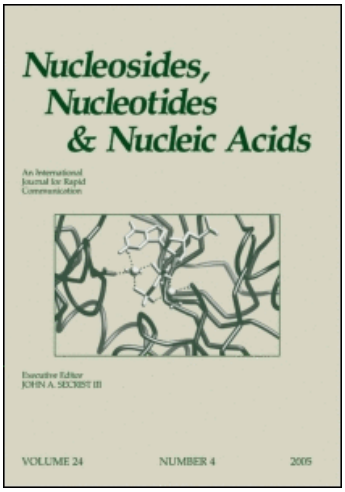

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Divalent Metal Ion-Catalyzed Pyrophosphate Bond Formation in Aqueous Solution. Synthesis of Nucleotides Containing Polyphosphate

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DIVALENT METAL ION-CATALYZED PYROPHOSPHATE BOND FORMATION IN AQUEOUS SOLUTION. SYNTHESIS OF NUCLEOTIDES CONTAINING POLYPHOSPHATE

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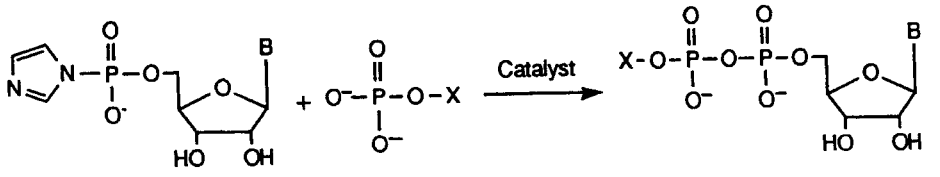
Abstract. Nucleotides containing polyphosphate were prepared from nucleoside-5'-phosphorimidazolide and nucleotides or phosphoryl compounds by divalent metal ion catalyst such as Mn^{2+}, Cd^{2+} or Mg^{2+} in aqueous solution.

Nucleotides bearing polyphosphate bond are widely distributed in living cells.[1] In the forms of ATP, nicotinamide-adenine dinucleotide, diadenosine polyphosphate,[2] diguanosine tetraphosphate,[3] cap portion of messenger RNAs[4] and uridine diphosphate glucose,[1] these compounds play essential roles in various biochemical processes as energy source in metabolism, building block in polynucleotide synthesis, coenzyme, mediator of DNA or protein synthesis and intermediate in carbohydrate metabolism.[1]

Several chemical methods have been developed for the preparation of these nucleotides bearing pyrophosphate bond.[5] The crucial step for their synthesis involves, in general, activation of nucleotides followed by reaction with phosphoryl compounds in an anhydrous medium. The synthetic methods, therefore, require tertiary alkyl ammonium ion as counter cation to solubilize the nucleotides and phosphoryl compounds in organic solvents. Further, protecting of base part and sugar hydroxyl groups of nucleotides is often needed for the solubilization and for the target reaction.

This paper is dedicated with respect to the late professor Tohru Ueda who contributed extensively to the nucleoside and nucleotide chemistry.

On the other hand, in biochemical processes, these compounds are synthesized in neutral aqueous medium from nucleoside 5'-triphosphate and nucleotides or phosphoryl compounds, catalyzed by nucleotidyl-transfer or phosphoryl-transfer enzymes which require either Mg^{2+} or Mn^{2+} ion as cofactor.[6] We initiated an investigation of nonenzymatic pyrophosphorylation in aqueous solution by using metal ion catalyst, to establish a new and simpler synthesis of the compounds of interest. Nucleoside 5'-phosphorimidazolide, an imidazole-activated nucleotide, was used in place of nucleoside 5'-triphosphate in the nonenzymatic reaction, as nucleoside 5'-triphosphate hydrolyzes easily by metal ion catalyst.[7] We have recently found that Mn^{2+} and Cd^{2+} ion catalyze the pyrophosphate bond formation from adenosine 5'-phosphorimidazolide and some nucleotides in aqueous solution.[8] To gain further information on the mechanism and synthetic utility of this reaction, we have examined the metal ion-catalyzed pyrophosphate bond formation from various nucleoside 5'-phosphorimidazolide and nucleotides under various conditions.



