The Facile Synthesis of 5′-Nucleotides by the Selective Phosphorylation of a Primary Hydroxyl Group of Nucleosides with Phosphoryl Chloride*

Tsuneo Sowa and Shunji Ouchi

Research Section, Foodstuff Plants, Asahi Chemical Industry Co., Ltd., Nobeoka, Miyazaki 882

(Received June 6, 1974)

5′-Nucleotides were directly prepared by selective phosphorylation with phosphoryl chloride in the presence of water and pyridine in acetonitrile. The rate of phosphorylation with phosphoryl chloride was markedly improved by the addition of suitable amounts of water and pyridine; the maximal formation of 5′-nucleotides was obtained by mixing these three reactants phosphoryl chloride, water, and pyridine in a molar ratio of 2:1:2 at a low temperature, followed by adding nucleosides corresponding to a one-fourth or one-fifth of the phosphoryl chloride. By mixing phosphoryl chloride, water, and pyridine in acetonitrile under controlled conditions, a hygroscopic complex (Complex 1), an active phosphorylating agent in the present phosphorylation of nucleosides, was isolated. It was identified as an adduct composed of tetrachloropyrophosphate and pyridinium chloride by means of its infrared spectra and by its chromatographic behavior. One of the proposed structures of the adduct is trichloropyrophosphopyridinium chloride. Both the selectivity in the 5′-hydroxyl group of the nucleosides and the yield of 5′-nucleotides increase upon the addition of pyridinium chloride in the phosphorylation of the unprotected nucleosides with tetrachloropyrophosphate. In the phosphorylation with tetrachloropyrophosphate, the H^+ derived from pyridinium chloride in acetonitrile is considered to increase the selectivity for the 5′-hydroxyl group by decreasing the reactivity of the hydroxyl groups in the ribose moiety of the nucleosides with the adduct. Similar results were obtained in the phosphorylation with phosphoryl chloride in the presence of pyridinium chloride in acetonitrile. Thus, several procedures for the selective phosphorylation of unprotected nucleosides to 5′-nucleotides are available.

Several attempts at the direct and selective phosphorylation of the 5′-hydroxyl group of nucleosides in the unprotected form have been made. Along those lines, 5′-nucleotides have been prepared from the corresponding nucleosides by direct phosphorylation with phosphoryl chloride in pyridine.[1,2] However, all of these methods are far from practical either in the laboratory or for commercial application because of the low conversion of nucleosides into nucleotides and because of the low selectivity in the formation of 5′-nucleotides.

Recently, Yoshikawa *et al.*[3] reported the preparation of 5′-nucleotides in good yields from the corresponding nucleosides by direct phosphorylation with phosphoryl chloride in trialkylphosphates; they also reported that, by the addition of an adequate amount of water to the reaction mixture, the competitive phosphorylation of the 2′ (or 3′)-hydroxyl group was strongly inhibited.

In the present paper, the authors will describe the methods of preparing the 5′-nucleotides by direct phosphorylation, with a complete selectivity for the primary hydroxyl group of nucleosides in acetonitrile, and will discuss the possible reaction mechanisms.

The nucleosides used in this experiment are adenosine (An–R), guanosine (Gn–R), inosine (Hx–R), xanthosine (Xn–R), uridine (Un–R), cytidine (Cn–R), 1-β-D-arabinofuranosyl uracil (Ara–Un), 1-β-D-arabinofuranosyl cytosine (Ara–Cn), 9-β-D-arabinofuranosyl adenine (Ara–An), 2′-deoxycytidine (Cn–dR), 2′-deoxyuridine (Un–dR), 8-bromoadenosine (Br–AnR), 8-hydroxyadenosine (8-OH–AnR), 8-mercaptoadenosine (8-SH–AnR), 8-bromoguanosine (8-Br–GnR), N^6-*n*-butyladenosine (N^6-Bu–AnR), 8,2′-*S*-anhydro(9-β-D-arabinofuranosyl) 8-mercaptoadenine (*S*-Anhyd–An), 2,2′-*O*-anhydro(1-β-D-arabinofuranosyl) cytosine hydrochloride (Anhyd–Cn), 2,2′-*O*-anhydro(1-β-D-arabinofuranosyl) uracil (Anhyd–Un), and 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICAR).

Results and Discussion

Phosphorylation of Unprotected Nucleosides. When inosine was subjected to the addition of phosphoryl chloride in the presence of water and pyridine in acetonitrile, inosine-5′-phosphate (5′-IMP) was exclusively obtained; the yield reached 92.4%. 5′-Nucleotides with the other base components listed in Table 1 could similarly be obtained with an almost complete selectivity in the 5′-hydroxyl group, but the nucleoside containing 8-bromoguanine was resistant to direct phosphorylation by phosphoryl chloride.

One of the advantages of the present phosphorylation method was distinctly demonstrated in the preparation of 5′-arabinonucleotides. Since the arabinonucleoside has no 2′,3′-*cis*-diol group, which could be easily protected with the isopropylidene or the benzylidene group, it has been difficult to prepare 5′-arabinonucleotides by a conventional phosphorylation method. For example, when Schreckel *et al.*[4] prepared 1-β-D-arabinofuranosyl cytosine 5′-monophosphate by the phosphorylation of 2′,3′-*O*-benzylidene cytidine with polyphosphoric acid, the yield amounted to only 10%. It was found that the present method was much superior to Schreckel's method for preparing 5′-arabinonucleotides because the phosphorylation of the arabinonucleosides could be accomplished directly without the introduction of any protecting groups to the secondary hydroxyl groups in the sugar moiety.

