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211. Morio Ikehara and Eiko Ohtsuka : Studies on Coenzyme Analogs. XIX.*1 Further Investigations of Phosphorylation Using Morpholinophosphorodichloridate and P^1-Diphenyl P^2-Morpholino Pyrophosphorochloridate.

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As discussed in the preceding paper,[1] it is of interest to attempt the phosphorylation of the unprotected nucleoside with the known phosphorylating agent, P^1-diphenyl P^2-morpholino pyrophosphorochloridate[2] in order to obtain the object material in much higher yield without complicated protection procedures. On the other hand to increase the phosphorylating power of morpholinophosphorodichloridate[3] would be necessary to raise the yield of the object material.

In this report, i) the phosphorylation of 9-erythrityladenine[4] and adenosine with P^1-diphenyl P^2-morpholino pyrophosphorochloridate and ii) the phosphorylation of isopropylideneadenosine by the use of Vilsmeier complex[5] derived from morpholinophosphorodichloridate and DMF*3 were described. Furthermore, in order to obtain a reference compound, the synthesis of 2′,3′-O-isopropylideneadenosine 5′-triphosphate by the usual procedure was also described.

9-Erythrityladenine was chosen as the starting material, because it was rather difficult to obtain 2′,3′-di-O-acetyl derivative by usual procedures. The phosphorylation on 4′-hydroxyl group would be much easier than the phosphorylation of 5′-hydroxyl of natural nucleoside, because the terminal hydroxyl on long-chain erythrityl moiety is more freely accessible for the attacking species than the sterically hindered 5′-hydroxyl of furanose ring.[6]

The reaction was carried out in dioxane solution by an analogous manner as described in the earlier report.[2] Due to the low solubility of erythrityladenine, the

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*1 Part XVIII. M. Ikehara, E. Ohtsuka, Y. Kodama : This Bulletin, **11**, 1456 (1963).

*2 Kita 12-jo, Nishi 5-chome, Sapporo (池原森男, 大塚栄子).

*3 Following abbreviations were used : DMF, dimethylformamide; AMP, adenosine monophosphate; DP, diphosphate; TP, triphosphate.

1) M. Ikehara, E. Ohtsuka, Y. Kodama : This Bulletin, **11**, 1456 (1963).
2) M. Ikehara, E. Ohtsuka : *Ibid.*, **10**. 997 (1962).
3) *Idem* : *Ibid.*, **11**, 435 (1963).
4) *Idem* : *Ibid.*, **11**, 1095 (1963).
5) A. Vilsmeier, E. Haack : Ber., **60B**, 119 (1927).

6) Though the difference of reactivity of these hydroxyl groups was not clearly proposed, it seems to be significant that the 5′-hydroxyl group of natural nucleoside is hindered sterically, in certain extent, by the neighboring base moiety. This effect may be appeared in debenzoylation of 9-(2′, 3′,5′-tri-O-benzoyl)-β-D-ribofuranosyl-6-mercaptopurine.[7]

7) J. J. Fox, I. Wempen, A. Hampton, I. L. Doerr : J. Am. Chem. Soc., **80**, 1669 (1958).

reaction time was prolonged to four days. At the end of the reaction, four spots corresponding to erythrityladenine (I), erythrityladenine monophosphoromorpholidate (II), diphosphoromorpholidate (III) and diphenyl phosphate were revealed by paper electrophoresis. In order to decompose phosphoromorpholidate *via* cyclic phosphates,[8] the reaction mixture was treated with *N* lithium hydroxide for 18 hours at room temperature. By this procedure the spots corresponding to II and III were diminished and the new spots having R_{AMP} 1.00 and 1.22 appeared. Chromatography on ion-exchanger column gave

