

Phosphorylation of Nucleotides with Inorganic Cyclo-Triphosphate

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Phosphorylation of nucleotides (nucleoside 3'- and 5'-monophosphates, and 2'-deoxynucleoside 5'-monophosphates) with inorganic sodium cyclo-triphosphate (P_{3m}) was studied in aqueous solutions under various conditions (mixing ratio of P_{3m} to nucleotides, pH, reaction temperature, and time). (1) Unprotected nucleoside 5'-monophosphates (5'-NMP's) were easily phosphorylated at the *cis*-2',3'-diol by P_{3m} to form selectively nucleoside 2',5'-bis(monophosphate) (2',5'-NDP's), nucleoside 3',5'-bis(monophosphate) (3',5'-NDP's), and nucleoside 2',3'-cyclic 5'-bis(monophosphate) (cNDP's). (2) The phosphorylation of 5'-NMP's was strongly dependent on mixing ratio, pH, reaction temperature, and time. Under conditions of high mixing ratios of P_{3m} to 5'-NMP's (5:1–10:1), high pH (12), and room temperature, 92–98% of 5'-NMP's was converted into 3',5'-NDP's and 2',5'-NDP's in roughly equimolar quantities. (3) Small quantities (5–8%) of cNDP's were formed at the initial stage of reaction of 5'-NMP's with P_{3m} but in the course of the reaction for a long period, cNDP's were hydrolyzed to 2',5'-NDP's and 3',5'-NDP's. (4) Nucleoside 3'-monophosphates (3'-NMP's) and 2'-deoxynucleoside 5'-monophosphates (dNMP's) could not be phosphorylated by P_{3m} , which indicates that the presence of hydroxyl groups at both 2'- and 3'-positions on nucleotides is indispensable for the phosphorylation of nucleotides with P_{3m} . (5) The mechanism of the formation of 2',5'-NDP's, 3',5'-NDP's, and cNDP's in the phosphorylation of 5'-NMP's with P_{3m} is discussed.

Phosphoryl chloride,^{1,2)} organic phosphorus compounds,^{1,3,4)} and polyphosphoric acid^{5–8)} have so far been used as phosphorylating agents of organic compounds, nucleosides, and bioorganic compounds. Since the phosphorylation with these phosphorylating agents is accompanied by various side reactions, introduction of protective groups is often required. In addition, the experimental operation of phosphorylation is extremely complicated because of its character of multi-stage reaction in organic solvents. Simple and selective phosphorylating agents have always been required.

In 1965–1969, Feldmann demonstrated that inorganic cyclo-triphosphate (P_{3m}) phosphorylated selectively some functional groups on organic compounds such as alkylamines,^{9,10)} alcohols,¹¹⁾ sugars,¹¹⁾ amino acids,¹²⁾ and phenol¹³⁾ without complicated handling. After Feldmann's work, several authors have tried to extend this technique to the phosphorylation of nucleosides.

Saffhill¹⁴⁾ reported that a treatment of unprotected adenosine with P_{3m} produced adenosine 2'- and 3'-monophosphates in high yield. On the other hand, Etaix and Orgel¹⁵⁾ reported that the reaction of 2'-deoxynucleosides with P_{3m} gave 2'-deoxynucleoside triphosphates in substantial yield, whereas a small amount of adenosine triphosphate and a large amount of adenosine 2'- and 3'-monophosphates were obtained by the reaction of adenosine with P_{3m} . There are some discrepancies in the kind and yield of products between the experimental results of Saffhill and those of Etaix and Orgel, because of some differences in their reaction conditions. Schwartz¹⁶⁾ produced a small amount of a mixture of 2'-deoxyadenosine 3'- and 5'-monophosphates by phosphorylating 2'-deoxyadenosine with P_{3m} .

The present authors¹⁷⁾ have extensively studied the phosphorylation of unprotected nucleosides with P_{3m} and its reaction mechanism. We reported that the hydroxyl groups at the 2'- and 3'-positions of ribonucleosides were selectively phosphorylated by the reaction of ribonucleosides with P_{3m} in an aqueous solution to form nucleoside 2'-, 3'-, and 2',3'-cyclic monophosphates in high yield (80–90%). We concluded on the basis of this mechanistic study that nucleoside 2'- and 3'-triphosphates as well as nucleoside 2',3'-cyclic monophosphates were produced as reaction intermediates.

The present authors have tried to phosphorylate nucleic acids and their related compounds with inorganic sodium cyclo-triphosphate (P_{3m}) to develop a selective phosphorylating agent for single-stage reaction in aqueous solution. In the present study, therefore, phosphorylation of nucleotides with P_{3m} , structures of reaction products, yields, and reaction mechanisms were investigated using HPLC and ³¹P NMR.

