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A New Reaction of Nucleotides with Cyanoacetylene

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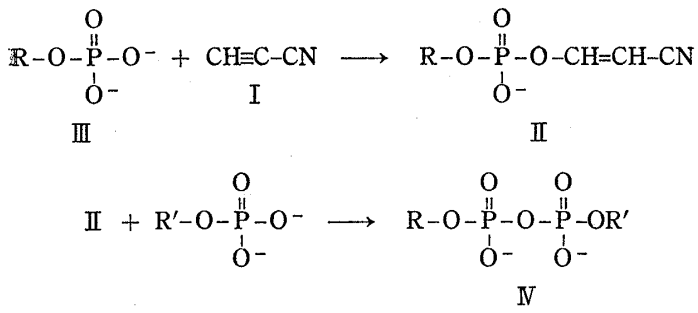
Cyanoacetylene reacted with cytidine 5′-phosphate and adenosine 5′- or 3′,5′-cyclic phosphate at their corresponding base moieties to afford a new type of heterocyclic nucleotides, 2-amino-7-(5-phospho-β-D-ribofuranosyl)-pyrimido[1,2-*c*]pyrimidin-5-ium-6(7H)-one (V) and 9-amino-3-(5- or 3,5-cyclic phospho-β-D-ribofuranosyl)-pyrimido[2,1-*i*]purin-6-ium (IX or XIII), respectively.

Cyanovinyl phosphate, an adduct of phosphoric acid and cyanoacetylene (I), has been known to undergo reaction with phosphoric acid or uridine in an aqueous solution to afford pyrophosphoric acid or uridine 5′-phosphate, respectively.[2] Accordingly, it is conceivable that an analogous adduct (II) of ribonucleoside 5′-phosphate (III) and I might be a useful intermediate for the synthesis of nucleotide coenzyme (IV) (see Chart 1). However, it turned out that the reaction of III with I did not proceed as expected, but a new type of cyclic compounds could be isolated from the reaction mixture. The present paper deals with the structure elucidation of the new compounds.



R: nucleoside residue
R′: sugar residue, nucleoside residue, *etc.*

Chart 1

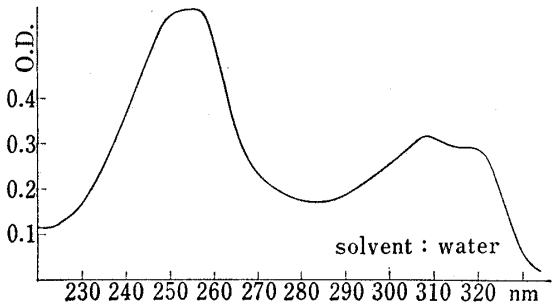


Fig. 1. UV Absorption Spectrum of 2-Amino-7-(5-phospho-β-D-ribofuranosyl)-pyrimido[1,2-*c*]pyrimidin-5-ium-6(7H)-one (V)

The reaction of I with tri-*n*-butylammonium[3] cytidine 5′-phosphate (5′-CMP) in 50% *tert*-butanol in the presence of mercuric chloride[4] at or below room temperature afforded pale-yellow scaly platelets (V). This compound gave a single ultraviolet spectrum (UV) absorbing spot and exhibited about half the mobility ratio of 5′-CMP on paper electrophoresis (phosphate buffer, pH 7.5)[5] and a positive color reaction with a periodate-benzidine test.[6] The elemental analyses were in good agreement with those for the 1:1 adduct of I and 5′-CMP.

1) Location: *Juso-honmachi, Yodogawa-ku, Osaka.*
2) J.P. Ferris, *Science*, **161**, 53 (1968); J.P. Ferris, G. Goldstein, and D.J. Beanlieu, *J. Am. Chem. Soc.*, **92**, 6598 (1970).
3) When the reaction was carried out at the same pH (9.0) with the excess of tri-*n*-butylamine to 5′-CMP as in the case of cyanovinyl phosphate,[2] no product could be isolated.
4) Mercuric chloride is known to be a catalyst for the addition reaction to triple bonds.
5) The compound II was also supposed to move half the distance of 5′-CMP.
6) M. Viscontini, D. Hoch, and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

