# Phosphorylation of Nucleosides with Sodium *cyclo*-Triphosphate

Mitsutomo TSUHAKO,* Mayumi FUJIMOTO, Shigeru OHASHI,†
Hiroyuki NARIAI,†† and Itaru MOTOOKA††
*Kobe Women's College of Pharmacy, Kitamachi, Motoyama, Higashinada-ku, Kobe 658*
†*Department of Chemistry, Faculty of Science, Kyushu University 33, Hakozaki, Higashi-ku, Fukuoka 812*
††*Department of Chemistry, Faculty of General Education, Kobe University, Tsurukabuto, Nada-ku, Kobe 657*
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Phosphorylation of nucleosides with sodium *cyclo*-triphosphate ($P_{3m}$) was studied under various conditions (mixing ratio of $P_{3m}$ to nucleoside, pH, reaction temperature and time). 1) Adenosine, cytidine, guanosine, and uridine were easily phosphorylated by $P_{3m}$ to form selectively nucleoside 2′-monophosphates, 3′-monophosphates, and 2′,3′-cyclic monophosphates. On the other hand, 2′-deoxyadenosine, 3′-deoxyadenosine, 2′-deoxycytidine, and thymidine could not be phosphorylated by $P_{3m}$. 2) The phosphorylation of adenosine, cytidine, guanosine, and uridine varied strongly, depending on the reaction conditions; mixing ratio, pH, reaction temperature and time. Under conditions of a high mixing ratio of $P_{3m}$ to nucleoside (5:1—10:1), high pH (12—14), high temperature (70—100 °C) for a short time (1—2 d) or low temperature (room temperature) for a long time (70—150 d), nucleoside 2′- and 3′-monophosphate could be obtained in high yield (about 50—100%). 3) At the initial stage of the phosphorylation reactions of adenosine, cytidine, and guanosine with $P_{3m}$, nucleoside 2′,3′-cyclic monophosphates were formed, though a small amount (about 1—3%), but in the course of the reaction for a long time, they were hydrolyzed to nucleoside 2′- and 3′-monophosphates. 4) 2′-deoxyadenosine, 3′-deoxyadenosine, 2′-deoxycytidine, and thymidine could not be phosphorylated by $P_{3m}$. This means that the presence of hydroxyl groups at the 2′- and 3′-positions of the ribose was necessary for the phosphorylation of nucleosides with $P_{3m}$. 5) Phosphorylation of nucleosides with short-chain phosphates (ortho-, pyro-, and triphosphate) could not be observed at all. 6) The mechanism of formation of nucleoside 2′-monophosphates, 3′-monophosphates, and 2′,3′-cyclic monophosphates in the phosphorylation of nucleosides with $P_{3m}$ was discussed.

Nowadays, as phosphorylating agents for nucleosides and related organic compounds are usually used phosphoryl chloride, polyphosphoric acid[1-4] and various organic phosphorus compounds.[5-8] However, the phosphorylation of nucleosides with such reagents is generally a tedious operation. Feldmann[9] has reported that sodium *cyclo*-triphosphate($P_{3m}$) reacted with alcohol or sugar in an alkaline solution to form its monophosphate or triphosphate derivatives. Also, Schwartz[10] and Saffhill[11] have reported that, by the phosphorylation of nucleosides with condensed phosphates, nucleosie 2′-, 3′-, and 5′-monophosphates were obtained. However, their formation conditions and the reaction mechanism are scarcely known at present. The present authors[12] have already reported that inosine was easily phosphorylated by $P_{3m}$ at 2′- and 3′-positions of the ribose to form selectively inosine 2′-monophosphate(2′-IMP) and 3′-monophosphate(3′-IMP). Accordingly, in the present study adenosine, 2′- and 3′- deoxyadenosine, cytidine, 2′-deoxycytidine, guanosine, uridine, and thymidine were phosphorylated with $P_{3m}$ under various experimental conditions, and the reaction products as well as the reaction mechanism were investigated.

## Experimental

*1) Phosphorylation of Adenosine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous sodium *cyclo*-triphosphate($P_{3m}$) solution were mixed 50 $cm^3$ of adenosine solutions of various concentrations ranging from 0.02 to 0.04 mol $dm^{-3}$, and each mixed solution (molar ratio $P_{3m}$: adenosine=5:1—10:1) was adjusted to the prescribed pH value (14, 12, 10, 7, and 2) by use of sodium hydroxide or hydrochloric acid solution and was allowed to react at 100 °C, 70 °C, and room temperature. Because the pH of the mixed solution decreased with the progress of the reaction, the pH of the solution was kept always at the prescribed value using sodium hydroxide solution.

*2) Phosphorylation of Cytydine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.5 mol $dm^{-3}$ aqueous $P_{3m}$ solution were mixed 50 $cm^3$ of aqueous cytidine solution of various concentrations ranging from 0.1 to 0.5 mol $dm^{-3}$, and each mixture ($P_{3m}$: cytidine=1:1—5:1) was allowed to react at pH 14—2, at room temperature and 70 °C.

