′ linkages seems to occur at bonds adjacent to free 2′-hydroxyl groups. As a side product, cyclic tri-(adenylic acid), which was formed by intramolecular condensation, was obtained in these reactions and in control reactions without template.

Keywords——poly (uridylic acid); hexaadenylate; nonaadenylate; 3′—5′-phosphodiester linkage; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; column chromatography; photoirradiation

The chemical condensation of oligonucleotides on a template polynucleotide in aqueous solution was first reported by Naylor and Gilham in 1966.[3] They attempted condensation of oligo(thymidylic acid) on a poly(A) template using water-soluble carbodiimide as the condensing reagent and obtained the dodecamer in 5% yield. Several papers have been published on the template-directed chemical condensation of oligonucleotides.[4–7] Uesugi and Ts'o[5] succeeded in polymerizing oligo(2′-O-methylinosinic acid) bearing 3′terminal phosphate group using poly(C) and a water-soluble carbodiimide in 40—70% yield. On the other hand, Orgel, Lohrmann, and their co-workers have reported detailed studies on condensations and polymerizations of mononucleotides in the presence of complementary polynucleotide templates (see the review by Orgel and Lohrmann, 1974[8]). They reported that the newly formed phosphodiester bond was mainly 2′—5′ when unprotected ribomononucleotides were used regardless of the method employed for the phosphate group activation.[9–11] Recently we examined the polymerization of adenylyl-(3′—5′)-adenosine cyclic 2′,3′-phosphate on a poly (U) template in water and obtained polymerized products in moderate yields (15—35 %).[7] However, in this case the newly formed phosphodiester bond was again exclusively

1) Part XLVII: M. Ikehara and T. Fukui, *Biochim. Biophys. Acta*, in press.
2) Location: *Suita, Osaka 565, Japan.*
3) R. Naylor and P.T. Gilham, *Biochemistry*, **5**, 2722 (1966).
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5) S. Uesugi and P.O.P. Ts'o, *Biochemistry*, **13**, 3142 (1974).
6) D.A. Asher and A.H. McHale, *Science*, **192**, 53 (1976).
7) S. Uesugi and M. Ikehara, *Biochemistry*, **16**, 493 (1977).
8) C.E. Orgel and R. Lohrman, *Acc. Chem. Res.*, **9**, 368 (1974).
9) J. Sulston, R. Lohrmann, L.E. Orgel, and H.T. Miles, *Proc. Natl. Acad. Sci. U.S.A.*, **59**, 726 (1968).
10) B.J. Weimann, R. Lohrmann, L.E. Orgel, H. Schneider-Bernloehr, and J.E. Sulston, *Science*, **161**, 387 (1968).
11) M. Renz, R. Lohrmann, and L.E. Orgel, *Biochim. Biophys. Acta*, **240**, 463 (1971).

2′—5′ and not the natural 3′—5′ linkage. Usher and McHale[6] obtained similar results starting from hexa (adenylic acid) having a cyclic 2′,3′-phosphate group. Therefore, for specific formation of the 3′—5′ phosphodiester linkage, some kind of protecting group at least on the terminal 2′-hydroxyl seems to be necessary. As we have been working on the synthesis of ribooligonucleotides and as it is very difficult to synthesize ribooligonucleotides with chain lengths longer than 10 by purely chemical methods, we were interested in the chemical joining of ribooligonucleotides on appropriate templates for the preparation of long ribooligonucleotides having natural phosphodiester linkages. Here we report polymerization reactions of a tri(adenylic acid) having a 2′-O-(*o*-nitrobenzyl)group and a 3′-phosphate group at the 3′-end (A–A–A_p^{NB}) in the presence of poly(U) template. The *o*-nitrobenzyl group can be removed photochemically with UV light (wavelength longer than 280 nm) at neutral pH at room temperature.[12,13]

Synthesis of A-A-A_p^{NB} (3)

The tri(adenylic acid) with an *o*-nitrobenzyl group on the terminal 2′-OH (3, A–A–A_p^{NB}) was synthesized by condensation using dicyclohexylcarbodiimide in pyridine of adenosine 3′-phosphate protected with monomethoxytrityl and benzoyl groups (1), with di(adenylic acid), in which all amino, hydroxyl and phosphate groups but the terminal 5′–OH were protected (2).

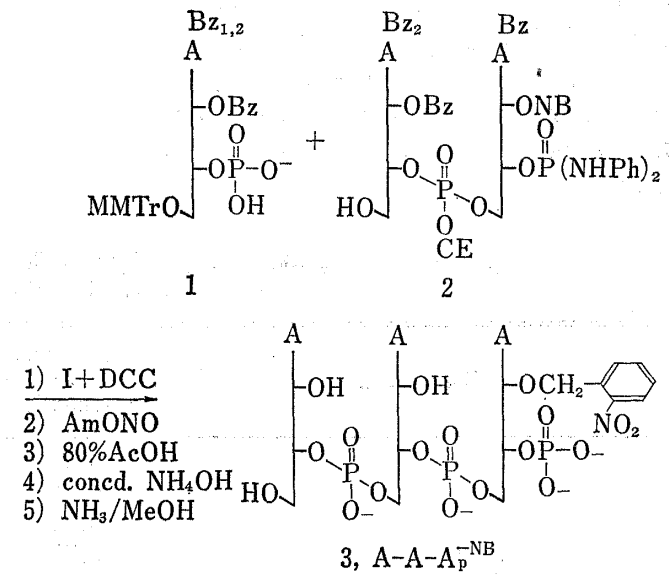

Fig. 1. Synthesis of A–A–A_p^{NB}

Abbreviations used are: MMTr, monomethoxytrityl: Bz, benzoyl; NB, *o*-nitrobenzyl; CE, cyanoethyl; Ph, phenyl; DCC, dicyclohexylcarbodiimide; AmONO, isoamylnitrite.