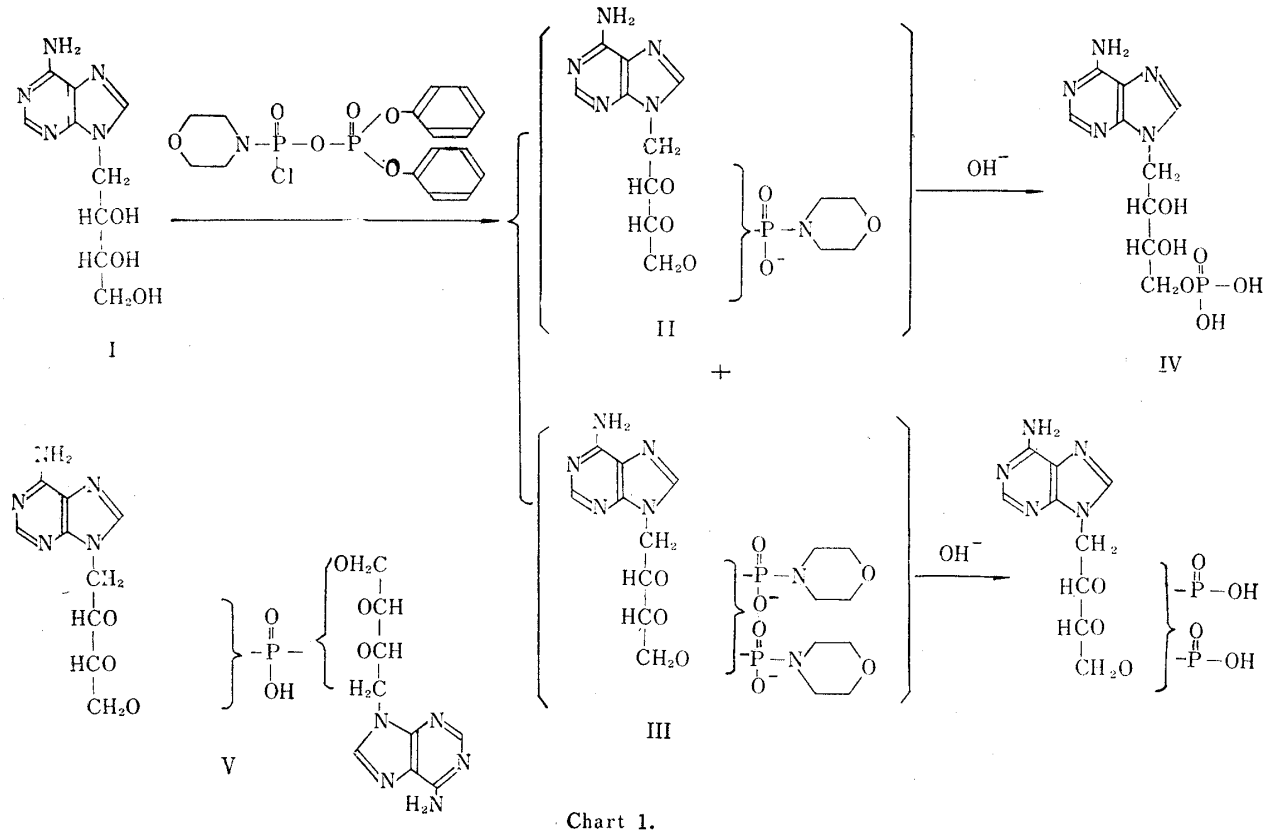

Chart 1.

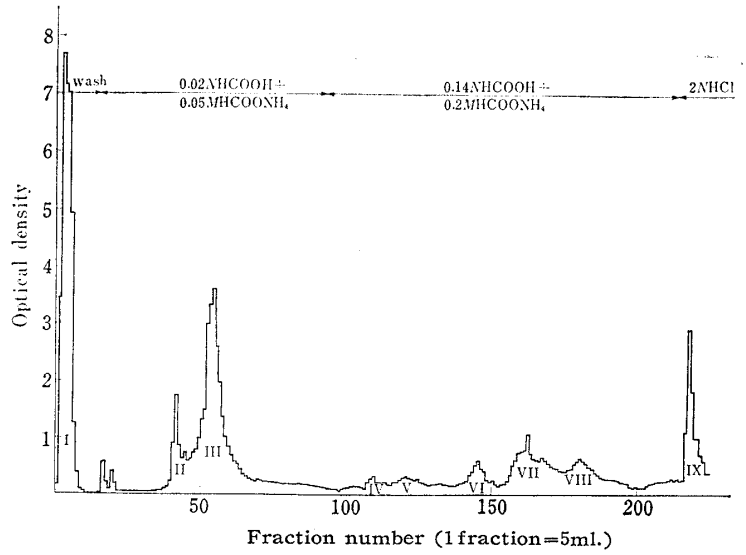

Fig. 1. Pattern of Chromatography of Phosphorylation of Erythrityladenine

8) T. Ueda: This Bulletin, 8, 459 (1960)

27% of 9-D-erythrityladenine, 3.4% of di-(9-D-erythrityladenine)-phosphate (V), 23% of 9-D-erythrityladenine monophosphate (IV), 22% of diphosphates and 11% of higher phosphates. The pattern of the chromatography was shown in Fig. 1.

