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145. **Mikio Honjo, Yoshiyasu Furukawa, and Kunio Kobayashi :**

Studies on Phosphorylation. II.*[1] Phosphorylation of Nucleosides with Organic Amine Salts of Phosphoric Acid.

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A number of phosphorylation methods of nucleosides by the use of activated phosphoric acid derivatives have been reported, but the phosphorylation with orthophosphate has so far been effected only biologically using yeast[1] or bacteria.[2] Bailly[3] reported the synthesis of glycerol monophosphate by heating glycerol with sodium dihydrogen phosphate under reduced pressure. We tried to apply this method to the phosphorylation of nucleosides.*[3]

When a mixture of isopropylidene inosine (referred to as Ip·HR) and sodium dihydrogen phosphate was heated at about 280° with stirring, it decomposed without melting. The use of sodium monohydrogen phosphate in place of sodium dihydrogen phosphate went into the same result. When Ip·HR was heated with sodium dihydrogen phosphate in DMF,*[4] isopropylidene IMP was formed in ca. 2% yield as determined by paper electrophoresis, but change of the solvent to *o*-chlorophenol (or DMSO) or change of the phosphate to potassium dihydrogen (or monohydrogen) phosphate brought about no phosphorylation. The failure of phosphorylation may be attributed to the low solubility of these phosphates. If this is the case, the use of more soluble organic amine salts of phosphoric acid might be more promising. In fact, the reaction of Ip·HR with mono(tri-*n*-butylammonium)phosphate in DMF afforded isopropylidene IMP in ca. 40～60% yield as determined by paper electrophoresis. Increased amount of the phosphate and prolonged reaction time raised the yield of phosphorylation to a certain extent, the use of *m*-cresol as solvent lowered the yield, and the use of *o*-chlorophenol

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*[1] Part I. K. Imai, T. Hirata, M. Honjo : Ann. Rept. Takeda Res. Lab., **23**, 1 (1964).

*[2] Juso-nishino-cho, Higashiyodogawa-ku, Osaka (本庄美喜男, 古川純康, 小林邦夫).

*[3] After completion of the present investigation, Ponnamperuma and Mack reported the syntheses of nucleotides by heating nucleosides with inorganic phosphates and the maximum yield of monophosphate was 20.6% (C. Ponnamperuma, R. Mack : Science, **148**, 1221 (1965)).

*[4] DMF : N,N-dimethylformamide, IMP : inosine monophosphate, DMSO : dimethylsulfoxide, DCC : dicyclohexylcarbodiimide, IDP : inosine diphosphate, UMP : uridine monophosphate, CMP : cytidine monophosphate, AMP : adenosine monophosphate, TMP : thymidine monophosphate, TDP : thymidine diphosphate.

1) P. Ostern, J. Terszakowec : Z. physiol. Chem., **250**, 155 (1937).

2) S. Sugiyama, T. Gomei : Abstracts of Papers, the Ann. Meeting of the Agr. Chem. Soc. of Japan (April, 1964), p. 185.

3) O. Bailly : Ann. Chim., (9) **6**, 96 (1916).

Table I. Phosphorylation of Ip·HR
Ip·HR : 0.1 mmole, Temp. : Reflux Phosphate :
Mono (tri-*n*-butylammonium) phosphate

No.	Phosphate (mmole)	Solvent (ml.)	Time (hr.)	% Phosphorylation
1	0.25	DMF (1)	6	40
			9	43
2	0.5	DMF (2)	2	47
			4	50
			6	58
3	0.5	DMF (1)	2	53
4	1.0	DMF (1)	2	57
5	0.5	*m*-Cresol (2)	2	21
6	0.5	*o*-Chlorophenol (2)	2	0
7	0.5	DMSO (2)	2	0

Ip·HR : Isopropylidene inosine

Table II. Phosphorylation of Ip·HR with Various Phosphates
Ip·HR : 0.1 mmole, Phosphate : 0.5 mmole, DMF : 1 ml.,
Time : 2 hr. (Reflux)

No.	Phosphate	% Phosphorylation
8	Di (tri-*n*-butylammonium) phosphate	66
9	Monoanilinium phosphate	31
10	Mono (triethylammonium) phosphate	45

Table III. Effect of Dehydrating Agents and Other Additives on Phosphorylation of Ip·HR
Ip·HR : 0.1 mmole, DMF : 1 ml., Mono (tri-*n*-butylammonium)
phosphate : 0.5 mmole, Time : 2 hr. (Reflux)