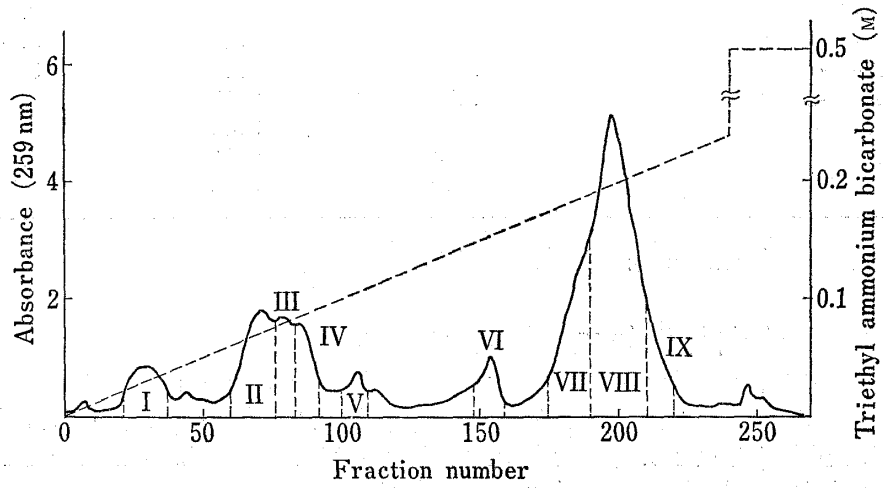

Fig. 2. Chromatography of the Products in the Synthesis of A–A–A_p^{NB} on a DEAE-Cellulose Column (1.7 × 46 cm)

Elution was carried out using a linear salt gradient of $Et_3NH_2CO_3$ (pH 7.5) (0—0.3M, total 6l). The major componenents in the fractions numbered as indicated above were A>p (fraction I), Ap (fractions II—IV), A-A_p^{NB} (fraction VI) and A-A-A_p^{NB} (fractions VII—IX).

12) A. Pachornik, B. Amit, and R.B. Woodward, *J. Am. Chem. Soc.*, **92**, 6333 (1970).
13) E. Ohtsuka, S. Tanaka, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **25**, 949 (1977).

After removal of all the protecting groups except for terminal 2'-O-(*o*-nitrobenzyl) group the products were separated by chromatography on a DEAE-cellulose column. The chromatogram is shown in Figure 2. The desired product, A–A–A_p^{-NB}, was eluted in fractions VII—VIII (*ca*, 60% from compound **2**). The trimer in fractions VIII was further purified by chromatography on a DEAE-Sephadex A-25 column to give pure A–A–A_p^{-NB}. This compound (**3**) was hydrolyzed completely by RNase M to give Ap and A_p^{-NB} in a 2:1 ratio Irradiation of the compound (**3**) gave A–A–Ap, which after dephosphorylation was digested completely by RNase M to give Ap and A in a 1.83:1.00 ratio. Hypochromicity calculated from ε(p) values of monomers and the trimer (**3**) was 19% which is comparable to that (16%) of A–A–A.[14] Hypochromicity obtained from RNase M digestion was 17%. The CD spectrum of this compound is rather similar to that of A–A–A, whereas the magnitude of the positive band in the long-wavelength region was reduced to about a half. Therefore, perturbation by the terminal *o*-nitrobenzyl group on adenine base stacking in A–A–A_p^{-NB} may not be serious. The properties of A–A–A_p^{-NB} and related compounds are summarized in Table I.

Table I. Paper Chromatography and Electrophoresis of Tri (Adenylic Acid) Derivatives and Realted Compounds

Compound	Paper chromatography *Rf*		Paper electrophoresis R_{-Aq-A}[a]
	Solvent A	Solvent B	
A	0.54	0.58	0
A>p	0.47	0.58	0.61
A_p	0.12	0.31	1.00
A_p^{-NB}	0.31		
A–A_p	0.07	0.24	1.02
A–A_p^{-NB}	0.13	0.38	1.02
A-A_p^{-NB}(NHPh)$_2$	0.72		0.27
A–A–A_p		0.12	1.04
A-A-A_p^{-NB}	0.06	0.24	1.03
A–A–A_p^{-NB}(NHPh)$_2$	0.49		0.54
A–A–A		0.26	0.62
A–A–A>p		0.24	0.88
A–A–A_p[b]	0.12	0.29	0.84
A–A–A_p^{-NB}[b]	0.20	0.41	0.83
A^{-NB}–A_p		0.38	0.98
A–A	0.23	0.44	0.35
A^{-NB}–A	0.40	0.58	0.33

a) Relative mobility to A_p(1.0) and A(0.0) is presented.
b) Cyclic tri(adenylic acid) derivatives are disignated by an underline.

Polymerization of A-A-A_p^{-NB} (Trimer/Poly(U)=1:2) at 0°

A polymerization reaction of A–A–A_p^{-NB} was first attempted with trimer-poly(U) ratio 1:2 at 0 °. A mixture of A–A–A_p^{-NB} (10 mM), poly(U) (20 mM), NaCl (1.5 M) and water-soluble carbodiimide (125 mM×3) was kept at 0 ° for 15 days. Each time when the carbodiimide was added, the pH of the solution was adjusted to 5.6. To remove salt and the carbodiimide from the reaction mixture, gel filtration on a Saphadex G-15 column was carried out. Elution with water gave the profile shown in Figure 4a. Peak I contained poly(U), the trimer and the polymerized products. Peak II was assumed to contain the carbodiimide and its derivatives because the UV spectra of these fractions showed no λ max around 260 nm but an end-absorption. The nature of the material in the peak III will be discussed

14) J. Brahms, A.M. Michelson, and K.E. Van Holde, *J. Mol. Biol.*, **15**, 467 (1966).