Monophosphate (IV) showed a single spot having Rf 0.39 on paper chromatogram in the solvent system, which was able to separate 2'- and 3'-AMP[9] (see Experimental). The position of phosphate group was assumed to be on 4'-hydroxyl group, because analogouscleavage of cyclic phosphate[10,11] occurred on secondary hydroxyl group. This conclusion was further supported by the direct comparison with a sample obtained in the phosphorylation of 2',3'-di-O-acetylerythrityladenine.[4]

The reaction of adenosine with P^1-diphenyl P^2-morpholino pyrophosphorochloridate was investigated as follows. In this case, in order to increase the solubility of adenosine, dioxane-DMF mixture was used. 48 hours' reaction at 20° gave 7.3% of adenosine, 1.8% of 5'-AMP-morpholidate, 3.5% of 5'-AMP, 1.9% of 2'-AMP, 4.6% of 3'-AMP and 17.4% ADP. The ratio of 5'-:2'-:3'-AMP was 45:16:39. From the results obtained in these experiments it was deduced that i) P^1-diphenyl P^2-morpholino pyrophosphorochloridate attack the primary hydroxyl group less specifically than lupetidylphosphorodichloridate[1] and ii) the phosphorylation occurred more specifically on the terminal hydroxyl group of long-chain sugar of erythrityladenine than on that of ribofuranosyl ring of adenosine.

The use of DMF as the reagent, which forms the Vilsmeier complex with phosphorylating agent, was investigated next. When equimolar amounte of DMF and morpholinophosphorodichloridate were mixed together, a complex salt was obtained as a white solid with simultaneous evolution of heat. Though the reaction of this salt with isopropylideneadenosine in dioxane gave six spots by paper electrophoresis, the extent of the reaction was extremely low, even after four days' reaction at room temperature. Then the solvent was changed to acetonitrile as described by Cramer.[12] After the overnight reaction at room temperature, ca. 50% of phosphorylation was attained. The reaction of this mixture with bis(tributylammonium)pyrophosphate in the presence or the absence of the additional DMF (1 equivalent of phosphorylating agent) was investigated. In the former case, 63 hours' reaction at room temperature gave much higher yield concerning with isopropylidene-ATP. Results were summarized in Table I together

Table I.

DMF	Isp-Ad (%)	Isp-AMP (%)	Isp-ADP (%)	Isp-ATP (%)
+	34	9	34	23
−	39	8	36	17

with that performed without addition of DMF. These results may be explained by assuming a disproportionation reaction of the resulting isopropylidene-ATP, which proceeded more rapidly in the absence of additional DMF.

In order to identify the resulting isopropylidene-ATP obtained in the experiments described above, 2',3'-O-isopropylideneadenosine was phosphorylated with P^1-diphenyl P^2-morpholino pyrophosphorochloridate by an usual procedure.[2] The structure of isopropylidene-ATP was confirmed by photometrical and elementary analyses and compared directly with those obtained above by paper electrophoresis and paper chromatography (see Experimental).

9) R. Markham, J.D. Smith: Biochem. J., **49**, 401 (1951).
10) R. Kuhn, H. Rudy: Ber., **68**, 383 (1935); R. Kuhn, H. Rudy, F. Weygand: *Ibid.*, **69**, 1543 (1936).
11) H.S. Forrest, A.R. Todd: J. Chem. Soc., **1950**, 3295.
12) F. Cramer, M. Winter: Chem. Ber., **94**, 989 (1961).

