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Studies of Phosphorylation. III. Selective Phosphorylation of Unprotected Nucleosides[1)]

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During an investigation of the factors affecting the reaction of 2′,3′-*O*-isopropylidene nucleosides with phosphoryl chloride, trialkyl phosphates were found to be powerful accelerators and useful solvents for the phosphorylation. The addition of a trialkyl phosphate to a large excess of phosphoryl chloride was markedly effective in promoting the phosphorylation; further, the lower alkyl phosphates, such as trimethyl and triethyl phosphates, were able to replace the excessive amount of phosphoryl chloride as solvents. When unprotected nucleosides were treated with phosphoryl chloride in trimethyl phosphate, the corresponding 5′-phosphates were mainly produced, together with a small amount of highly-phosphorylated products, in good yields. On the contrary, the treatment of 5′-*O*-acetyl nucleosides in a similar manner gave the corresponding 2′(or 3′)-nucleotides in low yields. The formation of the 2′(or 3′)-phosphate was strongly inhibited by the addition of a small amount of water to the reaction mixture. Thus, the selective phosphorylation of unprotected nucleosides to the 5′-nucleotides in a one-step procedure was accomplished.

It has been reported, in a previous paper,[2)] that 5′-nucleotides can be conveniently prepared in high yields by the treatment of 2′,3′-*O*-isopropylidene nucleosides with a large amount of phosphoryl chloride in the absence of any organic solvent. When a small amount of water was added to posphoryl chloride prior to the reaction, the yield of nucleotides was very much increased.

A similar effect was also observed upon the addition of alcohols. The reaction of *t*-butyl alcohol with an excessive amount of phosphoryl chloride gives hydrogen phosphorodichloridate according to Eq. (1):[2)]

$$t\text{-}C_4H_9OH + POCl_3 \rightarrow t\text{-}C_4H_9Cl + HOP(O)Cl_2 \quad (1)$$

On the other hand, it has been reported that the reaction of a primary alcohol with phosphoryl chloride proceeds satisfactorily at a low temperature according to Eq. (2):[3)]

$$ROH + POCl_3 \rightarrow ROP(O)Cl_2 + HCl \quad (2)$$

Therefore, an alkyl phosphorodichloridate may be considered to function as a potential accelerator for the phosphorylation as well as hydrogen phosphorodichloridate.

In the presence of an excessive amount of an alcohol, this reaction is known to proceed further to form the dialkyl phosphorochloridate and the trialkyl phosphate. The accelerating effect of these compounds, particularly the latter, on the reaction rate has been studied and found to be positive, as will be discussed later. It has also been found that an excessive amount of phosphoryl chloride may be replaced by a trialkyl phosphate as a solvent.

The treatment of an unprotected nucleoside with phosphoryl chloride in a trialkyl phosphate gives the phosphorylated products in a high yield. Analysis of the products showed that the phosphorylation always occurred preferentially on the hydroxyl group at the 5′-position in nucleosides. The selective phosphorylation of an unprotected nucleoside with phosphoryl chloride in a trialkyl phosphate has already been briefly communicated.[1)] This paper will deal with an extensive study of the factors affecting the reaction of nucleosides with phosphoryl chloride and the phosphorylation of nucleosides in the presence of a trialkyl phosphate.

Results and Discussion

The effect of the addition of alcohols on the phosphorylation of 2′,3′-*O*-isopropylideneinosine with phosphoryl chloride is shown in Fig. 1. Although the yield of inosine 5′-phosphate was not affected by the addition of *t*-butyl alcohol, the yield was significantly decreased with an increase in the amount of methyl or of isopropyl alcohol. The disadvantage of the latter cases may be due to the production of a high concentration of hydrogen chloride according to Eq. (2). In order to eliminate the influence of hydrogen chloride, methyl phosphorodichloridate,

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2) M. Yoshikawa and T. Kato. This Bulletin, **40**, 2849 (1967).

3) G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley & Sons, New York (1950), p. 211.

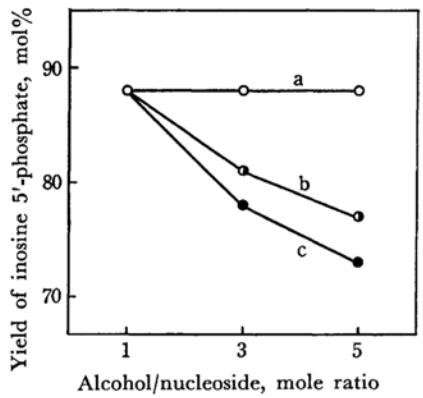

Fig. 1. Influence of addition of alcohols.
Experimental condition: 2′,3′-*O*-Isopropylideneinosine, 2.0 g; $POCl_3$, 10 m*l*; Temperature, 5°C
a: *t*-Butyl alcohol, b: *i*-Propyl alcohol, c: Methyl alcohol