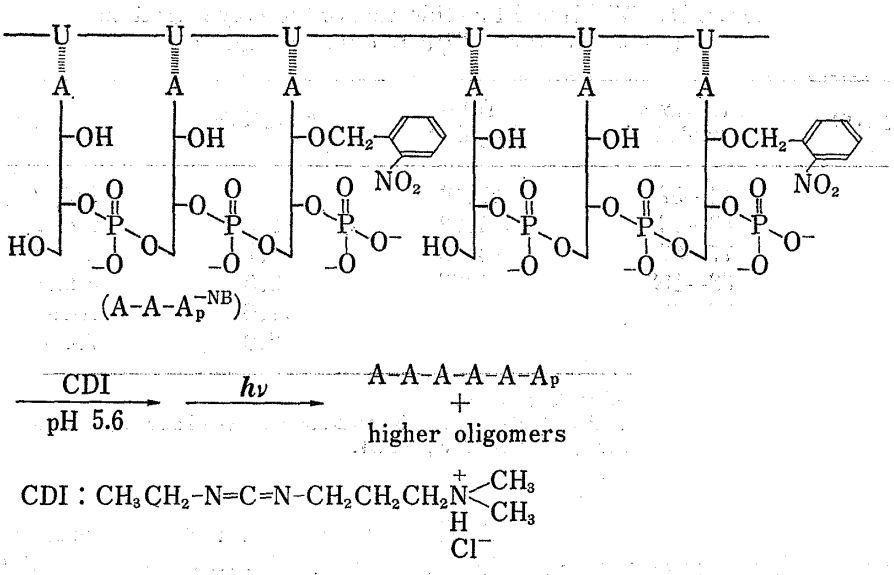

Fig. 3. Chemical Polymerization of A-A-A$_p^{-NB}$ on a Poly(U) Template

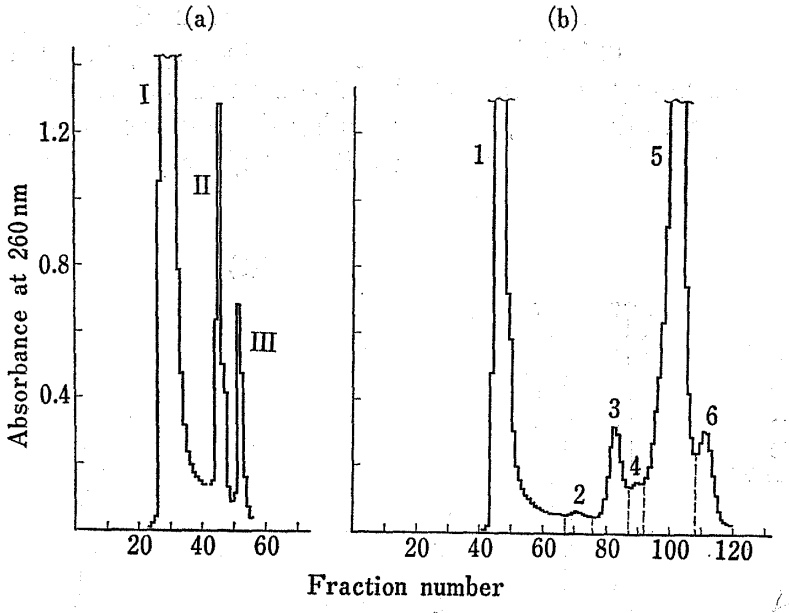

Fig. 4. Gel Filtration of the Polymerization Products (A-A-A$_p^{-NB}$/Poly(U) = 1 : 2, at 0 °)

a) Elution profile in the desalting step on a column (1.6×88 cm) of Sephadex G-15. Elution was carried out with water. 3.28 A_{260} units were recovered in the peak III (tube number 52—56). The materials in the peak I (tube no. 27—43) were separated in the following step after phonto-irradiation.

b) Elution profile in the separation step on a column (2.3×86 cm) of Sephadex G-50. Elution was carried out with 0.1 M $Et_3NH_2CO_3$. The yields and identification of these peaks are shown in Table II.

later. The materials in peak I were separated by chromatography on a Sephadex G-50 column after removal of the *o*-nitrobenzyl groups by photo-irradiation. Elution with 0.1 M $Et_3NH_2CO_3$ buffer (pH 7.5) gave the profile shown in Figure 4b. Six different peaks were also observed in a chromatogram (not shown here) which was recorded using a flow cell in a spectrophotometer. Yields and identification of these peaks are shown in Table II. Peak I was identified as poly(U) from its position and UV spectrum. The compounds in peaks

Table II. Yields and Identification of the Polymerization Products (A-A-A_p-^{NB}/Poly (U) = 1 : 2) at 0 °

Peak No.[a]	Fractions pooled	Total A_{260} units	% ratio[b]	Identification
1	44—67	73.19		Poly (U)
2	68—76	1.29	2.3	9-mer
3	77—87	4.83	8.7	6-mer
4	88—108	1.96	3.5	6-mer
5	93—108	39.68	71.6	A-A-Ap
6	109—115	4.43	8.0	A-A-A>p

a) The peak numbers are shown in Figure 4b.
b) The ratio of A_{260} units to that of total adenine oligonucleotides recovered including that in the desalting step, in which 5.9% is recovered in peak III.