*3) Phosphorylation of Guanosine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous $P_{3m}$ solution was mixed 50 $cm^3$ of 0.04 mol $dm^{-3}$ guanosine solution and the mixture ($P_{3m}$: guanosine=5:1) was allowed to react, similarly to the experiments of adenosine and cytidine series.

*4) Phosphorylation of Uridine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.05—0.5 mol $dm^{-3}$ aqueous $P_{3m}$ solutions was mixed 50 $cm^3$ of 0.05 mol $dm^{-3}$ aqueous uridine solution, and each mixed solution ($P_{3m}$:uridine=1:1—10:1) was allowed to react at pH 12 and 70 °C.

*5) Phosphorylation of Thymidine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous $P_{3m}$ solution was mixed 50 $cm^3$ of 0.04 mol $dm^{-3}$ aqueous thymidine solution, and the mixed solution ($P_{3m}$: thymidine=5:1) was allowed to react at pH 12—14 and 70 °C.

*6) Phosphorylation of 2′- or 3′-Deoxyadenosine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous $P_{3m}$ solution was mixed 50 $cm^3$ of 0.04 mol $dm^{-3}$ aqueous solution of 2′- or 3′-deoxyadenosine, the mixture was adjusted to pH 12 with 6 mol $dm^{-3}$ sodium hydroxide solution and was allowed to react at 70 °C.

*7) Phosphorylation of 2′-Deoxycytidine with* cyclo-*Triphosphate.* With 50 $cm^3$ of 0.5 mol $dm^{-3}$ aqueous $P_{3m}$ solution was mixed 50 $cm^3$ of 0.1 mol $dm^{-3}$ aqueous 2′-deoxycytidine solution, and the mixture was allowed to react at pH 12 and 70 °C.

*8) Phosphorylation of Nucleosides with Ortho-, Pyro-, or Triphosphate.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous solution of a short-chain sodium ortho-, pyro-, or triphosphate was mixed 50 $cm^3$ of 0.04 mol $dm^{-3}$ aqueous solutions of adenosine, cytidine, guanosine, uridine and thymidine, and each mixture was allowed to react at pH 12 and 70 °C, similarly to the phosphorylation of nucleosides with $P_{3m}$.

*Chemicals.* Sodium *cyclo*-triphosphate hexahydrate, $Na_3P_3O_9 \cdot 6H_2O$, was prepared by the procedure described in the previous paper[13] and recrystallized five times from an aqueous solution. Guaranteed grade adenosine, 2′- and 3′-deoxyadenosine, cytidine, 2′-deoxycytidine, guanosine, uridine, and thymidine from Sigma Chemical Company were used without purification.

*Anion-exchange Chromatography.* A column of 1.5×64 cm packed with an anion-exchange resin Dowex 1×2 (formate form) of 200—400 mesh was employed. The eluent used in the experiments of adenosine series was 0.3 mol $dm^{-3}$ formic acid solution, pH being about 2.1. The sample solution (1 $cm^3$) was injected into a column, and the eluate was fractionated in 10-g fractions with a fraction collector of the weight type. Adenosine, adenosine 2′-monophosphate (2′-AMP), adenosine 3′-monophosphate(3′-AMP), and 2′, 3′-cyclic monophosphate(2′, 3′-cAMP) were determined by means of spectrophotometry at 260 nm.

In experiments of cytidine series, a mixture of 0.05 mol $dm^{-3}$ formic acid with equal volume of 0.01 mol $dm^{-3}$ sodium formate solution was used as the eluent. The pH of the mixed solution was about 3.0. The absorbance was measured at 279 nm.

In experiments of guanosine series, an equal volume mixture of 1 mol $dm^{-3}$ formic acid and 0.15 mol $dm^{-3}$ sodium formate solution was used as the eluent. The pH of the mixed solution was about 2.7. The absorbance was measured at 255 nm.

The eluent used in experiments of uridine and thymidine series was an equal volume mixture of 1 mol $dm^{-3}$ formic acid and 0.1 mol $dm^{-3}$ sodium formate solution. The pH of the mixed solution was about 2.5. The absorbance was measured at 260 and 267 nm in experiments of uridine and thymidine series, respectively.

*High Performance Liquid Chromatography (HPLC).*[12] HPLC was carried out by use of a Shimadzu LC-3A, under the following conditions: packing material Lichromosorb $H_2N$ (5 μm, E. Merck), column size 250×4.6 mm I.D., pressure 70 kg/$cm^2$, column temperature 40 °C, eluent 0.015 mol $dm^{-3}$ ammonium dihydrogen orthophosphate, flow rate 1.0 $cm^3$/min.

