N-Alkylpyrazinecarbothionamide——To a solution of 1 mol. of N-alkylpyrazinecarboamide in 1.8 mol. of xylene was added 0.23 mol. of phosphor pentasulfide and 0.71 mol. of dry K_2S at 100° with violent agitation. Heating was continued for 5 hr. while stirring and the reaction mixture was immediately filtered. The residue was extracted with boiling benzene. The benzene solution was combined with the filtrate and evaporated to dryness. The remainder was several times recrystallized from EtOH. Results are shown in Table III.

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Summary

Methylpyrazine (I) and 2-picoline (VI) were converted to N,N-dimethylpyrazinecarbothionamide and N,N-dimethylpicolinethionamide (II and VII) respectively on fusion with sulfur in dimethylformamide. Iodine was found effective as a catalyst for the reaction. Besides the ordinal products (II and VII), the corresponding demethylated products were also isolated, thus indicating that the demethylation reaction, presumably due to the action of sulfur, was involved. The demethylation reaction was confirmed by the formation of N-methylpyrazinecarbothionamide and N-methylpicolinethionamide (III and VIII) from II and VII respectively on fusion with sulfur. II was 4 times more effective than pyrazinecarboamide against *M. tuberculosis* and its toxity was LD_{50} 1180± 15 mg./kg.

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78. **Morio Ikehara and Eiko Ohtsuka** : Studies on Coenzyme Analogs. XIV.*1 A New Phosphorylating Agent, Morpholinophosphorodichloridate.

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The recent progress in the field of nucleotide chemistry is due, at least partly, to the development of phosphorylating agent by many investigators.[1] In the authors' laboratory, various types of nucleotides were synthesized in order to elucidate the mechanism of action of several enzymes with their substrates. Analogs of nucleoside 5′-mono- and -triphosphate were synthesized from various nucleoside analogs as the substrate of snake venom 5′-nucleotidase[2,3] and myosin ATPase.[4,5] The protecting group of phosphorylating agent utilized for nucleotide synthesis in the past were removed either by hydrogenolysis or by alkaline hydrolysis. In order to phosphorylate

*1 Part XIII. M. Ikehara, A. Yamazaki, T. Fujieda : This Bulletin, **10**, 1075 (1962).

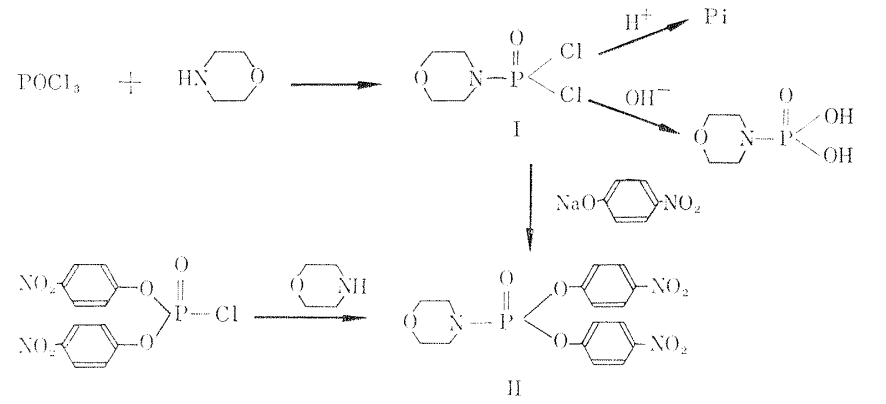
*2 Kita 12-jo, Nishi 5-chome, Sapporo (池原森男, 大塚栄子).

1) H. G. Khorana : "Some Recent Development in the Chemistry of Phosphate Esters of Biological Interest," p. 13, John Wiley & Sons, Inc., New York (1961).
2) Y. Mizuno, M. Ikehara, T. Ueda, A. Nomura, E. Ohtsuka, F. Ishikawa, Y. Kanai : This Bulletin, **9**, 338 (1961).
3) Y. Mizuno, M. Ikehara, A. Nomura, Y. Kanai, T. Fujieda : Abstracts of Papers presented at the 14th Symposium for Enzymatical Chemistry, p. 70 (1962).
4) M. Ikehara, E. Ohtsuka, S. Kitagawa, K. Yagi. Y. Tonomura : J. Am. Chem. Soc., **83**, 2679 (1961).
5) N. Azuma, M. Ikehara, E. Ohtsuka, Y. Tonomura : Biochim. Biophys. Acta, **60**, 104 (1962).

alkali-labile nucleosides, such as nebularine,[6] the protecting group should be removed in neutral or mild acidic medium.

