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Studies of Phosphorylation. I. Phosphorylation of 2′,3′-*O*-Isopropylidene Nucleoside by Phosphoryl Chloride*[1]

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5′-Nucleotides could be conveniently prepared in high yields by the phosphorylation of 2′, 3′-*O*-isopropylidene nucleosides with phosphoryl chloride in the absence of any organic solvent. The rate of phosphorylation in a large excess of phosphoryl chloride was markedly promoted by the partial hydrolysis of phosphoryl chloride with a small amount of water. The undesirable cleavage of the glycosidic linkage in nucleoside, which was observed particularly in a strongly acidic medium caused by the addition of excessive water, could be suppressed by the addition of ethers or amines in a phosphoryl chloride solution. 2′, 3′-*O*-Isopropylidene derivatives of adenosine, inosine, and guanosine gave the corresponding 5′-nucleotides in good yields, whereas that of xanthosine reacted very slowly, unlike the above nucleosides. All attempts at the phosphorylation of 2′, 3′-*O*-isopropylidene-2-mercaptoinosine were unsuccessful. The influence of substituents at the 2-position in purine bases on phosphorylation was investigated by comparing the reactivity between the above-mentioned nucleosides and their methylated derivatives, such as 2-dimethylaminoinosine, 2-methoxyinosine, and 2-methylthioinosine; it was found that these methylated nucleosides were readily phosphorylated to give the corresponding 5′-nucleotides in nearly quantitative yields.

Recently a number of reagents for the phosphorylation of nucleosides, such as dialkyl and diaryl phosphorochloridates,[1,2] tetra-*p*-nitrophenyl pyrophosphate,[3] *O*-benzylphosphorous *O*, *O*-diphenyl phosphoric anhydride,[4] tetrachloropyrophosphate[5] and morpholinophosphorodichloridate,[6] have been proposed. However, all of these reagents have some drawback, such as difficulty of preparations or complexity of the removal of the protective groups from the products. It seemed desirable to improve the phosphorylation method to make it simpler and more convenient, and with a higher yield. The simplest phosphorylation with phosphoryl chloride has, however, been reported to give a very poor yield of the desired product.[7-9] 2′, 3′-*O*-Isopropylideneguanosine gave guanosine 5′-phosphate (5′-GMP) in only a 20% yield, along with 5′-pyro- and -triphosphates.[10,11]

The present series of studies was undertaken to solve, for industrial purposes, the above problems and to obtain extensive knowledge about the reaction of nucleosides with phosphoryl chloride.

*1 Most of this study was presented at the 17th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1964.

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10) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, **1949**, 2476.
11) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **79**, 3747 (1957).

Experimental

Identification of the Reaction Products. Paper chromatography and paper electrophoresis were mainly used for this purpose. One-dimensional and two-dimensional paper chromatographies were performed overnight by the ascending technique on Toyo Roshi No. 51 paper (40×40 cm), with Solvent 1 (*n*-propyl alcohol : concentrated ammonium hydroxide : water (20 : 12 : 3)) and Solvent 2 (*n*-butyl alcohol : acetic acid : water (4 : 1 : 1)). Paper electrophoresis was carried out on the same paper for 2 hr with 10% acetic acid at a potential gradient of 30 V/cm.

The locations of the products were detected by means of ultraviolet light (253 mμ) and were identified by a comparison of the R_f values and the electrophoretic mobilities with those of authentic samples. Nucleotides were confirmed by Hanes and Isherwood spray, followed by ultraviolet irradiation,[12] and nucleosides, by a sodium periodate-benzidine test.[13] The R_f values and the electrophoretic mobilities of nucleosides and nucleotides are summarized in Table 1.

Quantitative Analysis of the Products. Authentic samples of known concentrations and the reaction mixture were developed on the same paper with Solvent 1 and Solvent 2, respectively. Each spot was excised and eluted with 0.1 N hydrochloric acid. The yields of the products were estimated by means of the relative absorption intensities of the eluates. The results obtained by Solvent 1 were in good agreement with those obtained by Solvent 2.

Preparation of 2′, 3′-*O*-Isopropylidene Nucleosides. Isopropylidene nucleosides were readily obtained in excellent yields by the method of Mori *et al.*[14]

A Typical Example. Phosphoryl chloride (61.5 g) was slowly added to 220 m*l* of acetone containing 3.6 g of water, and the mixture was kept at 15°C. Inosine (26.8 g) was then stirred into the solution in several portions at 15°C. After 30 min, the reaction mixture was added, drop by drop, into a 2.5 N sodium hydroxide solution, with care to maintain the pH between 9.0 and 9.5; then the solution was cooled to 10°C. The precipitated sodium phosphate was filtered off and washed with 50% aqueous acetone. The filtrate and washings were combined, and the solution was concentrated to about 200 m*l*. The solution was cooled, and the pH was adjusted to about 7 with 2 N hydrochloric acid. Crystalline precipitates were filtered off, washed with water, and dried. The 2′, 3′-*O*-isopropylideneinosine (25.0 g 81% yield) obtained was recrystallized from water.