Catalyst : Mn^{2+}, Cd^{2+}, Mg^{2+}

This present paper reports the results of these studies. Mechanistic role of the metal ion in this reaction is also described briefly.

Experimental

Materials: Adenosine-, uridine-, cytidine-, guanosine- and inosine 5'-monophosphate (pA, pU, pC, pG and pI) were purchased from Seikagaku Kogyo; adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), guanosine 5'-diphsophate (GDP) and guanosine 5'-triphosphate (GTP), from Boeringer; nuclease P1, from Seikagaku Kogyo; and venom phosphodiesterase and bacterial alkaline phosphatase, from Worthington. 7-Methylguanosine 5'-monophosphate (p7mG) was prepared from guanosine 5'-monophosphate and methyl iodide by modification of the published procedure.[9] Nucleoside 5'-phosphorimidazolides (ImpN) were prepared from nucleoside 5'-monophosphate and imidazole using triphenylphosphine and dipyridyl disulfide as a condensing agent as described previously.[10] ImpNs were isolated as a sodium salt in 80-90 %

yields. Analysis of the freshly prepared ImpN by HPLC showed only one UV absorbing compound. All other chemicals were of reagent grade.

High Performance Liquid Chromatography(HPLC): HPLC was performed with a Hitachi 638 apparatus using a RPC-5 column (4 mm x 25 cm) or with a Wakosil 5C18 column (4 mm x 25 cm). RPC-5 was prepared from fine granular polychlorotrifluoroethylene and Adogen 464 as described earlier.[11] HPLC on a RPC-5 column was eluted with a linear gradient from 0 to 10 mM of $NaClO_4$ solution in 2 mM Tris-acetate and 0.1 mM EDTA at pH 7.5 in 30 min at a flow rate of 1.0 ml/min. HPLC on a Wakosil 5C18 column was eluted with a linear gradient from 2.4 to 20 % methanol in 0.1 M triethylammonium acetate at pH 7.3 in 30 min at a flow rate of 1.0 ml/min. The eluate was monitored by UV absorption at 260 nm or 280 nm.

Spectrometric Method: UV absorption spectra were taken on a Hitachi 3200 instrument. ^1H NMR spectra were measured by a Varian Gemini 200 FT NMR spectrometer using sodium trimethylsilylpropanoate-d_4 (TSP-d_4) as an internal standard. The sample for ^1H NMR was passed through a Dowex 50 WX8 (Na^+ form) to remove a trace amount of transition metal ion, evaporated under vacuum twice with D_2O and dissolve in D_2O at pD 6.7 in 0.01 M phosphate buffer. ^{31}P NMR spectra were recorded on a Hitachi R-90H FT NMR spectrometer at 36.438 MHz using 85 % H_3PO_4 as an external standard. The sample was dissolved in 30 % D_2O buffered with 0.01 M Tris-HCl (pH 7.8).

Standard Procedure for the Pyrophosphate Bond Formation: Reactions were carried out in a Eppendorf tube. The samples were prepared on an ice bath, agitated vigorously and kept at 25 °C for various times. A typical reaction mixture (0.1 ml) contained 20 mM inosine 5'-phosphorimidazolide (ImpI), 20 mM or 60 mM pI and 20 mM metal chloride in 0.25 M N-ethylmorpholine-HCl buffer (pH 7.0). The reaction mixture was treated with 30 ul of 0.2 M versenol solution to remove the metal ion as versenol-metal chelate and stored in a freezer until analysis by HPLC. HPLC was performed with two different column systems as described above. Identification of peaks corresponding to ImpI, pI and diinosine 5',5"-diphosphate (IppI) was confirmed by coinjection with authentic samples.[12] Yields were calculated from the peak integrals of the HPLC chromatogram. Correction for the hypochromicity of IppI was made on the yield data. Hypochromicity of IppI was estimated from the ratio of UV absorption before and after digestion with venom phosphodiesterase. The estimated hypochromicity of IppI was 11 %