To obtain a sufficient amount of nucleoside polyphosphate by a relatively short and easy route, one step procedure from nucleoside to polyphosphate should be explored. For this purpose, morpholino residue was chosen as the protective group, which was hydrolized by mild acidic treatment. Furthermore, phosphoromorpholidate residue reacted successively with phosphates[7] to afford pyrophosphate linkage.

Morpholinophosphorodichloridate (I) was synthesized by the direct condensation of phosphoryl chloride with morpholine. In order to avoid exclusively the bifunctional reaction, which might afford dimorpholinophosphorochloridate,[8] 1.5 equivalent of morpholine was introduced gradually into phosphoryl chloride in benzene solution. On fractional distillation, morpholinophosphorodichloridate was found in the middle fraction, b.p. 125~127° at 10 mm./Hg. This was colorless oil having characteristic odor and hydrolyzed gradually by atmospheric moisture. The structure of I was confirmed by leading it to morpholino-di(*p*-nitrophenyl) phosphate (II), m.p. 148~150°, which was synthesized also from di(*p*-nitrophenyl)phosphorochloridate[9] and morpholine.[10] By



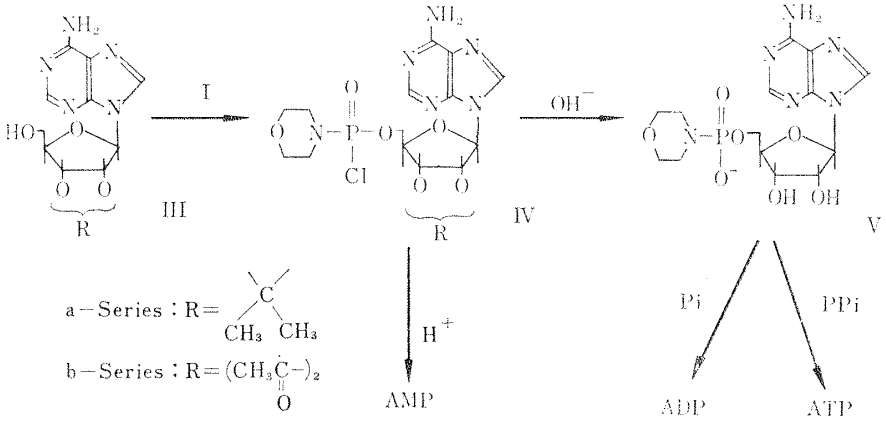


Chart 1.

6) M.P. Gordon, V.S. Weliky, G.B. Brown : J. Am. Chem. Soc., **79**, 3245 (1957).
7) J.G. Moffatt, H.G. Khorana : *Ibid.*, **83**, 649 (1961).
8) H.A.C. Montgomery, J.H. Turnbull : J. Chem. Soc., **1958**, 1963.
9) The synthesis of this substance was reported recently by T. Ukita, H. Hayatsu : J. Am. Chem. Soc., **84**, 1879 (1962).
10) Unpublished experiments by H. Takagi, to which authors thanks are due.

treatment with dilute hydrochloric acid at 100° for 30 minutes, compound I liberated inorganic phosphate, which was identified by the paper chromatography. Alkaline hydrolysis with *N* lithium hydroxide gave a spot corresponding to morpholino-dihydrogenphosphate.[11] These results confirmed compound I as morpholinophosphorodichloridate.

The bifunctional reaction might be circumvented by using this reagent I, though the reagent I may be less reactive than *p*-nitrophenylphosphorodichloridate,[12] a typical bifunctional reagent, because of the electron-releasing tendency of morpholino residue. This was demonstrated by the reaction of I with excess aniline. The resulting product was solely morpholinophosphorochloranilidate, m.p. 100~101°.