Similarly, 2′-deoxyuridine, 2′-deoxycytidine, 2,2′-*O*-

* Most of this study was presented at the 22nd Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1969.

Table 1. Direct phosphorylation of nucleosides

	H_2O (mmol)	Reaction time, hr	Yield of 5′-nucleotide, mol %	Selectivity of 5′-nucleotide, %
Gn-R	25	6	89.5	93.0
An-R	25	4	91.3	92.9
Hx-R	28	4	92.4	93.3
Xn-R	24	6	90.0	99.4
Un-R	25	6	86.3	98.1
Cn-R	20	2	98.6	100.0
Ara-Un	25	6	84.8	90.0
Ara-Cn	20	2	97.5	99.1
Ara-An	25	4	90.4	93.4
2′-An-dR[a]	25	4	—	—
2′-Cn-dR	20	2	95.1	98.4
2′-Un-dR	25	4	85.1	90.6
8,2′-*S*-Anhyd-AnR	25	4	98.8	100.0
2,2′-*O*-Anhyd-AnR	25	5	81.3	94.3
8-Br-AnR	25	4	84.3	91.7
8-OH-AnR	26	4	91.1	93.7
8-SH-AnR	26	6	80.0	92.8
N^6-Bu-AnR	25	4	88.8	93.4
8-Br-GnR	25	6	25.1	—
AICAR[b]	27	1	76.9	—

Experimental conditions:
CH_3CN 10 ml, $POCl_3$ 44 mmol, C_5H_5N 48 mmol
H_2O listed in above Table, nucleoside 10 mmol at 2 °C.

a) Adenine was produced in almost quantitative yield.
b) AICNR-5′MP was produced as a by-product.

anhydro(1-β-D-arabinofuranosyl) uracil, and 8,2′-*S*-anhydro(1-β-D-arabinofuranosyl)-8-mercaptoadenine, all with no 2′,3′-*cis*-diol group, could be easily phosphorylated to prepare the corresponding 5′-nucleotides in good yields. All attempts at the phosphorylation of 2′-deoxyadenosine and 2′-deoxyguanosine failed because of the lability of their glycosidic linkage and their low solubilities.

The phosphorylation of 5-amino-1-β-D-arabinofuranosylimidazole-4-carboxamide (AICAR) under the present conditions gave 5-amino-1-β-D-arabinofuranosylimidazole-4-carboxamide 5′-phosphate (AICAR-5′-MP).[5] In addition to the expected 5′-phosphomonoester, its dehydrated derivative, 1-β-D-arabinofuranosyl-4-cyano-5-aminoimidazole 5′-phosphate (AICNR-5′-MP, λ_{max} at pH 2.0, 240—242 nm),[6] was detected on the paper electrophoresis of the phosphorylation mixture. AICAR-5′MP was readily isolable from AICNR-5′MP by precipitating the former as its free acid from an acidic aqueous solution at pH 2.5.

In the present phosphorylation, the amounts of the three reagents were held to a definite value. That is, phosphoryl chloride, water, and pyridine were mixed in the molar ratio of 2: 1: 2 at a low temperature, and then nucleosides corresponding to one-fourth or one-fifth of the phosphoryl chloride was added. No reaction was observed without water, and the amount of water was thought to be critical. As is shown in Fig. 1, the formation of inosine 5′-phosphate obviously depended on the amount of water in the reaction

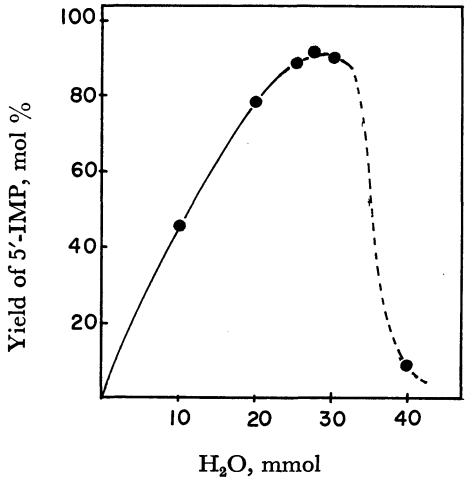

Fig. 1. Effect of water.
Experimental conditions: CH_3CN 10 ml, $POCl_3$ 44 mmol, C_5H_5N 48 mmol, H_2O: figured above, inosine 10 mmol at 2 °C.

media. When about 0.6 mol of water per mole of phosphoryl chloride was added, the yield of inosine 5′-phosphate rose to 90%. Either an increase or a decrease in the amount of water remarkably decreased the formation of inosine 5′-phosphate.