Experimental

Chemicals. Sodium cyclo-triphosphate hexahydrate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$, was prepared by the procedure described in our previous paper¹⁸⁾ and recrystallized twice from an aqueous solution. Guaranteed grade adenosine 5'-monophosphate (5'-AMP), adenosine 3'-monophosphate (3'-AMP), 2'-deoxyadenosine 5'-monophosphate (dAMP), cytidine 5'-monophosphate (5'-CMP), cytidine 3'-monophosphate (3'-CMP), 2'-deoxycytidine 5'-monophosphate (dCMP), guanosine 5'-monophosphate (5'-GMP), guanosine 3'-monophosphate (3'-GMP), uridine 5'-monophosphate (5'-UMP), uridine 3'-monophosphate (3'-UMP), and thymidine 5'-monophosphate (dTMP) were purchased from Yamasa Shoyu Co., Ltd. (Tokyo, Japan) and Sigma

Chemical Company.

Phosphorylation of Nucleotides with Inorganic Cyclo-triphosphate (P_{3m}). The initial concentration of P_{3m} was fixed at a constant value of 0.5 mol dm^{-3} and those of nucleotides were varied from 0.5 to 0.05 mol dm^{-3} . Values of pH of mixed solutions were adjusted to desired values (14, 12, 10, 7, and 5) with 6 mol dm^{-3} sodium hydroxide solution or hydrochloric acid. Mixed solutions were allowed to react at room temperature (20 – 25°C) or at specified temperatures (50 and 70°C) by use of a thermostated bath controlled within $\pm 2^\circ\text{C}$. As the pH of the mixtures handled would gradually decrease with progress of reaction, it was kept at the prescribed value using sodium hydroxide solution during reaction.

HPLC Measurement. HPLC analysis was carried out with a JASCO HPLC-800 system (Tokyo, Japan). A column ($250 \times 4.6 \text{ mm i. d.}$) was packed with an anion exchanger (MCI gel, CDR-10, Mitsubishi Chemical Industry, Tokyo, Japan), and the column temperature was maintained at 40°C .

The linear gradient elution technique was used for the adenosine ($5'$ -AMP, $3'$ -AMP, and dAMP) and cytidine ($5'$ -CMP, $3'$ -CMP, and dCMP) systems, whereas the isocratic elution technique was used for the separation of guanosine ($5'$ -GMP and $3'$ -GMP), uridine ($5'$ -UMP and $3'$ -UMP), and dTMP systems. All eluents contained ammonium sulfate, dipotassium hydrogenphosphate, and 4% (v/v) acetonitrile.

The eluent pH was kept at 10. The UV absorbance of an effluent was monitored continuously at 260 nm for the adenosine, guanosine, and uridine systems, 270 nm for the dTMP system, and 280 nm for the cytidine system.

^{31}P NMR Measurement. Pulse FT ^{31}P NMR spectra were recorded at room temperature by use of Varian XL-VX 300 (121 MHz) and Varian XL-200 (81 MHz) spectrometers. Orthophosphoric acid (85%) was used as an external standard.

Results and Discussion

Phosphorylation of $5'$ -AMP with P_{3m} . An aqueous reaction mixture of P_{3m} (0.5 mol dm^{-3}) and $5'$ -AMP (0.05 mol dm^{-3}) was allowed to react at pH 12 and room temperature. The pH of the mixed solution decreased gradually and reached about 9.9 after 1 d. Thus, the pH of the mixture was kept at 12 using sodium hydroxide solution during reaction.

Figure 1 shows the results of HPLC of the reaction products in the phosphorylation of $5'$ -AMP with P_{3m} at mixing ratio 10:1 (0.5 mol dm^{-3} : 0.05 mol dm^{-3}), pH 12, and room temperature. With progress of reaction, the peak of $5'$ -AMP (retention time about 9 min) was reduced and the three peaks of the reaction products were observed at the retention times of about 26,