No.	Additive (mg.)	Phosphorylated products[a] (%)	
2	none	5′-IMP (47)	
11	$ZnCl_2$ (100)	0	
12	$CuSO_4$ (100)	5′-IMP (46)	
13	Mg_2SO_4 (100)	5′-IMP (5)	
14	P_2O_5 (50)	0	
15	benzonitrile (100)	5′-IMP (56),	5′-IDP (9)
16	DCC (100)	5′-IMP (45),	5′-IDP (24)
17	phenylisocyanate (100)	5′-IMP (64),	5′-IDP (11)

[a] Isopropylidene group was removed as described under Experimental.

or DMSO effected no phosphorylation (Table I). Di(tri-*n*-butylammonium) phosphate also gave good yield of the phosphorylation, but monoanilinium- or mono(triethylammonium)phosphate reduced the yield (Table II). With the aim of improving yield, addition of dehydrating agents such as zinc chloride, cupric sulfate, magnesium sulfate or phosphorus pentoxide was studied, but the yield was rather poor. Addition of benzonitrile, DCC, or phenylisocyanate resulted in the formation of 5′-IDP besides 5′-IMP (Table III), and the reaction of di(tri-*n*-butylammonium)pyrophosphate or tri-*n*-butylammonium polyphosphate with Ip·HR produced 5′-IMP and 5′-IDP (Table IV).

Table IV. Phosphorylation of Ip·HR with Pyro- and Polyphosphates
Ip·HR : 0.1 mmole, Phosphate : 0.5 mmole,
DMF : 1 ml., Time : 2 hr. (Reflux)

No.	Phosphate	Phosphorylated products[a] (%)
18	di (tri-*n*-butylammonium) pyrophosphate	5′-IMP (64), 5′-IDP (21)
19	tri-*n*-butylammonium polyphosphate[b]	5′-IMP (64), 5′-IDP (27)

[a] See Table III.
[b] Polyphosphoric acid : Approx. $H_6P_4O_{13}$ (Wako Pure Chem. Ind., Ltd.)

Reaction of inosine with mono(tri-*n*-butylammonium)phosphate in DMF produced 2′(3′)-IMP, 5′-IMP and 2′(3′),5′-IDP in the yield of 20%, 10%, and 20%, respectively, but addition of metaboric acid[*1] or boron trioxide for protecting 2′- and 3′-hydroxyl groups gave no satisfactory results.

Isopropylidene-adenosine, -guanosine, -cytidine, -uridine or thymidine was phosphorylated with mono(tri-*n*-butylammonium)phosphate in DMF. It is interesting to note that nucleosides having a hydroxyl group at their purine or pyrimidine moiety are more easily phosphorylated than those having an amino group at the corresponding position. Phosphorylation of isopropylidene cytidine followed by removal of the protecting group produced 5′-UMP besides 5′-CMP (Table V). This may be due to the deamination of cytosine moiety during the reaction in DMF. The resulting 5′-AMP, -IMP, and -CMP were all isolated and identified with authentic samples after purification by ion-exchange chromatography. TMP formed by phosphorylation of thymidine was found to contain 60% of 5′-TMP and 40% of 3′-TMP as determined by the assay with bull semen 5′-nucleotidase.

Table V. Phosphorylation of Various Nucleosides
Nucleoside : 0.1 mmole, DMF : 1 ml., Mono (tri-*n*-butylammonium) phosphate : 0.5 mmole, Time : 2 hr. (Reflux)

No.	Nucleoside	Phosphorylated products[a] (%)
20	Ip-inosine	5′-IMP (53)
21	Ip-uridine	5′-UMP (58)
22	Ip-guanosine	5′-GMP (46)
23	Ip-adenosine	5′-AMP (38)
24	Ip-cytidine	5′-CMP (39), 5′-UMP (8)
25	thymidine	TMP (41), 3′, 5′-TDP (40)

[a] See Table III.
Ip- : Isopropylidene-

Deoxyadenosine, deoxyinosine, and deoxycytidine were not phosphorylated under similar conditions and decomposed to give the corresponding bases.

Studies on phosphoryation of nucleosides using labelled phosphates are now in progress.

Experimental

Method——Paper electrophoresis (PE) was carried out on Whatman filter paper No. 1 at 22 v./cm. for 1 hr. using following buffers. A : 0.05*M* borate buffer (pH 9.2), B : 0.05*M* phosphate buffer (pH 7.5), C : 0.05*M* citrate buffer (pH 3.7).

Phosphorylation of Ip·HR——A mixture of Ip·HR and the phosphate in the solvent was refluxed and subjected to PE (A). The UV-absorbing spot corresponding to isopropylidene-IMP (R_{IMP}[*5]$=0.75$) was eluted

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[*5] R_{IMP} : mobility ratio to 5′-IMP.

from the filter paper with 1*N* NH_4OH and the amount of isopropylidene-IMP was determined spectrophotometrically at 260 mμ. The results were given in Table I, II.