Yoshikawa *et al.*[3] reported that 5′-nucleotides were selectively obtained by treating the unprotected nucleosides with phosphoryl chloride in trialkylphosphates in the presence of a half amount of water per mole of phosphoryl chloride. In the present phosphorylation, however, there was no reaction without pyridine, the amount of pyridine was thus thought to be critical, too. When an amount of pyridine equimolar to phosphoryl chloride was added, the best result was obtained, as is shown in Fig. 2. The reaction temperature also influenced the phosphorylation yield and selectivity toward the 5′-hydroxyl group. In the present phosphorylation, the maximal formation of 5′-nucleotides was obtained in the reaction at a temperature between 0 and 2 °C (Fig. 3). A reaction at a higher temperature brought about an unfavorable formation of inosine 2′ (or 3′), 5′-diphosphate, and of hypoxanthine

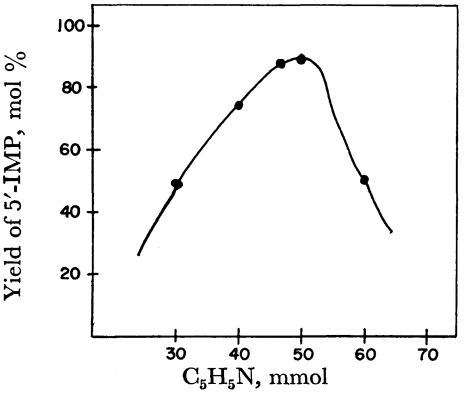

Fig. 2. Effect of pyridine on the direct phosphorylation of nucleosides.
Experimental conditions: CH_3CN 10 ml, $POCl_3$ 44 mmol, H_2O 28 mmol; C_5H_5N: figured above, inosine 10 mmol at 2 °C.

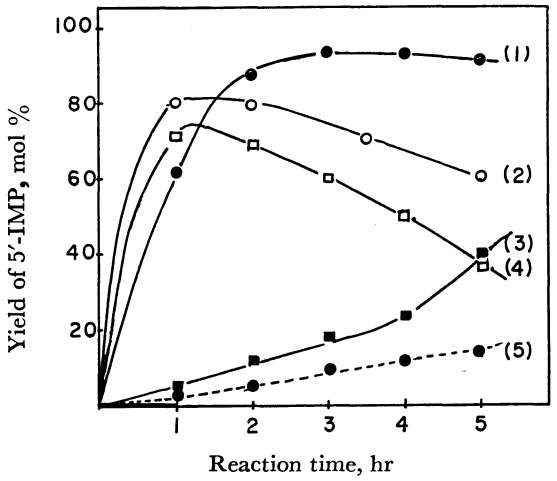

Fig. 3. Effect of reaction temperature.
Experimental conditions: CH_3CN 10 ml, $POCl_3$ 44 mmol, H_2O 28 mmol, C_5H_5N 48 mmol, inosine 10 mmol.
reaction temp.: (1) at 2 °C, (2) at 10 °C, (3) at 20 °C, (4) inosine 2′ (or 3′),5′-diphosphate produced at 20 °C, (5) hypoxanthine produced at 20 °C.

due to the cleavage of the glycosidic linkage.

Possible Mechanisms of the Selective Phosphorylation of the Unprotected Nucleoside. In the present phosphorylation described above, it was requisite to maintain the molar ratio of phosphoryl chloride, water, and pyridine at 2: 1: 2. On the basis of the evidence obtained so far, the real phosphorylating agent was assumed to be a species different from phosphoryl chloride. Phosphoryl chloride was partially hydrolyzed to dichlorophosphoric acid,[7] which was then further hydrolyzed provided an excess of water was present:

$$POCl_3 + H_2O + C_5H_5N \longrightarrow POCl_2(OH) + C_5H_5NHCl$$

In the present phosphorylation, the amount of water was strictly controlled, and only one-half of phosphoryl chloride was hydrolyzed to dichlorophosphoric acid. Moreover, in this hydrolysis only one mole of water and of pyridine was utilized; the stoichiometry of these reactants effected a phosphomonoester formation shown to have a molar ratio of phosphoryl chloride, water, and pyridine not of 1: 1: 1 but of 2: 1: 2. This seems to show that dichlorophosphoric acid might not be a phosphorylating agent. It is known that dichlorophosphoric acid is capable of reacting with phosphoryl chloride to give tetrachloropyrophosphate:[13]

$$HOPOCl_2 + POCl_3 + C_5H_5N \longrightarrow P_2O_3Cl_4 + C_5H_5NHCl$$

In this formation of tetrachloropyrophosphate from dichlorophosphoric acid, one mole of phosphoryl chloride and of pyridine were utilized, and the amounts of phosphoryl chloride, water, and pyridine were in the molar ratio of 2: 1: 2. Thus, the real species acting for the phosphorylation was considered to be tetrachloropyrophosphate, which dichlorophosphorylated the 5′-hydroxyl group in the reaction with the nucleoside. The pyrophosphate linkage in the tetrachloro-derivative of pyrophosphoric acid is extremely sensitive to hydrolysis with a base. The necessity to keep the amount of water limited and to keep the temperature low during the phosphorylation has been explained by the above discussion.

