Breslauer, K. J., Sturtevant, J. M., & Tinoco, I. (1975) *J. Mol. Biol. 99*, 549–565.
Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Part 3, p 1219, W. H. Freeman, San Francisco.
Dickerson, R. E., & Drew, H. R. (1981) *J. Mol. Biol. 149*, 761–786.
Drew, H. R., & Dickerson, R. E. (1981) *J. Mol. Biol. 151*, 535–556.
Hasnoot, C. A. G., de Bruin, S. H., Berendsen, R. G., Janssen, H. G. J. M., Binnendijk, T. J. J., Hilbers, C. W., van der Marel, G. A., & van Boom, J. H. (1983) *J. Biomol. Struct. Dyn. 1*, 115–129.
Lilley, D. M. J. (1985) *Nucleic Acids Res. 13*, 1443–1465.
Marky, L. A., Blumenfeld, K. S., Kozlowski, S., & Breslauer, K. J. (1983) *Biopolymers 22*, 1247–1257.
Patel, D. J., Kozlowski, S. A., Ikuta, S., Itakura, K., Bhatt, R., & Hare, D. R. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 197–206.
Patel, D. J., Kozlowski, S. A., Hare, D. R., & Reid, B. (1985) *Biochemistry 24*, 926–935.
Summers, M. F., Byrd, R. A., Gallo, K. A., Samson, C. J., Zon, G., & Egan, W. (1985) *Nucleic Acids Res. 13*, 6375–6386.
Wemmer, D. E., Chou, S. H., Hare, D. R., & Reid, B. R. (1985) *Nucleic Acids Res. 13*, 3755–3772.
Zon, G., & Thompson, J. A. (1986) *Biotechniques 1*, 22–32.

Template-Directed Polymerization of Oligoadenylates Using Cyanogen Bromide†

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ABSTRACT: Cyanogen bromide (BrCN) condensed oligoadenylates [oligo(A)] on a poly(uridylic acid) [poly(U)] template in an aqueous solution. Imidazole and divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Fe^{2+} were required for the condensation. Chain length of oligo(A) and reaction temperature affected the coupling yield. Hexaadenylate [$(pA)_6$] was converted to $(pA)_{12}$, $(pA)_{18}$, $(pA)_{24}$, $(pA)_{30}$, $(pA)_{36}$, $(pA)_{42}$, and $(pA)_{48}$ in a 68% overall yield for 20 h at 25 °C. The coupling yield increased with increase in the poly(U) concentration. Five- to sevenfold molar excess of uridylyl residues of poly(U) to adenylyl residues of oligo(A) gave the best yield (68%). Metal ions affected the formation of linkage isomers of the phosphate bonds: The 2′,5′- and 3′,5′-phosphodiester bonds were predominant in the presence of Co^{2+}, Zn^{2+}, and Ni^{2+} and the 5′,5′-pyrophosphate bond was predominant in the presence of Mn^{2+}. In particular, Ni^{2+} gave the highest ratio of the 3′,5′-phosphodiester bond (30%). *N*-Cyanoimidazole (**1**), *N*,*N*′-iminodiimidazole (**2**), and *N*-carboxamidoimidazole (**3**) were formed in a reaction of imidazole with BrCN in an aqueous solution. **1** and **2** had much the same condensing activity for the polymerization of adenylates as BrCN. A reaction pathway was proposed in which **1** and **2** are not only intermediates for the production of **3** but also the true condensing agent in the coupling reaction of oligo(A). Phosphorimidazolide derivative was detected in a reaction of 5′-AMP with either **1** or **2**. The condensation would proceed by way of *N*-cyanoimidazole–phosphate adduct, the phosphorimidazolide derivative, or both.

A few studies of nucleic acid condensation in an aqueous solution have been reported in the last two decades. It is not easy to form a phosphodiester bond in an aqueous solution, because the competition between water and nucleosidic hydroxy groups for an activated phosphate group would prevent the condensation. One of the possible ways to solve this problem is to use a complementary template for holding the two reacting termini of short oligonucleotides close to each other. This approach was first tried by Naylor and Gilham (1966). They condensed hexathymidylic acid on a poly(A)[1] template using water-soluble carbodiimide as a condensing agent and obtained a dimerized product in a 5% yield. Uesugi and Ts'o (1974) succeeded in the polymerization of oligo(2′-*O*-methylinosinic acid) using a poly(C) template and water-soluble carbodiimide. Until now, water-soluble carbodiimide has been almost exclusively used for nucleic acid synthesis in an aqueous solution as a condensing agent. Ibanez et al. (1971) have reported that cyanamide condensed a mononucleotide in a neutral aqueous solution. Since then, it has joined to prebiotic condensing agents.

In contrast to these block condensations, Orgel and his co-workers (Lohrmann & Orgel, 1978; Bridson & Orgel, 1980; Inoue & Orgel, 1983) have reported a series of studies of mononucleotide polymerization in the presence of a complementary polynucleotide as a template. They used active nucleoside 5′-phosphorimidazolide as a starting material and obtained polymerized products with chain lengths of up to 40 in sufficient yield. Divalent metal ions added as a catalyst affected the formation of the phosphodiester bond linkage

† This paper is dedicated to Prof. Morio Ikehara for the occasion of his retirement from Osaka University in March, 1986.

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[1] Abbreviations: BrCN, cyanogen bromide; HCN, hydrogen cyanide; DISN, diiminosuccinonitrile; poly(A), poly(adenylic acid, poly(U), poly(uridylic acid); poly(C), poly(cytidylic acid); oligo(G), oligo(guanylic acid); oligo(A), oligo(adenylic acid); $(pA)_4$, tetraadenylate; $(pA)_5$, pentaadenylate; $(pA)_6$, hexaadenylate; 2′-AMP, adenosine 2′-phosphate; 3′-AMP or Ap, adenosine 3′-phosphate; 2′,3′-cyclic AMP, adenosine cyclic 2′,3′-phosphate; A, adenosine; pAp, adenosine 3′,5′-diphosphate; A(2′)p(5′)Ap, adenylyl(2′-5′)adenosine 3′-phosphate; A(5′)pp(5′)A, P^1,P^2-bis(5′-adenosyl)diphosphate; 5′-AMP or pA, adenosine 5′-phosphate; Na_2EDTA, disodium ethylenediaminetetraacetate; C_{18}, octadecylsilane; HPLC, high-performance liquid chromatography; T_m, melting temperature; Tris, tris(hydroxymethyl)aminomethane; AUFS, absorbance units full scale.

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isomers. In a poly(C)–guanosine 5′-phosphorimidazolide system, Pb^{2+} predominantly formed the 2′,5′-linkage and Zn^{2+} led to the preferential formation of the 3′,5′-linkage (Lohrmann et al., 1980), whereas in a poly(U)–adenosine 5′-phosphorimidazolide system, Pb^{2+} preferentially produced the 3′,5′-linkage (Sleeper et al., 1979). The condensation of 5′-imidazolides of dinucleotides on polynucleotide templates have also been studied, and the coupling yield depended on the kind of linkage isomers of dinucleotides (Lohrmann & Orgel, 1979a,b).