5 and 6 were identified as A–A–A_p and A–A–A>p by direct comparison with authentic samples in paper chromatography and electrophoresis. A–A–A>p (10% of the trimer) is assumed to be formed by partial debenzylation and cyclization during the reaction and/or work-up in the presence of the carbodiimide. The compounds in peak 2—4 were identified as the polymerized products, nonamer (9-mer) and hexamer (6-mer), by T_m measurement of the complex with poly(U). The T_m's of 1A : 2U complexes of the oligomers from peaks 2,3,4 and 5 were 53.5, 44.4 and 14 °, respectively, in 0.01 M $MgCl_2$–0.01 M sodium cacodylate buffer (pH 7.0) maintaining the oligomer strand concentration at 2.5×10^{-6} M. The $1/T_m$ (K) *vs.* $1/n$ plot gave a straight line, thus confirming the correctness of the chain length (n) estimations. The linear relationship between $1/T_m$ and $1/n$ has been predicted by theoretical

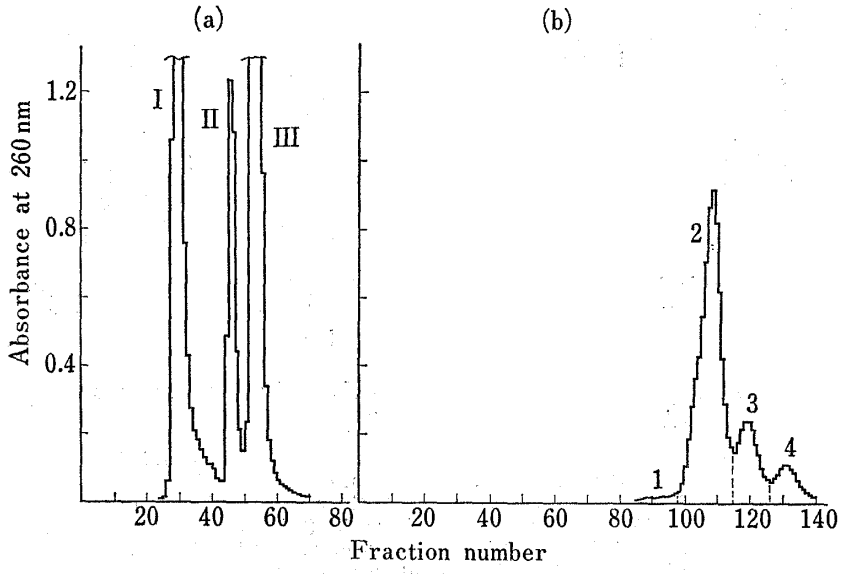

Fig. 5. Gel Filtration of the Control Reaction Products (without Poly(U), at 0 °)

a) Elution profile in the desalting step on a column of Sephadex G-15 under the same conditions as described in Fig. 4. 35.70 A_{260} units were recovered in the peak III (tube no. 53—64). Materials in the peak I (tube no. 27—42, 27.39 A_{260} units) were separated in the following step after photo-irradiation.
b) Elution profile in the separation step on a column of Sephadex G-50 under the same conditions as described in Fig. 4. The yields and identification of these peaks are as follows: Peak I (tube no. 85—98), 0.562 A_{260} units, 6-mer; peak 2 (tube no. 99—115), 17.73 A_{260} units, A-A-A_p, peak 3 (tube no. 116—126), 4.56 A_{260} units, A-A-A>p; peak 4 (tube no. 127—138), 2.38 A_{260} units, A-A-A.

analysis and has been confirmed experimentally in many polymer-oligomer complexes.[15] The 6-mer from peak 4 is assumed to contain a terminal 2′,3′-cyclic phosphate group as in the case of A–A–A>p from peak 6. In conclusion, under the present conditions at 0 ° for 15 days, about 14% of the trimer was polymerized to give 6-mer (12%) and 9-mer (2%).

Next, the effect of the template on the polymerization was examined by a control reaction without poly(U). The elution profiles of the desalting step and the separation step are shown in Figure 5a and 5b. In the desalting step, it was noted that peak III was larger than peak I. The peak I was analyzed by gel filtration on a Sephadex G-50 column. Only 1% of the total adenine oligonucleotides was eluted in the 6-mer region. The T_m of its complex with poly(U) was 41.5 ° under the same condition as described earlier. Thus it was proved that the polymerization was indeed template dependent.

To examine the newly formed phosphodiester linkage, the 6-mer from peak 3 (1 A_{260} unit) was digested with RNase M and the products were analyzed by paper chromatography in solvent D. About 7% of the total A_{260} units recovered was found in a spot (*Rf* 0.11) below Ap (*Rf* 0.23). On dephosphorylation of the compound in this spot, it showed the same mobility as that of A–A in paper electrophoresis. Therefore, the original spot must be A–Ap with a 2′—5′ phosphodiester linkage in the chain. Furthermore, examination of the trimer from peak 5 (2 A_{260} units) by the same treatment revealed that this oligomer also contained the same extent of 2′—5′-linkage. These results strongly suggest that the phosphodiester bond adjacent to a free 2′-hydroxyl may be activated by a large excess of carbodiimide and isomerize to form a 2′—5′ linkage. The newly formed phosphodiester linkage must be exclusively 3′—5′, because adjacent 2′-OH's were protected. Even if A–A–A_p^{NB} was partial-

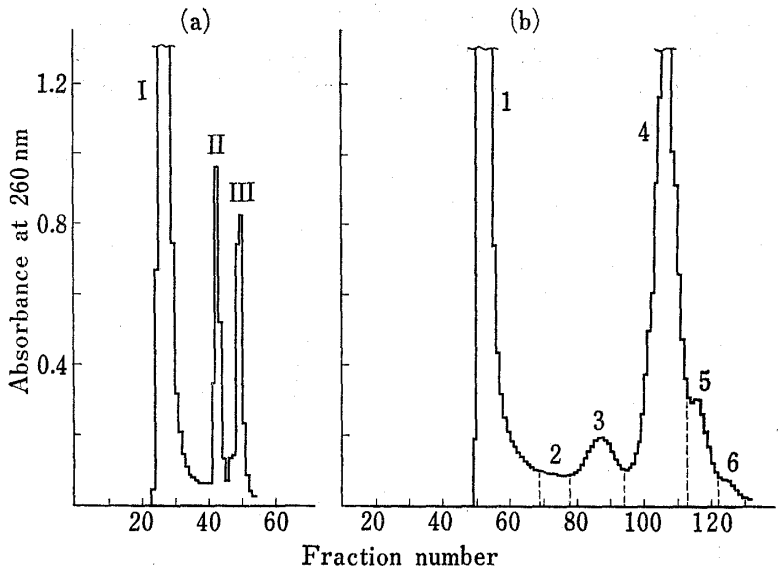

Fig. 6. Gel Filtration of the Polymerization Products (A–A–A_p^{NB} Poly(U)=1:2, at 15 °)