The nuclear magnetic resonance (NMR) spectrum clearly indicated the presence of two pairs of vinyl protons, out of which one pair of the protons appeared at δ 6.64 and 8.26, and the other at δ 6.96 and 8.77, respectively. These two sets of protons could be ascribed to the 5- and 6-protons of cytosine nucleus and the vinyl protons originated from cyanoacetylene. However, the UV absorption spectrum (Fig. 1) was quite different from that of 5′-CMP, and gradually changed in an acidic or basic solution even at room temperature, and in a neutral solution with a slower rate. The IR spectrum pattern at a 1600—1700 cm^{-1} region was also quite different from that of 5′-CMP. These data strongly suggest that V has a labile base moiety in the molecule. Treatment of V with a non-specific phosphatase and purification of the hydrolysate with ion-exchange chromatography using Amberlite CG-50 (H^+ form) afforded the dephosphorylated nucleoside (VI) as a yellow powder, which was characterized by the elemental analyses, UV- and NMR-spectra. All these findings clearly demonstrate that cyanoacetylene did not undergo reaction with the phosphoric acid moiety, but with the cytosine nucleus of 5′-CMP. The nucleoside VI moved to the cathode on paper electrophoresis (phosphate buffer, pH 7.5), demonstrating that the molecule possesses a positive charge in a neutral solution. It can thus be understandable that the nucleotide V moved half a distance of 5′-CMP[5] by paper electrophoresis. The compound V was assigned the 2-amino-7-(5-phospho-β-D-ribofuranosyl)-pyrimido[1,2-*c*]pyrimidin-5-ium-6(7H)-one structure. A possible interpretation for the formation of the adduct would be as follows: the N-3 of 5′-CMP, which is nucleophilic,[7] could first attack the β-carbon[8] of cyanoacetylene to form a pyrimidinyl-