Then the phosphorylation of 2',3'-O-isopropylidene adenosine (IIIa) was investigated. Compound IIIa, morpholinophosphorodichloridate, and 2,6-lutidine were brought into reaction in dioxane solution at 75~80° for 3~4 hours in the molar ratios 1:1:1, 1:1:2, or 1:2:1. On the paper chromatogram a spot of 2',3'-O-isopropylideneadenosine 5'-phosphoromorpholinochloridate (IVa) in a yield of 50~60% and a spot of unreacted isopropylideneadenosine were observed.

The yield of IVa was not improved by raising reaction temperature or by changing the ratio of the reactants. The reaction mixture was evaporated and hydrolyzed with water (pH 2.0) at 100° for one hour. Addition of barium hydroxide gave a crude barium salt of adenosine 5'-monophosphate (AMP), which was purified by reprecipitation from ethanol-water mixture. This material was photometrically 81% pure (37% yield as pure AMP) and gave a single spot on the paper chromatographical and the paper electrophoretical tests.

6-Dimethylamino-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl)purine[13] was phosphorylated by essentially the same procedure as IIIa and gave 27% of barium salt of 6-dimethylamino-9-β-D-ribofuranosylpurine 5'-phosphate. This material was estimated as 83% pure by photometry and as 78% by ion-exchanger chromatography.

Adenosine 5'-triphosphate (ATP) was synthesized from 2',3'-di-O-acetyladenosine (IIIb) as the starting material, because of the ease of the alkaline hydrolysis of its acetyl group after triphosphate synthesis. Compound IIIb, morpholinophosphorochloridate, and 2,6-lutidine were reacted in dioxane solution at 75° for 4 hours in the molar ratio of 1:1:2. The extent of the reaction was estimated by the paper chromatography to be about 50%. The next problem was the method of hydrolysis of chloridate residue and acetyl group without cleavage of morpholidate linkage in resulting adenosine 5'-phosphoromorpholidate by alkaline treatment. The treatment with aqueous lithium hydroxide was unsuccessful and it cleaved the morpholidate linkage and converted IVb into AMP. The treatment with methanol saturated with ammonia gave phosphoromorpholinoamidate, which hardly reacted with phosphate in the successive reaction, though in some cases 5'-morpholidate was produced by the hydrolysis of chloridate residue in the presence of trace amount of water. The following two step hydrolysis gave the most satisfactory result. The chloridate of IVb was first hydrolyzed with equivalent amounts of water and tributylamine dissolved in methanol and the reaction mixture was combined with methanol saturated with ammonia. The quantitative conversion of IVb to adenosine 5'-phosphoromorpholidate (V) was observed by this procedure. Compound V was identified by the paper chromatograpy and the paper electrophoresis with an authentic sample synthesized by the method of Khorana.[7]

11) A crystalline material appeared during storage of I in a refrigerator under exclusion of moisture for several months, and its migratory value on electrophoretic paper (RAMP 1.15) was equal to that of mono-substituted phosphoric acid. This indicates that the material is morpholinophosphate.
12) A.F. Turner, H.G. Khorana : J. Am. Chem. Soc., **81**, 4651 (1959).
13) M. Ikehara, E. Ohtsuka, F. Ishikawa : This Bulletin, **9**, 27 (1961).

The residue obtained by the evaporation of reaction mixture was thoroughly dried by codistillation with pyridine. This residue was taken up in pyridine and caused to react with tris(tributylammonium)pyrophosphate for 7 hours at room temperature. At this stage, the residue was often incompletely solubilized in pyridine.*3 In such a case the addition of N,N'-dicyclohexyl-4-morpholine carboxamidine and mechanical stirring will be recommended. The column chromatography of the product on Amberlite ion-exchanger by concave gradient elution with lithium chloride-hydrochloric acid solution gave the following results: recovered nucleoside 35.3, AMP 28.8, AMP-morpholidate 9.2, and ATP 21.3 %. The same analysis after 24 hours' reaction gave: AMP 46, ADP*4 2.1, and ATP 11.7 %. As stated by Khorana,[7] disproportionation of ATP to lower phosphates was observed albeit in small amount.