Hydrogen cyanide (HCN) is present in interstellar space and the atmosphere of Titan and Jupiter. It is readily synthesized by ultraviolet light, spark discharge, and shock wave from gas mixtures that have been suggested as models for the atmosphere of the primitive Earth. We were interested in the role of HCN and its derivatives in chemical evolution, in particular, the role of diiminosuccinonitrile (DISN) as a prebiotic condensing agent. DISN has been readily synthesized by the oxidation of diaminomaleonitrile, a tetramer of HCN (Ferris et al., 1979, 1982). Therefore, we initiated a program of nucleotide condensation using DISN. Ferris et al. (1984) have recently shown that DISN and cyanogen bromide (BrCN) caused the conversion from 3′-AMP or 2′-AMP to 2′,3′-cyclic AMP in the presence of divalent metal ions and imidazole. Quite recently, we have found that BrCN and DISN brought about the coupling reaction of oligo(A) in an aqueous solution (Kanaya & Yanagawa, 1985). To gain further information, we have examined, in detail, the intermolecular condensation of oligo(A) on a poly(U) template using BrCN as a model compound for DISN. In this paper, we present a detailed account of the experiments.

Materials and Methods

General Methods. Poly(A) and poly(U) were purchased from Yamasa Shoyu Co. Ltd. BrCN, imidazole, salts of metal ions, and sodium perchlorate were from Wako Pure Chemical Industries, Ltd. Nitrate salts of metal ions were used except for $MnCl_2 \cdot 4H_2O$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ or when noted otherwise. Buffers were prepared by titrating imidazole with nitric acid. All other compounds used were of reagent grade. Ultraviolet spectra were measured on a Gilford 2400-2 automatic recording spectrophotometer and a Hitachi 220A spectrophotometer. pH was measured with a Hitachi-Horiba F-7 pH meter. IR spectra were measured in KBr, unless noted otherwise, on a Hitachi 260-50 infrared spectrophotometer. ^1H and ^{13}C NMR spectra were obtained with a Bruker WM 400-MHz spectrometer using Me_4Si as internal standard at ambient temperature. Mass spectra were determined on a Shimadzu GC-MS 7000 mass spectrometer by electron impact at 70 eV. High-performance liquid chromatography (HPLC) was carried out on two systems: a Waters system consisting of a M-6000 A solvent pump, U6K injector, 660 solvent programmer, and 440 absorbance detector and a Jasco system equipped with a Tri Rotar-VI solvent pump, Uvidec-100 VI variable-wavelength UV monitor, AS-L350 intelligent processor, and Sic Chromatocoder 11 (Sic Instruments Co. Ltd.).

Preparation of Oligo(A)s. Oligo(A)s with chain lengths from 1 to 10 were prepared by nuclease SW (Seikagaku Kogyo Co., Ltd.) digestion of poly(A). Poly(A) (10 mg) was incubated with 14 units of nuclease SW (Mukai, 1965) in an aqueous solution (2 mL) containing 50 mM sodium carbonate buffer, pH 10.3, 100 mM NaCl, and 2 mM magnesium acetate at 37 °C. An adequate amount of the reaction mixture was taken out and dissolved in 100 mM NaCl and 10 mM Tris-HCl buffer, pH 7.0, every 10 min, and the increase in UV absorbance at 260 nm was monitored. When the increase reached 30%, the reaction was stopped by neutralization with Tris-HCl buffer, pH 7.0, followed by extraction with 90% phenol. The aqueous layer was dialyzed against 1 mM Tris-HCl, pH 7.0, to remove phenol, followed by HPLC using MCI-GEL CQA-30S (0.76 × 60 cm; Mitsubishi Chemical Industries Ltd.). A linear gradient of 0.3–1.0 M ammonium formate was applied over 1 h at a flow rate of 1 mL/min. Effluent was detected at 270 nm. Peaks containing oligo(A) were collected, desalted by dialysis against 1 mM Tris-HCl, pH 7.0, lyophilized, and stored at −20 °C.

Template-Directed Polymerization of Oligo(A)s Using BrCN. A reaction was carried out in a 0.28-mL Eppendorf tube in a total volume of 20 μL at 4, 20, and 40 °C. Details of the composition of the reaction mixture and the incubation time are given in figure legends. A reaction was terminated by addition of enough Na_2EDTA to make complexes with all divalent metal ions, and then the reaction mixture was lyophilized to remove the residual BrCN. After redissolution, the pH was adjusted to 8.0 with 1 M Tris-HCl buffer, pH 8.5. RNase A [Sigma; 0.25 mg/μmol of poly(U)] was added to digest excess poly(U), and then the solution was incubated at 37 °C for 8 h. HPLC of the polymerized products of oligo(A) was performed on a RPC-5 column (0.4 × 25 cm; Nishio Kogyo Co. Ltd.) by using a linear gradient of 2–200 mM $NaClO_4$ in 10 mM Tris–acetate buffer, pH 7.5, in 40 min at a flow rate of 1 mL/min. The eluate was monitored at 260 nm with an absorbance detector connected to a recorder equipped with a data processor to determine each peak area quantitatively, which was corrected by using molar extinction coefficients of nucleic acids (Brahms et al., 1966). The efficiency of a reaction was estimated by comparing each peak area of the products in the chromatogram with the peak area of a corresponding starting material.

Hydrolysis of Dimerized Products of Tetraadenylate with RNase T_2. For identification of the polymerized products of tetraadenylate [$(pA)_4$] on a poly(U) template using BrCN, the reaction mixture was applied to a CQA-30S column (0.4 × 25 cm) equilibrated with 0.3 M ammonium formate. The elution was carried out by a linear gradient of ammonium formate (0.3–1.0 M) over 40 min at 70 °C. The flow rate was 1 mL/min. The compounds eluted at 39, 44, and 45.3 min were collected separately, dialyzed against 1 mM Tris-HCl, pH 7.0, and lyophilized. They were then redissolved in 10 μL of 50 mM ammonium acetate, pH 4.5, containing 20 % (v/v) methanol and treated with RNase T_2 (0.02 unit; Seikagaku Kogyo Co., Ltd.; Uchida, 1966). After incubation for 30 min at 37 °C, the amount of Ap, A, A(2′)p(5′)Ap, and pA(5′)pp(5′)Ap resulting from the hydrolysis was determined by HPLC performed on a C_{18} column (MCI-GEL ODS 1 MU, 0.46 × 15 cm) by using a mobile phase of 50 mM triethylammonium acetate containing 5% CH_3CN. Nuclease P_1 (Seikagaku Kogyo Co., Ltd.) digestion was carried out in an aqueous solution (10 μL) containing 0.1 mM oligo(A), 20 mM sodium acetate, pH 5.5, and 230 μg of nuclease P_1 at 37 °C for 40 min. The digested products, pA and A(5′)pp(5′)A, were analyzed in the same way as described above. A(2′)p(5′)Ap as an authentic sample was a gift from Dr. Sawai (University of Tokyo), and pA(5′)pp(5′)Ap was obtained by RNase T_2 digestion of p<A(5′)pp(5′)A>p purchased from P-L Biochemicals. A(5′)pp(5′)A was obtained from Sigma Chemical Co. A molar absorption coefficient of pA(5′)pp(5′)Ap was taken from published data (ε 27 700; Christie et al., 1953).

Reaction of Imidazole with BrCN. A solution of BrCN (320 mg, 3 mmol) in 30 mL of 0.2 M imidazole–HNO_3, pH

6.2, was stirred for 24 h at 20 °C. HPLC of the reaction products was performed on a C_{18} reverse-phase column (Develosil, Nomura Chemical; 1.0 × 25 cm) with solvent systems A (3% AcCN/H_2O) and B (50% AcCN/H_2O). The elution conditions were 0% B for 30 min followed by a linear gradient to 100% B in 40 min. The flow rate was 2 mL/min, and the column effluent was monitored at 230 nm. Analysis by HPLC revealed four major peaks at 5, 13, 22, and 56 min.