2′, 3′-*O*-Isopropylidene derivatives of unnatural nucleoside were kindly supplied by Dr. Akihiro Yamazaki of the Ajinomoto Co., Inc.

Phosphorylation of 2′, 3′-*O*-Isopropylideneinosine by Levene's Procedure.[8] 2′, 3′-*O*-Isopropylideneinosine (1.0 g) was dissolved in a mixture of 17 m*l* of pyridine and 2.5 m*l* of benzene, after which the moisture in the solution was distilled off, together with the benzene. The resulting solution was stirred into 0.33 g of freshly-distilled phosphoryl chloride in 2 m*l* of anhydrous pyridine over a 7-min period at −10°C. After being kept for 1 hr at −10°C, the reaction mixture was poured into ice water, and the pH of the solution was adjusted to 9 with a 10 N sodium hydroxide solution. The resulting solution was evaporated to remove the pyridine under reduced pressure and then acidified to pH 1.5 with hydrochloric acid. The protective group of the 5′-nucleotide was eliminated by warming the solution at 70°C for 1 hr. When the solution was analyzed by paper chromatography, the resulting solution was found to contain inosine 5′-phosphate, inosinyl (5′-5′) inosine, and hypoxanthine in yields of 80%, 18%, and 2% respectively.

Phosphorylation by Phosphoryl Chloride. *A Typical Example.* To a mixture of freshly-distilled phosphoryl chloride (10 m*l*) and 0.058 m*l* of water, 1.0 g of 2′, 3′-*O*-isopropylideneinosine was gradually added, after which the mixture was maintained at 5°C for 4 hr with stirring. Ether (200 m*l*) was added to the mixture to remove the excessive phosphoryl chloride, and the precipitate, 2′, 3′-*O*-isopropylideneinosine 5′-phosphorodichloridate, was separated by centrifuge. The product was dissolved in about 10 m*l* of ice water, and the aqueous solution was adjusted to pH 1.5 with a sodium hydroxide solution and warmed at 70°C for 30—45 min to remove the protective group. The resulting solution was analyzed by paper chromatography.

Preparation of Dichlorophosphoric Acid. Water (5.4 g) was slowly added to 61.5 g of freshly-distilled phosphoryl chloride at 0°C; the mixture was kept at room temperature for 16 hr, and then the hydrogen chloride formed and the unchanged phosphoryl chloride were removed under reduced pressure. The density of the residual syrup was 1.75 at 25°C (the value reported by Grunze was 1.69[15] and that of 93% dichlorophosphoric acid by Van Wazer was 1.77[16]), and the phosphorus content was 24.2%, which is close to that of dichlorophosphoric acid.

Results and Discussion

Reinvestigation of Levene's Method.[8] The phosphorylation of 2′, 3′-*O*-isopropylideneinosine with phosphoryl chloride gave inosine 5′-phosphate (5′-IMP) in an 80% yield when the moisture in pyridine was kept under 0.1%.[17] During the phosphorylation, the intermediate, 2′, 3′-*O*-isopropylideneinosine 5′-phosphorodichloridate, was allowed to react with the starting nucleoside to give inosinyl (5′-5′) inosine[18] as the by-product in about a 20% yield.

This method has the fault that the by-product

12) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
13) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).
14) H. Mori *et al.*, presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Sapporo, July, 21, 1964, p. 169.; Ajinomoto Co., U. S. Pat. 3201388 (1965).
15) H. Grunze and E. Thilo, *Angew. Chem.*, **70**, 73 (1958).
16) J. R. Van Wazer and E. Fluck, *J. Am. Chem. Soc.*, **81**, 6360 (1959).
17) T. Kato, *et al.*, presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April, 1, 1963.
18) N. Muramatsu and T. Takenishi, *J. Org. Chem.*, **30**, 3211 (1965).

gives difficulty in the isolation of pure inosine 5'-phosphate. In addition, 2',3'-*O*-isopropylideneguanosine was scarcely phosphorylated because of its lower solubility in pyridine.