If tetrachloropyrophosphate is the species causing the selective phosphorylation at the 5′-hydroxyl group, dichlorophosphoric acid should be produced as an intermediate during the reaction. The transient formation of dichlorophosphoric acid is further indicated by the following evidence. The reaction of *t*-butanol with phosphoryl chloride is known to give dichlorophosphoric acid:[3,7]

$$POCl_3 + t\text{-}C_4H_9OH \longrightarrow POCl_2(OH) + t\text{-}C_4H_9Cl$$

If water provides a hydroxyl group to substitute for a chlorine atom in phosphoryl chloride, and if dichlorophosphoric acid is then actually formed, *t*-butanol might be utilized in place of water. Since *t*-butanol acts as an acceptor of the chlorine atom (as in the above equation), the molar ratio of phosphoryl chloride, *t*-butanol, and pyridine for the preparation of tetrachloropyrophosphate should be kept at 2: 1: 1. In the present study, in fact, under these conditions, 5′-nucleotides were similarly obtained by the direct phosphorylation of common nucleosides, in good agreement with the results obtained in the phosphorylation with phosphoryl chloride in the presence of water and pyridine, as is shown in Table 2. Though it was shown

Table 2. Direct phosphorylation by $POCl_3$ in the presence of alcohol and pyridine

Compd	Alcohol[a]	Reaction time, hr	Yield of 5′-*N*-tide, mol %	Selectivity of 5′-*N*-tide %
Hx–R	MeOH	4	94.4	96.4
Hx–R	EtOH	6	87.7	95.5
Hx–R	*t*-BuOH	6	93.8	95.1
Hx–R	*n*-BuOH	8	10.0	—
An–R	MeOH	4	85.6	93.9
Gn–R	MeOH	7	80.4	88.1
Cn–R	MeOH	6	98.2	100
Un–R	MeOH	7	76.6	89.6

Experimental conditions: CH_3CN 10 ml, $POCl_3$ 44 mmol, alcohol 24 mmol, C_5H_5N 24 mmol, nucleoside 10 mmol, at 2 °C.
a) MeOH: methanol, EtOH: ethanol, *t*-BuOH: *t*-butyl alcohol.

that *t*-butanol could be replaced by methanol or ethanol without affecting the reaction yield and selectivity** in the phosphorylation, its isomeric *n*-butanol gave poor results. *n*-Butanol was shown not to produce dichlorophosphoric acid, but to provide its phosphomonoester by reacting with phosphoryl chloride; it was not considered to be suitable for the preparation of tetrachloropyrophosphate in the presence of pyridine.

However, when the unprotected nucleosides were subjected to a reaction with tetrachloropyrophosphate in acetonitrile, it was found that the reaction went

** Selectivity of 5′-nucleotides is defined as the molar ratio (%) of 5′-nucleotides to all of the phosphorylated product.

Table 3. Direct phosphorylation with $P_2O_3Cl_4$

Compd.	Reaction time, hr	Yields of product, mol %		Selectivity of 5′-*N*-tide %
		5′-*N*-tide	by-pro[a]	
An–R	0.5	81.4	16.6	83.3
Gn–R	0.5	61.7	33.5	64.8
Hx–R	0.5	80.5	19.5	80.5
Xn–R	2.0	94.1	t	100
Un–R	0.5	90.3	8.6	91.3
Cn–R	0.5	60.3	37.7	62.5

Experimental conditions:
nucleoside 10 mmol, $P_2O_3Cl_4$ 24 mmol, CH_3CN 10 ml
reaction temp. at 2 °C
a) nucleoside 2′ (or 3′), 5′-diphosphate

essentially to completion within half an hour, that the 5′-nucleotides, except for uridine 5′-phosphate and xanthosine 5′-phosphate, were obtained in yields between 60 and 80%, and that considerable amounts of the phosphorylated by-products, nucleoside 2′ (or 3′), 5′-diphosphate, were formed (Table 3). Similar results had been obtained by the direct phosphorylation of nucleosides with tetrachloropyrophosphate in trialkylphosphate.[3] Therefore, it can not be concluded that tetrachloropyrophosphate acts alone in the efficient selective phosphorylation of the 5′-hydroxyl group of nucleosides.

When phosphoryl chloride, water, and pyridine were mixed in acetonitrile at a low temperature and when the reaction mixture was then treated with dry ether, hygroscopic crystals (Complex 1) were obtained; they began to soften at 40 °C and were completely molten at 50—51 °C. The thin-layer chromatography of this hygroscopic product showed a different behavior from tetrachloropyrophosphate (Fig. 4). The infrared spectra of the product was not the same as that of tetrachloropyrophosphate. Moreover, a crystalline product (Complex 2) which has been prepared by reacting tetrachloropyrophosphate with pyridinium chloride in the molar ratio of 1:2 showed the same hygroscopic

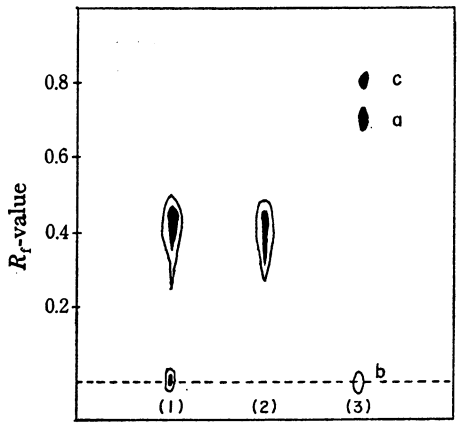

Fig. 4. Thin-layer chromatography
Solvent: ethylene glycohol dimethyl ether.
(1) Complex 1, (2) Complex 2, (3) authentic samples
a: $P_2O_3Cl_4$, b: $C_5H_5N \cdot HCl$, c: $POCl_3$.
●: detected with the Allen method.
○: detected with U.V. light.