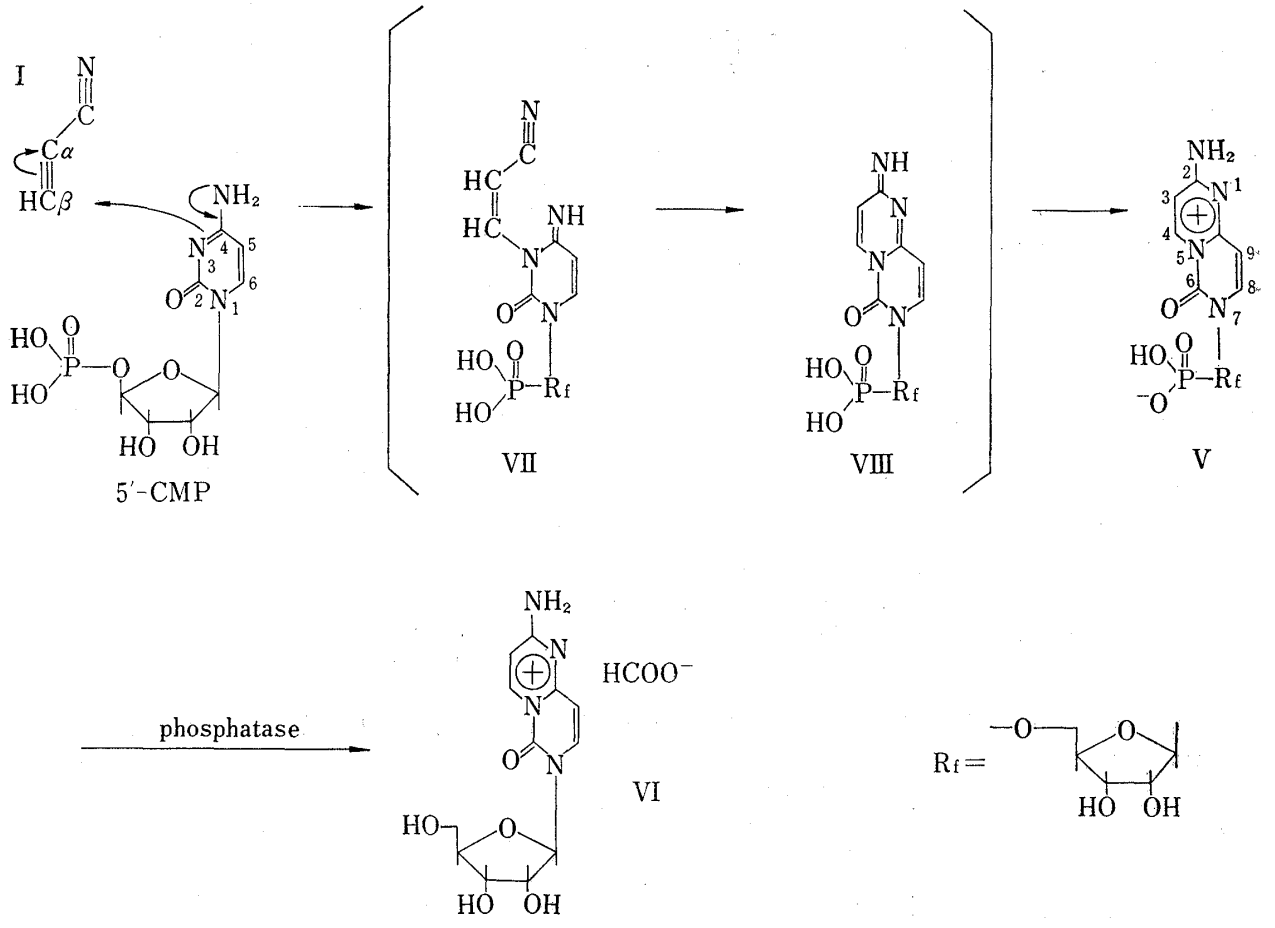


Chart 2

7) P. Brookes and P.D. Lawly, *J. Chem. Soc.*, **1962**, 1348.
8) K. Matsumura, T. Saraie, Y. Kawano, N. Hashimoto, and K. Morita, *J. Takeda Res. Lab.*, **30**, 475 (1971).