a) Elution profile in the desaltng step on a column of Sephadex G-15 under the same conditions as described in Fig. 4. 6.56 A_{260} units were recovered in the peak III (tube no. 49—60). The materials in the peak I (tube no. 25—41) were separated in the following step after photo-irradiation.
b) Elution profile in the separation step on a column of Sephadex G-50 under the same conditions as descrived in Fig. 4. The yield and identification of these peaks are shown in Table III.

15) P.O.P. Ts'o, "Basic Principles in Nucleic Acid Chemistry," Vol. 2, ed. by P.O.P. Ts'o., Academic Press, New York, 1974, p. 305.

ly deprotected in the reaction mixture, the resulting A–A–A_p would be quickly converted to inactive A–A–A>p.

Polymerization of A-A-A_p^{-NB} (Trimer/Poly(U)=1: 2) at 15°

In this case the reaction time was halved to 7 days to avoid possible breakdown of oligomers. The elution profiles of the desalting step and the separation atep sre shown in Fig. 6a and 6b. Yields and identification of the products are summarized in Table III.

TABLE III. Yields and Identification of the Polymerization Products (A-A-A_p^{-NB}/Poly (U)=1: 2) at 15 °

Peak No.[a]	Fractions pooled	Total A_{260} units	% ratio[b]	Identification
1	40—59	80.89		Poly (U)
2	60—68	2.81	5.0	6-mer
3	69—84	6.33	11.3	6-mer
4	85—103	34.79	61.9	A-A-A_p
5	104—112	5.64	10.0	A-A-A>p
6	113—123	1.41	2.5	A-A-A

a) The peak numbers are shown in Figure 6b.
b) The ratio of A_{260} units to that of total adenine oligonucleotides recovered including that in the desalting step, in which 9.4% is recovered in peak III.

Peaks 2 and 3 contained the polymerized products and the combined yield was about 16%. The compounds in these peaks were identified as the 6-mers because the complexes with poly(U) showed Tm's at 44° (peak 2) and 42.5° (peak 3) and no transition was observed above 50°. The 6-mer in peak 2 might be eluted after dissociating from a complex with poly(U) during passage through the column of Sephadex G-50. The poor resolution may be due to the lower temperature, at which the gel filtration was carried out, compared with that of the

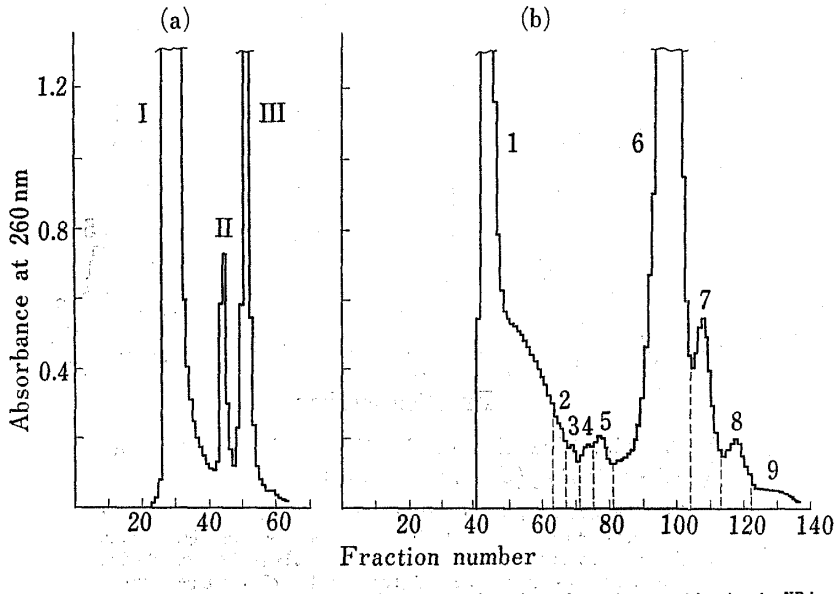

Fig. 7. Gel Filtration of the Polymerization Products (A–A–A_p^{-NB}/ Poly(U)=1: 1, at 0 °)

a) Elution profile in the desalting step on a column of Sephadex G-15 under the same conditions as described in Fig. 4. 21.36 A_{260} units were recovered in the peak III (tube no. 50—60). The materials in the peak I (tube no. 26—42) were separated in the following step after photo-irradiation.
b) Elution profile in the separation step on a column of Sephadex G-50 under the same conditions as described in Fig. 4. The yields and identification of these peaks are shown in Table IV.

first polymerization at 0°. The yield of this polymerization at 15° for 7 days is about the same as that of the polymerization at 0° for 15 days. This means that raising the temperature from 0° to 15° doubles the rate of polymerization. Though the 9-mer was not detected in this experiment, it could have been retained in the poly(U) peak.