Table 1. Effect of addition of methyl phosphorochloridates or trimethyl phosphate

Additive	Yield (mol%)			
	Inosine 5′-phosphate		Guanosine 5′-phosphate	
	1 hr	2 hr	2 hr	6 hr
none	26	70	7	14
CH_3OPOCl_2	44	88	12	51
$(CH_3O)_2POCl$	92	92	78	86
$(CH_3O)_3PO$	93	91	80	87

Experimental condition: 2′,3′-*O*-Isopropylidene nucleoside, 6.5 mol; $POCl_3$, 10 m*l*; Additive, 13 mmol; Temperature, 5°C

dimethyl phosphorochloridate and trimethyl phosphate were synthesized and added similarly to the phosphorylation mixture. Table 1 shows the results of the phosphorylation of 2′,3′-*O*-isopropylidene nucleosides in the $POCl_3$-$(RO)_nPOCl_{3-n}$ systems. Although the reaction of a nucleoside with methyl phosphorodichloridate scarcely proceeds at all under these conditions, the phosphorylation of a nucleoside with phosphoryl chloride was markedly promoted in the presence of the phosphorodichloridate. The increasing effect of those additives on the reaction rate is arranged in the order: $ROP(O)Cl_2 < (RO)_2P(O)Cl < (RO)_3PO$. An especially interesting result observed here was a sharp increase in the rate of phosphorylation with an increase in the amount of trimethyl phosphate. 2′,3′-*O*-Isopropylidene nucleosides were moderately soluble in anhydrous trimethyl phosphate. Thus, an excessive amount of phosphoryl chloride was advantageously replaced by trimethyl phosphate as the solvent.

In a first stage, the reaction mixture of a 2′,3′-*O*-isopropylidene nucleoside and phosphoryl chloride was a suspension in trimethyl phosphate. After the reaction had been completed, the suspension containing the corresponding 5′-phosphorodichloridate became clear. Thus, the phosphorylation reactions were successfully achieved; the results are summarized in Table 2. In the case of the xanthosine derivative, the phosphorylation was insufficiently, as in the previous method.

Table 2. Phosphorylation of 2′,3-*O*-isopropylidene nucleosides in trialkyl phosphate*

Isopropylidene nucleoside	$POCl_3$		$P_2O_3Cl_4$	
	Time hr	Yield of 5′-nucleotide mol%	Time hr	Yield of 5′-nucleotide mol%
	in $(CH_3O)_3PO$			
Inosine	2	92	0.5	95
Guanosine	1	90	1	92
Adenosine	1.5	90	2	90
Xanthosine	12	15	6	19
Uridine	14	98	6	86
Cytidine	4	98	6	94
AICAR**	4	76		
	in $(C_2H_5O)_3PO$			
Inosine	0.5	93	1	93
Guanosine	4	88	8	76
Adenosine	2	91	2	91
Uridine	14	95	9	82
Cytidine	12	94	6	89
	in $(n\text{-}C_4H_9O)_3PO$			
Inosine	6	78	2	93
Guanosine	8	25	8	54

* Experimental condition: 2′,3′-*O*-Isopropylidene nucleoside, 6.5 mmol; $POCl_3$ or $P_2O_3Cl_4$, 13 mmol; Trialkyl phosphate, 65—98 mmol; Temperature, −5°C
** 4-Amino-5-carbamoyl-1-β-D-ribofuranosylimidazole

For the reaction, triethyl phosphate and trimethyl phosphate are both suitable as solvents, but the former is less effective than the latter. The reaction rate is affected by the size of the alkyl groups in trialkyl phosphates, as is shown in Fig. 2, particularly in the phosphorylation of 2′,3′-*O*-isopropylideneguanosine with phosphoryl chloride. That is, the yield of guanosine 5′-phosphate decreases as the alkyl group becomes larger. This phenomenon is supposed to be concerned primarily with the solubility and the reactivity of the nucleoside derivative. The more reactive 2′,3′-*O*-isopropylideneinosine was well phosphorylated also in tri-*n*-butyl phosphate, but a prolonged reaction time was required for its completion in comparison with the phosphorylation in trimethyl phosphate.