Reactions of other ImpN with pN, ppN or pppN were carried out in the same way and analyzed by HPLC as described above. When no authentic sample was available, the peak corresponding to dinucleoside 5',5"-polyphosphate was collected by preparative HPLC, and its structure was identified by NMR. The structure was further confirmed by enzyme and alkaline digestion. Venom phosphodiesterase hydrolyzed pyrophosphate bond of dinucleoside 5',5"-polyphosphate. Digestion with venom phosphodiesterase was carried out at 37 ° for 2.5 h in a mixture containing 0.9-1.7 ODU of substrate, 0.05 M tris-acetate (pH 8.8), 0.2 units of enzyme and 0.01 M $MgCl_2$. On the other hand, both bacterial alkaline phosphatase and alkaline solution were inactive towards dinucleoside 5',5"-polyphosphate, as expected. Digestion with bacterial alkaline phosphatase was carried out for 2.5 h at 37 ° in a mixture (50 ul) containing the substrate (1-2 ODU), 0.1 M Tris-HCl (pH 8.05), 0.001 M $MgCl_2$ and 0.01 units of enzyme. Alkaline hydrolysis was carried out in a mixture (30 ul) containing the substrate (0.4-0.9 ODU) in 0.5 M NaOH solution for 1 d at room temperature.

Synthesis and Purification of Diadenosine 5',5"-polyphosphates: A reaction mixture (24 ml) contained 25 mM ImpA (0.1 M x 6.0 ml), 25 mM ADP (0.1 M x 6.0 ml), 25 mM $CdCl_2$ (0.25 M x 2.4 ml) and 0.1 M N-ethylmorpholine-HCl buffer (pH 7.0, 1.0 M x 2.4 ml). The reaction was run at 25 °C for 4 d. The reaction mixture was treated with chelex-100 to remove the Cd^{2+} ion and applied to a QAE-Sephadex A-25 column (2.5 cm x 40 cm). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer, 0.1 M (1.0 l)-0.6 M (1.0 l). Appropriate UV absorbing fractions were collected and lyophilized to give pure ApppA as a triethylammonium salt (189 mg, 27 % yield estimated from UV absorption at 260 nm).

The reaction of ImpA with ATP was carried out by $CdCl_2$ catalyst in the same way as described above. AppppA was isolated in 23 % yield calculated from UV absorption at 260 nm.

Synthesis and Purification of Cap Portion of mRNA, 7mGpppG: A reaction mixture (0.6 ml), which contained 50 mM ImpG (0.125 M x 0.24 ml), 50 mM 7-methylguanosine 5'-diphosphate (0.25 M x 0.12 ml), 50 mM $CdCl_2$ (0.25 M x 0.12 ml) in 0.2 M N-ethylmorpholine buffer (pH 7.0), was stirred for 4 d at 25 °. The reaction mixture was treated with 0.25 M Versenol buffer (0.15ml) to remove the Cd^{2+} ion as Versenol-Cd^{2+} chelate. The formation of 7mGpppG was checked by HPLC. The reaction mixture was, then, applied to a DEAE-Toyopearl column (10 mm x 30 cm), and the column was eluted with a linear gradient of triethylammonium bicarbonate buffer, H_2O (0.4 l)-0.5 M (0.4 l). UV absorbing

TABLE 1 Formation of NppN from ImpN and pN Catalyzed by Metal Ions

	Yield (% based on the starting ImpN)	
catalyst	IppI	CppC
Mg^{2+}	12	12
Ca^{2+}	8	6
Mn^{2+}	28	13
(Fe^{2+})	4	6
Co^{2+}	11	7
Ni^{2+}	5	4
Cu^{2+}	8	2
Zn^{2+}	13	5
Cd^{2+}	15	14
Ba^{2+}		2
Hg^{2+}	2	4
None	2	1

Reactions were run at 25 °C for 4 d using 20mM ImpN, 60mM pN, 20mM metal chloride in 0.2M N-ethylmorpholine-HCl buffer (pH 7.0).

fractions corresponding to 7mGpppG were collected and lyophilized to give HPLC pure product as a triethylammonium salt (10 mg, 191 ODU at 260 nm, 37 % yield estimated from UV absorption).