Identification of 5′-IDP——From the each reaction mixture of the experiments No. 15~19 (Table III, IV), DMF was evaporated *in vacuo* respectively, and the residue was dissolved in H_2O and applied to a column of Dowex-1 (Cl^-; 5 ml.). After washing with H_2O, the column was eluted with 0.06*N* HCl to give two UV-absorbing fractions. The second fraction was heated at 70° for 30 min. to remove isopropylidene group and desalted by treating with activated charcoal. The substance thus obtained was identified as 5′-IDP from the following evidences. i) PE (B, C) gave a single UV-absorbing, periodate-benzidine[4] positive spot corresponding to 5′-IDP. ii) Total P/inosine = 1.95~2.10, labile P/inosine = 0.90~1.05. iii) The UV-spectra of the substance at pH 2, 7, 12 were identical with those of inosine.

Phosphorylation of Various Nucleosides——A mixture of nucleoside (0.1 mmole) and $Bu_3N \cdot H_3PO_4$ (0.5 mmole) in DMF (1 ml.) was refluxed for 2 hr. and DMF was evaporated *in vacuo*. The aqueous solution of the residue was applied to a column of Dowex-1 (Cl^-, 5 ml.) and washed with H_2O, and the yield of the product was calculated from TOD[*6] of the nucleotide fraction which was eluted with 0.06*N* HCl. Each nucleotide was heated at 70° for 30 min. to remove isopropylidene group and identified as 5′-nucleotide by PE (A, C) after desalting with activated charcoal.

Isolation of 5′-IMP——A mixture of Ip·HR (155 mg., 0.5 mmole) and $Bu_3N \cdot H_3PO_4$ (5 mmoles) in DMF (10 ml.) was refluxed for 5 hr. and DMF was evaporated *in vacuo*. The residue was dissolved in H_2O (50 ml.), adjusted to pH 1.5 with HCl, heated at 70° for 30 min., desalted with activated charcoal (2 g.), and applied to a column of Dowex-1 ($HCOO^-$, 5 ml.). After washing with H_2O, the column was eluted with 1*N* HCOOH (300 ml., TOD 2250, 63% yield), and the eluate was desalted with activated charcoal (2 g.) and concentrated. EtOH was added to the concentrated solution to give a white crystalline powder (120 mg., UV purity 80%, 52% yield). The product gave a single UV-absorbing spot corresponding to 5′-IMP on PE (A, C) and its UV-spectra at pH 2, 7, 12 were identical with those of inosine.

Isolation of 5′-AMP——A mixture of isopropylidene adenosine (155 mg., 0.5 mmole) and $Bu_3N \cdot H_3PO_4$ (5 mmoles) in DMF (10 ml.) was refluxed for 5 hr. and the mixture was worked up as described above for 5′-IMP and applied to a column of Dowex-1 ($HCOO^-$, 5 ml.). After washing with H_2O, the column was eluted with 0.1*N* HCOOH (300 ml., TOD 2950, 41% yield), and the eluate was concentrated to ca. 50 ml. *in vacuo* and acetone (250 ml.) was added to give colorless needles, m.p. 190~195° (65 mg., 38% yield). The product gave a single UV-absorbing spot corresponding to 5′-AMP on PE (A, C) and its UV-spectra at pH 2, 7, 12 were identical with those of adenosine.

Isolation of 5′-CMP——A mixture of isopropylidene cytidine (125 mg., 0.45 mmole) and $Bu_3N \cdot H_3PO_4$ (4.5 mmoles) in DMF (10 ml.) was refluxed for 5 hr. and the reaction mixture was worked up as described above for 5′-IMP and applied to a column of Dowex-1 ($HCOO^-$, 5 ml.). After washing with H_2O, the column was eluted with 0.01*N* HCOOH (300 ml., TOD 1300, 41% yield), and the eluate was evaporated to dryness *in vacuo*. The residue was dissolved in H_2O (1 ml.), and EtOH (1 ml.) was added to give colorless needles, m.p. 228~230° (decomp.) (55 mg., 35% yield). The product gave a single UV-absorbing spot corresponding to 5′-CMP on PE (A, C) and its UV-spectra at pH 2, 7, 12 were identical with those of cytidine. The ion-exchange column was further eluted with 0.5*N* HCOOH (300 ml., TOD 500, 10% yield), and the eluate was evaporated to dryness *in vacuo*. The residue showed a single UV-absorbing spot corresponding to 5′-UMP on PE (A, C) and its UV-spectra at pH 2, 7, 12 were identical with those of uridine.