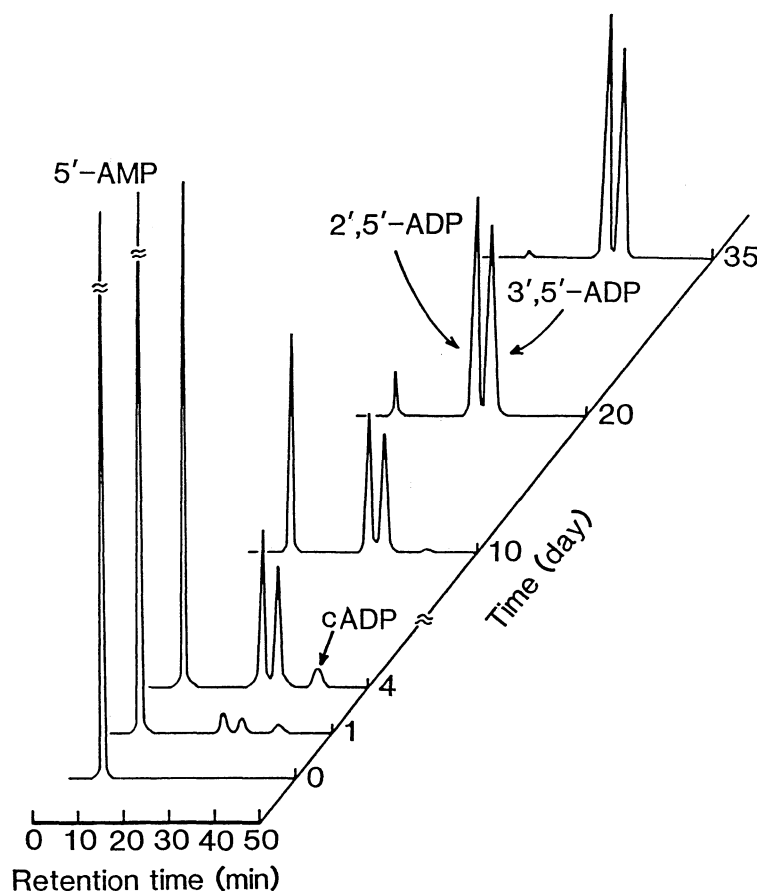


Fig. 1. Phosphorylation of $5'$ -AMP with P_{3m} .
 P_{3m} : $5'$ -AMP=10:1 (0.5 mol dm^{-3} : 0.05 mol dm^{-3}), pH 12, room temperature.

30, and 38 min, respectively. These reaction products were identified to be adenosine 2',5'-bis(monophosphate) (designated as 2',5'-ADP), adenosine 3',5'-bis(monophosphate) (3',5'-ADP), and adenosine 2',3'-cyclic 5'-bis(monophosphate) (cADP) by comparing with results of HPLC and ^{31}P NMR analyses of the authentic samples. As can be seen from the result of HPLC, two main products (2',5'-ADP and 3',5'-ADP) and a by-product (cADP) were produced in the phosphorylation of 5'-AMP with $\text{P}_{3\text{m}}$ in an aqueous solution. cADP was produced at the initial stage of reaction, and it gradually decreased with reaction time and finally disappeared after 13 d. On the other hand, the amounts of 2',5'-ADP and 3',5'-ADP increased with elapse of reaction time and reached 46.1 and 46.3% after 20 d, respectively. Under any reaction conditions, the amounts of 2',5'-ADP and 3',5'-ADP were nearly equal to each other.

To establish the structure of phosphorylated products, ^{31}P NMR spectra were measured. Figure 2 shows the ^{31}P NMR spectra for the reaction mixture of $\text{P}_{3\text{m}}$ and 5'-AMP at molar ratio 10:1, pH 12, and room temperature. In the ^1H -decoupling spectrum, four peaks due to the reaction products were observed at $\delta=+4.9$, $+4.7$, $+4.6$, and $+4.4$. By comparing with the chemical shifts of the authentic samples, the peaks at $\delta=+4.9$ and $+4.7$, and at $\delta=+4.6$ and $+4.4$ were

attributed to 3',5'-ADP and 2',5'-ADP, respectively. The peaks at $\delta=+4.9$ and $+4.7$ split into a doublet and a triplet, respectively, in the ^1H -coupling spectrum. Thus, it was found that the peaks at $\delta=+4.9$ and $+4.7$ were attributable to the phosphorus atoms at the 3'- and 5'-positions of ribose in 3',5'-ADP, respectively, and the peaks at $\delta=+4.4$ and $+4.6$ were attributable to the phosphorus atoms at the 2'- and 5'-positions in 2',5'-ADP, respectively. A singlet peak corresponding to the 2',3'-cyclic phosphate of cADP was observed at $\delta=+20.5$ and split into a triplet due to the two protons of 2'- and 3'-positions of ribose in the ^1H -coupling. The singlet peak at $\delta=-21.4$ corresponds to the starting material, $\text{P}_{3\text{m}}$, the peaks at $\delta=-4.0$ and -4.3 were assigned to the terminal phosphorus atoms of triphosphate (P_3) produced by the hydrolysis of $\text{P}_{3\text{m}}$ and those at $\delta=-18.2$, -18.4 , and -18.6 to the middle phosphorus atom of P_3 . The singlet peaks at $\delta=+5.4$ and -4.6 were attributed to ortho (P_1) and pyrophosphates (P_2), respectively.