Results

Imidazole-activated nucleotide, nucleoside 5'-phosphorimidazolide hydrolyzes spontaneously in aqueous solution in the absence of catalyst. Previously we showed that Mn^{2+} and Cd^{2+} ions have a high activity for the pyrophosphate bond formation from ImpA and pA.

To apply the pyrophosphate bond formation to the preparation of other nucleotides, we examined catalytic activity of various metal ions in the dicytidine 5',5"-diphosphate (CppC) formation from ImpC and pC. TABLE 1 shows the yield of CppC, along with the yield of IppI from ImpI and pI. The activity of metal ions in the CppC and IppI formation was in the same order as that in the AppA formation, though the yields of CppC and IppI were a few times lower than that of AppA. Mn^{2+} and Cd^{2+} ions had higher activity in these pyrophosphate bond formations than any other metal ions examined.

TABLE 2 Effect of Temperature on the AppA Formation by Mn^{2+} Ion Catalyst

Time \ Temp	Yield (% based on ImpA) 4°C	25°C	50°C
2 h			36
4 h			41
8 h			42
1 d		38	35
2 d		43	34
4 d	35	45	
8 d	43		

Reactions of 20mM ImpA and 20mM pA were conducted in the presence of 20mM $MnCl_2$ in 0.2M N-ethylmorpholine-HCl buffer (pH 7.0).

TABLE 2 lists the yield data of Mn^{2+} ion-catalyzed AppA formation conducted at 4, 20 and 50 °C. The yield of AppA was 42-45 %, independent of the reaction temperature. The pyrophosphorylation of ImpA competes with hydrolysis of ImpA. The results indicate that increment of the reaction temperature accelerated greatly both the pyrophosphorylation and the hydrolysis of ImpA at the same rate. At 50 °C, the reaction was completed in less than 8 h. At this temperature, the product AppA degraded gradually in prolonged times.

The effect of concentration of Mn^{2+} ion on the AppA formation is shown in FIG. 1. The formation of AppA was maximum at an equimolar amount of Mn^{2+} ion to ImpA, and decreased as the molar ratio of Mn^{2+} to ImpA decreased. The pyrophosphate bond formation probably takes place stoichiometrically in the metal complex composed of Mn^{2+} ion, ImpA and pA.

The pH and a buffer component of the reaction medium had a considerable effect on the pyrophosphate bond formation , in which Mn^{2+} or Mg^{2+} ion was used as catalyst, as shown in FIG. 2. We used N-ethylmorpholine-HCl buffer and 2,6-lutidine-HCl buffer at pH 6.5-8.0 and 5.5-7.0, respectively. They have very little coordinating ability to metal ions. The buffer solution such as tris(hydroxymethyl)aminomethane-HCl which can coordinate to the metal ion inhibits the pyrophosphate bond formation, as the reaction takes place in the coordination sphere of the metal ion. The yield of AppA by Mn^{2+} ion catalyst was lower in 2,6-lutidine buffer than in N-ethylmorpholine buffer at the same pH. On the other hand, the yield of AppA by Mg^{2+} ion catalyst was almost the