N-Cyanoimidazole (**1**) was eluted as a peak with a retention time of 13 min. The eluate was freeze-dried, dissolved in acetonitrile, and flash chromatographed on silica gel. **1** was eluted with acetonitrile. The eluate was crystallized from benzene to give colorless needles: mp 59–60 °C; ^1H NMR ($CDCl_3$) δ 7.16 (1 H, d, H-4), 7.29 (1 H, t, H-5), 7.94 (1 H, br s, H-2); ^{13}C NMR ($CDCl_3$) δ 104.65 (cyano carbon), 119.98 (C-4), 130.70 (C-5), 138.92 (C-2); IR (KBr) 3160, 3130, 2270, 1725, 1690, 1640, 1530, 1470, 1285, 1225, 1060, 1000, 850, 750, 630, 590, 485 cm^{-1}; UV_{max} (AcCN) 218 nm (ϵ 4.4 × 10^3); mass spectrum, *m/z* 93 (M^+), 68, 66, 41, 40. Anal. Calcd for $C_4H_3N_3$: C, 51.61; H, 3.25; N, 45.14. Found: C, 51.75; H, 3.23; N, 44.71. These spectral data were identical with those of an authentic sample that was synthesized from imidazole and BrCN in benzene at 60 °C (Giesemann, 1955; Hagan, 1985).

N,N'-Iminodiimidazole (**2**) was eluted from the C_{18} column as a peak with a retention time of 22 min: mp 97–99 °C; ^1H NMR ($CDCl_3$) δ 7.22 (1 H, br s, H-4), 7.35 (1 H, br s, H-5), 7.97 (1 H, br s, H-2), 8.71 (2 H, br s, =NH); ^1H NMR ($CDCl_3$ + 1 drop of D_2O) δ 7.20 (1 H, t, H-4), 7.26 (1 H, t, H-4'), 7.29 (1 H, t, H-5), 7.39 (1 H, t, H-5'), 7.91 (1 H, t, H-2), 8.02 (1 H, t, H-2'); ^{13}C NMR ($CDCl_3$) δ 118.14 (imino carbon), 121.82 (C-4), 131.21 (C-5), 134.95 (C-2); IR (KBr) 3160, 3110, 1685, 1655, 1475, 1410, 1330, 1230, 1215, 1110, 1030, 960, 905, 830, 765, 650 cm^{-1}; UV_{max} (AcCN) 201 nm (ϵ 1.14 × 10^4) 235 nm (shoulder); mass spectrum, *m/z* 161 (M^+), 147, 146, 120, 94, 93, 68, 41, 40. Anal. Calcd for $C_7H_7N_5$: C, 52.16; H, 4.38; N, 43.46. Found: C, 52.01; H, 4.48; N, 43.43.

N-Carboxamidoimidazole (**3**) was eluted from the C_{18} column as a peak with retention time of 56 min: mp 91–93 °C; ^1H NMR (CD_3OD) δ 7.10 (1 H, t, H-4), 7.67 (1 H, br s, H-2), 7.74 (1 H, t, H-5); IR (KBr) 3320, 3120, 1690, 1655, 1640, 1475, 1370, 1320, 1245, 1210, 1160, 1110, 1000, 875, 740, 685, 650 cm^{-1}; UV_{max} (H_2O) 205 nm (ϵ 3.9 × 10^3), 241 (2.4 × 10^3); mass spectrum, *m/z* 111 (M^+), 69, 68, 41, 40. Anal. Calcd for $C_4H_5N_3O$: C, 43.24; H, 4.54; N, 37.83. Found: C, 43.10; H, 4.51; N, 37.66.

Time Course of the Reaction of BrCN with Imidazole in an Aqueous Solution. For detection of products from a reaction of imidazole with BrCN, an aqueous solution (1 mL) containing 7 mM BrCN and 200 mM imidazole nitrate buffer, pH 6.2, was analyzed by HPLC. The reaction mixture (5 μL) was applied to a C_{18} column (0.46 × 15 cm) equilibrated with 3% aqueous CH_3CN solution at adequate intervals of time. The elution condition was the same solution for 20 min followed by a linear gradient to 50% CH_3CN in 20 min at a flow rate of 1 mL/min. Yield of the compounds (**1**, **2**, and **3**) was calculated by comparing peak areas of each compound with that of a corresponding standard material that was isolated from a reaction mixture of imidazole and BrCN.

Results and Discussion

Condensation of Oligo(A) Using BrCN in an Aqueous Solution. $(pA)_4$ was condensed with BrCN on a poly(U) template in the presence of imidazole and divalent metal ions at 4, 25, and 40 °C. After the polymerization reaction for 20 h, the products were analyzed by HPLC performed on a RPC-5 column. Figure 1 shows the elution profile.

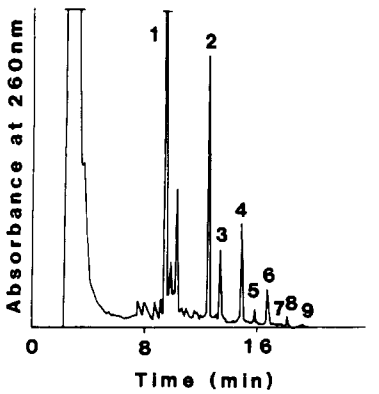


FIGURE 1: HPLC of the products in the polymerization of $(pA)_4$ on a reverse-phase RPC-5 column. The reaction mixture (1.2 mL) contained 0.15 mM $(pA)_4$, 0.75 mM poly(U), 10 mM $MnCl_2$, 200 mM imidazole nitrate buffer, pH 6.0, and 15 mM BrCN and was incubated at 20 °C for 20 h. Ten microliters of the reaction mixture was applied to HPLC, which was carried out by using linear gradient elution from 2 to 200 mM $NaClO_4$ in 10 mM Tris–acetate buffer, pH 7.5, over a period of 40 min. Pressure, 80 kg/cm^2; flow rate, 1 mL/min; UV detector, 260 nm; AUFS, 0.064. Identification and a retention time (minutes) of peaks were as follows: peak 1 (9.7), $(pA)_4$; 2 (12.7), ApApApA(5')pp(5')ApApApA; 3 (13.5), pApApApA(2')p(5')ApApApA + pApApApA(3')p(5')ApApApA; 4 (15.0), $(pA)_{12}$; 5 (16.0), $(pA)_{12}$; 6 (16.9), $(pA)_{16}$; 7 (17.9), $(pA)_{16}$; 8 (18.3), $(pA)_{20}$; 9 (19.5), $(pA)_{20}$. Peaks, 4, 6, and 8 contain pyrophosphate linkages and peaks 5, 7, and 9 contain 2',5'- and 3',5'-phosphodiester linkages.

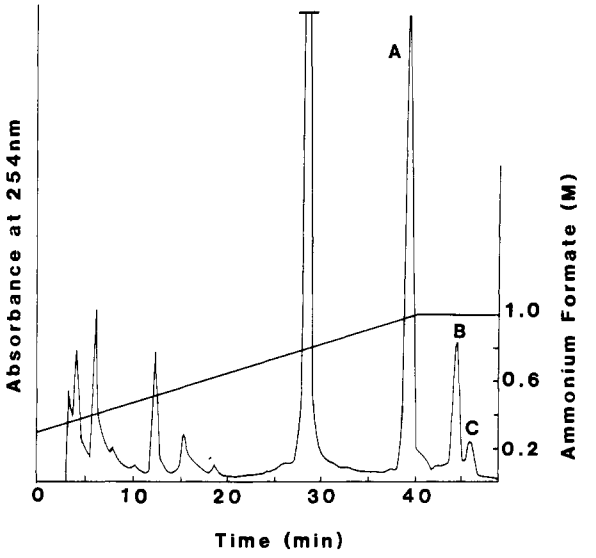


FIGURE 2: HPLC of the products in the polymerization of $(pA)_4$ on an anion-exchange CQA-30S column. Elution was carried out by using a linear gradient of ammonium formate (0.3–1.0 M) over 40 min at 70 °C. Pressure, 85 kg/cm^2; flow rate, 1 mL/min; UV detector, 254 nm; AUFS, 0.5. The compound eluted at 28 min was the starting material, $(pA)_4$. The identification of peaks A, B, and C is described under Results and Discussion.