Polymerization of A-A-A_p^{-NB} (Trimer/Poly(U)=1: 1) at 0°

Alternatively, polymerization reaction, in which the concentration of the trimer was doubled to make a 1: 1 mixture of the trimer and poly(U), was attempted at 0° for 15 days. If a 1: 1 complex is formed under this condition, it might be a less hindered substrate than the triple stranded complex, in which the trimer is associated with two strands of poly(U). The elution profile for the desalting step and the separation step are shown in Fig. 7a and 7b. Yields and identification of the products are summarized in Table IV. It may be noted that

Table IV. Yields and Identification of the Polymerermization Products (A-A-A_p^{-NB}/Poly (U)=1: 1) at 0°

Peak No.[a]	Fractions pooled	Total A_{260} units	% ratio[b]	Identification
1	41—63	75.57		Poly (U)
2	64—67	2.60		Unidentified
3	68—71	1.82	1.5	6-mer
4	72—75	1.93	1.5	6-mer
5	76—81	2.89	2.3	6-mer
6	82—104	81.06	65.0	A-A-A_p
7	105—113	9.82	7.9	A-A-A>p
8	114—122	3.97	3.2	A-A-A
9	123—138	1.97	1.6	Unidentified

a) The peak numbers are shown in Figure 7b.
b) The ratio of A_{260} units to that of total adenine oligonucleotides recovered including that in the desalting step, in which 17.1% is recovered in peak III.

the peak III in the desalting step is much larger than those on the earlier polymerization reactions. Peaks 3—5 contained the polymerized products and the combined yield was about 5%. The compounds in these peaks were identified as the 6-mers because the complexes with poly(U) showed T_m's at 42° (peak 3), 44° (peak 4) and 43.5° (peak 5) and again no transition was observed above 50 °C. The poor resolution may be caused by reasons similar to those in the second polymerization. Peak 2 showed no significant transition over the temperature range 0° to 80° in 0.01 M $MgCl_2$–0.01 M sodium cacodylate (pH 7), whereas poly(U) itself showed a T_m of a self-complex at around 5° with hyperchromicity of ca. 30% under the same conditions. Upon addition of poly(U), a solution of peak 2 material gave a T_m at 41.5° with a very small hyperchromicity showing that a trace of the 6-mer was present in peak 2. The yield of this polymerization and the absolute amount of the polymerized products were smaller than those in the polymerization with trimer-poly(U) (1: 2). These poor results may be due to side reactions of the adenine oligonucleotide and probably also of poly(U).

The Side Product in the Peak III of the Dealting Step

In all reactions including the control reaction at 0°, three major peaks were observed in the elution profile of the desalting step with Sephadex G-15. The first and the third peaks contained adenine oligonucleotides. The UV spectrum of the peak III exhibited a λ_{max} at 259 nm which is significantly higher than that (258 nm) of the ordinary adenine oligonucleptides.

The compounds in the peak III were purified by successive paper chromatography in solvent C and B (or D). Two bands (*Rf* 0.43 and 0.31) were obtained in about equal amounts

in the second paper chromatogram in solvent B. In paper electrophoresis, these compounds showed similar mobilities which were different from that of A–A–A. The compound (1 A_{260} unit) from the low *Rf* band was hydrolyzed completely to Ap with RNase M. This compound was resistant to acidic hydrolysis with 0.1 N hydrochloric acid at 37° for 30 min, showing that it does not contain a terminal 2′,3′-cyclic phosphate group.[7]

The compound (1 A_{260} unit) from the high *Rf* band was also hydrolyzed with RNase M but gave rise to Ap and A_p^{-NB}-Ap, which were identified by paper electrophoresis. All these operations were carried out without precautions to avoid exposure to light. When the Peak III from the control reaction was carefully protected from light the high *Rf* compound was obtained predominantly and it gave adenosine and A_p^{-NB}-A[13] in 1:1.7 residue ratio after successive digestion with RNase M and alkaline phosphatase.

From the results described above, it was concluded that the original compound in peak III of the desalting step was a cyclic tri(adenylic acid)(the high *Rf* compound) with a nitrobenzyl group on a 2′-hydroxyl in one of the adenylic acid residues. Upon exposure of this compound to the light, it may have been converted to a cyclic tri(adenylic acid)(the low *Rf* compound) without protecting group. The properties of the latter compound in paper chromatography, paper electrophoresis and circular dichroic spectra are the same as those of the cyclic tri(adenylic acid) reported by Ohtsuka *et al.*[16]

The CD spectrum of the cyclic tri(adenylic acid) showed $[\theta]$min -3.0×10^4 at 267 nm, $[\theta]$max 0.3×10^4 at 248 nm and $[\theta]$min -0.9×10^4 at 219 nm in 0.1 M sodium chloride–0.01 M sodium phosphate buffer (pH 7) at 15°. This spectrum is entirely different from that of the linear tri(adenylic acid). The properties of the cyclic tri(adenylic acid) and related compounds are included in Table I.

Conclusion

Polymerization reactions of A–A–A_p^{-NB} were tried under three different sets of conditions. In the first polymerization, the trimer (10 mM) and poly(U)(20 mM) were treated with the carbodiimide at 0 °C for 15 days to give the polymerized products in about 14% yield. In the second polymerization, the same components were treated at around 15 ° for 7 days to give the polymerized products in about 16% yield. In the third polymerization, the trimer(20 mM) and poly(U)(20 mM) were treated in the same way as in the first polymerization to give the polymerized products in about 5% yield. The relatively poor yeilds in these reactions may not be due to instability of the trimer-poly(U) complexes. The A–A–A_p-2poly(U) and A–A–A_p^{-NB}-2poly(U) complexes showed T_m at 16 ° and 13.5 ° in 1.5 M NaCl–0.01 M sodium cacodylate buffer (pH 7) with oligomer strand concentration at 1.1×10^{-5} M. The reduction of T_m by the introduction of the nitrobenzyl group on the terminal 2′-hydroxyl of A–A–Ap was only 2.5 ° under these cinditions. The oligomer strand concentrations ($3—6\times10^{-3}$ M) in the polymerization mixtures are several hundred times higher than in these T_m measurements. The T_m of the polymerization mixture may be much higher than 15 °. The yields of peak III in the desalting step provide further support for stable complex formation in the polymerization mixture. In the control reaction without poly(U) the cyclic tri(adenylic acid) was obtained in high yield (*ca*, 57%). Upon complex formation with poly (U), the trimer may be protected against intramolecular condensation between 3′-phosphate group and 5′–OH. In the first polymerization at 0 °C and the second polymerization, the yield of the cyclic trimer were about 6% and 9%, respectively. These figures suggest that the trimer has been protected by complex formation with poly(U) almost equally at 0 ° and 15 °. In the third polymerization, the yield of the cyclic trimer was 17%. If we assume that only a 1:2 complex is formed in the reaction mixture and that the remaining trimer

16) E. Ohtsuka, H. Tsuji, and M. Ikehara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1022 (1974).