*Thin-layer Chromatography (TLC).* Plates used were DC-Alufolin cellulose $F_{254}$ and DC-Fertigplatten cellulose F (E. Merck). As the developer was used a mixture solution consisting of saturated ammonium sulfate, 1 mol $dm^{-3}$ ammonium acetate solution, and 2-propanol, in the ratio of 80:18:12.

## Results and Discussion

*Phosphorylation of Adenosine with $P_{3m}$.* With 50 $cm^3$ of 0.2 mol $dm^{-3}$ aqueous $P_{3m}$ solution was mixed 50 $cm^3$ of 0.02 mol $dm^{-3}$ aqueous adenosine solution ($P_{3m}$:adenosine=10:1) and the mixture was allowed to react at pH 12 and 70 °C. The pH of the mixture decreased gradually and reached to about 7.5 after 1 d. This fact suggests that $P_{3m}$ reacted with water or adenosine to form linear phosphates or their derivatives. As an example, results of anion-exchange chromatography of the reaction products are shown in Fig. 1. As can be seen from Fig. 1, after 1 d peaks corresponding to adenosine 2′-monophosphate(2′-AMP), adenosine 3′-monophosphate(3′-AMP), and adenosine 2′, 3′-cyclic monophosphate(2′, 3′-cAMP) were observed at about 700, 1070, and 1200 $cm^3$ of the effluent, respectively. Reaction products of adenosine with $P_{3m}$ were only 2′-AMP, 3′-AMP, and 2′, 3′-cAMP. On the other hand, the peak of adenosine appeared at 70 $cm^3$ of the effluent. The products obtained in the reaction were identified to be 2′-AMP, 3′-AMP, and 2′, 3′-cAMP, by comparing their data of anion-exchange chromatography, HPLC, and TLC with those of the authentic samples, as well as by chemical analysis of the reaction products. Also, it is found that the amounts of 2′-AMP and 3′-AMP increase with the reaction time.

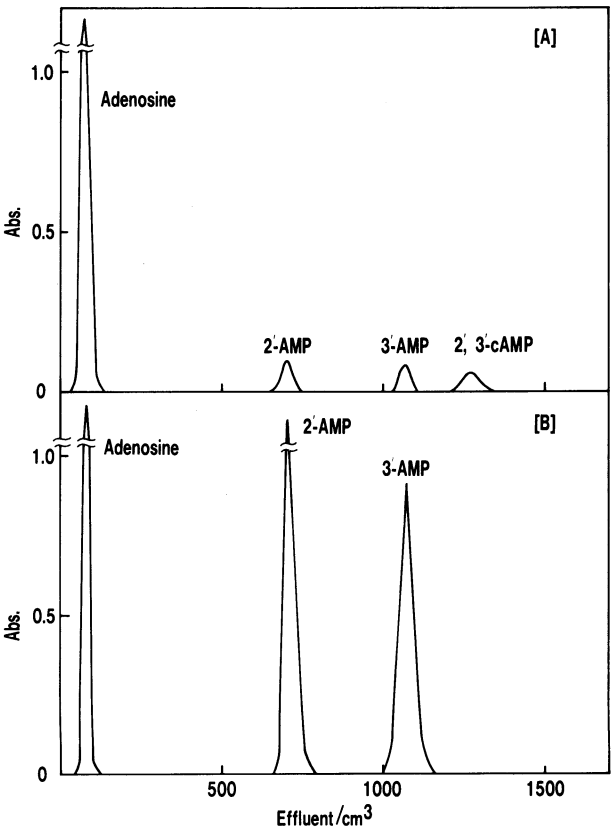


Fig. 1. Elution patterns for the reaction products of $P_{3m}$ with adenosine at 70 °C and pH 12.
$P_{3m}$: Adenosine=10:1, (A): After 1 d, (B): After 17 d.

In Fig. 2, the amounts of 2′-AMP, 3′-AMP, and 2′, 3′-cAMP, formed by the reaction of $P_{3m}$ with adenosine (mixing ratio=10:1) at pH 12 and 70 °C, are plotted as a function of time. With the progress of the reaction, amounts of 2′-AMP and 3′-AMP increased gradually to maximum values, about 37 and 45% after 17—25 d respectively, and then decreased slowly. On the contrary, the amount of 2′,3′-cAMP was largest (about 3%) after 1 d, and decreased rapidly with the reaction time, and finally became entirely zero after 8 d. On the other hand, with the increase of 2′-AMP and 3′-AMP formation, the starting material(adenosine) decreased.

Further, after a long reaction time (more than 30 d) the amounts of decomposition products of adenosine, 2′-AMP, and 3′-AMP increased gradually.

Figure 3 shows the change of the amounts of 2′-AMP and 3′-AMP in the reaction of $P_{3m}$ with adenosine under conditions of mixing ratio 5:1, pH 12, and room temperature. The decrease in the mixing ratio as well as the lowering of the reaction temperature caused to decrease extremely the formation rate and the yields of products. Further, formation of 2′,3′-cAMP observed at the initial stage of the reaction, was so little that it was omitted in Fig. 3.