The identification of each peak was performed as follows: The reaction mixture of $(pA)_4$ was applied to a CQA-30S column (0.4 × 25 cm). As shown in Figure 2, three peaks were eluted at 39 (A), 44 (B), and 45.3 min (C), and the peaks were collected separately. The enzymatic digest of each compound was analyzed by HPLC performed on a C_{18} column. The compound from peak A gave Ap, A, and pA(5')pp(5')Ap in a 2.0:1.0:0.8 ratio after RNase T_2 digestion and pA and A-(5')pp(5')A in a 3.0:0.9 ratio after nuclease P_1 digestion. Accordingly, the compound from peak A was identified as

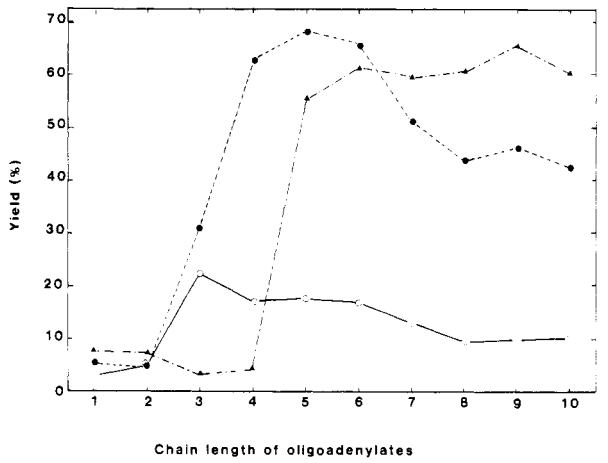


FIGURE 3: Effect of chain length of oligo(A)s on their coupling reactions. The reaction mixture (20 μL) contained 0.15 mM $(pA)_n$ (n = 1–10), 0.75 mM poly(U), 50 mM $MnCl_2$, 200 mM imidazole nitrate buffer, pH 6.0, and 50 mM BrCN and was incubated for 20 h at 4 (○), 20 (●), or 40 °C (▲).

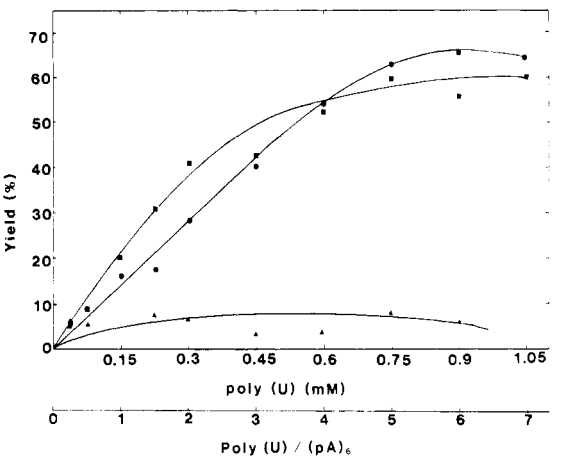


FIGURE 4: Effect of poly(U) as a template on the coupling reaction of $(pA)_6$. The reaction mixture (20 μL) contained 0.15 mM $(pA)_6$, 0.038–1.05 mM poly(U), 10 mM $MnCl_2$, 200 mM imidazole nitrate buffer, pH 6.0, and 10 mM BrCN and was incubated for 20 h at 4 (▲), 25 (■), and 40 °C (●).

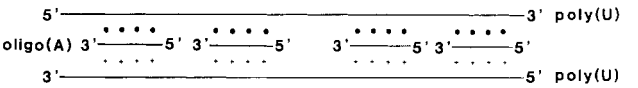


FIGURE 5: Possible interaction between oligo(A) and poly(U). (·) Watson–Crick base pairs; (+) Hoogsteen base pairs.

ApApApA(5′)pp(5′)ApApApA, in which $(pA)_4$ was dimerized by a pyrophosphate linkage. Because the compound from peak B gave pAp, Ap, A, and an undigested 2′,5′-linked dimer [A(2′)p(5′)Ap] in a 0.9:4.1:1.0:1.9 ratio after RNase T_2 digestion, it was identified as pApApApA(2′)p(5′)ApApApA, in which $(pA)_4$ was dimerized by a 2′,5′-linkage. On the other hand, the compound from peak C gave pAp, Ap, and A in a 0.9:5.7:1.0 ratio after RNase T_2 digestion, and it was identified as a 3′,5′-linked dimer, $(pA)_8$.

The compound from peak A on the CQA-30S column chromatogram (Figure 2) was eluted at peak 2 on the RPC-5 column chromatogram (Figure 1). The compounds from peaks B and C in Figure 2 could not be separated on a RPC-5 column and eluted at peak 3 (Figure 1). The compounds from peaks 5, 7, and 9 in Figure 1 corresponded to the trimer, tetramer, and pentamer of $(pA)_4$, respectively. The retention time of these peaks agreed with those of authentic $(pA)_{12}$, $(pA)_{16}$, and $(pA)_{20}$ obtained by poly(A) digestion with nuclease SW.

Effect of Chain Length of Oligo(A)s on Their Coupling Reactions. Figure 3 shows the effect of the chain length of starting oligo(A)s on their coupling reactions at 4, 20, and 40 °C at an oligo(A):poly(U) ratio of 1:5. With chain lengths greater than three, the tetramer, pentamer, and hexamer gave optimal coupling at 20 °C; in particular, the pentamer gave the best yield. However, chain lengths greater than six gave lower yields. On the other hand, at 40 °C the yield increased markedly for the pentamer and reached a maximum (about 60%) for the hexamer. No decrease in yield was observed at chain length greater than six. At 4 °C, the coupling yield was very low and the trimer gave the best yield (20%). A little decrease in the yield was observed at chain lengths larger than three.

Sawai (1982) has reported that the T_m of $A(pA)_2$·poly(U) complex in 0.1 M NaCl solution was 25.7 °C and the T_m of $A(pA)_4$·poly(U) complex was 40.2 °C. A marked increase in coupling yields in the trimer at 20 °C and in the pentamer at 40 °C in Figure 3 indicates that the tightness of binding between oligo(A) and poly(U), in other words, the T_m value, relates to the coupling yield of oligo(A) in the presence of poly(U). Thus, it appears likely that the formation of a duplex between oligo(A) and poly(U) is necessary to achieve a satisfactory yield in the coupling reaction. The reason why the yield decreased with oligomers longer than $(pA)_7$ at 20 °C might be due to the reduction of the ease of moving of oligo(A) on a poly(U) template. We mainly used both $(pA)_4$ and $(pA)_6$ in the following experiments, because $(pA)_4$, $(pA)_5$, and $(pA)_6$ gave sufficient coupling yields at 20 °C.