Phosphorylation of Nucleoside Without a Basic Solvent. Aryl phosphates have been synthesized by heating phenols with phosphoryl chloride in the presence of a catalyst, such as a metal halide.[19] However, this method had not been applied in the nucleotide field because of the lability of the glycosidic linkage, especially in purine nucleosides. In fact, it was observed that the nucleoside was almost decomposed under the same conditions. A similar treatment at a lower temperature, however, converted 2',3'-*O*-isopropylidene nucleoside to the corresponding 5'-nucleotide, and the maximum yield was obtained at 0—15°C (Fig. 1). As the reaction progressed, the clear reaction medium was turned into gel.

Surprisingly, the addition of a small amount of water in phosphoryl chloride increased the solubility of nucleoside and powerfully accelerated the phosphorylation. The 0.5 to 1.0 range of the mole ratio of water to the nucleoside gave the best results, as is shown in Fig. 2. At a higher mole ratio of water, the yield decreased significantly because of the cleavage of the glycosidic linkage. As a result of the partial hydrolysis of phosphoryl chloride, the gelation of the medium was prevented. It was desirable that the mole ratio of phosphoryl chloride to nucleoside be more than fifteen; this was best both for the phosphorylating agent and the diluent (Fig. 3). In this method, unlike the

TABLE 1. R_f VALUES AND ELECTROPHORETIC MOBILITIES OF NUCLEOSIDES AND NUCLEOTIDES

Compound	R_f Value		Mobility
	Solvent 1	Solvent 2	
Inosine 5'-phosphate	0.15	0.02	7.0
Inosine	0.46	0.24	1.0
Hypoxanthine	0.46	0.32	—
Adenosine 5'-phosphate	0.20	0.03	1.3
Adenosine	0.59	0.27	−7.6
Adenine	0.53	0.35	—
Guanosine 5'-phosphate	0.14	0.02	3.4
Guanosine	0.40	0.22	−1.5
Guanine	0.35	0.27	—
Xanthosine 5'-phosphate	0.17	0.02	5.5
Xanthosine	0.40	0.21	1.0
Xanthine	0.35	0.30	—
2-Mercaptoinosine	0.40	0.35	0.5
2-Dimethylaminoinosine 5'-phosphate	0.20	0.12	2.7
2-Dimethylaminoinosine	0.53	0.33	−2.6
2-Methoxyinosine 5'-phosphate	0.18	0.10	4.7
2-Methoxyinosine	0.46	0.34	−0.3
2-Methylthioinosine 5'-phosphate	0.20	0.12	5.3
2-Methylthioinosine	0.52	0.40	0.5
Cytidine 5'-phosphate	0.18	0.05	0.6
Cytidine	0.56	0.15	−8.4
Cytosine	0.56	0.27	—
Uridine 5'-phosphate	0.17	0.07	5.4
Uridine	0.46	0.31	0.7
Uracil	0.52	0.44	—

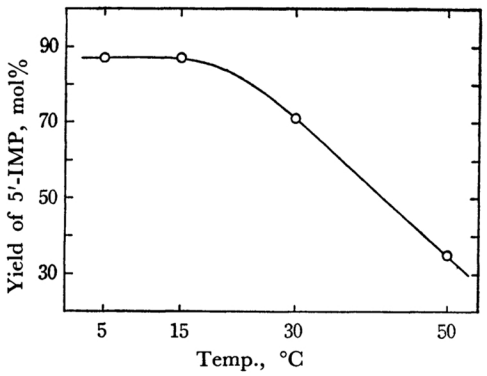

Fig. 1. Influence of reaction temperature.
Experimental condition:
Ip-HxR*2: 10 mmol, $POCl_3$: 150 mmol
H_2O: 5 mmol

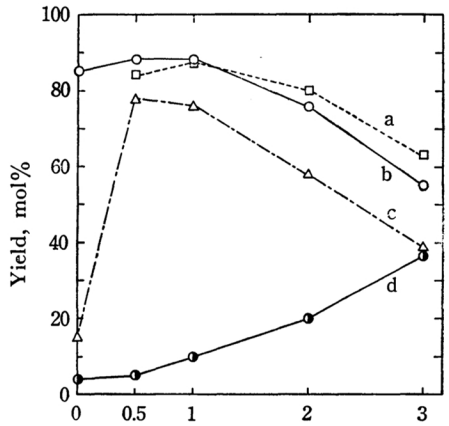

Fig. 2. The effect of addition of water.
Experimental condition:
2',3'-*O*-isopropylidene nucleoside: 1 g
$POCl_3$: 10 ml, 5°C, 4 hr (5'-IMP and 5'-AMP) or 6 hr (5'-GMP)

a : 5'-AMP
b : 5'-IMP
c : 5'-GMP
d : hypoxanthin produced in the case of Ip-HxR

19) For example see L. L. Shmidt, É. A. Talts and É. É. Iokhannes, *J. Gen. Chem. USSR (Eng. Transl.)*, **33**, 1251 (1963).