The amounts of 2′-AMP, 3′-AMP, and 2′, 3′-cAMP obtained by the phosphorylation of adenosine with $P_{3m}$ under various conditions are summarized in Table 1. As can be seen, the phosphorylation of adenosine with $P_{3m}$ could not proceed at pH 7 or less, namely in neutral or acidic medium. The larger the ratio of $P_{3m}$ to adenosine, the higher the pH and the higher the reaction temperature are, the larger are the amounts of 2′-AMP and 3′-AMP formed.

The phosphorylation of 2′-deoxyadenosine, 3′-deoxyadenosine, and adenine (purine base of adenosine) with $P_{3m}$ were also investigated under conditions similar to those for the phosphorylation of adenosine with $P_{3m}$. However, these compounds could not be phosphorylated at all. Results of the phosphorylation of 2′- and 3′-deoxyadenosine are also shown in Table 1 for reference.

Based on above-described results, 2′-AMP and 3′-AMP could be selectively obtained by the phosphorylation of adenosine with $P_{3m}$ under conditions of high mixing ratio ($P_{3m}$:adenosine=5:1—10:1), high pH (pH 10—14), and high temperature (70—100 °C). Also, 2′, 3′-cAMP was formed in a short period (1—3 d) after the reaction, but it was easily decomposed with time and thus its amount was so little as about 3% at the highest. Based on the fact that 2′,3′-cAMP is easily hydrolyzed under the present experimental conditions to form 2′-AMP and 3′-AMP, 2′, 3′-cAMP formed at the initial stage of the reaction is considered to be hydrolyzed.

From the above results, the optimum conditions for the phosphorylation of adenosine with $P_{3m}$ are found to be mixing ratio 10:1, pH 12, and 70 °C. Further, the fact that 2′- and 3′-deoxyadenosine cannot be phosphorylated at all with $P_{3m}$ may suggest that the presence of hydroxyl groups at both 2′- and 3′-positions of the

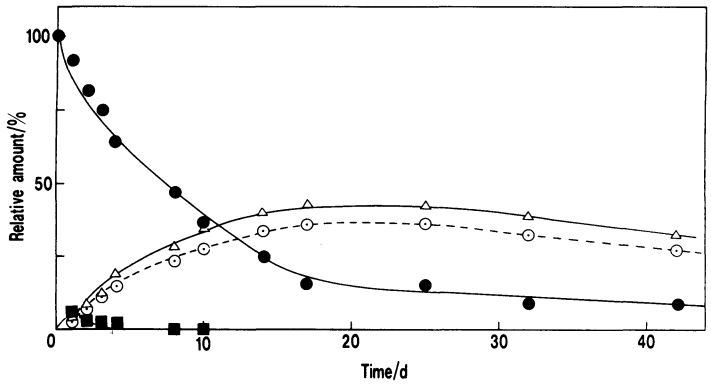


Fig. 2. Change of the amounts of 2′-AMP, 3′-AMP, 2′,3′-cAMP, and adenosine in the reaction of adenosine with $P_{3m}$.
$P_{3m}$: adenosine=10 : 1, pH 12, 70 °C.
--⊙--; 2′-AMP, —△—; 3′-AMP, —■—; 2′,3′-cAMP, —●—; adenosine.

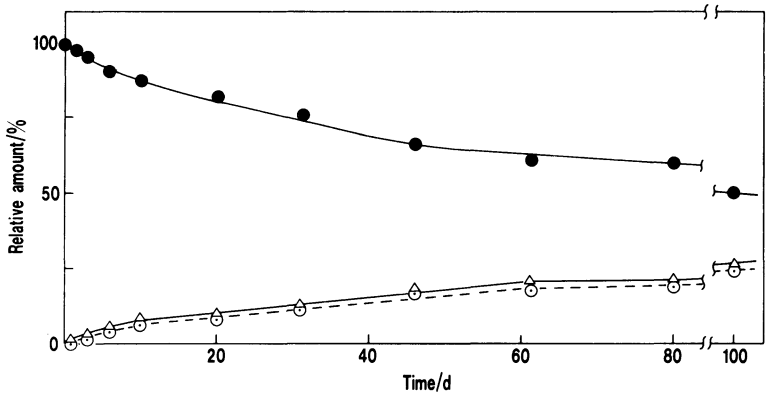


Fig. 3. Change of the amounts of 2′-AMP, 3′-AMP, and adenosine in the reaction of adenosine with $P_{3m}$.
$P_{3m}$: adenosine=5 : 1, pH 12, room temp.
--⊙--; 2′-AMP, —△—; 3′-AMP, —●—; adenosine.