Effect of the Concentration of Poly(U) on the Coupling Reaction of $(pA)_6$. The coupling yields of $(pA)_6$ were examined at different poly(U) concentrations varying from 0.0375 to 1.05 mM at 4, 25, and 40 °C (Figure 4). The yield was dependent on the poly(U) concentration at 25 and 40 °C. The yield increased with the increase in the poly(U) concentration. When 5–7 times molar excess of poly(U) to oligo(A) was used, the maximum yield was about 60–70%. However, no effect of the poly(U) concentration was observed in the yield at 4 °C.

When poly(A) and poly(U) are mixed in a ratio of 1:2, a poly(U)·poly(A)·poly(U) triple helix is formed because the adenine heterocycle is simultaneously able to engage in both Watson–Crick and Hoogsteen base pairs (Blake et al., 1967). The structure of the ternary complex consists of an antiparallel poly(A)·poly(U) double helix with Watson–Crick base pairs and the third poly(U) strand running parallel to the poly(A) strand (Arnott & Selsing, 1974). In the present experiment, a Hoogsteen-type base pair might be formed in some places when the poly(U) concentration was raised. At a $(pA)_6$ to poly(U) ratio of 1:5, $(pA)_6$ is supposed to hybridize sparsely to poly(U) as shown in Figure 5. The high concentration of poly(U) would present many reaction fields at which oligo(A)s are able to couple with one another.

Next, we should inquire into the concept of "sliding". When the poly(U) concentration was 5–7 times that of $(pA)_6$, the best coupling yield was obtained. How could the hexaadenylate molecules that might thinly exist on the poly(U) template come close to each other and combine with one another with the aid of BrCN? Pörschke (1974) has reported that "slidomers" play an important role in the formation and dissociation of double-helical complexes from short complementary homopolymers. Chen et al. (1985) have also introduced "sliding" to explain the formation of oligomers one unit longer than a template. They considered that newly formed oligo(G) should slide on the template of oligo(dC). We

Table I: Effect of Metal Ions on Coupling Reaction of $(pA)_6$[a]

metal ion	yield of oligo(A) (%) 12-mer	18-mer	24-mer	30-mer	total yield (%)
Mn^{2+}	24.6	3.9	0.8		29.3
Co^{2+}	16.2	1.9	1.1	0.3	19.5
Ni^{2+}	11.5	4.0	1.9		17.4
Cu^{2+}	4.9	3.5	1.0		9.4
Zn^{2+}	7.9	2.6			10.5
Mg^{2+}	7.4	1.3	0.8		9.5
Fe^{2+}	5.8	4.5	1.2		11.5
metal free	0.5				0.5

[a] The reaction mixture (20 μL) contained 0.15 mM $(pA)_6$, 0.15 mM poly(U), 200 mM imidazole nitrate buffer, pH 6.0, 15 mM BrCN, and 10 mM divalent metal ions and was incubated at 25 °C for 6 h.

supposed that oligo(A) molecules should slide on a poly(U) template and when they come close to each other, the condensation could be caused with BrCN. In the case of an A-U base pairing, the sliding seems to be easier than in the case of G-C pairing, because an A-U pair is formed by two hydrogen bonds, whereas a G-C pair is formed by three hydrogen bonds.

Effects of Imidazole, BrCN, and Divalent Metal Ions on the Coupling Reaction of $(pA)_6$. The effect of imidazole on the coupling reaction of $(pA)_6$ was examined in the range of 0–2.0 M. Sufficient yields (about 60%) were obtained at the concentration of 0.05–0.4 M, and the optimal concentration was at about 0.2 M. Imidazole was essential for the reaction, because when it was removed from a reaction mixture, no oligomer was obtained.

The effect of the concentration of BrCN on the coupling reaction of $(pA)_6$ was also examined in the range of 0–100 mM. BrCN gave sufficient yields in the range of 30–80 mM. BrCN has been widely used as an activating agent for polysaccharide resins (Axen et al., 1967; Lowe, 1979; Porath, 1974), nucleosides and nucleotides (Ferris & Yanagawa, 1984), and as a chemical reagent for the restrictive digestion of proteins at the position of methionine (Gross & Witkop, 1962). Here it is worthwhile to have found that BrCN could efficiently produce intermolecular bonds between the two molecules of nucleic acid in an aqueous solution.

Divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and Fe^{2+} had an appreciable effect on the coupling reaction of $(pA)_6$ (Table I). The effectiveness of divalent metal ions on the overall yield of the coupling reaction was in the order $Mn^{2+} > Co^{2+} > Ni^{2+} > Fe^{2+} > Zn^{2+} > Mg^{2+} > Cu^{2+}$. Among the metal ions Mn^{2+} gave the best yield of the coupling reaction. When metal ions were removed from the reaction mixture, the yield was only 0.5% and the product was nothing but a dimerized one. Figure 6 shows the effect of Mn^{2+} on the coupling reaction of $(pA)_6$. The best yield was obtained at 0.05 M.

Formation of Linkage Isomers in the Dimerized Products of $(pA)_4$. The dimerized products of $(pA)_4$ (peaks 2 and 3 in Figure 1) were found to be linked by three kinds of phosphodiester bonds, 5′,5′-, 2′,5′-, and 3′,5′-linkages. As these dimers were separately eluted from the CQA-30S column (Figure 2) at 70 °C, the molar ratio of the three linkage isomers could be determined by comparing the corresponding peak areas. It is expected that there is no difference among the molar absorption coefficients of the three dimers of $(pA)_4$ at 70 °C, because intra- and intermolecular interactions of oligo(A) such as base stacking or hydrogen bonding should be disrupted at 70 °C. The reason why the three linkage isomers could be separated by column chromatography is as follows: when a mixture of poly(U) and oligo(A) was applied

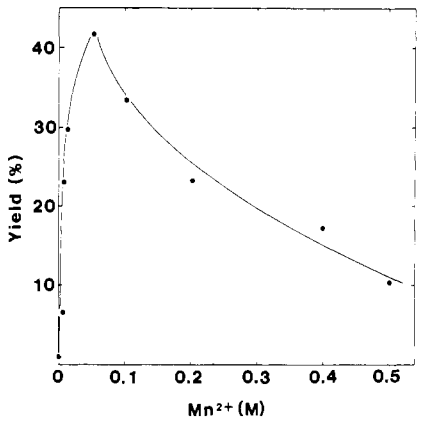


FIGURE 6: Effect of Mn^{2+} on the coupling reaction of $(pA)_6$. The reaction mixture (20 μL) contained 0.15 mM $(pA)_6$, 0.75 mM poly(U), 0–0.5 M $MnCl_2$, 200 mM imidazole nitrate buffer, pH 6.0, and 15 mM BrCN and was incubated for 6 h at 25 °C.