(50%) is free, the yield of the cyclic trimer should have been much higher than 17%. Therefore, in the 1:1 mixture of the trimer and poly(U) under the present conditions, both 1:1 and 1:2 complexes may be formed. This situation might cause side reactions on poly(U) and the trimer and result in a reduction of the yield of polymerization.

A possible reason for the relatively low yields of polymerization in this system is steric interference of a nitrobenzyl group to prevent close contact between the activated 3′-phosphate group and the 5′–OH of the adjacent molecule. If this is the case, a smaller protecting group would be desirable. Polymerization of oligo(inosinic acids) having methyl groups on 2′-hydroxyls occurs in high yeilds under similar conditions.[5] In addition, the use of oligomer with a preactivated terminal phosphate group may be necessary to avoid isomerization of the phosphodiester linkages adjacent to free 2′–OH groups. It may also be noted that the isomerization occurred in linear oligomers, which might have been incorporated in the complex with poly(U), but was not observed in the cyclic trimer which might have been derived from free trimer.

Experimental

General Methods——Paper chromatography was performed on Whatman 1 paper by the descending technique using the following solvent systems: solvent A, 2-propanol-concentrated ammonium hydroxide–water (7:1:2, v/v); solvent B, 1-propanol-concentrated ammonium hydroxide–water (55:10:35, v/v); solvent C, ethanol–1 M ammonium acetate (pH 7.5) (7:3, v/v); solvent D, 1-propanol–concentrated ammonium hydroxide–water (6:1:3, v/v). Paper electrophoresis was performed on Toyo filter paper No. 51A at 35 V/cm in 0.05 M $Et_3NH_2CO_3$ buffer (pH 7.5). UV absorption spectra were obtained on a Hitachi 124 spectrophotometer. For T_m measurements, a Hitachi 124 spectrophotometer, equipped with a Komatsu Solidate SPDH-124 thermostatted cell, was used. The temperature of stirred solution was measured directly by a Shibaura MGB-III thermister. The molar extinction coefficients (ε) determined by Brahms *et al.*[14] for oligo (adenylic acids) were used for oligomers with corresponding chain lengths in these experiments. CD spectra were recorded on a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. The molar ellipticity, $[\theta]$, is presented in terms of per residue values. *Escherichia coli* alkaline phosphatase (BAPF) was purchased from Worthington Biochemical Co. The incubation was in 0.1 M NH_4HCO_3 at 37° for 3—4 hr with the enzyme (1.5 units/ml). RNase M was a generous gift from Dr. M Irie and incubation was carried out in 0.1 M ammonium acetate (pH 5) at 37° for 4 hr with the enzyme (0.2 mg/ml). Snake venom phosphodiesterase was purchased from Worthington Biochemical Co. and the incubation was carried out in 0.1 M NH_4HCO_3 at 37° for 3 hr with the enzyme (0.2 mg/ml). A mixture (*ca.* 1:1) of N,N,2′-*O*-tribenzoyl- and N,2′-O-dibenzoyl-5′-O-monomethoxytrityladenosine 3′-phosphates (**1**) was synthesized by monomethoxytritylation and subsequent benzoylation of adenosine 3′-phosphate according to procedures similar to those described by Lapidot and Khorana.[17] The dinucleotide (**2**, shown in Figure 1) was synthesized by Ohtsuka *et al.*[18]

Adenylyl-(3′—5′)-adenylyl-(3′—5′)-2′-O-(*o*-nitrobenzoyl)adenosine 3′-Phosphate (A-A-A_p^{NB}, 3)——A mixture of freshly precipitated N,O-benzoylated derivatives of pyridinium 5′-*O*-monomethoxytrityladenosine 3′-phosphate (**1**, 0.136 mmol), the dinucleotide (**2**, shown in Figure 1, 132 mg, 0.092 mol), in which 5′-terminal hydroxyl is free and the other functional groups are fully protected, and pyridinium Dowex 50×8 (138 mg) was rendered anhydrous by repeated evaporation (4 times) with pyridine and was treated with dicyclohexylcarbodiimide (1.36 mmol) in anhydrous pyridine (2 ml) at 26° for 16 days. 50% aqueous pyridine was added and the carbodiimide extracted with *n*-pentane. After 16 hr at room temperature, the mixture was filtered to remove dicyclohexylurea and the resin. After evaporation with added pyridine, the anhydrous residue was treated with isoamyl nitrite (1.1 ml) in pyridine (2 ml)–acetic acid (2 ml) at 24—26° for 14 hr. The reaction mixture was evaporated to dryness and the residual pyridine was removed by repeated evaporation with toluene (4 times). The residue was dissolved in 80% aqueous acetic acid (20 ml)–acetic acid (2 ml) and kept at 30° for 5 hr. After checking by thin-layer chromatography in $CHCl_3$–methanol (20:3) that detritylation was complete the solvent was evaporated to dryness and the residue was evaporated with added *n*-butanol (5 times). The residue was dissolved in methanol (8 ml)–pyridine (0.8 ml). To the cooled mixture at 0°, concentrated NH_4OH (1 ml) was added. After 5 min at 0°, volatile materials were evaporated quickly and the residue was evaporated with added pyridine. The anhydrous residue was treated with methanolic ammonia saturated at 0° (50 ml) at 30° for 20 hr. After careful evaporation of the volatile materials, the residue was suspended in aqueous pyridine (25%, 50 ml) and filtered to remove insoluble materials. The