Table 1. Phosphorylation of adenosine, 2′-deoxyadenosine, and 3′-deoxyadenosine with $P_{3m}$

| Reaction conditions | | | | Yield/% | | |
|---|---|---|---|---|---|---|
| Mixing ratio | pH | Temp/°C | Time/d | 2′-AMP | 3′-AMP | 2′,3′-cAMP |
| $P_{3m}$: Adenosine | | | | | | |
| 5 : 1 | 14 | 70 | 1 | 10 | 11 | 0 |
| | 12 | 100 | 1 | 13 | 17 | 0 |
| | 12 | 70 | 4 | 6 | 7 | 1 |
| | | | 21 | 29 | 36 | 0 |
| | 12 | room | 20 | 9 | 9 | 0.6 |
| | | | 100 | 24 | 26 | 0 |
| | 10 | 70 | 20 | 7 | 9 | 0.4 |
| | | | 64 | 13 | 17 | 0 |
| | 7 | 70 | 35 | 0 | 0 | 0 |
| | 2 | 70 | 35 | 0 | 0 | 0 |
| 10 : 1 | 12 | 70 | 1 | 2 | 3 | 3 |
| | | | 17 | 37 | 45 | 0 |
| $P_{3m}$: 2′-Deoxyadenosine | | | | | | |
| 5 : 1 | 12 | 70 | | — | 0 | — |
| $P_{3m}$: 3′-Deoxyadenosine | | | | | | |
| 5 : 1 | 12 | 70 | | 0 | — | — |

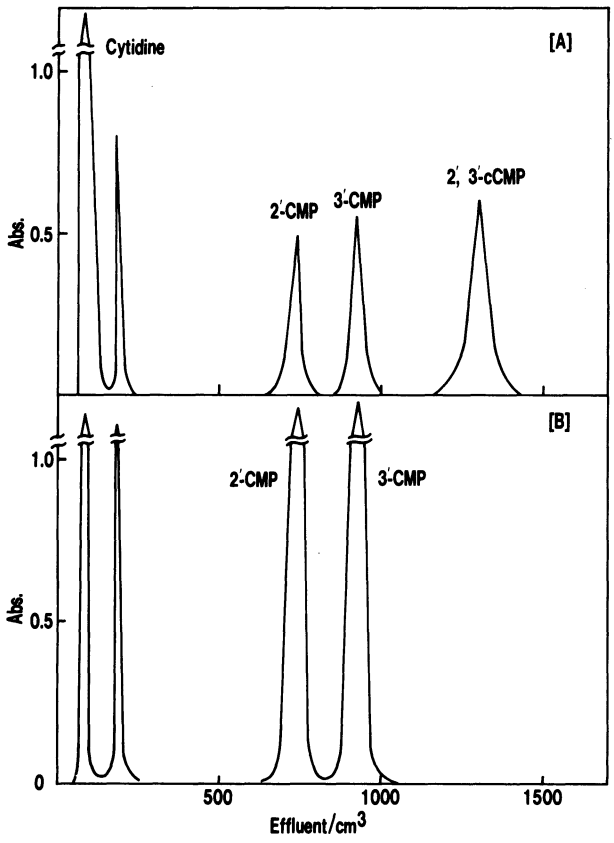


Fig. 4. Elution patterns for the reaction products of $P_{3m}$ with cytidine at 70 °C and pH 12.
$P_{3m}$: Cytidine = 1 : 1, (A): After 3 d, (B): After 9 d.

ribose is required for the phosphorylation of nucleosides with $P_{3m}$.

*Phosphorylation of Cytidine with $P_{3m}$.* In Fig. 4 are shown the results of anion-exchange chromatography of the reaction products in the phosphorylation of cytidine with $P_{3m}$. Peaks corresponding to cytidine 2′-monophosphate(2′-CMP), cytidine 3′-monophosphate(3′-CMP), and cytidine 2′,3′-cyclic monophosphate(2′,3′-cCMP) appeared at about 740, 930, and 1300 $cm^3$ of the effluent, respectively. These products were identified by comparing their data of anion-exchange chromatography, HPLC, and TLC with those of the authentic samples. The peak of cytidine appeared also at about 80 $cm^3$ of the effluent. Further, a large peak (about 180 $cm^3$) appeared between those of cytidine and 2′-CMP was proved to correspond to the decomposition product of cytidine by the experiment, in which cytidine alone was treated at pH 12 and 70 °C. As the decomposition product is, however, not identified yet and is beyond the purpose of the present study, it is not described here.

The reaction products of the phosphorylation of cytidine with $P_{3m}$ were only 2′-CMP, 3′-CMP, and 2′, 3′-cCMP. In the reaction for a long time, 2′-CMP and 3′-CMP were formed markedly.