Table II: Effect of Metal Ions on Formation of Linkage Isomers of Octaadenylate[a]

metal	linkage isomers (%) 5′,5′	2′,5′	3′,5′	(2′,5′ + 3′,5′)/5′,5′
Ni^{2+}	8.7	61.3	30.0	10.0
Co^{2+}	23.2	58.9	17.9	3.3
Zn^{2+}	39.5	44.0	16.5	1.5
Mn^{2+}	71.6	22.4	6.0	0.4
Cu^{2+}	48.5	30.5	21.1	1.1

[a] The reaction mixture (1.2 mL) contained 0.15 mM $(pA)_4$, 0.75 mM poly(U), 10 mM divalent metal ions, 200 mM imidazole nitrate buffer, pH 6.0, and 15 mM BrCN and was incubated at 20 °C for 20 h.

to a CQA-30S column and the elution of oligo(A) was performed, the poly(U) was kept in the column. A difference in the affinities of three dimers for the poly(U) led to good separation. When the poly(U) was digested with RNase A prior to the application, three dimers of $(pA)_4$ were eluted from the column at a lower salt concentration and no definite separation was observed. Table II summarizes the molar ratio of the 2′,5′- and 3′,5′-phosphodiester bonds and the 5′,5′-pyrophosphate bond formed with BrCN in the presence of Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, and Cu^{2+}. In the presence of Mn^{2+}, the formation of a 5′,5′-pyrophosphate linkage was predominant (71.6%), whereas in the presence of Ni^{2+}, Co^{2+}, and Zn^{2+}, most of the resulting linkage was 2′,5′- and 3′,5′-phosphodiester bonds. In particular, it is noteworthy that Ni^{2+} gave a 3′,5′-linkage of 30% and a 2′,5′-linkage of 61%. The effectiveness of metal ions on the formation of a 3′,5′-linkage was in the order $Ni^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+}$. On the other hand, the effectiveness of metal ions on the formation of a 2′,5′-linkage was in the order $Ni^{2+} > Co^{2+} > Zn^{2+} > Cu^{2+} > Mn^{2+}$. This was just the reverse order as that seen in the formation of the 5′,5′-linkage. These results suggest that divalent metal ions markedly affected the formation of the linkage isomers of phosphate bonds.

It has been shown that metal ions influence the conformation of nucleic acids. Shin (1973) demonstrated that divalent metal ions such as Ni^{2+}, Co^{2+}, and Zn^{2+} affected the formation of the ordered structure of poly(U). Of the divalent metal ions, Ni^{2+} was most effective in producing the ordered structure of poly(U). The stimulatory effect of metal ions on the formation of the ordered structure of poly(U) increased in the order $Ni^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Cu^{2+}$. This order was in good agreement with that of a stimulatory effect of the metal ions other than Cu^{2+} on the formation of 2′,5′- and 3′,5′-phosphodiester bonds. In particular, Ni^{2+} was most effective

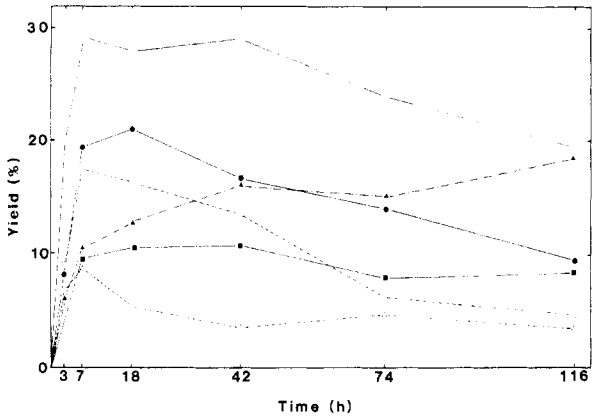


FIGURE 7: Time course of the coupling reaction of $(pA)_6$ in the presence of different divalent metal ions. The reaction mixture (20 μL) contained 0.25 mM $(pA)_6$, 0.15 mM poly(U), 200 mM imidazole nitrate buffer, pH 6.0, 15 mM BrCN, and 10 mM divalent metal ions, i.e., Mn^{2+} (○), Co^{2+} (●), Ni^{2+} (□), Zn^{2+} (▲), Cu^{2+} (■), and Mg^{2+} (△) and was incubated at 25 °C.

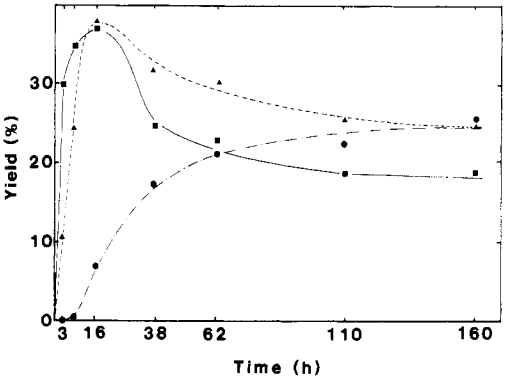


FIGURE 8: Time course of the coupling reaction of $(pA)_6$ at 4, 25, and 40 °C. The reaction mixture (20 μL) contained 0.15 mM $(pA)_6$, 0.15 mM poly(U), 100 mM Mn^{2+}, 200 mM imidazole nitrate buffer, pH 6.0, and 15 mM BrCN and was incubated at 4 (●), 25 (▲), and 40 °C (■).

in both cases. This fact probably indicates that the structure of poly(U) as a template affected the formation of three linkage isomers; that is, when poly(U) took an ordered structure like a helix, it promoted the formation of 2′,5′- and 3′,5′-phosphodiester bonds. This speculation is quite reasonable, because a 2′,5′- or 3′,5′-linkage must be formed between two oligo(A)s that form a line one behind another on a poly(U) strand. On the other hand, in the case of the formation of a 5′,5′-pyrophosphate linkage, one of the two oligo(A)s is unable to hybridize to the same poly(U) strand with an ordered structure, because two 5′-terminal phosphate groups of oligo(A) must face each other. There is the possibility of the formation of a pyrophosphate linkage when poly(U) strands assume a random coil.

Metal ions such as Zn^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} possess a fairly strong affinity for both the base and phosphate moieties of nucleic acids. The extent of the conformational change of nucleic acids caused with metal ions depends on the relative intensity of the binding of metal ions to the base and phosphate moieties. In the case of Cu^{2+}, the binding to the base moiety predominates, and it prevents the stacking of bases and the formation of ordered structures of nucleic acids (Eichhorn, 1973). In our experiments, Cu^{2+} gave the lowest yield in the coupling reaction of $(pA)_4$ with BrCN (Table I) and many unidentified peaks were observed on the RPC-5 column chromatogram (data not shown). The appearance of these peaks may be due to the abundance of poly(U) random structures caused with Cu^{2+}.

Mn^{2+} gave the best yield of the coupling reaction of $(pA)_4$; however, 72% of the dimerized products had a 5′,5′-pyrophosphate linkage (Table II). Shin (1973) has shown that Mn^{2+} had little stimulatory effect on the formation of an ordered structure of poly(U). It might indicate that moderate disruption of the poly(U) ordered structure with Mn^{2+} caused an increase in the formation of pyrophosphate linkages. Bridson and Orgel (1980) reported that Zn^{2+} was an efficient catalyst for the oligomerization of guanosine 5′-phosphorimidazolide on a poly(C) template, and the oligomeric products were predominantly 3′,5′-linked. This was consistent with the fact that Zn^{2+} exists in an active site of DNA and RNA polymerase (Mildvan, 1974). In our experiments using BrCN, the molar ratio of the 3′,5′-linkage formed in the presence of Zn^{2+} was only 17% (Table II). This was inconsistent with the result obtained by Bridson and Orgel (1980). This discrepancy may be due to a difference between G-C and A-U pairings and a difference in interaction between nucleotides and metal ions.

Time Course of the Coupling Reaction of $(pA)_6$ in the Presence of Divalent Metal Ions. The time course of the coupling reaction of $(pA)_6$ was examined in the presence of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+} (Figure 7). Of all the metal ions, Mn^{2+} gave the best yield of the coupling reaction, whereas Mg^{2+} gave the lowest yield. The yields reached the maximum after 18 h except in the case of Zn^{2+} and then gradually decreased. On the contrary, Zn^{2+} continued to promote the coupling reaction even after 42 h. In the presence of Mn^{2+}, Co^{2+}, and Ni^{2+}, the dimerized product linked with a pyrophosphate bond markedly decreased and that linked with a 2′,5′- or 3′,5′-phosphodiester bond increased a little in 116 h. On the other hand, in the presence of Zn^{2+} the dimerized product linked with a pyrophosphate bond decreased a little but that linked with a 2′,5′- or 3′,5′-phosphodiester bond increased a little in 116 h (data not shown). Therefore, we assumed that the decrease in the yield after 18 h is mainly due to the hydrolysis of the pyrophosphate bond. Moreover, these results suggest that metal ions play important roles in not only the formation of the phosphate bonds but also the degradation of the phosphate bonds.