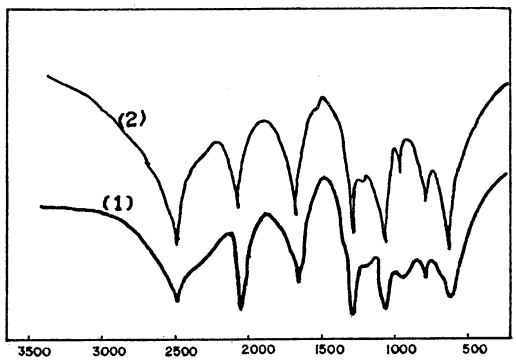

Fig. 5. Infrared spectra of Complex 1 and Complex 2.
(1) Complex 1, (2) Complex 2

properties, and its chromatographic behavior was quite similar to that of Complex 1. The identity of Complex 1 with Complex 2 was further confirmed by a comparison of their infrared spectra (Fig. 5). The infrared spectra of Complex 1 and Complex 2 both included a broad strong band due to P=O stretching at 1280 cm^{-1}, a broad band associated with the P–O–P group at 980 cm^{-1} and three fairly sharp bands at 2500, 2200, and 1680 cm^{-1} (–C=N$^+$–stretching). The fact that both Complex 1 and Complex 2 absorb ultraviolet light strongly suggests the presence of pyridine as a constituent of both these complexes.

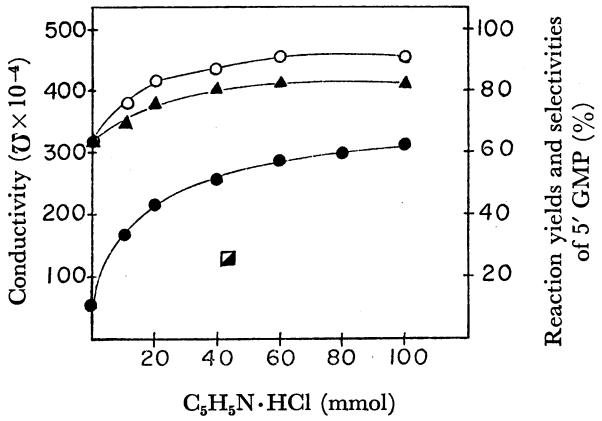

Fig. 6. Effect of pyridinium chloride.
Experimental conditions: $P_2O_3Cl_4$ 22 mmol, CH_3CN 10 ml, at 0 °C.
Phosphorylation of guanosine was performed by adding guanosine (10 mmol) into the above mixture.
○: selectivity of 5′-guanylic acid (5′ GMP), ●: reaction yield of 5′ GMP, ▲: conductivity, ◪: conductivity in the absence of $P_2O_3Cl_4$.

Figure 6 shows the conductivity of mixtures of tetrachloropyrophosphate with varying amounts of pyridinium chloride in acetonitrile. The conductivity increases with the addition of pyridinium chloride to four times the initial value when pyridinium chloride equivalent to twice the molar amount of tetrachloropyrophosphate is present. Similar results were obtained on the addition of water after the mixing of phosphoryl chloride and pyridine in acetonitrile, and the same conductivity was obtained when water was

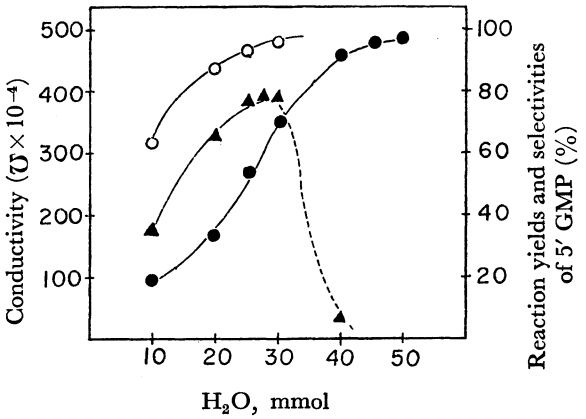

Fig. 7. Effect of water on conductivity, the reaction yield and selectivity of 5′ GMP.
Experimental conditions: $POCl_3$ 22 mmol, C_5H_5N 48 mmol, CH_3CN 10 ml, at 2 °C.
The phosphorylation was performed by adding guanosine (10 mmol) into the above solution.
●: conductivity, ○: selectivity of 5′ GMP, ▲: reaction yield of 5′ GMP.

added in half the molar amount of phosphorus oxychloride (Fig. 7). On the other hand, the conductivity of pyridinium chloride was only 105×10^{-4} ℧ at the same concentration in anhydrous acetonitrile, as is plotted in Fig. 6. The results obtained suggest that the active phosphorylating agent formed by the reaction of phosphoryl chloride with pyridine containing water is an adduct of tetrachloropyrophosphate with pyridinium chloride, which is readily dissociated into charged species in solution.

In order to test the potency of the adduct for selective phosphorylation, a common nucleoside was reacted with tetrachloropyrophosphate in the presence of varying amounts of pyridinium chloride. A typical example is shown in Fig. 6. Both the reaction yield and the selectivity of phosphorylation were obviously influenced by the amount of pyridinium chloride. The results presented in Table 4 showed a good agreement with those obtained in the mixture of phosphoryl chloride, water, and pyridine.