Yields of 2′-CMP, 3′-CMP, and 2′, 3′-cCMP in the phosphorylation of cytidine or 2′-deoxycytidine with $P_{3m}$ are summarized in Table 2. At pH 14 and 70 °C, cytidine was phosphorylated with $P_{3m}$ in a short time (10—24 hr), namely about 55% of the starting material (cytidine) was phosphorylated after 1 d. With the elapse of the reaction time, however, 2′-CMP and 3′-CMP once formed were hydrolyzed, and as the result, the amounts of them decreased rapidly. At the same reaction temperature, the phosphorylation of cytidine with $P_{3m}$ became slower with the decrease of pH, and the phosphorylation did not proceed under acidic conditions. 2′, 3′-cCMP was easily formed at the initial stage of the reaction, but with the elapse of reaction time, 2′, 3′-cCMP was hydrolyzed to form 2′-CMP and 3′-CMP. 2′,3′-cCMP was well formed at pH 12 and room temperature (about 4.2%).

Table 2. Phosphorylation of cytidine and 2′-deoxycytidine with $P_{3m}$

| Reaction conditions | | | | Yield/% | | |
|---|---|---|---|---|---|---|
| Mixing ratio | pH | Temp/°C | Time/d | 2′-CMP | 3′-CMP | 2′,3′-cCMP |
| $P_{3m}$: Cytidine | | | | | | |
| 1 : 1 | 14 | 70 | 0.3 | 16 | 24 | 0.6 |
| | | | 0.9 | 22 | 32 | 0 |
| | | | 3 | 7 | 5 | 0 |
| | 12 | 70 | 3 | 1 | 2 | 2.6 |
| | | | 14 | 17 | 24 | 0 |
| | | | 21 | 17 | 23 | 0 |
| | 12 | room | 4 | 8 | 11 | 4.2 |
| | | | 65 | 35 | 45 | 0.2 |
| | 10 | 70 | 5 | 1 | 2 | 1.3 |
| | | | 16 | 4 | 7 | 0 |
| | 7 | 70 | 7 | 0.4 | 0.6 | 0.2 |
| | | | 26 | 0.4 | 1.6 | 0 |
| | 2 | 70 | 14 | 0 | 0 | 0 |
| 5 : 1 | 12 | 70 | 3 | 4 | 6 | 2 |
| | | | 7 | 24 | 35 | 0 |
| $P_{3m}$: 2′-Deoxycytidine | | | | | | |
| 5 : 1 | 12 | 70 | | — | 0 | — |

At pH 12 and room temperature after a long time(65 d), about 80% of cytidine was phosphorylated to form about 35 and 45% of 2′-CMP and 3′-CMP, respectively. As can be seen from Table 2, at such a high pH as 14 and at high temperature (70 °C), 2′-CMP and 3′-CMP once formed were easily hydrolyzed.

The optimum conditions for the phosphorylation of cytidine with $P_{3m}$ are either at pH 12 and room temperature for a long time (65—70 d) or at pH 14 and 70 °C for a short time (12—24 h). Cytidine, differing from adenosine series, was found to be phosphorylated even at pH 7, though small in amount. On the other hand, the phosphorylation of 2′-deoxycytidine with $P_{3m}$ could not be observed at all, similarly to the cases of 2′-deoxyadenosine and 3′-deoxyadenosine. For the phosphorylation of cytidine, the presence of hydroxyl groups at both 2′- and 3′-positions of the ribose was also suggested to be necessary. The basis of this conclusion will be discussed later in detail.

*Phosphorylation of Guanosine with $P_{3m}$.* An example of the results of anion-exchange chromatography of the reaction products obtained in the phosphorylation of guanosine with $P_{3m}$ is shown in Fig. 5-(A). Guanosine was easily phosphorylated with $P_{3m}$ to form chiefly guanosine 2′-monophosphate(2′-GMP) and guanosine 3′-monophosphate (3′-GMP). Also in the phosphorylation of guanosine, the formation of guanosine 2′, 3′-cyclic monophosphate(2′,3′-cGMP), though small in amount, was observed at the initial stage of the reaction. Amounts of 2′-GMP and 3′-GMP obtained under various conditions are summarized in Table 3. The optimum conditions for the phosphorylation of guanosine with $P_{3m}$ are found to be the mixing ratio 5:1, pH 12, room temperature, and long reaction period (70 d).

*Phosphorylation of Uridine with $P_{3m}$.* Figure 6 shows the results of HPLC of the reaction products in the phosphorylation of uridine with $P_{3m}$. Comparing the data with those of the authentic samples, the main reaction products were uridine 2′-monophosphate (2′-UMP) and uridine 3′-monophosphate(3′-UMP).