The analogous reaction of V with tributylammonium phosphate also gave ADP in 18 % yield estimated by the paper electrophoresis.

Thus morpholinophosphorodichloridate could be used as a phosphorylating agent of various types of nucleoside and its analogs and phosphorylated product could be further derived to polyphosphate by the reaction with inorganic phosphate salt without special purification, when suitably protected nucleoside was used.

The experiments for increasing the low phosphorylating power of this reagent are now in progress in our laboratory.

Experimental

Morpholinophosphorodichloridate——22.4 g. (0.26 mole) of freshly disteilled morpholine was added dropwise with stirring into a solution of 25.6 g. (0.17 mole) of freshly distilled $POCl_3$ dissolved in 100 cc. of dry benzene. Temperature was maintained below 20° by occasional cooling by ice-water after 2.5 hr's reaction a white precipitate (morpholine hydrochloride) appeared and was filtered off. The filtrate and washings (by dry benzene) were combined and evaporated in a reduced pressure. The residual yellowish oil was subjected to fractional distillation *in vacuo*. After distillation of a small amount of $POCl_3$, a colorless oil $b.p_{10}$ 125~127° was obtained (yield ca. 10 g.). This oil was divided into small portions, sealed in glass tubes, and stored in a refrigerator. The third fraction of this distillation was a colorless crystal, $b.p_3$ 175~178°, m.p. 64~75° (yield ca. 4 g.). This was dimorpholinophosphorochloridate. Above 180° at 3 mm. a brown resinous substance (ca. 3 g.) remained as a residue.

Di(*p*-nitrophenyl)phosphoromorpholidate——i) 161 mg. (0.5 mmole) of well dried sodium *p*-nitrophenolate was suspended in a dry benzene solution (20 cc.) containing 102 mg. (0.5 mmole) of morpholinophosphorodichloridate. The whole reaction mixture was refluxed for 3 hr. The resulting white yellow precipitate was removed by filtration and the filtrate was evaporated *in vacuo*. The residual oil was triturated with a small amount of Et_2O. A white amorphous substance thus obtained was recrystallized from benzene-petr. ether. Colorless cubes, m.p. 148~150° (yield 50 mg.). *Anal.* Calcd. for $C_{16}H_{16}O_8N_3P$: N, 10.03; P, 7.58. Found: N, 10.21; P, 7.47.

UV: λ_{max}^{EtOH} 279, λ_{min}^{EtOH} 238 mμ. Mixed melting point test with dimorpholino-*p*-nitrophenylphosphate. synthesized from dimorpholinophosphorochloridate and sodium *p*-nitrophenolate, m.p. 116~118° (*Anal.* Calcd. for $C_{14}H_{20}O_6N_3P$: N, 11.75; P, 8.65. Found: N, 11.65; P, 8.65.), showed m.p. 100°.

ii) 50 mg. of di(*p*-nitrophenyl)phosphorochloridate was reacted in 10 cc. of dry benzene with excess morpholine. The solvent was evaporated and the residue was taken up in benzene. Concentration of the solvent and addition of petr. ether gave colorless crystals, m.p. 148~150°. This material was identical with the sample obtained in i).

Acidic Hydrolysis of Di(*p*-nitrophenyl)phosphoromorpholidate——20 mg. of di(*p*-nitrophenyl)phosphoromorpholidate was dissolved in 5 cc. of EtOH-*N*HCl (1:1) and heated at 100° for 30 min. The solvent was evaporated *in vacuo* and colorless needles were collected by filtration. The material had m.p. 168° and gave m.p. 168~170° by the mixed melting point test with authentic di-*p*-nitrophenylphosphate.

Hydrolysis of Morpholinophosphorodichloridate——i) 2 mg. of morpholinophosphorodichloridate was heated in 2 cc. of *N*HCl at 100° for 30 min. Paper chromatography (iso-PrOH: 1% $(NH_4)_2SO_4$ = 3:2)

*3 When triethylamine was used instead of tributylamine for alkaline hydrolysis of IV the residue was a thick oil and unsuitable for the next reaction.