In contrast with this excellent ability of trialkyl phosphates, the analogous trialkyl phosphorothioates and triaryl phosphates do not fulfil these conditions. The utilization of other polar organic

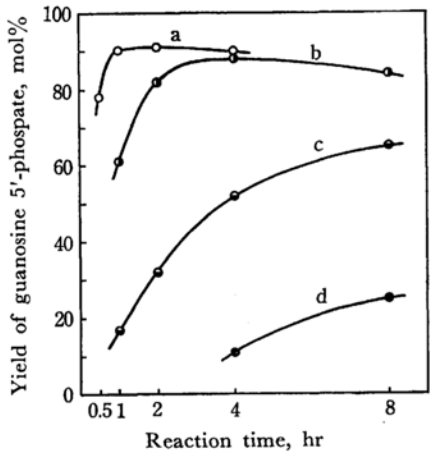

Fig. 2. Influence of alkyl group of trialkyl phosphate.
Experimental condition: 2',3'-*O*-Isopropylideneguanosine, 2.1 g; $POCl_3$, 1.2 m*l*; Trialkyl phosphate, 10 m*l*; Temperature, −5°C
a: Trimethyl phosphate, b: Triethyl phosphate, c: Tri-*β*-chloroethyl phosphate, d: Tri-*n*-butyl phosphate

solvents possessing S→O, N→O, or C=O groups, such as dialkyl sulfates, dialkyl sulfites, dialkyl sulfones, dialkyl sulfoxides, alkyl nitrates, alkyl nitrites, dialkyl carbonates, and alkyl ketones, gave generally unsatisfactory results.

Trialkyl phosphates were also attractive solvents in the phosphorylation of nucleosides with pyrophosphoryl tetrachloride. The yields of 5'-nucleotides were slightly better than those with phosphoryl chloride, particularly in the case of 2',3'-*O*-isopropylideneguanosine.

An attempt at the selective phosphorylation of unprotected nucleosides was made for the purpose of establishing the manufacturing method of 5'-nucleotides. In the previous experiments, nucleoside 5'-phosphates have been produced together with the 2'(or 3')-phosphate[4] or the 2'(or 3'), 5'-diphosphate,[5] and the yields have been reported to be low.[6] The method reported by Ikehara involving the use of 2,6-lupetidyl phosphorodichloridate is better, but it is still not satisfactory in either yield or selectivity.[7]

When treated with phosphoryl chloride in trialkyl phosphates, naturally-occurring nucleosides gave the corresponding nucleotides in nearly quantitative yields without any prior protection of the 2'- and 3'-hydroxyl groups; the products consisted mainly of the 5'-phosphates, accompanied by a small amount of such highly-phosphorylated products as the 2'(or 3'),5'-diphosphate and 2',3',5'-triphosphate (Table 3). Thus, this reaction proceeds selectively at the 5'-position. The competitive phosphorylation of the 2'(or 3')-hydroxyl group was strongly inhibited in the acidic medium; this was easily achieved by the addition of an adequate amount of water to the mixture, as is shown in Table 3. This result is of interest in comparison with the result reported by Barker and Foll[4] that the addition of a small amount of water tends to decrease the mole ratio of the 5'-phosphate in the products of the phosphorylation of adenosine with phosphoryl chloride in pyridine. As another example, 5'-*O*-acetylinosine was phosphorylated to give the corresponding 2'(or 3')-nucleotide in only a 22% yield without any prior treatment with water; moreover, it did not react under the acidic conditions, as has been described above.

Thus, the synthesis of 5'-nucleotides was emancipated from such complicated treatments as the prior protection of the nucleoside sugar, the extreme dehydration of the solvent required in the case of pyridine, or the dissolution of a nucleoside in a solvent before the phosphorylation.

It should be noted that xanthosine, which is generally unaffected by any chemical phosphorylation, was easily converted to the corresponding 5'-phosphate. Thus, this method can be applied generally to the synthesis of naturally-occurring ribonucleotides and offers a convenient procedure for industrial purposes.

In comparison with phosphoryl chloride, pyrophosphoryl tetrachloride yielded large amounts of the 2'(or 3'),5'-di- and 2',3',5'-triphosphates together with the 5'-phosphate because of its stronger reactivity. In this case, the highly-phosphorylated products were proved to be *O*-phosphate in the sugar moiety by comparison with the ultraviolet absorption spectra of the corresponding 5'-phosphate. The improvement of this technique may make an effective synthesis of a nucleoside diphosphate possible.

For the synthesis of deoxynucleoside 5'-phosphates, the phosphorylation of 3'-*O*-acetyl 2'-deoxynucleosides has been adopted, usually *via* 3'-*O*-acetyl 5'-*O*-trityl 2'-deoxynucleosides.[8] The direct treatment of thymidine with dibenzyl phosphorochloridate, followed by hydrogenolysis, has been reported to produce mainly thymidine 3',5'-diphosphate, together with some 5'-phosphate.[9] However, the phosphorylation of deoxynucleosides at a lower temperature, carried out by the use of the method described above, gave mainly the cor-

4) G. R. Barker and G. E. Foll, *J. Chem. Soc.*, **1957**, 3798.
5) F. Cramer, G. W. Kenner, N. A. Hughes and A. R. Todd, *ibid.*, **1957**, 3297.
6) J. M. Gulland and G. I. Hobday, *ibid.*, **1940**, 746.
7) M. Ikehara, E. Ohtsuka and Y. Kodama, *Chem. Pharm. Bull. (Tokyo)*, **11**, 1456 (1963).
8) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, **1953**, 951.
9) C. A. Dekker, A. M. Michelson and A. R. Todd, *ibid.*, **1953**, 947.