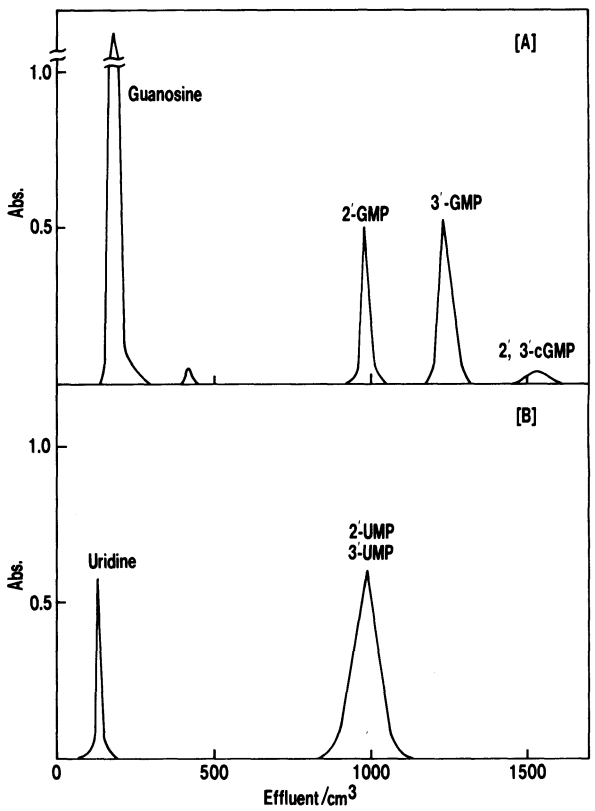


Fig. 5. Elution patterns for the reaction products of $P_{3m}$ with guanosine or uridine at 70 °C and pH 12.
(A); $P_{3m}$: Guanosine=5 : 1, after 14 d.
(B); $P_{3m}$: Uridine=10 : 1, after 4 d.

Table 3. Phosphorylation of guanosine with $P_{3m}$

| Reaction conditions | | | | Yield/% | | |
|---|---|---|---|---|---|---|
| Mixing ratio | pH | Temp/°C | Time/d | 2′-GMP | 3′-GMP | 2′,3′-cGMP |
| $P_{3m}$: Guanosine | | | | | | |
| 5 : 1 | 12 | 70 | 25 | 21 | 23 | 2.0 |
| | 12 | Room | 2 | 3 | 4 | 3.2 |
| | | | 70 | 39 | 44 | 0 |
| | 10 | 70 | 25 | 9 | 11 | 0 |
| | 7 | 70 | 34 | 0 | 0 | 0 |
| | 2 | 70 | 30 | 0 | 0 | 0 |

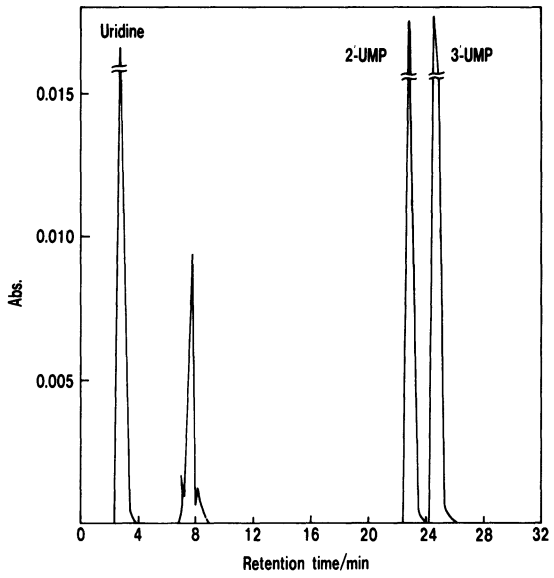


Fig. 6. Elution profile of the reaction products of $P_{3m}$ with uridine at 70 °C and pH 12.
$P_{3m}$: Uridine = 10 : 1, after 49 d.

Three small peaks, appeared between those of the starting material (uridine) and 2′-UMP, were proved to be those of decomposition products of uridine, based on the experiment carried out with uridine alone under the same conditions (pH 12 and 70 °C). On the other hand, 2′-UMP and 3′-UMP was not separated by anion-exchange chromatography under the above-described conditions, as shown in Fig. 5-(B). Further, the formation of 2′, 3′-cUMP could not be ascertained by means of HPLC and anion-exchange chromatography. It is not clear yet whether 2′, 3′-cUMP was not formed originally or 2′, 3′-cUMP once formed at the initial stage of the reaction, being very unstable, was easily hydrolyzed. It may be reasonable, however, to consider that the latter is more probable, similarly to the case of adenosine, cytidine, and guanosine series.

In Table 4 are shown the results of phosphorylation of uridine with $P_{3m}$ under various conditions. Under the conditions of $P_{3m}$: uridine = 10 : 1, pH 12, and room temperature, uridine is phosphorylated with $P_{3m}$ completely (100%) after 140 d. Thus, the starting material (uridine), 2′-UMP, and 3′-UMP were not hydrolyzed at all at room temperature.

*Phosphorylation of Thymidine with $P_{3m}$.*[14] Thymidine, one of 2′-deoxynucleosides, was not phosphorylated at all under above-described conditions. This fact may be explained similarly to the above-described cases of 2′- and 3′-deoxyadenosine and 2′-deoxycytidine. The reasons will be discussed later in detail.

