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Mg(II) ION-MEDIATED CONVERSION OF MONO- AND OLIGONUCLEOTIDES TO 5'-POLYPHOSPHATES IN AQUEOUS SOLUTION

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5'-Polyphosphates of mono- and oligonucleotides were prepared from the corresponding 5'-monophosphates with phosphorotriimidazolide or phosphorotribenzimidazolide mediated by Mg(II) or Mn(II) ion in aqueous solution.

Nucleoside 5'-di- and 5'-triphosphates, oligonucleotides 5'-polyphosphates and unsymmetrical diesters of pyrophosphate, such as coenzymes, play essential roles in many biological systems[1]. Their syntheses are of particular significance and have been carried out by various chemical and enzymatic methods[2].

Conventionally, they are prepared via activation of the 5'-monophosphate of mono- and oligonucleotides, followed by reaction with ortho- or pyrophosphate. Nucleotides activated with 5'-phosphoromorpholidate[3], 5'-phosphoroimidazolide[4] and 5'-phosphorothioate[5] are used as intermediates for the synthesis. The methods, however, require anhydrous condition, and tertiary alkylammonium ions are used as counter cations for solubilizing the nucleotides and phosphates in organic solvent. Therefore, microscale synthesis of the polyphosphates is difficult by these methods, as a very small amount of water is hard to excude in a small scale reaction . To overcome this difficulty, Kozarich has used diimidazolide orthophosphate for the pyrophosphorylation of 5'-nucleotides[6], instead of activation of nucleoside 5'-phosphates, though the reaction was carried out in strictly anhydrous solvent.

Previously, we reported that Mn(II) and Cd(II) ions catalyzed the pyrophosphate bond formation from nucleoside 5'-phosphoroimidazolide and nucleotides or phosphates in aqueous solution[7]. As activation of nucleoside 5'-phosphate with imidazole is required in this method, and thus submicroscale preparation is difficult, we explored the metal-ion mediated pyrophosphorylation in aqueous solution and found that phosphorotriimidazolide worked satisfactorily in aqueous solution in the presence of Mg(II) or Mn(II) ion.

The phosphorylating agent, phosphorotriimidazolide (PIM)[8] or phosphorotribenzimidazolide (PBIM)[9], was prepared from phosphorous oxychloride and imidazole or benzimidazole in tetrahydrofuran by a modification of the method of Cramer *et al*[10]. A typical pyrophosphorylation reaction was carried out in a reaction mixture (100 µl) containing 10 mM adenosine 5'-monophosphate (pA), 0.5 M PIM, 0.5 M $MgCl_2$ and 0.2 M N-ethylmorpholine buffer (pH 7.0) at 50 °C for 8h. The reaction mixture was treated with Versenol solution to complex the Mg(II) ion as Versenol-Mg(II) chelate and analyzed by HPLC on a RPC-5 and a ODS-silica gel columns. The HPLC profile of the reaction mixture is shown in Fig 1., which demonstrates the formation of a series of adenosine 5'-polyphosphates containing phosphate residue of 1 to 6. The structures of adenosine 5'-di-, 5'-tri- and 5'-tetraphosphates were confirmed by comparison of the HPLC with those of the authentic samples in two column systems. Yields of adenosine 5'-di- (ppA), 5'-tri- (pppA) and 5'-tetraphoshates (p4A) were 29, 34 and18 %, respectively, based on the starting pA. We presume that the peaks of longer retention times correspond to the 5'-poly-phosphates with more than five phosphate residues, though the authentic adenosine 5'-polyphosphates are not available. Diadenosine 5,5'-polyphosphates[7] (Ap3A and Ap4A) were also formed in a small amount in the reaction. Ap3A and Ap4A are likely formed by coupling of pA and imidazolide of adenosine 5'-di- and 5'-triphosphates, respectively, which are formed from pA and PIM. We postulate that hydrolysis of PIM produces di- and monoimidazolides of orthophosphate, which coordinate to the Mg(II) ion and react with the nucleotide in the coordination sphere of the Mg(II) ion to form the pyrophosphate bond as shown in the following scheme.

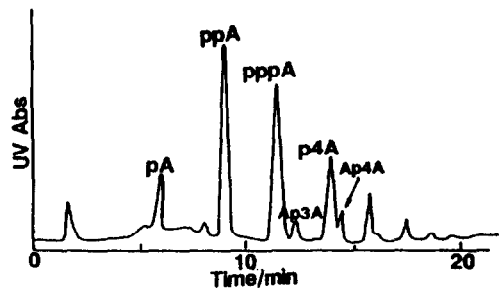
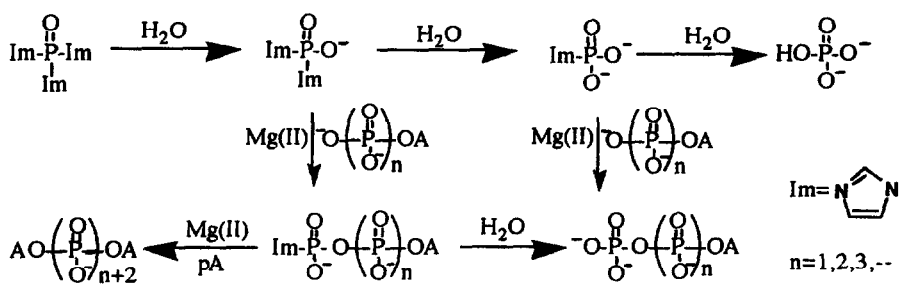


Fig. 1 HPLC Profile of the Products from pA with PIM in the Presence of Mg(II) at 50°C for 8 h.