Phosphorylation of Thymidine——A mixture of thymidine (25 mg., 0.1 mmole) and $Bu_3N \cdot H_3PO_4$ (0.5 mmole) in DMF (10 ml.) was refluxed for 2 hr. and DMF was evaporated *in vacuo* from the reaction mixture. The residue was dissolved in H_2O and applied to a column of Dowex-1 ($HCOO^-$, 5 ml.), which, after washing with H_2O, was eluted with 0.08*M* ammonium formate (pH 4.5) (200 ml., TOD 370, 41% yield) and then with 0.2*M* ammonium formate (pH 4.5) (300 ml., TOD 310, 40% yield). Both fractions were desalted with activated charcoal and subjected to PE (C) to give a single spot corresponding to TMP and TDP, respectively, and their UV-spectra at pH 2, 7, 12 were identical with those of thymidine. The first fraction was treated with bull semen 5′-nucleotidase and the digest determined by PE (B) to contain 60% of thymidine. The second fraction was identified as 3′,5′-TDP from the following evidences. Total P/thymidine = 2.05, labile P/thymidine = 0.0.

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Summary

Inosine 5′-phosphate has been synthesized by heating isopropylidene-inosine with mono(or di)(tri-*n*-butylammonium)-, mono(triethylammonium)-, or monoanilinium

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[*6] TOD = optical density at 260 mμ × ml.

4) M. Viscontini, D. Hoch, P. Karrer: Helv. Chim. Acta, 38, 642 (1955).

phosphate in dimethylformamide followed by removal of the protecting group. Similarly, from isopropylidene-adenosine, -guanosine, -cytidine and -uridine, the corresponding 5′-nucleotides were obtained and from thymidine, its mono- and di-phosphate. Furthermore, inosine 5′-phosphate and 5′-diphosphate were prepared by heating isopropylidene inosine with tri-*n*-butylammonium-pyrophosphate or -polyphosphate.

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146. Takenari Nakagome, Akira Kobayashi, Atsuko Misaki, Toshiaki Komatsu[*1], Toru Mori, and Seiichi Nakanishi[*2]: Synthesis of Pyridazine Derivatives. XI.[*3] Synthesis of N^1-4-Pyridazinylsulfanilamide Derivatives.[*4]

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In an earlier paper1) of this series, the synthesis of a series of N^1-4-pyridazinylsulfanilamides including N^1-(3,6-dimethoxy-4-pyridazinyl)sulfanilamide1,2) (XII) was reported. A superior chemotherapeutic activity3) of XII prompted further study on the synthetic procedure1) of XII. The present paper describes the new finding which have been revealed in the course of the study.

It has already been reported1) that 4-amino-3,6-dichloropyridazine (I), on treatment with one molecular proportion of sodium methoxide or caustic alkali in methanol, yielded 3-methoxy compound (II) in good yield, and with an excess of the reagent at an elevated temperature it gave two products, *i.e.*, 4-amino-3,6-dimethoxypyridazine (III) and an alkali-soluble by-product which afforded 4-amino-6-chloro-3(2*H*)pyridazinone (IV) after recrystallization from water.

Thin-layer chromatographic study of the crude alkali-soluble product showed an additional spot besides (IV). It was found possible to separate the crude mixture, by fractional acidification of the alkaline solution of the mixture, with the result that less acidic 4-amino-6-methoxy-3(2*H*)pyridazinone (V), m.p. 276~277° was obtained in addition to the known 4-amino-6-chloro-3(2*H*)pyridazinone (IV), m.p. 300~301°, in a ratio of 1:9. Structural elucidation of V is dealt with later. By heating with methyl alcoholic sodium methoxide or caustic alkali, V was obtained from III, but not from IV. These experiments indicate that mechanisim of the reaction is such that II is first formed from I, and then the replacement of chlorine atom and the demethylation in II take place side by side to yield III and IV respectively, and subsequently part of III suffers cleavage of methoxy group at 3 position to give V.

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[*3] Part X. T. Nakagome: Yakugaku Zasshi, **83**, 934 (1963).

[*4] Presented at the 85th Annual Meeting of the Pharmaceutical Society of Japan at Tokushima, Oct. 28, 1965.

1) Part VI. T. Nakagome, T. Hayama, T. Komatsu, Y. Eda: Yakugaku Zasshi, **82**, 1103 (1962).

2) M. Yanai, T. Kuraishi, T. Kinoshita: *ibid.*, **81**, 708 (1961).

3) Presented at the 9th Annual Meeting of the Japan Society of Chemotherapy. Chemotherapy, **9**, 411 (1961) (abstract).