In Fig. 3, the amounts of 2',5'-ADP, 3',5'-ADP, and cADP, formed in the reaction of $\text{P}_{3\text{m}}$ with 5'-AMP at mixing ratio 10:1, pH 12 and room temperature, are plotted as a function of time. While the starting material (5'-AMP) decreased with reaction time, each amount of 2',5'-ADP and 3',5'-ADP gradually increased up to the same constant value of approximately 47%. The yield of 2',5'-ADP was nearly equal to that of 3',5'-ADP at any reaction times. The yield of cADP reached about 5% after 1 d and decreased to zero after 13 d.

The influence of pH on the reaction of $\text{P}_{3\text{m}}$ with 5'-AMP is shown in Fig. 4. The phosphorylation of 5'-AMP with $\text{P}_{3\text{m}}$ proceeded faster in a high alkaline region, e.g., at pH 12, than at pH 10 and less. The phosphorylation did not proceed in an acidic medium (pH 5) at all. This fact may be explained in terms of

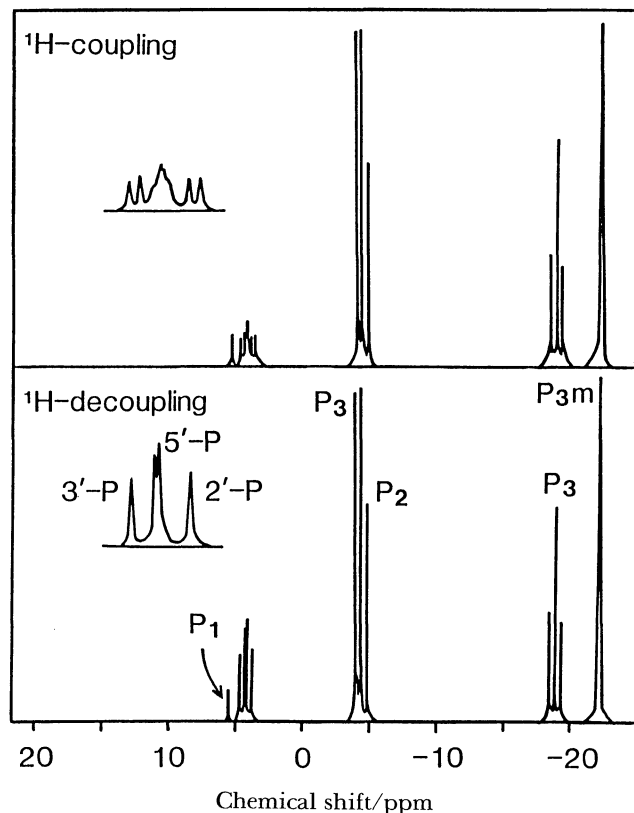


Fig. 2. ^{31}P NMR of the reaction products.
 $\text{P}_{3\text{m}}$: 5'-AMP=10:1 (0.5 mol dm $^{-3}$: 0.05 mol dm $^{-3}$),
 pH 12, room temperature, after 30 d.

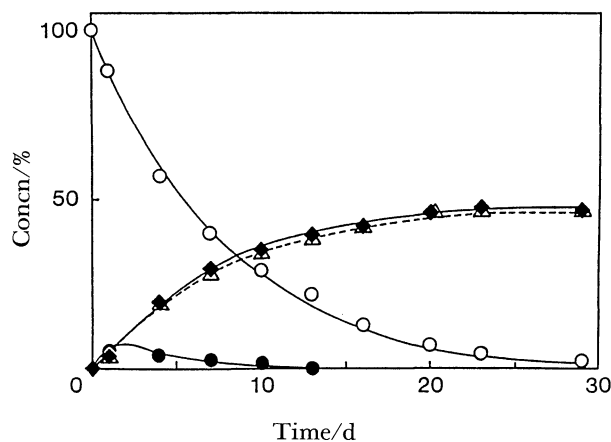


Fig. 3. Changes of the amounts of products in the reaction of $\text{P}_{3\text{m}}$ with 5'-AMP.
 $\text{P}_{3\text{m}}$: 5'-AMP=10:1 (0.5 mol dm $^{-3}$: 0.05 mol dm $^{-3}$),
 pH 12, room temperature.
 ○; 5'-AMP, ◆; 2',5'-ADP, △; 3',5'-ADP, ●; cADP.

such a sequence that the hydroxyl group ($-OH$) at the 2'- or 3'-position of ribose in 5'-AMP¹⁹) is partially dissociated into O^- which nucleophilically attacks the phosphorus atom of P_{3m} .