Table 3. Direct phosphorylation of unprotected nucleosides in trialkyl phosphate

Nucleoside	$POCl_3$					$P_2O_3Cl_4$			
	R of $(RO)_3PO$	H_2O mmol	Time hr	Yield of nucleoside-phosphate mol% 5′-mono	higher	R of $(RO)_3PO$	Time hr	Yield of nucleoside-phosphate mol% 5′-mono	higher
Inosine	CH_3CH_2	0	2	68	18	CH_3	1	54	46
Inosine	CH_3CH_2	2.0	2	91	8				
Guanosine	CH_3	0	6	85	9	CH_3	4	59	41
Guanosine	CH_3	2.0	6	90	5				
Adenosine	CH_3CH_2	0	6	84	11	CH_3CH_2	4	57	33
Xanthosine	CH_3	0	9	80	5	CH_3	12	64	22
Uridine	CH_3	0	12	89	8	CH_3	6	73	25
Cytidine	CH_3CH_2	0	1	88	8	CH_3	0.5	86	13
Deoxyinosine	CH_3	0	5	73	6				
Deoxycytidine	CH_3	0.5	6	64	19				
AICAR	CH_3	0	4	91	5				

Experimental condition: Nucleoside, 2 mmol; $POCl_3$ or $P_2O_3Cl_4$, 4 mmol (6 mmol in the case of inosine and guanosine); Trialkyl phosphate, 5 m*l*, Temperature, 0°C

responding 5′-phosphates (Table 3).

Most nucleosides are fairly soluble in trimethyl and triethyl phosphates, while those nucleosides are insoluble or only slightly soluble in pyridine, which has been used hitherto. The effect of trialkyl phosphates may be, at least partly, due to the higher solubility of nucleosides in these phosphates. This idea is supported by the difficulty of the phosphorylation in tri-*n*-butyl phosphate, in which the solubility of nucleosides is fairly low. Interaction between a trialkyl phosphate and the phosphorylating reagent may also play a role. It is considered that the interaction between a trialkyl phosphate and phosphoryl chloride is possible; an ionized structure may be formed according to the following equation:

$$(RO)_3PO + POCl_3 \rightarrow [(RO)_3P\text{-}O\text{-}\overset{\overset{O}{\|}}{P}Cl_2]^+ \; Cl^-$$

The ionized structure may be postulated as an active phosphorylating agent.

Experimental

Identification of the Products on the Paper Chromatograms. The location of the nucleoside phosphate on the chromatograms was detected by means of ultraviolet light, and it was identified by a comparison of the R_f value with that of an authentic sample. Ribonucleoside diphosphate was confirmed further by a negative periodate-benzidine test, and deoxyribonucleoside phosphate by a Hanes and Isherwood spray.[10]

Phosphorylation of 2′,3′-O-Isopropylidene Nucleoside. *Method A.* Into a cold mixture of freshly-distilled phosphoryl chloride and an alcohol or some other additive, such as methyl phosphorodichloridate, dimethyl phosphorochloridate, or trimethyl phosphate, a 2′,3′-*O*-isopropylidene nucleoside was added with stirring. After a predetermined period the reaction mixture was poured into ether, and then the precipitate was separated by centrifuge. The product was dissolved carefully in 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. The resulting solution was analyzed by the paper chromatographical method described in a previous report.[2]

Method B. After a few moles of phosphoryl chloride (or pyrophosphoryl tetrachloride) per mole of a nucleoside had been mixed with a trialkyl phosphate, a 2′,3′-*O*-isopropylidene nucleoside was added to the cold mixture and the mixture was stirred. The reaction mixture was then treated in the same way as in Method A.

Selective Phosphorylation of an Unprotected Nucleoside. An unprotected nucleoside was phosphorylated according to Method B. An adequate amount of water was added to a mixture of phosphoryl chloride and a trialkyl phosphate before the addition of a nucleoside. The phosphorylation mixture was hydrolyzed in ice water after ether treatment and was then immediately analyzed by the paper chromatographical method.

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10) C. S. Hanes and F. A. Isherwood, *Nature*, **164** 1107 (1949).