Time Course of the Condensation of $(pA)_6$ at 4, 20, and 40 °C. The yield of the coupling reaction of $(pA)_6$ with BrCN at 20 and 40 °C reached its maxima in 16 h and then gradually decreased (Figure 8). The decrease in yield was greater at 40 °C than at 25 °C. The reaction at 4 °C had a lag time of about 3 h and reached a plateau in 110 h, at which time the yield was much the same as that at 25 °C. The coupling reaction with BrCN was much faster than water-soluble carbodiimides, by which the reaction was performed at 0 °C for several weeks to get sufficient yield (Uesugi & Ikehara, 1977).

Effect of pH on the Coupling Reaction of $(pA)_6$. Effect of pH on the coupling reaction of $(pA)_6$ was examined in the range of pH 3.5–9.0: 0.2 M sodium acetate buffer was used in the range of pH 4.1–5.9; 0.2 M imidazole nitrate in the range of pH 6.0–7.0; 0.2 M Tris–acetate in the range of pH 7.8–8.7. The optimal pH was pH 6.25. The yield was low below pH 5.9 or above pH 7.8.

Formation of N-Cyanoimidazole, N,N′-Iminodiimidazole, and N-Carboxamidoimidazole in a Reaction of Imidazole with BrCN and Their Condensing Activities. To investigate a possible mechanism for the coupling reaction of oligo(A) on a poly(U) template, we tried to find products in a reaction of imidazole with BrCN. Reaction products were analyzed

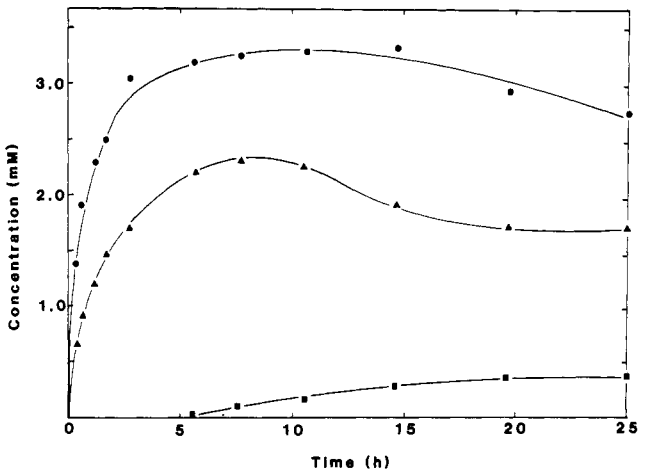
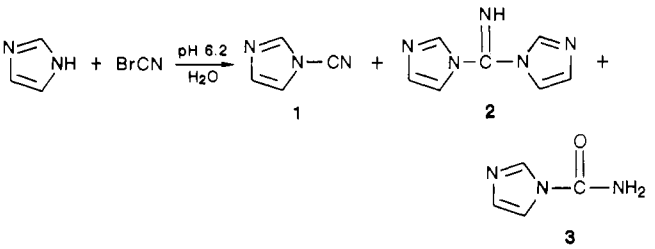


FIGURE 9: Time course of formation of imidazole derivatives from imidazole and BrCN in an aqueous solution: *N,N'*-iminodiimidazole (●), *N*-cyanoimidazole (▲), and *N*-carboxamidoimidazole (■).

by HPLC performed on a C_{18} column. Four peaks appeared in the chromatogram at 2.0, 5.5, 9.0, and 33.2 min. Four compounds were isolated from the reaction mixture by preparative HPLC and identified by their structure analyses with mass, IR, and NMR spectra: imidazole nitrate from peak 1 (retention time, 2.0 min); *N*-cyanoimidazole (**1**) from peak 2 (retention time, 5.5 min); *N,N'*-iminodiimidazole (**2**) from peak 3 (retention time, 9.0 min); *N*-carboxamidoimidazole (**3**)



from peak 4 (retention time, 33.2 min). It was confirmed that **1** and **2** were unstable and gradually converted to **3** in an aqueous solution. The conversion was promoted with an acid catalyst such as silica gel. Figure 9 shows the time course of the formation of **1**, **2**, and **3**. **1** and **2** were formed rapidly from imidazole and BrCN in an aqueous solution. Their formation attained a maximum in 5–10 h and then gradually decreased. **3** appeared after 5 h and its formation then gradually increased. **1**, **2**, and **3** were obtained from imidazole and BrCN in 25%, 39%, and 5% yields, respectively, after the reaction for 25 h. About 70% of BrCN was converted to **1**, **2**, and **3**.

Table III shows a condensing activity of **1**, **2**, and **3** for the coupling reaction of $(pA)_4$ and $(pA)_6$. **1** and **2** had much the same activity for the coupling reaction as BrCN in the presence of imidazole. **1** and **2** were less effective in the absence of imidazole. BrCN showed no activity in the absence of imidazole. **3** had no activity even in the presence of imidazole. **1** and **2** contain a carbodiimide bond (—N=C=N—). The same bond was found in water-soluble carbodiimides or cyano derivatives such as cyanamide and dicyandiamide, known as prebiotic condensing agents for nucleic acid and peptide syntheses (Steinman et al., 1964, 1965; Schimpl et al., 1965). An active central carbon atom of carbodiimide can be easily attacked with a nucleophilic oxygen atom of a phosphate group to form a chemical bond between an oxygen and a carbon atom. Then the phosphorus atom could be activated by releasing electrons to the carbodiimide.

A Possible Mechanism for the Coupling Reaction with BrCN. *N*-Cyanoimidazole (**1**) was first formed from imidazole and BrCN in an aqueous solution, and then an addition of one or more imidazole molecules to **1** resulted in the formation of *N,N'*-iminodiimidazole (**2**). **1** and **2** had a condensing activity for the coupling reaction of oligo(A). It is estimated that BrCN cannot condense the nucleic acids in the absence of imidazole, because the total conversion yield of **1** and **2** from BrCN was about 70%, and the lack of imidazole in the reaction mixture resulted in no polymerization (Table III).

Table III: Coupling Reaction of $(pA)_4$ and $(pA)_6$ with BrCN, *N*-Cyanoimidazole, and *N,N'*-Iminodiimidazole in the Presence or Absence of Imidazole[a]

coupling agent	imidazole	yield (%) from $(pA)_4$	yield (%) from $(pA)_6$
BrCN	+	54.1	67.6
	−	0	0
N-cyanoimidazole	+	60.8	77.3
	−	44.0	47.5
N,N'-iminodiimidazole	+	64.5	63.5
	−	18.5	13.7
N-carboxamidoimidazole	+	0	0

[a] The reaction mixture (20 μL) contained 0.15 mM $(pA)_4$ or $(pA)_6$, 0.75 mM poly(U), 10 mM $MnCl_2$, 200 mM imidazole nitrate buffer, pH 6.0, or 200 mM sodium acetate buffer, pH 6.0, 10 mM BrCN, *N*-cyanoimidazole, *N,N'*-iminodiimidazole, or *N*-carboxamidoimidazole and was incubated at 20 °C for 20 h.