We have further studied the phosphorylation of 5'-AMP at varying temperatures and mixing ratios, with the pH of reaction mixture maintained at pH 12. The yields of the 2',5'-ADP, 3',5'-ADP, and cADP thus obtained are summarized in Table 1. The effect of reaction temperature on maximum yield was examined for the reaction of 5'-AMP with P_{3m} at a mixing ratio of 5:1. As can be seen in Table 1, the best yield was obtained at room temperature after 40 d. Due to

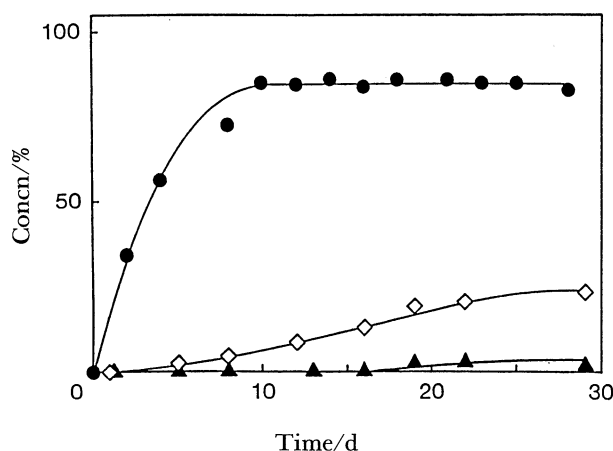


Fig. 4. Changes of the amounts of products in the reaction of P_{3m} with 5'-AMP.

P_{3m} : 5'-AMP=5:1 (0.5:0.1 mol dm⁻³), 50 °C, ●; pH 12, ◇; pH 10, ▲; pH 7.

the hydrolysis of the reaction products, the maximum yield obtained at higher temperatures is lower than that obtained at room temperature. The effect of the mixing ratio of P_{3m} to 5'-AMP was tested as listed in Table 1. The ratio of P_{3m} to 5'-AMP of 10:1 (0.5 mol dm⁻³:0.05 mol dm⁻³) is better than that of 1:1 (0.5 mol dm⁻³:0.5 mol dm⁻³). The yield at a mixing ratio of 10:1 was, however, not so much different from that at 5:1.

The optimum conditions for the phosphorylation of 5'-AMP with P_{3m} are found to be mixing ratio 5:1–10:1, pH 12, room temperature, and reaction period 35–41 d. The total yields of 2',5'-ADP and 3',5'-ADP under the optimum condition were about 96.5%. On the other hand, cADP was produced only at the initial stage of reaction under any reaction conditions and its yield was about 5–6% which was much lower than those of 2',5'-ADP and 3',5'-ADP. The amount of cADP gradually decreased with elapse of reaction time. This is because cADP is easily hydrolyzed to give 2',5'-ADP and 3',5'-ADP.

The phosphorylation of 3'-AMP or dAMP with P_{3m} was also investigated under various conditions. However, these compounds could not be phosphorylated with P_{3m} at all under any conditions. This fact may suggest that the presence of the hydroxyl groups at both the 2'- and 3'-positions of ribose in 5'-AMP is required for the phosphorylation of nucleotides with P_{3m} .

Phosphorylation of 5'-CMP with P_{3m} . HPLC separations of the reaction products in the phosphorylation of 5'-CMP with P_{3m} gave the three peaks due to cytidine 2',3'-cyclic 5'-bis(monophosphate) (designated as cCDP), cytidine 2',5'-bis(monophosphate)

Table 1. Yields of 2',5'-ADP, 3',5'-ADP, and cADP

Reaction conditions				Yield/%		
Mixing ratio	pH	Temp/°C	Time/d	2',5'-ADP	3',5'-ADP	cADP
P _{3m} :5'-AMP						
1:1 (0.5 M:0.5 M)	12	50	3	9.3	8.1	5.6
			20	28.3	26.2	0
5:1 (0.5 M:0.1 M)	12	Room	1	3.1	2.6	4.7
			41	49.3	47.2	0
		50	2	14.3	15.8	4.0
			14	41.1	45.1	0
	10	50	29	9.6	11.5	2.4
			7	50	29	0.6
	12	70	1	4.8	5.3	4.0
			6	30.2	34.0	0
10:1 (0.5 M:0.05 M)	12	Room	1	3.6	3.3	4.9
			35	47.6	47.4	0
	12	50	2	17.1	18.9	2.7
			18	42.1	46.4	0
P _{3m} :3'-AMP						
10:1 (0.5 M:0.05 M)	12	Room	24	0	0	0
P _{3m} :dAMP						
10:1 (0.5 M:0.05 M)	12	Room	25	—	0	—