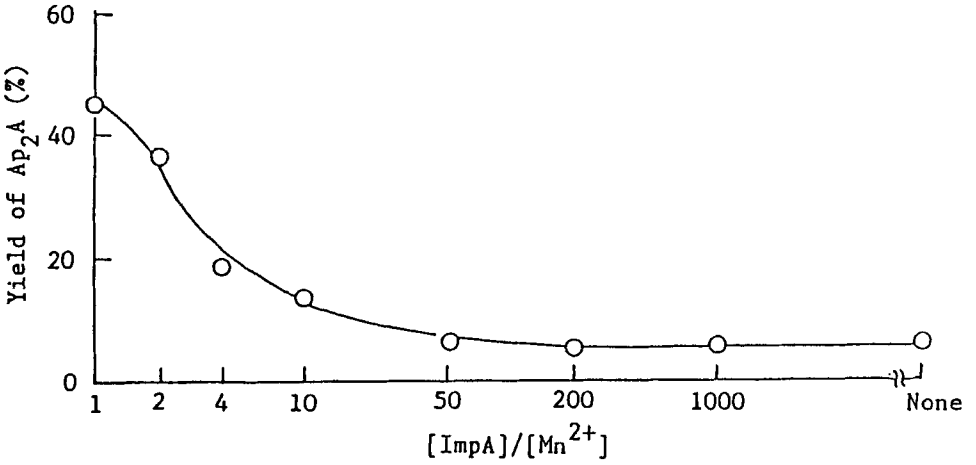


FIG. 1 Effect on Mn^{2+} Concentration on the AppA Formation: The reactions were run at 25 °C for 4 d in 0.2 M N-ethylmorpholine-HCl buffer (pH 7.0). 20 mM ImpA and 20 mM pA were used in the reaction.

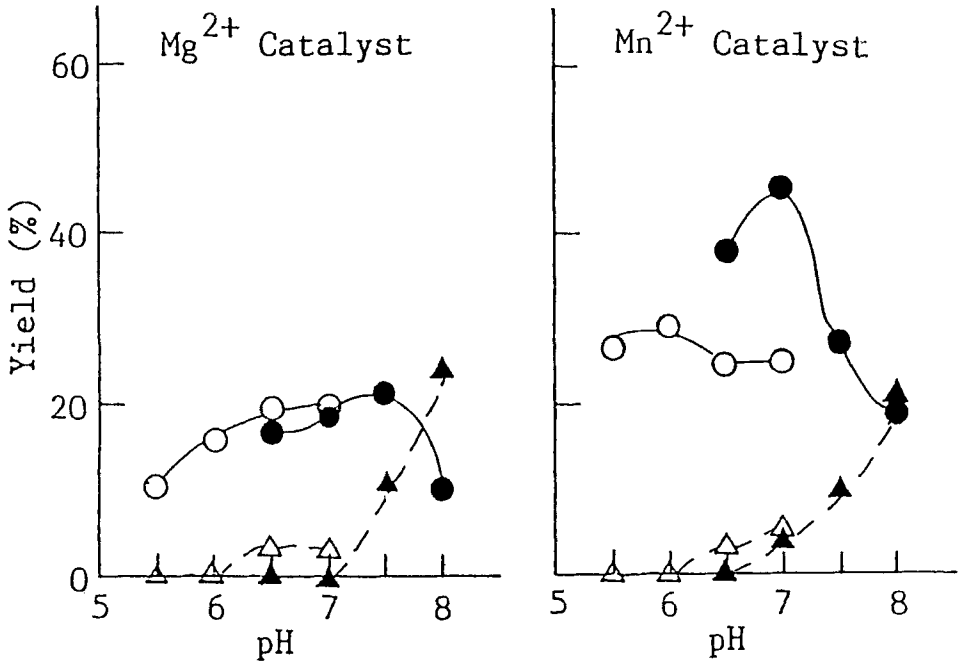


FIG. 2 Effect of pH on the AppA Formation. The reactions were run at 25 °C for 4 d in 0.2 M N-ethylmorpholine buffer (●, ▲) or in 0.2 M 2,6-lutidine buffer (○ , Δ) using 20 mM ImpA, 20mM pA and 20 mM metal chloride.
● , ○ ; AppA. ▲ , Δ ; ImpA