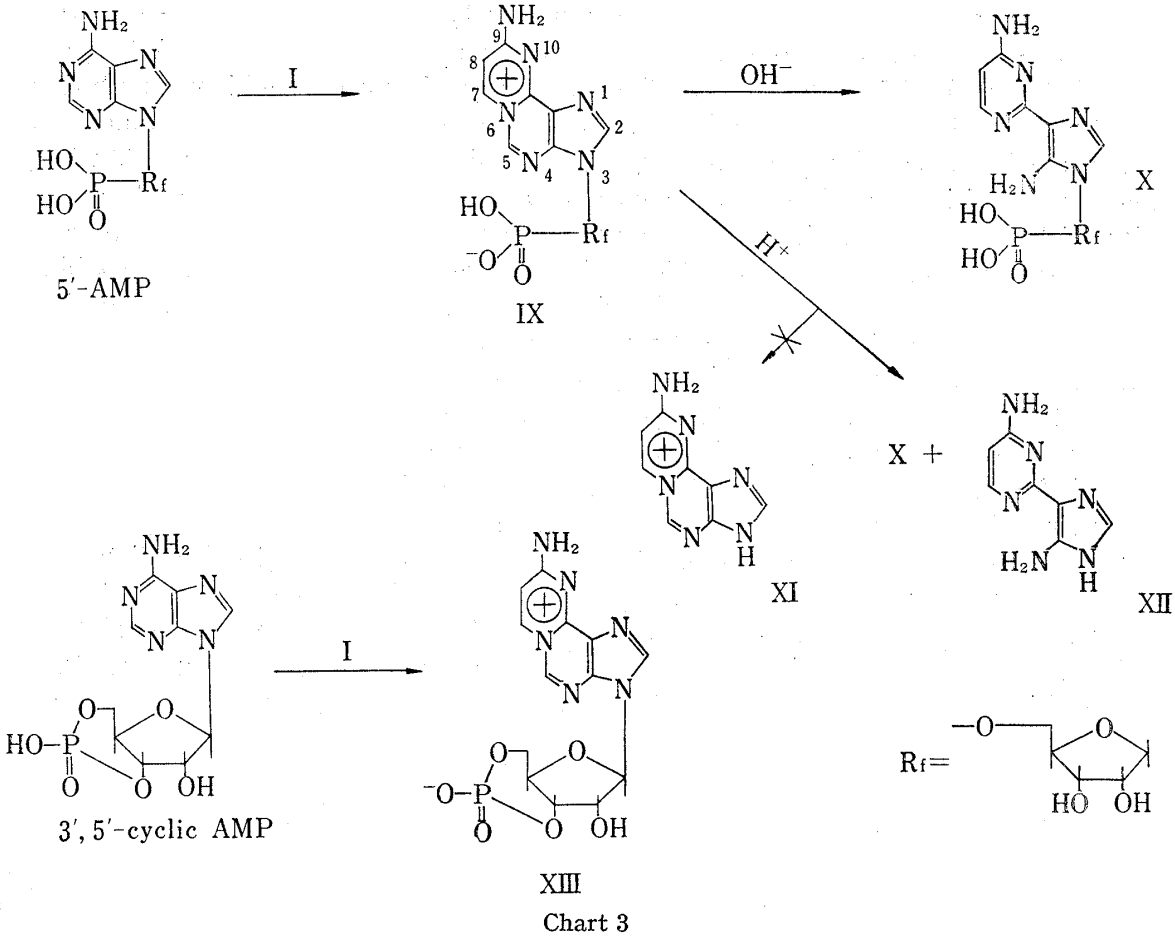


Chart 3

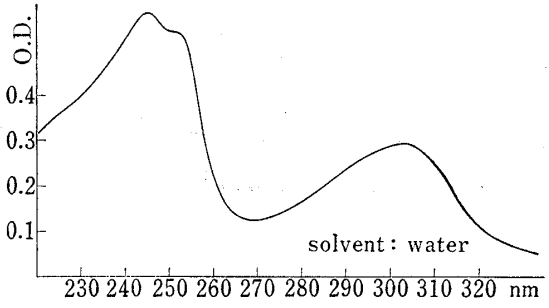


Fig. 2. UV Absorption Spectrum of 9-Amino-3-(5-phospho-β-D-ribofuranosyl)-pyrimido[2,1-*i*]purin-6-ium (IX)

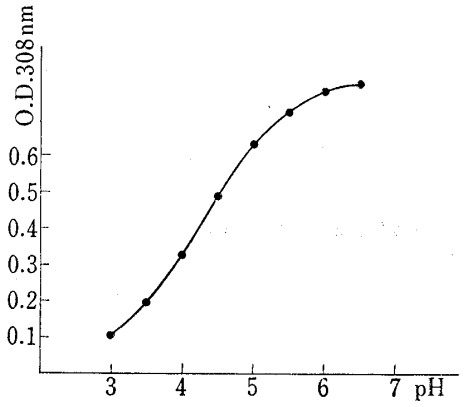


Fig. 3. pH Dependence of the Reaction of 5′-CMP with Cyanoacetylene

acrylonitrile (VII), which in turn would be cyclized to afford VIII,[9] and this nucleotide would finally be isomerized to V (Chart 2).

A similar reaction of cyanoacetylene (I) with tri-*n*-butylammonium adenosine 5′-phosphate (5′-AMP) afforded colorless crystals (IX), which were proved to be the 1:1 adduct of I and 5′-AMP on the basis of the elemental analyses ($C_{13}H_{15}O_7N_6P \cdot 1/2H_2O$), the UV (different from that of 5′-AMP, Fig. 2) and NMR (two vinyl protons at δ 7.21 and 8.86) spectra, and the

9) Recently, Sanchez reported a similar reaction of cyanoacetylene with the amino-oxazoline derivative to yield arabinofuranosyl cytosine.[10]

10) R.A. Sanchez and L.F. Orgel, *J. Mol. Biol.*, **47**, 531 (1971); D.H. Shannahoff and R.A. Sanchez, *J. Org. Chem.*, **38**, 593 (1973).