*4 ADP stands for adenosine 5'-diphosphate.

of the reaction mixture showed a spot at Rf 0.54, which was visualized by $HClO_4$-molibdate spray.[14] The Rf value was identical with that of authentic inorganic phosphate.

ii) 2 mg. of morpholinophosphorodichloridate was incubated in *N* LiOH and 1 cc. of dioxane for 1 hr. at room temperature. Paper chromatography (iso-PrOH : 1% $(NH_4)_2SO_4$=2:1) of the reaction mixture gave two spots, Rf 0.43 and 0.62. The latter seemed to be morpholinophosphate and the former was inorganic phosphate (Rf 0.44).

Morpholinophosphorochloroanilidate——100 mg. of morpholinophosphorodichloridate was dissolved in benzene containing 500 mg. of aniline. After heating at 50° for 30 min., aniline hydrochloride was filtered off and the filtrate was evaporated *in vacuo*. Recrystallization of this residue from Et_2O-petr. ether gave white leaflets, m.p. 100~101°. The qualitative test showed halogen and phosphorus as components of the material. *Anal.* Calcd. for $C_{10}H_{14}ON_2ClP$: N, 11.42; P, 12.68. Found : N, 10.67; P, 13.4.

Adenosine 5′-Monophosphate——Into a solution of 190 mg. (1 mmol.) of morpholinophosphorodichloridate dissolved in 4 cc. of anhyd. dioxane was added 154 mg. (0.5 m mol.) of 2′,3′-O-isopropylideneadenosine and 54 mg. (0.5 mmol.) of 2,6-lutidine. Reaction mixture was stirred by the magnetic stirrer at 80° for 3 hr. under exclusion of moisture. In some experiments reaction was carried out at 75° for 4 hr. It was found that both supernatant and precipitate contained nucleotide material by the paper chromatography (iso-PrOH : 1% $(NH_4)_2SO_4$=2:1). Following UV absorbing spots were cut out, eluted by H_2O, and estimated quantitati vely by the absorption at 260 mμ : isopropylideneadenosine Rf 0.88, isopropylidene-AMP-morpholidate (chloridate residue was hydrolyzed during migration) Rf 0.63. At this stage the extent of the reaction was 50~60% in all experiments.

The solvent was evaporated in a reduced pressure and 10 cc. of H_2O was added until pH was adjusted to 2.0. This solution was heated at 100° for 1 hr. in order to hydrolyze morpholidate and isopropylidene group. The whole was concentrated *in vacuo* and finally codistilled with benzene. The residue thus obtained was taken up in 5 cc. of H_2O and adjusted to pH 6.5 with $Ba(OH)_2$. The resulting precipitate(mainly $Ba_3(PO_4)_2$) was removed by centrifugation, pH was adjusted to 7.0, and 2 volumes of EtOH was added. After whole was stored in a refrigerator for 30 min. the resulting precipitate collectedby centrifugation. Washing with aqueous EtOH, EtOH, and Et_2O, and finally drying over P_2O_5 at 3 mm. Hg. gave 174 mg. of AMP Ba salt. Purity estimated photometrically on the weight basis ($\varepsilon_{260}=15\times10^3$) was 58%. Reprecipitation from EtOH-H_2O(2:1) gave 110 mg. of 81% pure AMP-Ba. Yield calculated as pure AMP was 37 %. This material was made free by the addition of equivalent amount of H_2SO_4 in the H_2O solution. Barium sulfate was filtered off and the filtrate was concentrated *in vacuo* to a small bulk. AMP·$2H_2O$ was obtained in the form of fine needles, m.p. 190~192°. Paper chromatography (iso-PrOH : 1% $(NH_4)_2SO_4$=2:1) Rf 0.30. Paper electrophoresis (0.05 mole triethylammonium bicarbonate, pH 7.5, 20 v/cm., 1 hr.) RAMP 1.00.