TABLE 3 NppN Formation from ImpN and pN Catalyzed by Mg^{2+}, Mn^{2+} or Cd^{2+}

	Yield (% based on the starting ImpN)						
Metal Ion	AppA	GppG	IppI	CppC	UppU	7mGppG*	7mGppG7m
Mg^{2+}	20	22	2	7	9	13	7
Mn^{2+}	40	40	28	17	11	36	14
Cd^{2+}	42	54	15	9	6	24	11
None	4	4	2	2	2	2	3

The reactions were run at 25 °C for 4 d using 20mM ImpN, 60mM pN and 20mM metal chloride in 0.2M N-ethylmorpholine-HCl buffer (pH 7.0).
* m7GppG was formed from Imp7mG and pG.

same in both the buffers at the same pH. These results indicate that 2,6-lutidine coordinates to the Mn^{2+} ion and interfere the coordination of ImpA and pA, suppressing the pyrophosphate bond formation, but not to the Mg^{2+} ion. The inhibitory effect of the 2,6-lutidine buffer was also observed in the case of Cd^{2+} ion-catalyzed pyrophosphate bond formation.

A neutral condition resulted in the most efficient pyrophosphate bond formation. Hydrolysis of ImpA was accelerated at lower pH below 6.0, as protonation of imidazole of ImpA made the phosphorimidazolide bond susceptible to nucleophilic attack by water. High pH above 7.5 stabilized the phosphorimidazolide bond and retarded the reaction of ImpA. A substantial amount of the starting ImpA remained unchanged at high pH.

TABLE 3 shows the effect of base part on the pyrophosphate bond formation from various ImpNs and pNs, where Ns are A, U, C, G, I and 7mG. The Mn^{2+}, Cd^{2+} or Mg^{2+} was used as catalyst. The Mn^{2+} ion demonstrated considerably high efficiency in the NppN formation, regardless of the difference of base part. On the other hand, the catalytic activity of the Cd^{2+} ion in the pyrophosphate bond formation varied depending on the base part.

Coordination of guanine to the Cd^{2+} ion seems to have contributed to the enhancement of the GppG formation. The NppN formation of purine bases took place more effectively than that of pyrimidine bases.

The stacking interaction between purine bases is stronger than the one between pyrimidine bases. The strong stacking interaction of purine bases can bring ImpN and pN closer in the coordination sphere of the metal ion. and promote the reaction between ImpN and pN. The presence of cationic ion on

TABLE 4 Synthesis of Diguanosine 5',5"-polyphosphates from ImpG and GDP or GTP

Metal ion	Yield (%)[a] GpppG[b]	GppppG[c]
Mg^{2+}	68	39
Mn^{2+}	84	45
Cd^{2+}	73	34
None	5	1

Reactions were run at 25 °C for 4 d in the presence of 20mM metal chloride in 0.2 M N-ethylmorpholine-HCl buffer (pH 7.0). a: Yields are expressed based on the starting GDP or GTP. b: 60 mM ImpG and 20 mM GDP were used in the reaction. c: 60 mM ImpG and 20 mM GTP were used in the reaction.

the guanine base by 7-methylation caused a decrease in the stacking interaction but an increase in the electrostatic repulsion between Imp7mG and p7mG. The coordination of 7-methylguanine nucleotide to the metal ion would be also suppressed by electrostatic repulsion. Thus the yield of 7mGppG7m or 7mGppG was very low compared with that of GppG. The low yield of nicotinamide adenine dinucleotide from ImpA and nicotinamide mononucleotide[8] is also attributable to the presence of cation on the nicotinamide.

Dinucleoside 5',5"-polyphosphate is known to have several biological activities such as inhibition of kinase reaction and stimulation of DNA synthesis for diadenosine 5',5"-tetraphosphate (AppppA),[13] and control of embryogenesis and stimulation of protein synthesis for diguanosine 5,5"-tetraphosphate (GppppG).[14] AppppA or ApppA was prepared from ImpA and ATP or ADP by the metal ion catalyst[8]. Similarly the reaction of ImpG with GTP or GDP by the metal ion catalyst gave GppppG or GpppG as shown in TABLE 4. Mn^{2+} and Cd^{2+} ion were effective catalysts also in these pyrophosphate bond formation. The yields of diadenosine 5',5"-polyphosphate and diguanosine 5',5"-polyphosphate by the Mn^{2+} ion catalyst were 45 to 84 % under the optimum condition. The yield in the reaction is comparable to the one by the conventional method[15] where anhydrous organic solvent was used as a medium.