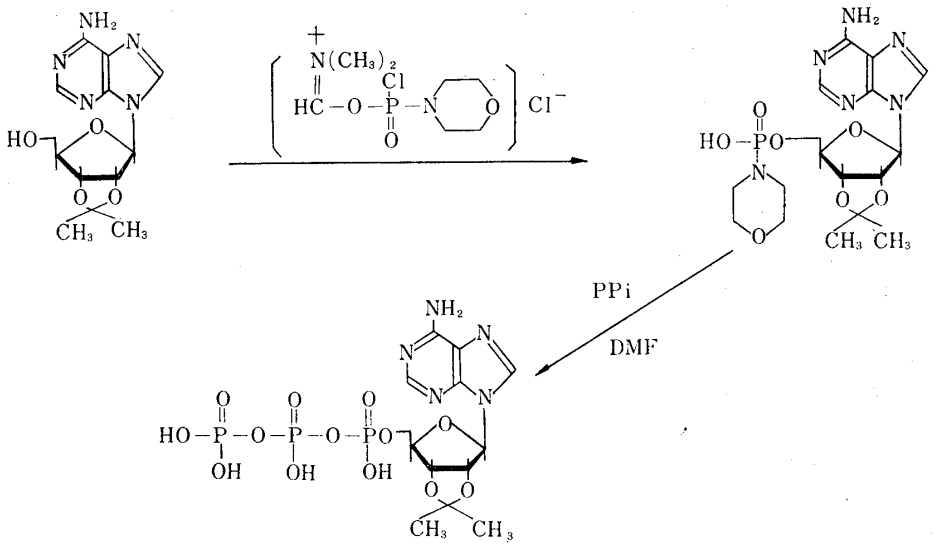

Chart 2.

Experimental

Phosphorylation of 9-D-Erythrityladenine——Morpholinophosphorodichloridate (41 mg., 0.2 m mole), diphenylphosphate (50 mg., 0.2 m mole) and 2,6-lutidine (43 μl., 0.6 m mole) were mixed and set aside for 15 min. under exclusion of moisture. Into a solution of P^1-diphenyl P^2-morpholino pyrophosphorochloridate thus obtained, 9-D-erythrityladenine (48 mg., 0.2 m mole) was added and the whole solution was kept in standing at room temperature for 48 hr. At this stage a large amount of 9-D-erythrityladenine remained unreacted. The reaction was continued for further 4 days under occasional shaking. An aliquot examined by paper electrophoresis (0.05 mole triethylammonium bicarbonate, pH 7.5, 20 v./cm., 1 hr.) 4 spots having R_{AMP} 0.00(I), 0.37(II), 0.70(diphosphate) and 1.00(III) were revealed by UV irradiation. Into the whole solution *N* LiOH (1 ml.) was added and the solution was set aside at room temperature for 18 hr. The spot (II) diminished after this treatment and new spots having R_{AMP} 1.00 (monophosphate) and 1.22 (diphosphate) were detected. The solution was adjusted to pH 1 with *N* HCl and diphenylphosphate was removed by the extraction with $CHCl_3$. pH was adjusted again to 8.5 and the solution was applied to a column of Dowex 1-X8 (formate, 100~200 mesh, 1.1×23 cm.). Each fractions were collected, evaporated *in vacuo*, and examined by paper electrophoresis. Results were shown in the following Table.

Table II.

Peak	Eluting buffer	TOD_{260}	Yield (%)		R_{AMP}	Compound
I	H_2O	640	27		0.00	Erythrityl-A
II	0.02*N* HCOOH + 0.05*M* $HCOONH_4$	80	3.4		0.32	Diester
III	〃	546	23		1.00	MP
IV	0.14*N* HCOOH + 0.2*M* $HCOONH_4$	26	1.1		—	
V	〃	60	2.6		—	
VI	〃	84	3.6,	22	—	DP
VII	〃	266	11		—	
VIII	〃	118	5.1		—	
IX	2*N* HCl	256	11		—	Higher P

9-D-Erythrityladenine 4′-MP showed Rf 0.39 by paper chromatography (solvent, saturated $(NH_4)_2SO_4$-H_2O-iso-PrOH=79:19:29). This sample was chromatographically identical with a sample obtained previously.[4]

Phosphorylation of Adenosine——Into a suspension of adenosine (134 mg., 0.5 m mole) in 2 ml. of DMF, P^1-diphenyl P^2-morpholino pyrophosphorochloridate (prepared from morpholinophosphorodichloridate (102 mg., 0.5 m mole), diphenyl phosphate (125 mg., 0.5 m mole) and 2,6-lutidine (0.114 ml., 1 m mole)) dissolved in 1 ml. of dioxane was added. The whole solution was kept in standing for 48 hr. at 20° under exclusion of moisture. Examination by paper electrophoresis revealed 3 spots having R_{AMP} 0.27

(adenosine), 0.65 (AMP-morpholidate), and 1.00 (AMP). The whole solution was separated from insoluble material (mainly adenosine) and pH was adjusted to 8.5. Chromatography on a column of Dowex 1-X8 (formate, 1.1×20 cm., 100~200 mesh) gave the following results: unadsorbed material 7.3%, AMP-morpholidate 1.8%, 5'-AMP 3.5%, 2'-AMP 1.9%, 3'-AMP 4.6% and diphosphates 17.4% (total 37.4%). The ratio obtained for 5'-:2'-:3'-AMP=45:16:39.