Although, at the present stage of the investigation, no definite determination of the structure of the adduct of tetrachloropyrophosphate with pyridinium chloride has been made, one proposed structure is trichloropyrophosphopyridinium chloride, which would be formed as in the following equations:

$$C_5H_5NHCl \rightleftarrows C_5H_5N^+H + Cl^- \quad (1)$$

$$C_5H_5N^+H \rightleftarrows C_5H_5N + H^+ \quad (2)$$

$$Cl_2P(O)-O-P(O)Cl_2 + C_5H_5N \longrightarrow \left[Cl_2P(O)-O-P(O)(Cl)-N^+C_5H_5\right] Cl^- \quad (3)$$

One possible hypothesis is that H^+ derived as indicated in the above equations increases the conductivity of the mixture in acetonitrile, and also increases the selectivity for the 5′-hydroxyl group by decreasing the reactivity of the hydroxyl groups in the ribose moiety of nucleosides with the adduct.

5′-Nucleotides could not be obtained by reacting nucleosides with phosphoryl chloride in the presence of water and strong bases, such as triethylamine, morpholine, or pyrolidine, in acetonitrile (Table 5). In the phosphoylation with tetrachloropyrophosphate,

Table 4. Direct phosphorylation with $P_2O_3Cl_4$ in the presence of $C_5H_5N\cdot HCl$

Compd	Reaction time, hr	Yield of 5′-*N*-tide, mol %	Selectivity of 5′-*N*-tide %
An–R	3.0	92.1	93.8
Gn–R	4.0	90.0	95.0
Hx–R	2.0	93.7	95.1
Xn–R	6.0	89.2	98.6
Un–R	2.0	87.2	90.8
Cn–R	1.5	98.2	98.2
Ara–Un	2.0	88.6	91.4
Ara–Cn	2.0	95.4	97.2

Experimental conditions: nucleoside 10 mmol, $P_2O_3Cl_4$ 22 mmol, $C_5H_5N\cdot HCl$ 44 mmol, CH_3CN 10 ml, at 2 °C.

Table 5. Effect of organic amine

Organic amine	pK_a value	Yields of the products, mol %			
		5′ IMP[1]	Hx′–R	Hx[2]	others[3]
γ-Picoline	5.18	91.3	2.7	1.0	5.0
2,6-Lutidine	5.03	90.4	3.6	0.8	5.2
Pyridine	6.02	92.8	1.5	0.8	4.9
Dimethylaniline	5.10	91.7	2.9	1.6	2.5
Pyrrole	11.3	31.7	40.2	28.1	—
Pyrrolidine		32.7	36.1	30.2	—
Morpholine	9.6	40.7	33.8	21.6	4.2
Triethylamine	10.9	28.8	69.7	1.6	—

Experimental Conditions:
CH_3CN 10 ml, $POCl_3$ 44 mmol, H_2O 28 mmol, organic amine 48 mmol, inosine 10 mmol, at 2 °C for 4 hr.
1) 5′-Inosinic acid. 2) Hypoxanthine. 3) Inosine 2′(3′), 5′-diphosphate.

Table 6. Direct phosphorylation by $P_2O_3Cl_4$ in the presence of organic amine hydrochloride

Org. amine hydrochloride	Reaction time, hr	Yields of product, mol %		Selectivity of 5′ IMP
		5′ IMP[a]	others[b]	
Pyridine	4	94.9	3.6	96.3
γ-Picoline	4	93.8	5.6	94.3
Morpholine	6	90.7	4.5	93.3
Pyrolidine	6	95.6	1.8	98.1
Triethylamine	6	89.6	3.6	93.1
Ethylamine	6	85.0	3.4	92.0

Experimental conditions:
inosine 10 mmol, $P_2O_3Cl_4$ 22 mmol, CH_3CN 10 ml, org. amine hydrochloride 44 mmol, at 2 °C.
a) 5′-Inosinic acid. b) Inosine 3′(or 2′),5′-diphosphate.

however, interesting evidence was obtained that the above organic amine hydrochloride could replace pyridinium chloride (Table 6). The reaction of morpholine with phosphoryl chloride is knwon to give morpholinophosphorodichloridate or dimorpholinophosphorochloridate.[8,9] This means that, in the reaction of the free base with phosphoryl chloride, no adducts were derived because the strong base is too reactive to permit the substitution of a chlorine atom by a hydroxyl group; thus, no formation of dichlorophosphoric acid as an intermediate occurs.

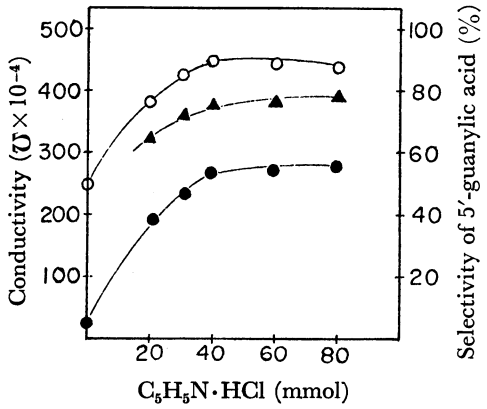

Fig. 8. Effect of pyridinium chloride on conductivity and selectivity of 5′-guanylic acid.
Experimental conditions: CH_3CN 10 ml, $POCl_3$ 44 mmol, at 2 °C.
The phosphorylation was performed by adding guanosine (10 mmol) into the above solution.
○: selectivity of 5′ GMP, ▲: reaction yield of 5′ GMP
●: conductivity.