17) Y. Lapidot and H.G. Khorana, *J. Am. Chem. Soc.*, **85**, 3867 (1963).
18) E. Ohtsuka, T. Tanaka, S. Tanaka, and M. Ikehara, *J. Am. Chem. Soc.*, **100**, 4580 (1978).

filtrate was diluted with water to 150 ml and applied to a column (1.7×46 cm) of DEAE-cellulose (bicarbonate form). After washing with water (1 l), elution was carried out with a linear gradient of $Et_3NH_2CO_3$ buffer (pH 7.5) (0—0.3 M, total 6 l). Fractions of 20 ml were collected at 18 min intervals. The chromatogram is shown in Figure 2. Fractions VII—IX contained the desired product (3, A–A–A_p^{NB}) and a trace of impurity. The yield was 2240 A_{259} units (62% from 2). For the polymerization reaction, the trimer in fraction VIII was further purified to remove a trace of impurity by chromatography on a column (11×24 cm) of DEAE-Sephadex A-25 (bicarbonate). Elution was carried out with a linear gradient of $Et_3NH_2CO_3$ buffer (0.1—0.35 M, total 5 l). Fractions of 20 ml were collected at 20 min intervals. The fractions containing pure A–A–A_p^{NB} (tube No. 138—150, 1064 A_{259} units) were combined and desalted by repeated evaporation with water. UV: λ_{max} (0.1 N HCl) 258 nm (ε (p) 14900), λ_{max} (H_2O), 258 nm (ε (p) 13100), λ_{max} (0.1 N NaOH), 258 nm (ε (p) 12900). Hypochromicity, obtained from RNase M[19] digestion and calculated from absorbances at the λ_{max}'s of the digest and control solution, was 17% at room temperature. The CD spectrum of this compound exhibited a pair of bands with opposite signs ($[\theta]_{max}$ 1.05×10^4 at 273 nm, $[\theta]_{min}$ -1.89×10^4 at 251 nm) in the main absorption band region and a weak ($[\theta]_{min}$ -0.2×10^4 at around 300 nm) and broad (290—380 nm) band in the absorption region of the nitrobenzyl group in 0.1 M KF-0.01 M sodium cacodylate buffer (pH 7.0) at 26 °C. The properties of A–A–A_p^{NB} and related compounds in paper chromatography and paper electrophoresis are summarized in Table I.

Polymerization using A-A-A_p^{NB}-poly (U) (1:2) Mixture at 0°——A solution of triethylammonium A–A–A_p^{NB} (65.5 A_{258} units) and poly (U) (92 A_{261} units) were mixed in a sterilized polypropylene tube and lyophilized. The residue was dissolved in sterilized water (0.5 ml) and NaCl (44 mg) and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (24 mg) were added. The pH of the solution was adjusted to 5.6 with 0.1 N HCl. After addition of $CHCl_3$ (5 μl), the reaction mixture was kept at 0° for 15 days. Additional carbodiimide (24 mg each) was added after 4 days and after 9 days, the pH being adjusted to 5.6 each time. After 15 days, 0.2 M $Et_3NH_2CO_3$ buffer (pH 7.5, 0.5 ml) was added to adjust the pH and the mixture was applied to a column (1.6×88) of Sephadex G-15 for desalting. Elution was carried out with water. Fractions of 2.65 ml were collected at 6 min intervals. The elution profile is shown in Figure 4a. During the operations described hitherto, precautions were taken to avoid exposure of the nitrobenzyl derivatives to sun-light. The fractions in peak I (tube No. 27—43) were combined and irradiated with a high-pressure mercury lamp through a pyrex filter at room temperature for 2 hr. After evaporation of the solvent, the residue was dissolved in 0.1 M $Et_3NH_2CO_3$ buffer (0.5 ml) and applied to a column (2.2×86 cm) of Sephadex G-50. Elution was carried out with 0.1 M $Et_3NH_2CO_3$ buffer. Fractions of 2.65 ml were collected at 12—15 min intervals. The absorbance of the eluant was also monitored continuously by a flow cell connected to a Hitachi 124 spectrophotometer. The elution pattern is shown in Figure 4b. The yields and the results of identification of the peaks are summarized in Table II. The UV spectrum of each fraction was checked and all adenosine derivatives showed λ_{max} at 258—259 nm.

Control Reaction without Poly (U) at 0°——The same reaction mixture as that in the reaction described above, except that poly(U) was omitted, was prepared and treated in the same manner as in the reaction containing the template. The elution profiles in the desalting step and the separation step are shown in Figure 5a and 5b.

Polymerization using A-A-A_p^{NB}-poly(U) (1:2) Mixture at 15°——The same reaction mixture as that in the reaction at 0° was prepared and kept in a water-bath at 15° for 7 days. The reaction mixture was treated in the same manner as in the reaction at 0°. The elution profile in the desalting step and the separation step are shown in Figure 6a and 6b. The yields and results of identification are summarized in Table III.

Polymerization using A-A-A_p^{NB}-poly(U) (1:1) Mixture at 0°——The same reaction mixture as that in the first experiment, except that the amount of A–A–A_p^{NB} was doubled, was prepared and kept at 0° for 15 days. The reaction mixture was treated in the same manner as in the first experiment. The elution profiles in the desalting step and the separation step are shown in Figure 7a and 7b. The yield and results of identification are summarized in Table IV.

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