*2 Ip-HxR represents 2',3'-*O*-isopropylideneinosine in table and figure.

TABLE 2. YIELDS OF 5'-NUCLEOTIDES

2',3'-*O*-Isopropylidene nucleoside		$POCl_3$ m*l*	H_2O m*l*	Reaction time, hr	Temp. °C	Yields of 5'-nucleotide, mol%
Inosine	1.0 (g)	10	0.058	4	5	88
Adenosine	1.0 (g)	10	0.058	6	5	87
Guanosine	1.0 (g)	10	0.056	7	10	90
Xanthosine	0.5 (g)	10	0.028	24	5	32
Cytidine	284 (mg)	5	0.018	16	5	90
Uridine	283 (mg)	5	0.018	32	5	76

TABLE 3. EFFECT OF PARTIALLY HYDROLYZED PHOSPHORYL CHLORIDE

Ip-HxR g	$POCl_3$ g	$HOP(O)Cl_2$ g	Temp. °C	Time hr	Yields of 5'-IMP mol%
3.1	26	1.35	5	4	90
3.1	26	4.05	5	4	91
3.1	26	6.75	5	4	92
3.1	0	17.5	5	4	30

TABLE 4. EFFECT OF AMOUNT OF *t*-BUTYL ALCOHOL*

t-Butyl alcohol (mol**)	0.5	1	2	4	8
5'-IMP (mol%)	81	85	87	85	85
5'-AMP (mol%)	—	91	—	89	90

* Experimental condition:
isopropylidene uncleoside: 6.5 mmol, $POCl_3$: 15 m*l*, temperature: 10°C, time: 4 hr

** Mole amount per one mole of 2',3'-*O*-isopropylidene nucleoside.

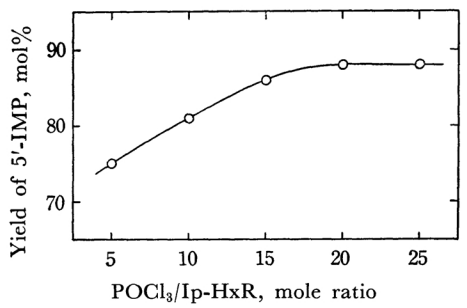

Fig. 3. Influence of amount of $POCl_3$.
Experimental condition:
Ip-HxR: 3.1 g, H_2O: 0.09 m*l*, 5°C, 6 hr

case of Levene's method, the formation of dinucleoside (5'-5') phosphates was found to be negligible, and all the naturally-occuring 5'-nucleotides except xanthosine 5'-phosphate were obtained from the corresponding nucleosides in good yields, as is shown in Table 2.

Dichlorophosphoric acid and hydrogen chloride are known to be the major products of the partial hydrolysis of phosphoryl chloride at low temperatures according to the following equation:[16,20]

$$POCl_3 + H_2O \rightarrow HOPOCl_2 + HCl$$

However, an attempt at the phosphorylation of nucleoside with dichlorophosphoric acid alone was unsuccessful, as may be seen in the last line of Table 3.

The formation of tetrachloropyrophosphate has been reported in the reaction of phosphoryl chloride with traces of water in pyridine.[11] However, this condensation in the absence of a base has been reported to require more drastic conditions.[21]

The addition of *t*-butyl alcohol instead of water to phosphoryl chloride was also effective in facilitating the phosphorylation. In contrast with the case of water, this effect did not vary with the amount of alcohol used (Table 4). The reaction of *t*-butyl alcohol with phosphoryl chloride seems to give *t*-butyl chloride and dichlorophosphoric acid according to the following equation:

$$POCl_3 + t\text{-}C_4H_9OH \rightarrow HOPOCl_2 + t\text{-}C_4H_9Cl$$

t-Butyl chloride was isolated from the reaction mixture and identified by gas chromatography.

The decomposition of nucleosides was prevented by adding a certain organic base together with water to the medium. When three moles of water were added to the nucleoside, the addition of two-thirds of a mole of tertiary amines to the water served to prevent the acidic cleavage of nucleosides (*cf.* Table 5). Primary and secondary amines were relatively inferior to tertiary amines for this purpose because the former tended to give less reactive dichloroamidates. Excessive

20) R. F. Hudson and G. Moss, *J. Chem. Soc.*, **1962**, 3599.

21) M. Becke-Goehring and J. Sambeth, *Angew. Chem.*, **69**, 640 (1957).