phosphoric acid liberation upon treatment with a non-specific phosphatase, as well as its behavior on paper electrophoreogram ($M_5'_{-AMP}$[11]$=0.5$) and the positive color reaction with a periodate-benzidine test.[6] The compound IX was thus assigned the 9-amino-3-(5-phospho-β-D-ribofuranosyl)-pyrimido[2,1-*i*]purin-6-ium structure. It decomposed easily in a basic solution and purification of the product with ion-exchange chromatography furnished colorless crystals (X). This substance was identified as 1-(5-phospho-β-D-ribofuranosyl)-4-(4-amino-pyrimidin-2-yl)-5-aminoimidazole on the basis of the elemental analyses ($C_{12}H_{17}O_7N_6P \cdot 1/2H_2O$) and the NMR spectrum (the lack of H-2 of purine nucleus). In an attempt to isolate the base moiety (XI), IX was subjected to acid hydrolysis. The products, however, turned out to be X and its base (XII) and even a trace of the expected XI was not isolated.

The reaction of I with tri-*n*-butylammonium adenosine 3′,5′-cyclic phosphate afforded 9-amino-3-(3,5-cyclic phospho-β-D-ribofuranosyl)-pyrimido[2,1-*i*]purin-6-ium (XIII) (Chart 3). These new heterocyclic nucleotides (V, IX and XIII) were strongly fluorescent under ultraviolet light.[12] The analogous reaction of I with tri-*n*-butylammonium uridine-, guanosine- or inosine 5′-phosphate did not afford the corresponding adduct.[14]

The reaction of I with 5′-CMP was examined at various pH values, and was found to be pH-dependent. When the reaction rates were plotted *vs.* pH values, there was observed an inflexion point near the pK_a (4.2) of cytosine nucleus (Fig. 3). This fact indicates that I reacts with the non-protonated form of 5′-CMP or -AMP. It is apparent that cyanoacetylene has a different reactivity from that of acrylonitrile, because the latter has been known to react with the deprotonated form of inosine, φ-uridine, uridine or guanosine.[15] One possible explanation of the difference would be as follows: the double bond of acrylonitrile is susceptible to the attack of strong nucleophiles and only the nucleotides which form anionic heterocycles in basic media undergo addition to acrylonitrile. The triple bond of cyanoacetylene is much more susceptible to nucleophilic attack and can be attacked, even in weakly acidic media, by the N-3 of 5′-CMP or the N-1 of 5′-AMP.

Experimental[16]

Paper Electrophoresis (PE)——PE was run on Whatman No. 1 paper at a constant voltage of 22 V/cm for 1.5 hr in the following buffers: 1, 0.05 M phosphate buffer, pH 7.5; 2, 0.05 M citrate buffer, pH 3.7.

2-Amino-7-(5-phospho-β-D-ribofuranosyl)-pyrimido[1,2-*c*]pyrimidin-5-ium-6(7H)-one (V)——To a solution of 5′-CMP (1.615 g, 5 mmoles), $(n\text{-Bu})_3N$ (0.925 g, 5 mmoles) and $HgCl_2$ (50 mg) in 50% *t*-BuOH (50 ml) was added cyanoacetylene (I) (2 ml). The mixture was left at 0° for 5 days to deposit pale yellow scaly platelets (860 mg, 45%), a part of which was recrystallized from H_2O. This compound decomposed above 200° without melting. UV $\lambda_{max}^{H_2O}$ nm: 255, 308. *Anal.* Calcd. for $C_{12}H_{15}O_8N_4P \cdot \frac{1}{2}H_2O$: C, 37.62; H, 4.21; N, 14.61; P, 8.10. Found: C, 37.57; H, 4.13; N, 14.68; P, 8.16. NMR (D_2O, 87°, 100 MHz) δ: 6.1 (1H, d, $J=3.6$ Hz, H_1'), 6.64, 6.96, 8.26, 8.77 (each 1H, d, $J=8.0$ Hz). The mother liquor of crude V was adsorbed on a column of Dowex-1 (formate, 50 ml) and the column was washed with H_2O and eluted with 0.01 N HCO_2H to recover 5′-CMP (530 mg, 33%). No addition product of I and ribose or phosphoric acid moiety of 5′-CMP was detected.