(2',5'-CDP), and cytidine 3',5'-bis(monophosphate) (3',5'-CDP). The main reaction products were identified to be 2',5'-CDP and 3',5'-CDP by comparing with the results of HPLC and ^{31}P NMR of the authentic samples. Although a small amount (about 5–8%) of cCDP was produced at the initial stage of reaction, it decreased rapidly with reaction time. The formation of 2',5'-CDP and 3',5'-CDP increasingly predominated with progress of reaction.

Table 2 lists the yields of 2',5'-CDP, 3',5'-CDP, and cCDP under various reaction conditions. In the reaction of 5'-CMP with $\text{P}_{3\text{m}}$ at mixing ratio 5:1 (0.5 mol dm $^{-3}$:0.1 mol dm $^{-3}$), pH 12, and room temperature for long time (48 d), about 92% of 5'-CMP was phosphorylated to form 2',5'-CDP (40%) and 3',5'-CDP (52%). The yield of 3',5'-CDP was always a little more than that of 2',5'-CDP, though both yields were nearly equal to each other. The phosphorylation proceeded for a short time (5 h) at 70 °C and high pH (14), and the total yields of 2',5'-CDP, and 3',5'-CDP reached about 66% at 5 h. The amounts of these products, however, gradually decreased with time, because they were easily hydrolyzed at pH 14 and 70 °C. The reaction rate was decreased by lowering the pH of the mixed solution. The reaction did not proceed at all in acidic medium (pH 5). Neither 3'-CMP, nor dCMP could be phosphorylated with $\text{P}_{3\text{m}}$ under the same conditions as the phosphorylation of 5'-CMP with $\text{P}_{3\text{m}}$.

The experimental results indicate that the optimum conditions for the phosphorylation of 5'-CMP with $\text{P}_{3\text{m}}$ are mixing ratio 5:1, pH 12, and room

temperature.

Phosphorylation of 5'-GMP with $\text{P}_{3\text{m}}$. The phosphorylation of 5'-GMP with $\text{P}_{3\text{m}}$ was carried out by the same method as that of the 5'-AMP and 5'-CMP systems. Figure 5 shows an example of HPLC analysis of the reaction products in the phosphorylation of 5'-GMP with $\text{P}_{3\text{m}}$. The products were guanosine 2',5'-bis(monophosphate) (2',5'-GDP) and guanosine 3',5'-bis(monophosphate) (3',5'-GDP). No guanosine 2',3'-cyclic 5'-bis(monophosphate) (cGDP) was obtained in the phosphorylation of 5'-GMP with $\text{P}_{3\text{m}}$ unlike those of the 5'-AMP and 5'-CMP systems. It is not clear yet whether cGDP was not formed originally or the cGDP once formed at the initial stage of the reaction was so unstable as to be immediately hydrolyzed to form 2',5'-GDP and 3',5'-GDP. However, it may be reasonable to consider that the latter is more probable, similarly to the case of the 5'-AMP and 5'-CMP systems.

Table 3 shows amounts of 2',5'-GDP and 3',5'-GDP obtained under various reaction conditions. The optimum conditions for the phosphorylation of 5'-GMP with $\text{P}_{3\text{m}}$ are found to be mixing ratio 5:1—10:1, pH 12, room temperature, and long reaction period (28 d). The yields of the reaction products (2',5'-GDP and 3',5'-GDP) under the optimum conditions were approximately 97%. Also, the yield of 2',5'-GDP was nearly equal to that of 3',5'-GDP under any conditions. On the other hand, 3'-GDP was not phosphorylated with $\text{P}_{3\text{m}}$ at all.