6-Dimethylamino-9-β-D-ribofuranosylpurine 5′-Monophosphate——Into a solution of 2.44 g. of morpholinophosphorodichloridate in 16 cc. of dioxane 2.0 g. of 6-dimethylamino-9-(2′,3′-O-isopropylidene)-β-D-ribofuranosylpurine[13] and 0.64 g. of 2,6-lutidine was added. Reaction was carried out at 75° for 4 hr. After the reaction, the precipitate was filtered off (ca. 1.0 g.), washings and filtrate were combined and acidified with 0.003*N*HCl to pH 2.0. The whole was heated at 100° for 45 min. and the solvent was removed *in vacuo* to a small bulk. pH of the solution was adjusted to 6.4 with $Ba(OH)_2$. Precipitate*5 thus appeared was removed by centrifugation. Filtrate was extracted with Et_2O (25 cc.), concentrated to a small bulk, and 2 volumes of EtOH, was added. 1050 mg. of precipitate was collected by centrifugation, washed with aqueous EtOH, EtOH and Et_2O, and finally dried over P_2O_5 at 3 mm.Hg. This material was 83% pure estimated photometrically on the weight basis (ε_{268}=18,300). Calculated from the pattern of ion-exchanger (Dowex-I, formate, eluted with HCOOH), purity was 78%. Paper chromatography, (solvent, iso-PrOH-1% $(NH_4)_2SO_4$; 3:2) Rf 0.30, which was identical with that of authentic specimen.[13]

Adenosine 5′-Triphosphate——116 mg. (0.33 mmol.) of 2′,3′-di-O-acetyladenosine, 68 mg. (0.33 mmol.) of morpholinophosphorodichloridate, and 83 mg. (0.66 mmol.) of 2,6-lutidine was reacted in 3 cc. of dioxane at 75° for 4 hr. with mechanical stirring. Into this mixture 5 cc. of MeOH containing 1.2 mmole of tributylamine and 0.06 cc. of H_2O was added. After 15 min., 5 cc. of MeOH previously saturated with ammonia at 0° was added and the reaction mixture was kept for standing overnight at room temperature. MeOH and ammonia was evaporated *in vacuo* and after the addition of 2 cc. of pyridine, solvent was again evaporated in reduced pressure. Into the residue 1.67 mmole of tris(tributylammonium)pyrophosphate, which was previously dried by azeotropical distillation with pyridine, in 5 cc. of pyridine was added. The whole was evaporated again to remove trace amount of H_2O and redissolved in 4 cc. of pyridine. Reaction was carried out for 7 hr. at room temperature with stirring by a magnetic stirrer. The analysis of this reaction mixture on Amberlite IRA-400 (X8, Cl^- form, 200~400

*5 When this precipitate contained nucleotide material, some desired product may be recovered by extraction with hot water and precipitation with 2 volumes of EtOH.

14) C. S. Hanes, F. A. Isherwood : Nature, **164**, 1107 (1949).

mesh) column (0.8×7 cm.) eluted with 0.003*N*HCl and 0.003*N*HCl+0.35*M*LiCl by concave gradient elution technique was as shown in the following table, together with the result at 24 hr's reaction period.

	Nucleoside (%)	AMP	AMP-morpholidate	ADP	ATP
7 hr.	35.3	28.8	9.2	0	21.3
24 〃	—	46.0	0	2.1	11.7

Each fraction was evaporated *in vacuo* below 20° and identified by the paper chromatography (on Toyo Roshi No. 51A) with the authentic samples.

Solvent	Adenosine	AMP	AMP-morpholidate	ADP	ATP
iso-PrOH-1% $(NH_4)_2SO_4$=3:2	0.48	0.31	0.48	0.20	0.12
iso-PrOH-NH_3-H_2O=7:1:3	0.45	0.07			

Adenosine 5′-Diphosphate——35 mg. of 2′,3′-di-O-acetyladenosine was reacted in the same condition as described above. Ammonia treated residue was further reacted with 0.5 mmole of tributylammonium phosphate at 10° for 18 hr. 16% of ADP was found by paper electrophoresis (condition was same as above).

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Summary

A new phosphorylating agent, morpholinophosphorodichloridate was synthesized from phosphorylchloride and morpholine. The reagent was used successfully for the synthesis of adenosine 5′-monophosphate and 6-dimethylamino-9-β-D-ribofuranosylpurine 5′-monophosphate. Starting from 2′,3′-di-O-acetyladenosine, adenosine 5′-di- and triphosphate were synthesized by phosphorylation with this reagent followed by the reaction with inorganic phosphate or pyrophosphate in fairy good overall yield.

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