Yields of adenosine 5'-polyphosphates under several conditions are listed in Table 1. The time course of the polyphosphates formation indicated that successive phosphorylation took place in the initial stage of the reaction forming the long polyphosphates until the phosphorylating agent was consumed, then gradual hydrolysis of the 5'-polyphosphates produced the 5'-mono, di- and triphosphates. The low reaction temperature retarded the rate of the phosphorylation and the hydrolysis of the polyphosphate. Thus the reaction at room temperature required a long reaction time for completion of the 5'-polyphosphate formation. Higher concentration of the phosphorylating agent gave higher yields of the 5'-polyphosphate. Mn(II) ion also mediated the polyphosphate formation, though it promoted the formation of diadenosine 5,5'-polyphosphate more effectively than Mg(II) ion. Mn(II) ion has been reported to mediate diadenosine 5,5'-polyphosphate formation from imidazolide of pA and adenosine 5'-mono, di- or triphosphate[7]. Without metal ions, hydrolysis of PIM to phosphate took place predominantly and a very small amount of adenosine 5'-diphsophate was obtained in addition to the starting pA.

Table 1. Synthesis of Adenosine 5'-Polyphosphates from the 5'-Monophosphate[a]

No.	Metal Ion	Time h	Temp °C	Yield (%)[b] pA	ppA	pppA	pnA[c]
1	Mg(II)	4	50	5	28	34	28
2		12		6	30	33	22
3		24		8	35	32	13
4		72		28	44	16	
5		168	22	9	25	43	12
6[d]				39	16	26	11
7[e]				10	31	34	13
8	Mn(II)	24	50	7	33	18	10
9		168	22	5	36	30	12
10	None	24	50	94	5		
11		120	22	98	2		

a, Reaction conditions are described in text except for No. 6 and 7. b, Based on the starting pA. c, n=4, 5, 6. d, pA (0.01 M), PIM (0.1 M) and $MgCl_2$ (0.1 M) were used. e, pA (0.05 M), PIM (0.5 M) and $MgCl_2$ (0.5 M) were used.

The use of PBIM in place of PIM increased the yield of adenosine 5' di- and triphosphates from pA, although it retarded the reaction rate. Thus ppA, pppA and p4A were formed in 26, 36 and 18 % yield, respectively, by the reaction of 0.01M pA, 0.5 M PBIM and 0.5 M Mg(II) at 50°C and pH 7.0 for 3d. When Mn(II) ion was used, the yields of ppA, pppA and p4A were 65, 17 and 3%, respectively, under the same reaction condition.

Similarly, Mg(II) ion mediated the conversion of uridine, cytidine and guanosine 5'-monophosphates with PIM to the corresponding 5'-polyphosphates as shown in Table 2.

The triphosphate, pppA2'p5'A2'p5'A or 2-5 A, is associated with the interferon's antiviral action, and activates 2-5 A dependent ribonuclease which degrades viral messenger RNA[11]. The 5'-monophosphate is devoid of

such biological activity, though the diphosphate has the same activity as the triphosphate[12]. Thus, we conducted pyrophosphorylation of 5'-monophosphate of 2',5'-triadenylate (pA2'p5'A2'p5'A) with PIM by Mg(II) ion in aqueous solution. The 5'-di-, 5'-tri- and 5'-tetra-phosphates of 2',5'-triadenylate were obtained in substantial yields.

Table 2. Phoshorylation of 5'-Nucleotides and 2',5'-Triadenylate[a]

pN	Time d	Yield (%)[b] pN	ppN	pppN	p_nN ($n \geq 4$)
pU	3	5	25	36	30
pC	3	3	25	33	35
pG	3	8	23	34	31
pG[c]	3	98	2		
pA2p5A2p5A	3	10	34	26	21
pA2p5A2p5A[c]	3	93	4	tr	
pA2p5A2p5A	7	14	48	29	8

a, Phosphorylation of the nucleotides (0.01M) was carried out at pH 7.0 and 22 °C with PIM (0.5M) in the presence of $MgCl_2$ (0.5M).
b, Based on the starting nucleotides.
c, Control experiment without $MgCl_2$.

This method provides a simple procedure for the synthesis of the polyphosphates of the mono- and oligonucleotides, especially for microscale synthesis, as the reaction proceeds in aqueous solution without protection and activation of the nucleotides.

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9 Phosphorotribenzimidazolide (PBIM): mp. 118-123°. IR; 1617, 1306. NMR; 7.55 (m, 2H), 7.74(m, 2H), 9.02(s, 1H).
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