Phosphorylation of 5'-UMP with $\text{P}_{3\text{m}}$. In the phosphorylation of 5'-UMP with $\text{P}_{3\text{m}}$, the reaction

Table 2. Yields of 2',5'-CDP, 3',5'-CDP, and cCDP

Reaction conditions				Yield/%		
Mixing ratio	pH	Temp/°C	Time/d	2',5'-CDP	3',5'-CDP	cCDP
P _{3m} :5'-CMP						
1:1 (0.5 M:0.5 M)	12	Room	5	4.9	6.0	6.3
			81	35.7	46.7	0
	12	50	1	2.1	3.5	5.0
			14	20.5	28.6	0
5:1 (0.5 M:0.1 M)	12	Room	3	2.3	4.0	6.3
			48	39.7	51.6	0
	12	50	1	2.9	4.6	4.1
			21	31.3	44.4	0
	12	70	3	2.6	5.4	5.0
			12	13.6	35.1	0
	10	50	14	2.7	2.9	4.5
			41	11.4	15.5	0
10:1 (0.5 M:0.05 M)	7	50	34	0.8	2.6	0
			5hr	28.3	38.0	0
	12	50	4	5.2	8.0	8.3
			24	32.4	46.8	0
P _{3m} :3'-CMP						
10:1 (0.5 M:0.05 M)	12	Room	21	0	0	0
P _{3m} :dCMP						
10:1 (0.5 M:0.05 M)	12	Room	15	—	0	—

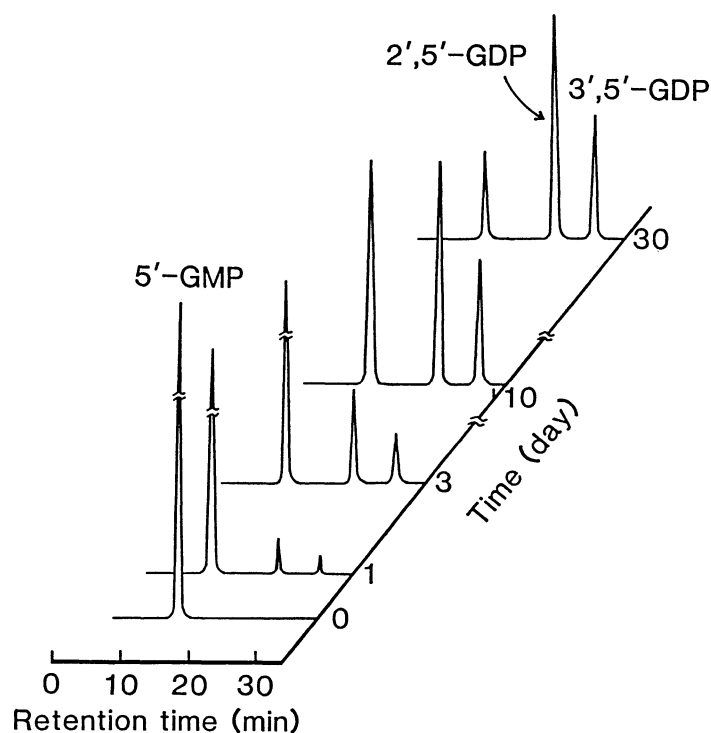


Fig. 5. Phosphorylation of 5'-GMP with P_{3m} .
 P_{3m} : 5'-GMP=1 : 1 (0.5 mol dm⁻³: 0.5 mol dm⁻³), pH 12,
 room temperature.

Table 3. Yields of 2',5'-GDP and 3',5'-GDP

Reaction conditions				Yield/%	
Mixing ratio	pH	Temp/°C	Time/d	2',5'-GDP	3',5'-GDP
P_{3m} : 5'-GMP					
1 : 1 (0.5 M : 0.5 M)	12	Room	28	49.7	40.3
	12	50	19	37.4	31.4
	12	70	6	29.5	28.0
5 : 1 (0.5 M : 0.1 M)	12	Room	28	47.6	49.7
	12	50	26	43.8	47.5
	12	70	17	32.4	36.7
10 : 1 (0.5 M : 0.05 M)	12	Room	28	46.8	50.0
	12	50	21	43.8	48.3
	12	70	22	37.6	38.4
	10	50	26	33.2	30.7
	7	50	25	0	0
P_{3m} : 3'-GMP					
10 : 1 (0.5 M : 0.05 M)	12	Room	21	0	0

products were identified to be uridine 2',5'-bis(monophosphate) (2',5'-UDP), uridine 3',5'-bis(monophosphate) (3',5'-UDP), and uridine 2',3'-cyclic 5'-bis(monophosphate) (cUDP) by comparing with the data of HPLC and ³¹P NMR of the authentic samples. Although cUDP was produced at the initial stage of the reaction, it disappeared with reaction time and the formation of 2',5'-UDP and 3',5'-UDP predominated instead.

Yields of 2',5'-UDP, 3',5'-UDP, and cUDP under

various reaction conditions are summarized in Table 4. The optimum conditions for the phosphorylation of 5'-UMP with P_{3m} are mixing ratio 5 : 1—10 : 1, pH 12, and room temperature. The total yields of 2',5'-UDP and 3',5'-UDP under the optimum conditions were about 93% at the highest. 3'-UMP was not phosphorylated with P_{3m} at all, similarly to 3'-AMP, 3'-CMP, and 3'-GMP.