The addition of pyridinium chloride to phosphoryl chloride in acetonitrile also increased the conductivity of the mixture (Fig. 8). This shows that a dissociable adduct, dichlorophosphopyridinium chloride, similar to trichloropyrophosphopyridinium chloride was probably present in this mixture, suggesting the selective formation of 5′-nucleotides by directly reacting nucleosides with phosphoryl chloride in the presence of pyridinium chloride. As expected, 5′-nucleotides were formed in yields between 85 and 98% from the corresponding nucleosides under the above conditions, as is shown in Table 7.

Table 7. Direct phosphorylation by $POCl_3$ in the presence of $C_5H_5N·HCl$

Compd.	Reaction time, hr	Yield of 5′-*N*-tide, mol %	Selectivity of 5′-*N*-tide, %
An–R	4	89.4	93.4
Gn–R	6	85.7	89.9
Hx–R	4	91.2	93.1
Xn–R	6	88.9	92.1
Cn–R	6	98.0	100
Un–R	6	95.4	98.3
Ara–Cn	6	96.3	100
Ara–Un	6	97.5	99.0

Experimental Conditions: nucleoside 10 mmol, $POCl_3$ 25 mmol, $C_5H_5N·HCl$ 48 mmol, CH_3CN 10 ml, at 2 °C.

The phosphorylation of nucleosides at the 5′-hydroxyl group was not much affected by the polarity of the solvents used in the present reactions. Among the solvents, acetonitrile was superior to the others in both the formation of 5′-nucleotides and the selectivity in

Table 8. Effect of the solvent

Solvent	Reaction time, hr	Yield of 5′-inosinic acid, mol %	Selectivity of 5′-inosinic acid, %
CH_3CN	4	93.5	95.2
CH_3NO_2	5	90.0	95.4
$(CH_3)_2C=O$[a]	4	74.3	83.3
THF[b]	6	71.3	98.0
Dioxane	8	78.9	84.3
CH_2Cl_2	4	89.7	94.6
DMF[c]	6	28.9	—
t-C_4H_9OH	6	1.7	—
$CHCl_3$	8	44.0	92.8

Experimental Conditions: organic solvent 10 ml, $POCl_3$ 44 mmol, H_2O 28 mmol, C_5H_5N 48 mmol, inosine 10 mmol, at 2 °C.
a) The reaction mixture was treated with acid to eliminate the protecting group. b) Tetrahydrofuran. c) Dimethylformamide.

Table 9. R_f-values and the electrophoretic mobilities of nucleosides and nucleotides

Compound	R_f values		Electrophoretic mobilities (cm)	
	Solv. A	Solv. B	pH 2.5	pH 8.8
An–R	0.17	0.51		4.5
An–R–5′MP	0.35	0.14	6.0	15.0
Gn–R	0.33	0.32		5.5
Gn–R–5′MP	0.46	0.19	7.0	16.0
Hx–R	0.44	0.33	−1.0	8.0
Hx–R–5′MP	0.67	0.10	13.0	17.5
Xn–R	0.35			14.0
Xn–R–5′MP	0.52			19.0
Cn–R	0.53	0.52		6.0
Cn–R–5′MP	0.74	0.11	−2.0	17.5
Un–R	0.54	0.51		6.0
Un–R–5′MP	0.69	0.14	8.0	17.0
Ara–Cn	0.65	0.33		−2.5
Ara–Cn–5′MP	0.72	0.13		15.5
Ara–Un	0.58	0.48		−1.0
Ara–Un–5′MP	0.78			15.0
Ara–An	0.19	0.45		−1.0
Ara–An–5′MP	0.38	0.13		13.0
8-Br–AnR	0.08	0.62		4.0
8-Br–AnR–5′MP	0.24	0.14		14.0
8-SH–AnR	0.14	0.68		5.9
8-SH–AnR–5′MP	0.30	0.21		16.5
8-OH–AnR	0.25	0.31		8.0
8-OH–AnR–5′MP	0.44	0.15		18.5
8,2′-*S*-Anhyd AnR	0.12	0.61		−1.0
8,2′-*S*-Anhyd–AnR–5′MP	0.23	0.18		10.0
2,2′-*O*-Anhyd–UnR	0.67	0.48		−2.0
2,2′-*O*-Anhyd–UnR–5′MP	0.81	0.09		17.0
AICAR	0.36	0.39	−10.0	
AICAR–5′MP	0.50	0.15	0	
AICNR–5′MP		0.20	6.5	

the 5′-hydroxyl group (Table 8). Nitromethane and methylene chloride, which dissolve nucleosides completely, are useful as reaction solvents in place of acetonitrile. However, no 5′-nucleotides were formed in dimethylformamide (DMF) or *n*-butanol, presumably because both the amide and alcohol were so reactive to phosphoryl chloride. In acetone, the isopropylidenation reaction took place in inosine at the same time to give 2′,3′-*O*-isopropylidene inosine 5′-phosphate in a 38% yield.

The methods described here simple, direct ways to effect phosphorylation with a complete selectivity to the primary hydroxyl group of nucleosides under mild conditions; they have been adapted to produce the 5′-nucleotides economically.