Phosphorylation of 2',3'-O-Isopropylideneadenosine by the Use of Vilsmeier Complex——i) DMF (44 mg., 0.6 m mole) was added to morpholinophosphorodichloridate (122 mg., 0.6 m mole) under cooling with ice H_2O. The whole solution changed to a white amorphous mass. After 2 hr. at room temperature, 6 spots were detected by paper electrophoresis: R_{AMP} 0.20 (adenosine), 0.52, 0.58 (AMP-morpholidate), 0.84, 1.00 (AMP), and 1.10. The extent of reaction proceeded, which was estimated by visual observation, was fairy low. Little difference was observed after 3 day's incubation at room temperature.

ii) Isopropylideneadenosine (93 mg., 0.3 m mole), triethylamine 61 mg., 0.6 m mole) and DMF (44 mg., 0.6 m mole) was added into 0.2 ml. of acetonitrile. The mixture was cooled to 0° and a solution of morpholinophosphorodichloridate (22 mg., 0.6 mmole) dissolved in 2 ml. of dioxane was added. After standing overnight at room temperature, an aliquot was examined by paper electrophoresis. 2 spots having R_{AMP} 0.12 (I) and 0.52 (II) were revealed by molibdate spray[13] and UV irradiation. The ratio of the amount of I to II estimated from the eluants of the above spots cut out from the paper was 0.5.

iii) Synthesis of isopropylidene-ATP: The reaction mixture obtained in the experiment described in ii) was separated from the insoluble material by filtration. A solution of DMF (22 mg.) and bis(tributylammonium)pyrophosphate (0.75 m mole) in pyridine (2 ml.) was added to a half of the filtrate. The solvent was evaporated under reduced pressure and the residue was taken up in 1 ml. of pyridine. Reaction was carried out at room temperature for 63 hr. under exclusion of moisture. An aliquot was applied to paper electrophoresis and eluants from the corresponding spot was estimated photometrically. Results were listed in Table I. To the rest of above filtrate a solution of bis-tributylammonium pyrophosphate (0.75 m mole) dissolved in 2 ml. of pyridine was added. This was concentrated to a half and kept in standing for 63 hr. at room temperature (see Table I).

2',3'-O-Isopropylidene-ATP——Into a dioxane (3 ml.) solution of 2',3'-O-isopropylideneadenosine (175 mg., 0.57 m mole), a dioxane (2 ml.) solution of P^1-diphenyl P^2-morpholino pyrophosphorochloridate (freshly prepared from morpholinophosphorodichloridate (232 mg., 1.14 m mole), diphenyl phosphate (285 mg., 1.14 m mole) and 2,6-lutidine (259 μ, 2.28 m mole) was added. In order to complete the phosphorylation, additional diphenyl phosphate (285 mg., 1.14 m mole) and 2,6-lutidine (388 μl., 3.42 m mole) was added. The reaction flask was tightly stoppered and stored in a desiccator for 72 hr. at room temperature (89% reaction was observed by paper electrophoresis). 2,6-Lutidine hydrochloride was removed by filtration, filtrate was evaporated *in vacuo*, codistilled twice with pyridine and dissolved again in 5 ml. of pyridine. Pyridine was evaporated to a half of its volume and into the solution a pyridine (7 ml.) solution of bis(tributylammonium)pyrophosphate (2.75 m mole) was added. The reaction was carried out at room temperature for 23 hr. under exclusion of moisture. After the addition of 30 ml. of H_2O, the whole solution was extracted with Et_2O (50 ml.×4). H_2O-layer (pH 6) was adsorbed on Norit A and desorbed with 50% EtOH containing 2% NH_3 (recovery 35%). Alkaline solution was concentrated *in vacuo* to 46.5 ml. (TOD_{260} 3278, pH 7.8) and applied to the top of Dowex 1-X8 (Cl'-form, 20×110 cm.) column. After the water-wash, 0.003*N* HCl+0.015*M* LiCl, 0.003*N* HCl+0.1*M* LiCl and 0.003*N* HCl+0.15*M* LiCl was used as eluting buffer. Isopropylidene-AMP, -ADP, and -ATP was eluted as the single peak. TP-fractions were collected and neutralized with LiOH and evaporated *in vacuo* to a small bulk. EtOH (2 volumes) and Me_2CO (20 volumes) was added and white precipitate was collected by centrifugation. The precipitate was washed with EtOH, EtOH-Et_2O (1:1, v/v) and Et_2O and dried over P_2O_5 at 3 mm./Hg. The yield of 2',3'-O-isopropylidene-ATP·Li_4 was 40 mg. Purity estimated photometrically on the weight basis was 26.5%. *Anal.* Calcd. for $C_{13}H_{16}N_5O_{13}P_3 \cdot Li$: Total P, 16.2, labile P, 10.8. Found: Total P, 15.1, labile P 10.9 (devided by purity). Total P-labile P-base=2.80: 1.95: 1.00 (theory, 3:2:1). Paper electrophoresis: migration distance 12.3 cm., R_{ATP} 1.00. Paper chromatography: Rf 0.623, R_{ATP} 1.03 (solvent, iso-PrOH-1% $(NH_4)_2SO_4$=2:1); Rf 0.601, R_{ATP} 0.98 (solvent, PrOH-NH_4OH-H_2O=60:30:10). These values were well coincided with those obtained from isopropylidene-ATP described in the proceding section.