2-Amino-7-(β-D-ribofuranosyl)-pyrimido[1,2-*c*]pyrimidin-5-ium-6(7H)-one·formate (VI)——To a solution of V (420 mg) in 1 M acetate buffer (pH 5.0, 60 ml) was added a preparation of non-specific phosphatase[17]

11) Mobility ratio to 5′-AMP.

12) Recent papers reported the reaction of adenosine, cytidine or their nucleotides with chloroacetaldehyde to give the corresponding fluorescent etheno derivatives.[13]

13) J.R. Barrio, J.A. Secrist III, and N.J. Leonard, *Biochim. Biophys. Res. Commun.*, **46**, 597 (1972); J.A. Secrist III, J.R. Barrio, N.J. Leonard, and G. Weber, *Biochemistry*, **11**, 3499 (1972).

14) Cyanoacetylene seems to react with all nucleotides in basic media, but the isolation of adducts was difficult[3] because of side reactions.

15) M. Yoshida and T. Ukita, *J. Biochem.*, **57**, 818 (1965).

16) All melting points were uncorrected. Unless otherwise stated, NMR spectra were measured at 60 MHz, using tetramethylsilane as external standards. Chemical shifts were expressed in δ values. Cyanoacetylene was kindly provided by Dr. N. Hashimoto of this laboratories.

(80 mg). The mixture was left at 37° for 2 hr, and then adsorbed on a charcoal (4 g) column. The column was washed with H_2O and eluted with pyridine–EtOH (1:1 v/v, 300 ml). The eluate was evaporated to dryness *in vacuo*. The residue was dissolved in H_2O and the solution was passed through a column of Amberlite CG-50 (H^+ form). The column was washed with H_2O and eluted with 0.01 N HCO_2H. Fractions containing VI were collected and evaporated to dryness *in vacuo* (bath temperature below 30°) to give a white powder (120 mg). This showed a single UV absorbing spot at $M_{Cytidine}=1.3$ (PE, buffer 2). UV $\lambda_{max}^{H_2O}$ nm: 254, 308. *Anal.* Calcd. for $C_{12}H_{16}O_7N_4$: C, 45.90; H, 4.75; N, 16.47. Found: C, 44.67; H, 4.54; N, 16.13. NMR (d_6-DMSO) δ: 5.86 (1H, H_1'), 6.54, 7.11, 8.54, 8.80 (each 1H, d, $J=8.0$ Hz); 9.54, 10.01 (each 1H, disappears with D_2O, NH).

9-Amino-3-(5-phospho-β-D-ribofuranosyl)-pyrimido[2,1-*i*]purin-6-ium (IX)——To a solution of 5′-AMP (3.47 g, 10 mmoles), $(n\text{-Bu})_3N$ (1.85 g, 10 mmoles) and $HgCl_2$ (110 mg) in 50% *t*-BuOH (100 ml) was added I (4 ml). The mixture was left at 0° for 5 days to deposit colorless needles (2.22 g, 55%), a part of which was recrystallized from H_2O. This compound decomposed above 210° without melting. UV $\lambda_{max}^{H_2O}$ nm: 245, 303. *Anal.* Calcd. for $C_{13}H_{15}O_7N_6P\cdot\frac{1}{2}H_2O$: C, 38.37; H, 3.96; N, 20.60; P, 7.62. Found: C, 38.93; H, 4.37; N, 20.55; P, 7.51. NMR (D_2O, 80°) δ: 6.36 (1H, d, $J=5.2$ Hz, H_1'), 7.21 (1H, d, $J=8.0$ Hz), 8.86 (1H, d, $J=8.0$ Hz), 8.87 (1H, s), 9.19 (1H, s).