Table 4. Phosphorylation of uridine with $P_{3m}$

| Reaction conditions | | | | Yield/% 2′(3′)-UMP |
|---|---|---|---|---|
| Mixing ratio | pH | Temp/°C | Time/d | |
| $P_{3m}$: Uridine | | | | |
| 1 : 1 | 12 | 70 | 36 | 29 |
| | 12 | room | 35 | 12 |
| | 2 | 70 | 35 | 0 |
| 5 : 1 | 12 | 70 | 10 | 78 |
| | 10 | 70 | 10 | 23 |
| | 7 | 70 | 12 | 7 |
| 10 : 1 | 12 | 70 | 4 | 84 |
| | 12 | room | 7 | 32 |
| | | | 140 | 100 |

*Phosphorylation of Nucleosides with Ortho-, Pyro-, and Triphosphate.* The phosphorylation of nucleosides with short-chain phosphates such as ortho-($P_1$), pyro-($P_2$), and triphosphate($P_3$) was also investigated under similar experimental conditions to those with $P_{3m}$, but the phosphorylation of nucleosides with short-chain phosphates was not observed at all.

*Mechanism of Phosphorylation of Nucleosides with $P_{3m}$.* The mechanism of the phosphorylation of adenosine, cytidine, guanosine, and uridine with $P_{3m}$ is summarized in Fig. 7. First, a hydrogen bond is formed between the hydroxyl group at the 2′- or 3′-position of ribose of nucleoside and an oxygen atom of $P_{3m}$. Then, by the attack of the corresponding oxygen atom of the hydroxyl group at 3′- or 2′-position upon a phosphorus atom of $P_{3m}$, the ring of $P_{3m}$ is opened to give an intermediate product, a triphosphate derivative of nucleoside. This triphosphate derivative, however, is immediately hydrolyzed to give nucleoside 2′- or 3′-monophosphate and $P_2$. On the other hand, the mechanism of formation of nucleoside 2′,3′-cyclic monophosphate may be as follows: As shown in Fig. 7, the intermediate, nucleoside 2′- or 3′- triphosphate formed first by the reaction of nucleoside with $P_{3m}$, is attacked by the hydroxyl group at 3′- or 2′- position, to give 2′,3′-cyclic monophosphate and $P_2$. Nucleoside 2′,3′-cyclic monophosphate thus formed is hydrolyzed to give 2′- and

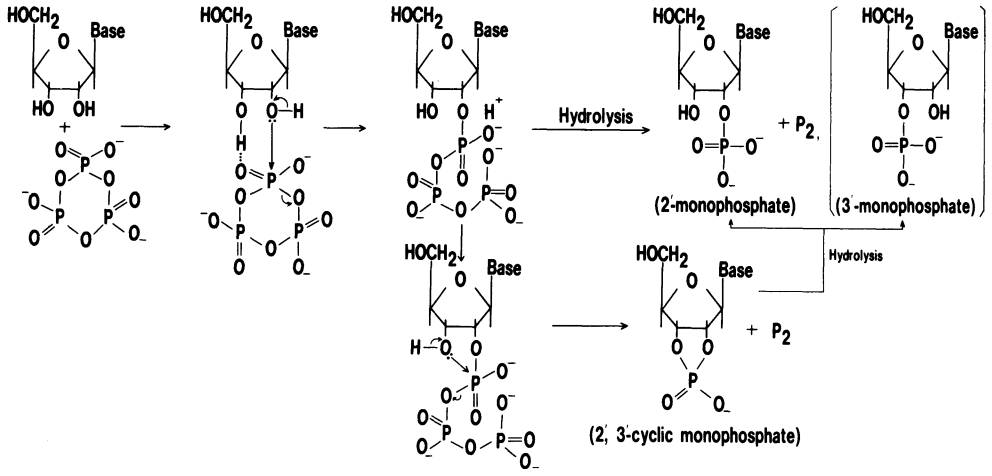


Fig. 7. Mechanism of phosphorylation of nucleosides with $P_{3m}$.

3'-monophosphate.

As can be seen from above-mentioned experimental results, nucleoside 2'- and 3'-monophosphates, formed by the phosphorylation of adenosine, cytidine, guanosine or uridine with $P_{3m}$, are nearly equal in amount. This means that the probability to form a hydrogen bond between the hydroxyl group at 2'- or 3'-position of the ribose and an oxygen atom of $P_{3m}$ are practically equal. Further, based on the fact that 2'-deoxyadenosine, 3'-deoxyadenosine, 2'-deoxycytidine, and thymidine cannot be phosphorylated with $P_{3m}$ at all, the presence of hydroxyl groups at both 2'- and 3'-positions of the ribose is suggested to be indispensable for the phosphorylation. In this connection, formation of the above-mentioned hydrogen bond between the hydroxyl group at 2'- or 3'- position and an oxygen atom of $P_{3m}$ is considered to be really possible in aqueous solution, because the bond length of P=O in crystalline sodium *cyclo*-triphosphate is 1.48 Å,[9] while that of C–C bond at 2'- and 3'-positions of ribose in nucleoside is 1.53 Å.[15]

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