Authors are indebted to Mr. A. Nomura of this Faculty for the assistance in the skillfull operation of automatic nucleic acid analyzer. Authors also wish to thank Mr. H. Uno for the synthesis of isopropylidene-ATP.

13) C. S. Hanes, R. A. Isherwood: Nature, **164**, 1107 (1949).

Summary

The phosphorylation of unprotected nucleoside, 9-D-erythrityladenine and adenosine, was attempted by the use of P^1-diphenyl P^2-morpholino pyrophosphorochloridate. Whereas in the former case 23% of 4'-monophosphate was obtained, the yield of 5'-AMP was very low. Isopropylideneadenosine was phosphorylated with Vilsmeier complex derived from morpholinophosphorodichloridate in 50% yield. The use of DMF in the reaction of isopropylidene-AMP-morpholidate was also investigated. 2',3'-O-Isopropylidene-ATP was synthesized and characterized.

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212. Morio Ikehara and Eiko Ohtsuka : Studies of Nucleosides and Nucleotides. XXI.*1 A New Synthesis of Thymidine 5'-Triphosphate and the Use of P^1,P^2-Di-(2-cyanoethyl)pyrophosphate in the Nucleoside Triphosphate Synthesis.

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The synthesis of nucleoside triphosphate by the use of P^1-diphenyl P^2-morpholino pyrophosphorochloridate was reported in the series of papers from this laboratory.[1~3] In these cases we used acetyl group for the protection of hydroxyl group of nucleosides and the acetyl group was removed after initial phosphorylation by the alkaline treatment prior to the reaction with pyrophosphate salt. However, in the case of 9-β-D-xylofuranosyladenine, the alkaline removal of 2'- and 3'-acetyl group after phosphorylation caused the cyclization of 5'-phosphoromorpholidate to 3'-hydroxyl and gave 3',5'-cyclic phosphate exclusively.[4] In order to circumvent this cyclization, the deprotection after the triphosphate formation would be necessary.

Conditions for alkaline treatment of nucleosides and phosphates appeared in the literature were listed in Table I, together with those obtained by us. The results of this investigation were applied to the synthesis of thymidine 5'-triphosphate.

3'-O-Acetylthymidine was phoshorylated with P^1-diphenyl P^2-morpholinopyrophosphorochloridate according to the procedure reported earlier.[1]

The resulting 3'-O-acetylthymidine 5'-morpholinophosphorochloridate was reacted with 5 equivalents of bis(tributylammonium) pyrophosphate in the presence of 1 equivalent of tributylamine. In this case the reaction could be expected to occur in the following 2 ways : i) reaction of phosphorochloridate with pyrophosphate salt and ii) reaction of phosphoromorpholidate residue with pyrophosphate. Though it is hard to

*1 Part XX : M. Ikehara, E. Ohtsuka, S. Kitagawa, Y. Tonomura : Biochim. Biophys. Acta, in press.
*2 Kita 12-jo, Nishi 5-chome, Sapporo (池原森男, 大塚栄子).
1) M. Ikehara, E. Ohtsuka : This Bulletin, **10**, 997 (1962).
2) Same as *1.
3) M. Ikehara, E. Ohtsuka : This Bulletin, **11**, 1353 (1963).
4) Unpublished experiments by M. Ikehara and E. Ohtsuka.