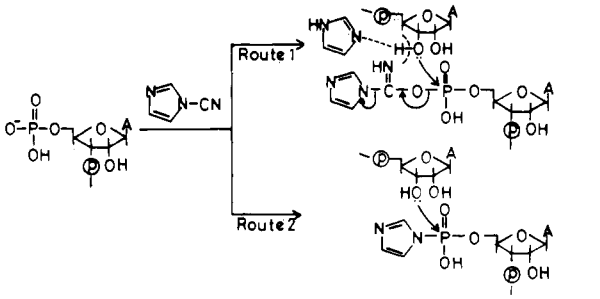


FIGURE 10: Possible reaction mechanism for formation of the activated phosphate derivatives and the phosphodiester bond.

We would like to discuss the reaction mechanism for activation of a phosphate group. Two mechanisms can be considered: One is a direct addition of the condensing reagent **1** or **2** to the phosphate group (route 1). Then, the phosphorus atom can be activated by releasing electrons toward an imidazole group through the newly formed P–O–C bond (Figure 10). The other is formation of phosphorimidazolide (route 2). We examined its formation from a reaction of pA with BrCN in imidazole nitrate buffer, pH 6.0. A small amount of adenosine 5'-phosphorimidazolide was formed only when a large excess of BrCN was used as a condensing agent. Thus, the latter possibility cannot be excluded. Metal ions might contribute to stabilization of the structure of a poly(U) template by binding to phosphate groups. In addition, metal ions might interact with a proton of a 2'- or 3'-hydroxy group of the ribose moiety through a hydrogen bond and increase the nucleophilicity of the oxygen atom (Figure 10). On the other hand, imidazole is a starting material of both **1** and **2**, and it may also be playing a role similar to the metal ions. This consideration is derived from the result that the lack of imidazole in the coupling reaction of oligo(A) with **1** and **2** caused a decrease in yield. Since imidazole can accept and donate a proton from one to another, it may be helpful to pull out a proton from a 2'- or 3'-hydroxy group when the oxygen atom of a hydroxy attacks an activated phosphorus atom.

In conclusion, as described above, the template-directed polymerization of oligo(A) occurred in the presence of BrCN, imidazole, and divalent metal ions at 4–40 °C. The dimerized

products of $(pA)_4$ had newly formed three kinds of phosphate bonds, 2′,5′-, 3′,5′-, and 5′,5′-linkages. BrCN was converted to *N*-cyanoimidazole and *N,N′*-iminodiimidazole, which were true condensing agents for the coupling reaction of oligo(A).

ACKNOWLEDGMENTS

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Registry No. **1**, 36289-36-8; **2**, 104619-51-4; **3**, 2578-41-8; oligo(A), 24937-83-5; poly(U), 27416-86-0; $(pA)_6$, 7619-61-6; $(pA)_{12}$, 104714-91-2; $(pA)_{18}$, 75433-19-1; $(pA)_{24}$, 75433-24-8; $(pA)_{30}$, 103106-41-8; $(pA)_{36}$, 103106-44-1; $(pA)_{42}$, 104714-92-3; $(pA)_{48}$, 104714-93-4; BrCN, 506-68-3; Mn^{2+}, 16397-91-4; Co^{2+}, 22541-53-3; Ni^{2+}, 14701-22-5; Cu^{2+}, 15158-11-9; Zn^{2+}, 23713-49-7; Mg^{2+}, 22537-22-0; Fe^{2+}, 15438-31-0; imidazole, 288-32-4.

REFERENCES

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509–521.
Axen, R., Porath, J., & Ernback, S. (1967) *Nature (London) 214*, 1302–1304.
Blake, R. D., Massoulie, J., & Fresco, J. R. (1967) *J. Mol. Biol. 30*, 291–308.
Brahms, J., Michelson, A. M., & Van Holde, K. E. (1966) *J. Mol. Biol. 15*, 467–488.
Bridson, P. K., & Orgel, L. E. (1980) *J. Mol. Biol. 144*, 567–577.
Chen, C. B., Inoue, T., & Orgel, L. E. (1985) *J. Mol. Biol. 181*, 271–279.
Christie, S. M. H., Elmore, D. T., Kenner, G. W., Todd, A. D., & Weymouth, F. J. (1953) *J. Chem. Soc.*, 2947–2953.
Eichhorn, G. L. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) pp 1210–1243, Elsevier, Amsterdam.
Ferris, J. P., & Yanagawa, H. (1984) *J. Org. Chem. 49*, 2121–2125.
Ferris, J. P., Edelson, E. H., Mount, N. M., & Sullivan, A. E. (1979) *J. Mol. Evol. 13*, 317–330.
Ferris, J. P., Hagan, W. J., Jr., Alwis, K. W., & McCrea, J. (1982) *J. Mol. Evol. 18*, 304–309.
Ferris, J. P., Yanagawa, H., Dudgeon, P. A., Hagan, W. J., Jr., & Mallare, T. E. (1984) *Origins Life 15*, 29–43.
Giesemann, H. (1955) *J. Prakt. Chem. 1*, 345–348.
Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.
Hagan, W. J., Jr. (1985) Ph.D. Thesis, Rensselaer Polytechnic Institute, New York.
Ibanez, J. D., Kimball, A. P., & Oro, J. (1971) *Science (Washington, D.C.) 173*, 444–446.
Inoue, T., & Orgel, L. E. (1983) *Science (Washington D.C.) 219*, 859–862.
Kanaya, E., & Yanagawa, H. (1985) *Nucleic Acids Symp. Ser. 16*, 181–184.
Lohrmann, R., & Orgel, L. E. (1978) *Tetrahedron 34*, 853–855.
Lohrmann, R., & Orgel, L. E. (1979a) *J. Mol. Evol. 12*, 237–257.
Lohrmann, R., & Orgel, L. E. (1979b) *J. Mol. Evol. 14*, 243–250.
Lohrmann, R., Bridson, P. K., & Orgel, L. E. (1980) *Science (Washington D.C.) 208*, 1464–1465.
Lowe, C. R. (1979) *Lab. Tech. Biochem. Mol. Biol. 7*, 346–347.
Mildvan, A. S. (1974) *Annu. Rev. Biochem. 43*, 357–399.
Mukai, J. I. (1965) *Biochem. Biophys. Res. Commun. 21*, 562–567.
Naylor, R., & Gilham, P. T. (1966) *Biochemistry 5*, 2722–2728.
Porath, J. (1974) *Methods Enzymol. 34*, 13–30.
Pörschke, D. (1974) *Biophys. Chem. 2*, 83–96.
Sawai, H. (1982) *Viva Origino 10*, 102–103.
Schimpl, A., Lemmon, R. M., & Calvin, M. (1965) *Science (Washington D.C.) 147*, 149–150.
Shin, Y. A. (1973) *Biopolymers 12*, 2459–2475.
Sleeper, H. L., Lohrmann, R., & Orgel, L. E. (1979) *J. Mol. Evol. 13*, 203–214.
Steinman, G., Lemmon, R. M., & Calvin, M. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 27–30.
Steinman, G., Lemmon, R. M., & Calvin, M. (1965) *Science (Washington, D.C.) 147*, 1574–1575.
Uchida, T. (1966) *J. Biochem. (Tokyo) 60*, 115–132.
Uesugi, S., & Ts'o, P. O. P. (1974) *Biochemistry 13*, 3142–3152.
Uesugi, S., & Ikehara, M. (1977) *Biochemistry 16*, 493–498.