Similarly, Cd^{2+} ion-catalyzed pyrophosphate bond formation between ImpG and 7-methylguanosine-5'-diphosphate in aqueous solution gave 7mGpppG, cap portion of mRNA, in 37 % yield . These compounds could be prepared in a large scale and isolated by anion-exchange chromatography in a pure form. The

TABLE 5 ^{1}H NMR of Dinucleoside 5',5"-Polyphosaphates

Compound	Chemical shift(coupling constant, Hz)							
	8-H	2-H	8-H[a]	6-H	5-H	1'-H	1'-H[b]	CH_3
AppA	8.17	8.03				5.95(5.2)		
ApppA	8.29	8.10				6.01(4.1)		
AppppA	8.38	8.14				6.03(5.8)		
UppU				7.92(8.2)	5.94(8.2)	5.96(4.3)		
IppI	8.27	8.13				6.00(5.5)		
CppC				7.91(7.5)	6.05(7.5)	5.98(3.9)		
GppG	7.94					5.79(5.4)		
GpppG	8.01					5.84(4.8)		
GppppG	8.07					5.87(4.8)		
7mGpppG	8.03		N.O.[c]			5.82(6.1)	5.92(2.6)	4.06
7mGppG	7.93		8.86[d]			5.74(5.9)	5.83(3.1)	4.02
7mGpp7mG			N.O.[c]				5.96(3.8)	4.12

a, 8-H of 7-methylguanine b, 1'-H of 7-methylguanine c, not obseved.
d, weak intensity

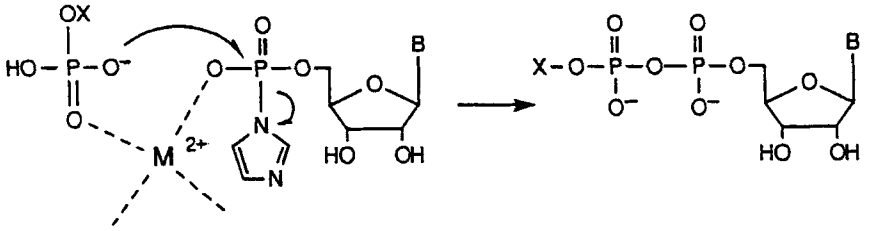
synthesis of cap portion of mRNA from new capping agents, Imp7mG or Impp7mG, will be published elsewhere.

Some ^{1}H NMR data of dinucleoside 5',5"-polyphosphates are listed in Table 5. Base and 1'-H protons of these compounds showed up-field shift compared to that of the corresponding nucleoside-5'-phosphates. The result is due to the ring current effect of the heterocyclic bases, which is caused by base-base stacking interaction in the dinucleoside 5',5"-polyphosphates. It is noteworthy that resonance peak of 8-H proton showed very weak intensity in the case of 7-methylguanine nucleotides. Methylation of N-7 position of guanine causes down-field shift and line broadening for the 8-H proton. The 8-H proton of 7-methylguanine may be exchangeable with solvent, D_2O, as acidity of 8-CH increases by the 7-methylation.

Discussion

The Mn^{2+}, Mg^{2+} and Cd^{2+} ions have high activity for the pyrophosphate bond formation. The mechanistic role of the metal ions in the catalytic action is ambiguous. We postulate, however, that the pyrophosphate bond formation takes place in the mixed ligand complex composed of ImpN, pN and the metal ion.