Experimental

The ultraviolet spectra were recorded on a Hitachi EPS-2T spectrophotometer, and the infrared spectra, on a Hitachi EPI-G2. The conductivity experiments were run on a Shimadzu conduction meter. The paper chromatography was carried out by the ascending technique on Toyo-Roshi No. 51 paper (40×40 cm), using the following solvent systems: Solvent A, isopropyl alcohol–saturated ammonium sulfate–1M-sodium acetate, 2: 79: 19, Solvent B, *n*-butanol–acetic acid–water 4: 1: 5. The paper electrophoresis of the product was performed on the same paper with a 0.05M-borate buffer (pH 8.8) and a 0.05M-citrate buffer (pH 2.5) at a potential gradient of 1.5 kV/cm, for 1 hr. The structure of 5′-nucleotides was confirmed by both a sodium periodate–benzidine test[10] and a 5′-nucleotidase of bull-seminal-plasma treatment.[11] Thin-layer chromatography was used for the identification of the reaction product obtained by mixing phosphoryl chloride, water and pyridine; it was carried out on Wakogel B-5F with Solvent C, ethyleneglycohol dimethyl ether, in a dry box. The location of the products on the layer was determined by the Allen method[12] and by ultraviolet absorption. The nucleotides were identified by a comparison of the R_f-values with those of the authentic samples, as summarized in Table 9.

Preparation of Tetrachloropyrophosphate. Tetrachloropyrophosphate was readily obtained by the method of Craft.[8] A mixture of phosphoric oxide (42.6 g, 0.15 mol) and phosphorus pentachloride (97.8 g, 0.47 mol) was heated at 105 °C for 8 hr, and then cooled and filtered. The solid was washed with carbon tetrachloride, and the combined filtrate and washings were distilled, giving tetrachloropyrophosphate (18.3 g, 31.3 %). Bp 101—103 °C/10 mmHg, d_4^{20}, 1.81.

Preparation of Complex 1. Into a mixture of freshly distilled phosphoryl chloride (20.2 g), water (1.18 g) pyridine (10.4 g), and acetonitrile (23 g) were vigorously stirred at a temperature of 2 °C. Dry ether (100 ml) was then added to the reaction mixture; the precipitate was separated by filtration, washed with ether, and then dried. The hygroscopic crystals (25.8 g) began to soften at 40 °C and was completely molten at 50—51 °C.

Preparation of Complex 2. The hygroscopic crystal (22.3 g) were prepared by mixing tetrachloropyrophosphate (15 g) and pyridinium chloride (13.8 g) in acetonitrile, followed by adding ethyl ether (300 ml) in essentially the same manner as has been described above. Mp 82—88 °C.

Phosphorylation of Nucleosides in the Unprotected Form.

Method A: To a mixture of freshly distilled phosphoryl chloride (44 mmol), water (28 mmol), pyridine (48 mmol), and acetonitrile (189 mmol), which was maintained at 2 °C with stirring, we added a nucleoside (10 mmol, dried at 120 °C for 4 hr), after which the mixture was maintained for 4 hr. The reaction mixture was poured into ice water and stirred further for 1 hr at 5 °C. The resulting solution was analyzed by the paper electrophoretic method described above. The results obtained are listed in Table 1.

Method B: A nucleoside (10 mmol) was dissolved in a mixture of tetrachloropyrophosphate (22 mmol) and acetonitrile (190 mmol), after which the resulting mixture was poured into ice water and analyzed by the paper-electrophoretic method. The results obtained are listed in Table 3.

Method C: A solution which contained a 5′-nucleotide was obtained by reacting the nucleoside (10 mmol) with a mixture of phosphoryl chloride (44 mmol), alcohol (24 mmol), and pyridine (24 mmol) in acetonitrile (190 mmol) in essentially the same manner as that described for Method A. The results obtained are listed in Table 2.

Method D: A solution which contained a 5′-nucleotide was obtained by reacting the nucleoside (10 mmol) with phosphoryl chloride (24 mmol) or tetrachloropyrophosphate (24 mmol) in the presence of pyridinium chloride (44 mmol) in acetonitrile (190 mmol) in the same way as in Method A. The results obtained are listed in Table 4 and Table 7.

References

1) J. M. Gulland and G. I. Hobday, *J. Chem. Soc.*, **1940**, 746.
2) G. R. Baker and G. E. Fall, *J. Chem. Soc.*, **1957**, 3798.
3) M. Yoshikawa, T. Kato, and T. Takenishi, This Bulletin, **42**, 3505 (1969).
4) A. M. Schrecker, J. A. R. Meed, and M. J. Urshel, *Biochem. Pharmacology*, **15**, 1443 (1966).
5) M. Kikkawa, *J. Patent Publication* 42–4348.
6) T. Meguro, *ibid.*, 41–1534.
7) M. Yoshikawa and T. Kato, This Bulletin, **40**, 2849 (1967).
8) H. A. C. Montogomery and J. H. Turnbell, *Proc. Chem. Soc.*, **1957** 178, *J. Chem. Soc.*, **1958** 1963.
9) M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull.*, **10**, 536 (1962), *ibid.*, **11**, 435 (1963).
10) M. Viscontini, D. Hoch, and P. Karrei, *Helv. Chim. Acta*, **38**, 642 (1955).
11) L. A. Leppel and R. J. Hilmae, *J. Biol. Chem.*, **188**, 655 (1951).
12) R. J. L. Allen, *Biol. J.*, **34**, 858 (1940).
13) R. W. Chambers, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **79**, 3747 (1957).