1-(5-Phospho-β-D-ribofuranosyl)-4-(4-amino-pyrimidin-2-yl)-5-aminoimidazole (X)——A solution of IX (407 mg, 1 mmole) in 1 N NaOH (15 ml) was heated at 100° for 1 hr. The reaction mixture was neutralized with Dowex-50 (pyridinium form, 10 ml), the resin filtered off and the filtrate was passed through a column of Dowex-1 (formate form). The column was washed with H_2O and eluted with 0.005 N HCO_2H. Fractions containing X were collected and evaporated to dryness *in vacuo*. The residue was dissolved in H_2O (3 ml) and the solution was heated, to which MeOH was added dropwise. The slightly turbid solution was kept in the refrigerator to afford colorless crystals (240 mg), mp 185—210° (decomp.). UV $\lambda_{max}^{H_2O}$ nm: 248, 326. *Anal.* Calcd. for $C_{12}H_{17}O_7N_6P\cdot\frac{1}{2}H_2O$: C, 36.30; H, 4.57; N, 21.08; P, 7.81. Found: C, 36.57; H, 4.88; N, 20.84; P, 7.80. $[\alpha]_D^{26}$ −80.9° (*c*, 1.02, 0.1 N NaOH). NMR (NaOD) δ: 5.59 (1H, d, $J=6.0$ Hz, H_1'), 6.32 (1H, d, $J=6.0$ Hz), 7.79 (1H, s), 8.12 (1H, d, $J=6.0$ Hz).

4-(4-Amino-pyrimidin-2-yl)-5-aminoimidazole (XII)——A solution of IX (880 mg, 2.16 mmoles) in 1 N HCl (40 ml) was heated at 100° for 40 min. The reaction mixture was evaporated to dryness *in vacuo* and the residue was dissolved in H_2O. The solution was passed through a column of Amberlite CG-50 (H^+ form, 50 ml) and the column was washed with H_2O. The effluent and washings were combined and evaporated again to dryness *in vacuo*. The residue was recrystallized from H_2O to afford X (450 mg). The column was further eluted with 0.05 N HCl, the eluate (190 ml, TOD_{260} 3000) was evaporated to dryness and the residue was dissolved in H_2O (1 ml). There was then added dropwise MeOH (20 ml) and $(CH_3)_2CO$ to give a white crystalline powder. This showed a single UV absorbing spot at $M_{Adenine}=2.2$ (PE, buffer 2). UV λ_{max}^{pH2} nm: 251, 318. *Anal.* Calcd. for $C_7H_8N_6\cdot 2HCl$: C, 33.74; H, 4.05; N, 33.74; Cl, 28.47. Found: C, 33.27; H, 4.00; N, 33.17; Cl, 28.66. NMR (d_6-DMSO) δ: 6.39 (1H, d, $J=7.5$ Hz), 7.82 (1H, s), 7.95 (1H, d, $J=7.5$ Hz).

9-Amino-3-(3,5-cyclic phospho-β-D-ribofuranosyl)-pyrimido[2,1-i]purin-6-ium (XIII)——To a solution of 3′,5′-cyclic AMP (3.29 g, 10 mmoles), $(n\text{-Bu})_3N$ (1.85 g, 10 mmoles) and $HgCl_2$ (110 mg) in 50% *t*-BuOH (100 ml) was added I (4 ml). The mixture was kept at 0° for 5 days to precipitate a brown solid, which was recrystallized from H_2O (200 ml) to afford colorless needles (0.5 g, 13%). This compound decomposed above 295° without melting. UV $\lambda_{max}^{H_2O}$ nm: 245, 302. *Anal.* Calcd. for $C_{13}H_{13}O_6N_6P$: C, 41.10; H, 3.44; N, 22.08; P, 8.16. Found: C, 40.71; H, 3.62; N, 22.24; P, 8.22.

The pH Depencence of the Reaction of I with 5′-CMP——To a solution of 5′-CMP (64.7 mg, 0.2 mmole) in 0.1 M citrate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5, each 5 ml) was added *t*-BuOH (5 ml), $HgCl_2$ (4 mg) and I (0.1 ml). The mixture was left at 20° for 3 hr. An aliquot (0.5 ml) was diluted with 0.1 M citrate buffer (pH 5.0, 10 ml). Each optical density at 308 nm was measured and the values were plotted *vs.* pH for illustration (Fig. 3).

Acknowledgement We are grateful to Dr. S. Tatsuoka, Director of this Division, for his encouragement throughout this work. Thanks are also due to the members of the Analytical Section of these Laboratories for microanalyses and NMR spectral mesaurements.

17) Partially purified from diastase (Wako Chemicals).