The ³¹P NMR parameters of the main reaction products (2',5'-NDP's and 3',5'-NDP's) in the phosphoryl-

Table 4. Yields of 2',5'-UDP, 3',5'-UDP, and cUDP

Reaction conditions				Yield/%		
Mixing ratio	pH	Temp/°C	Time/d	2',5'-UDP	3',5'-UDP	cUDP
P _{3m} : 5'-UMP						
1:1 (0.5 M:0.5 M)	12	Room	1	2.2	2.1	7.6
			55	37.5	38.8	0
	12	50	1	4.9	5.2	7.6
			25	28.2	27.5	0
	12	70	1	6.4	7.4	5.4
			24	16.6	16.8	0
5:1 (0.5 M:0.1 M)	12	Room	1	1.5	1.5	6.3
			55	45.3	47.0	0
	12	50	1	2.9	3.1	6.7
			37	40.2	39.0	0
	12	70	1	4.0	4.5	6.0
			26	26.3	25.5	0
10:1 (0.5 M:0.05 M)	12	Room	6	11.1	10.8	6.9
			60	46.1	47.0	0
	12	50	4	10.1	10.7	6.0
			35	40.3	39.1	0
	12	70	3	4.6	5.3	5.1
			31	28.0	27.6	0
	10	50	8	5.5	6.1	7.3
			30	19.3	21.1	4.5
	7	50	30	2.0	1.6	1.9
	P _{3m} : 3'-UMP					
10:1 (0.5 M:0.05 M)	12	Room	21	0	0	0

Table 5. ³¹P NMR Parameters of Phosphorylated Products (2',5'-NDP's and 3',5'-NDP's)

Product	δ (2'-P)	δ (3'-P)	δ (5'-P)	³ J _{P(2')H} /Hz	³ J _{P(3')H} /Hz
2',5'-ADP	4.4	—	4.6	6.88	—
3',5'-ADP	—	4.9	4.7	—	7.29
2',5'-CDP	4.0	—	4.1	6.81	—
3',5'-CDP	—	4.2	4.1	—	7.26
2',5'-GDP	3.7	—	4.0	6.80	—
3',5'-GDP	—	4.2	4.0	—	7.21
2',5'-UDP	4.1	—	4.2	6.80	—
3',5'-UDP	—	4.4	4.3	—	7.25

ation of 5'-NMP's with P_{3m} are shown in Table 5. The measurements of ³¹P NMR were made at pH 12.

Mechanism of Phosphorylation of 5'-NMP's with P_{3m}. In the reaction of P_{3m} and 5'-NMP's, cis-2',3'-diol on nucleotides is phosphorylated to form 2',5'-NDP's, 3',5'-NDP's, and cNDP's. Considering that dNMP's and 3'-NMP's are not phosphorylated, the presence of hydroxyl groups at both 2'- and 3'-positions is indispensable for the phosphorylation of 5'-NMP's with P_{3m}.

From the above-mentioned experimental results, the authors propose the reaction mechanism shown in Fig. 6. The hydrogen bond is first formed between the hydroxyl group at the 2'- or 3'-position on nucleotides and an oxygen atom of P_{3m}, e.g., the hydrogen bond of 3'-OH with P_{3m} in Fig. 6. Closely related hydrogen-bonded intermediates have been postulated.^{13,17)} Then, the lone electron pair on the oxy-

gen atom of another hydroxyl group (2'-OH in Fig. 6) nucleophilically attacks the phosphorus atom of P_{3m} to generate nucleoside 2'-triphosphates as an unstable intermediate, via the ring opening of P_{3m}. The roles of the 2'- and 3'-hydroxyl groups in hydrogen bonding and nucleophilic attack could equally be reversed, of course. An intramolecular attack of unphosphorylated hydroxyl group (3'-OH in Fig. 6) on a phosphorus atom of nucleoside 2'-triphosphates occurred to produce cNDP's as an intermediate. The cNDP's thus formed are quickly hydrolyzed to give 2',5'-NDP's and 3',5'-NDP's. The yields of 2',5'-NDP's were nearly equal to those of 3',5'-NDP's, which shows that the rate of hydrolysis at the 2'-position of cNDP's is almost the same as that at the 3'-position. Such a fact is well-known in the hydrolysis of RNA in an alkaline solution.²⁰⁾

In the 5'-GMP system, however, guanosine 2',3'-