Table 5. Effect of addition of amines*

Amine	Yields of products, mol%		
	5′-IMP	Hypo-xanthine	Inosine
—	55	38	4
n-Butylamine	67	17	5
Cyclohexylamine	67	22	9
Aniline	60	18	5
Di-*n*-butylamine	78	10	5
Diphenylamine	79	11	2
N-Methylaniline	80	5	7
Pyrrolidine	79	7	4
Tri-*n*-butylamine	92	3	0
Tri-*n*-propylamine	86	7	2
Triethylamine	86	8	6
N,*N*-Dimethylaniline	92	3	0
Pyridine	88	7	4
Morpholine	70	22	6
N-Methylmorpholine	91	5	0

* Experimental Condition:
Ip-HxR: 10 mmol, $POCl_3$: 170 mmol, H_2O: 30 mmol, amine: 20 mmol, temperature: 5°C, time: 6 hr

Table 6. Effect of addition of ethers*

Ether	Yields of products, mol%		
	5′-IMP	Hypo-xanthine	Inosine
Diethylether	75	16	4
Diisopropylether	78	12	5
1,4-Dioxane	65	25	4
Dimethyleneglycol dimethylether	78	23	5

* Ethers were used in place of amines in same manner as in Table 5.

quantities of amines in relation to water, however, seemed to inhibit the protonation of nucleosides and to decrease their solubilities in phosphoryl chloride.

It can be clearly seen from Table 6 that the presence of ethers in the reaction media is also effective in reducing the decomposition of nucleosides, but the effect is not as marked as those of tertiary amines.

Reactivity of 2-Substituted Purine Nucleosides to Phosphoryl Chloride. The relatively lower yields of 5′-GMP as compared with those of 5′-IMP and 5′-AMP (adenosine 5′-phosphate) in Fig. 2 were attributable to the short reaction period used so as to give maximum yields. When the addition of water to phosphoryl chloride was omitted, 2′,3′-*O*-isopropylideneguanosine was unusually inactive toward phosphoryl chloride, whereas 2′,3′-*O*-isopropylideneinosine was readily phosphorylated. The best results were obtained when 0.5—1.0 mol of water was added to a nucleoside in phosphoryl chloride as is shown in Fig. 2. 2′,3′-*O*-Isopropylidenexanthosine was less susceptible to the phosphorylation, yielding only 30% of xanthosine 5′-phosphate (5′-XMP), even after a much longer reaction period. All attempts at the phosphorylation of 2-mercaptoinosine failed.

As has been mentioned by Chambers *et al.*,[11] the lower reactivity of guanosine may be due to the intramolecular hydrogen bond between the amino group at the C-2 position in guanine and the 5′-hydroxyl group in the ribose moiety. The same reason may serve for the unusual inertness of xanthosine and 2-mercaptoinosine, which seem more likely to form a stable hydrogen bond between the 5′-hydroxyl group and the N-3 hydrogen of an amide-type structure. To verify this phenomenon, we examined the phosphorylation of some other nucleosides, the substituents of which at the C-2 position were methylated. As expected, it was found that such methylated nucleosides as 2′,3′-*O*-isopropylidene derivatives of 2-dimethylaminoinosine, 2-methoxyinosine, and 2-methylthioinosine were readily phosphorylated and gave the corresponding 5′-nucleotides in nearly quantitative yields, as is shown in Fig. 4. All of the results are consistent with the Chambers explanation.

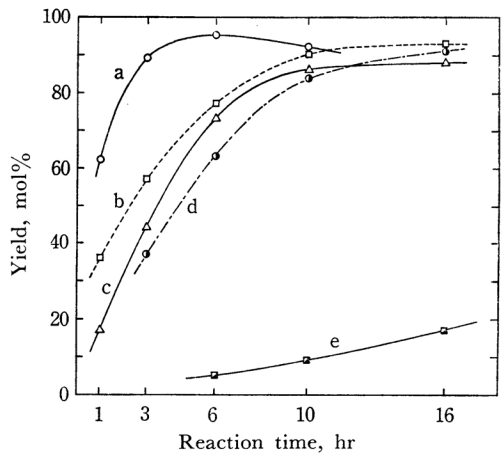

Fig. 4. Phosphorylation of 2-substituted nucleosides.

Experimental condition:
2′,3′-*O*-isopropylidene nucleoside: 2 mmol, $POCl_3$: 10 m*l*, H_2O: 0.018 m*l*, 5°C
a : 2-dimethylaminoinosine 5′-phosphate
b : 2-methoxyinosine 5′-phosphate
c : guanosine 5′-phosphate
d : 2-methylthioinosine 5′-phosphate
e : xanthosine 5′-phosphate

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