The phosphorimidazolide of ImpN and phosphate group of the phosphate donor could be brought into closer by coordination to the metal ion. Nucleophilic attack of the phosphate on the proximate phosphorimidazolide of ImpN gives the pyrophosphate bond by displacing imidazole, as shown in the scheme. Thus the metal ion plays a role of a template in the reaction. The metal ion also neutralizes negative charge of the substrates working as a pseudo-proton and enhance the nucleophilic reaction. The Mn^{2+} and Mg^{2+} ion have a high affinity with the phosphate[16] and can bring the phosphate group and ImpN closer. The Ca^{2+} and Ba^{2+} ion also have high affinity with the phosphate, but they have a larger ionic radius than Mg^{2+} and Mn^{2+} ions.[17] The ionic radius of Mg^{2+} is similar to that of Mn^{2+} ion.[17] The Mg^{2+} ion forms complexes of tetrahedral or octahedral structure, while the Mn^{2+} ion forms complexes of octahedral structure preferentially.



Proposed Scheme for the Metal Ion-Catalyzed Pyrophosphate Bond Formation.

The ionic radius and coordination mode of the metal ion are the main determinants of the structure and reactivity of the metal complex, which seems important for the catalytic action in the pyrophosphate bond formation. Thus either the Mg^{2+} and Mn^{2+} ion appears to be appropriate for the pyrophosphate bond formation. The Zn^{2+} ion, which appears to have an appropriate size but, undesirably, binds to the base part and preferentially forms tetrahedral complexes,[17] showed low activity in the pyrophosphate bond formation. These results suggest that the octahedral Mg^{2+} or Mn^{2+} ion complex works as an intermediate in the pyrophosphate bond formation.

The high activity of the Cd^{2+} ion in the pyrophosphate bond formation is puzzling, as the character of Cd^{2+} ion is different from that of Mg^{2+} or Mn^{2+} ion. The Cd^{2+} ion is a rather soft metal ion and coordinates to both the phosphate and base part of nucleotides as the Zn^{2+} ion.[16] The activity of the Cd^{2+} ion in the pyrophosphate bond formation varied depending on the base part. Coordination of the base part to the Cd^{2+} ion may have affected the efficiency of the pyrophosphate bond formation.

In conclusion, we think that the roles of the metal ion in the pyrophosphate bond formation are (a) the organization of the substrates by coordination, and (b) the activation of the substrates by coordination.

The difference of the yield depending on the base part suggests the importance of the stacking interaction between the nucleotides. The stacking interaction of aromatic rings enhances the molecular association by 1.5 kcal/mol.[18] The heterocyclic base-stacking interaction in the complexes promotes the association between the nucleotides, enhancing the pyrophosphate bond formation. The low yields of NppN in cases of pyrimidine and 7-methylguanine nucleotides are attributable to the weak stacking interactions of pyrimidine bases[18] and 7-methylguanine. The lower yield of IppI compared with that of AppA or GppG should be due to the fact that the stacking interaction of inosine is weaker than that of adenosine or guanosine. Sigel et al. reported that the association constant of the inosine system is lower than that of guanosine or adenosine system, both in a free state and a metal complex state.[18] The importance of the stacking-interaction in the pyrophosphate bond formation is also illustrated by the fact that the reaction of ImpA with pyrophosphate or orthophosphate by the metal ion catalyst gave low yield of ATP or ADP,[8] as stacking interaction between the substrates is impossible.

The Mg^{2+} ion is essential for the transferases and kinases which catalyzes nucleotidyl or phosphate transfer.[6] The pyrophosphate bond formation is catalyzed by these enzymes. The Mg^{2+} ion can be replaced with Mn^{2+} ion without losing the activity of the enzymes. The action of Mg^{2+} and Mn^{2+} ion in the pyrophosphate bond formation in our nonenzymatic reaction may have similar function in the enzymatic reactions.

The present metal ion-catalyzed pyrophosphate bond formation is applicable to the synthesis of nucleotides containing polyphosphates, though the yields of the pyrophosphorylation were modest to considerably high. The advantage of this method over the conventional ones is the simpler and short procedure, as the reaction proceeds in neutral aqueous solution and no protecting group is required.

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