then warmed at 50° for 30 min. After addition of 2 ml. of 0.5*N* tartaric acid, the fluorescence intensities were measured.

The fluorometry of PAL: To 1 ml. of the reacted solution were added 5 ml. of phosphate buffer (0.2*M*, pH 7.4), and 0.1 ml. of 0.05*M* KCN or 0.1*M* phosphate buffer, and the mixtures were warmed at 50° for 120 min. The fluorescence intensities were measured after addition of 1 ml. of 0.6*M* Na_2CO_3.

A separation of PALP and PAL was not carried out in this case, instead, as references pure PALP and PAL solutions were treated by the same procedure.

We are grateful to Dr. Y. Mano of Faculty of Medicine, University of Tokyo for supplying the acid phosphatase solution.

Summary

The specificity of the previously reported fluorometric method of determination of PALP in biological materials was investigated. The fluorescence excitation and emission spectra, the change in fluorescence intensity with pH, the electrophoresis, the paper chromatography, and the hydrolysis with acid phosphatase were examined with the PALP fraction obtained from mouse liver. The results showed that the method had high specificity for the determination of PALP in liver.

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68. **Akira Nohara, Kin-ichi Imai, and Mikio Honjo:**

Synthesis of the Glucose Analogs of Inosine-5′ Phosphate.

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In the previous paper*2 we have recorded the synthesis of several compounds related to 5′-IMP*3 and interesting observations that the presense of 2′- and/or 3′-hydroxyl group(s) in the ribose moiety was not prerequisite for a flavor enhancing activity of some 5′-nucleotides, and the monophosphate grouping on position 5′ was essential for the activity. In an extention of our studies on the structure -flavor enhancing activity relationship, we have undertaken the synthesis of the nucleotides analogs whose sugar moiety was D-glucose, in place of D-ribose.

To this end, 6-benzamido-9-(2′, 3′, 4′-tri-O-acetyl-6′-diphenylphosphoryl-β-D-glucopyranosyl)purine (I) appeared the most attractive starting material, because compound I has already been reported[1] and in addition it is quite safe to conclude that I is a β-anomer on the basis of a comparatively large coupling constant (ca. 10 c.p.s.) of $H_{1'}$.[2]

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*3 5′-IMP, inosine-5′ phosphate; 5′-AMP, adenosine-5′ phosphate; 3′-AMP, adenosine-3′ phosphate; 2′-AMP, adenosine-2′ phosphate; 2′,3′-cyclic AMP, adenosine-2′,3′-cyclic phosphate; 3′,5′-cyclic AMP, adenosine-3′,5′-cyclic phosphate; AG, 9-β-D-glucopyranosyladenine.

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Hydrolysis of I was carried out according to Matsui and Nobuhara's procedure[1] (1 *N* lithium hydroxide in dioxane at room temperature for 45 minutes and then at 100° for additional 3 hours). The reaction mixture was fractionated into three parts by the use of Dowex-1 (formate form) resin (see Fig. 1). Fraction 1 as well as 2 contained a single ultraviolet absorbing material having M-value identical with that of 5′-AMP ($M_{5'-AMP}$ 1.0*4) on paper electrophoresis (acetate, pH 9.2) and therefore the compounds eluted were assumed to be isomeric 9-β-D-glucopyranosyladenine phosphates. The subsequent effluent (Fraction 3) exhibited a positive color reactlon for phosphorus (ferric-chloride-sulfosalicylic acid reagent[3]) and $M_{5'-AMP}$ value was found to be 0.56. The ultraviolet absorption spectra of this fraction were again indistinguishable from those of 5′-AMP and nuclear magnetic resonance (NMR) spectrum lacked the signals due to aromatic protons. Accordingly, to the compound from Fraction 3 has been assigned the 9-β-D-glucopyranosyladenine-4′,6′-cyclic phosphate structure (II).[4] The 3′,6′-cyclic phosphate structure can be ruled out from the point of view of other studies.[5~7]

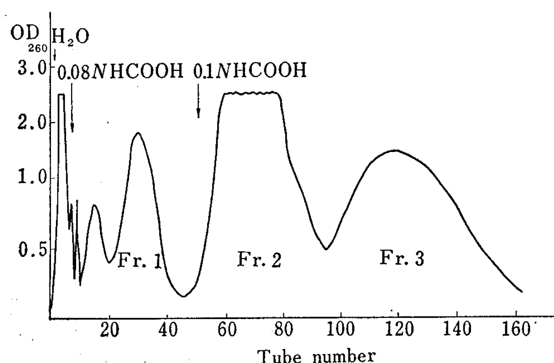

Fig. 1. Fractionation of Alkaline Hydrolysis Products of 6-Benzamido-9-(2′,3′,4′-tri-*O*-acetyl-6′-diphenylphosphoryl-β-D-glucopyranosyl)purine (I) on Dowex-1 X-8 (formate) Column (20 ml. in each tube)

It has been reported that the column chromatography of a mixture of 5′-,2′-,3′-AMP and 2′,3′-cyclic AMP by the use of Dowex-2 (formate form) resin may elute them in the following delaying order; 5′-,2′-AMP and a mixture of 3′-AMP+2′, 3′-cyclic AMP[8] (eluting system being formic acid). There is, therefore, an ample possibility that the glucose derivatives could be eluted in the following delaying order; 9-β-D-glucopyranosyladenine-6′ phosphate (III) (Fraction 1),-4′ phosphate (IV) (Fraction 2) and -4′,6′-cyclic phosphate (II) (Fraction 3).

With this consideration in mind, both Fraction 1 and 2 were deaminated with nitrous acid and treated separately with Dowex-50 (hydrogen form) resin. Each solution of the resulting glucose phosphate has been enzymatically assayed with glucose-6-phosphate dehydrogenase.*5 It was shown by this experiment that the product derived from Fraction 1 alone was positive to the reaction. Accordingly the 9-β-D-glucopyranosyladenine-6′ phosphate (III) structure of this compound has been unambiguously determined.

Hydrolysis of I with 1 *N* lithium hydroxide (at room temperature) followed by removal of phenyl group (by heating) afforded III and IV. Our explanation of the product distribution (II, III and IV) in the alkaline hydrolysate of I is as follows. Alkaline hydrolysis of I first gives 6-benzamido-9-(β-D-glucopyranosyl-6′-phenyl hydrogen phosphoryl) purine (V) which in turn gives a comparatively more stable 4′,6′-cyclic phosphate (VI).[9] The more drastic hydrolysis-coditions led to the removal of the benzoyl group on N^6 and simultaneous decyclization of the 4′,6′-cyclic phosphate to

*4 The ratio of the migration distance of a sample to that of 5′-AMP.

*5 Glucose-6-phosphate dehydrogenase was kindly provided by Professor O. Hayaishi of Kyoto University.

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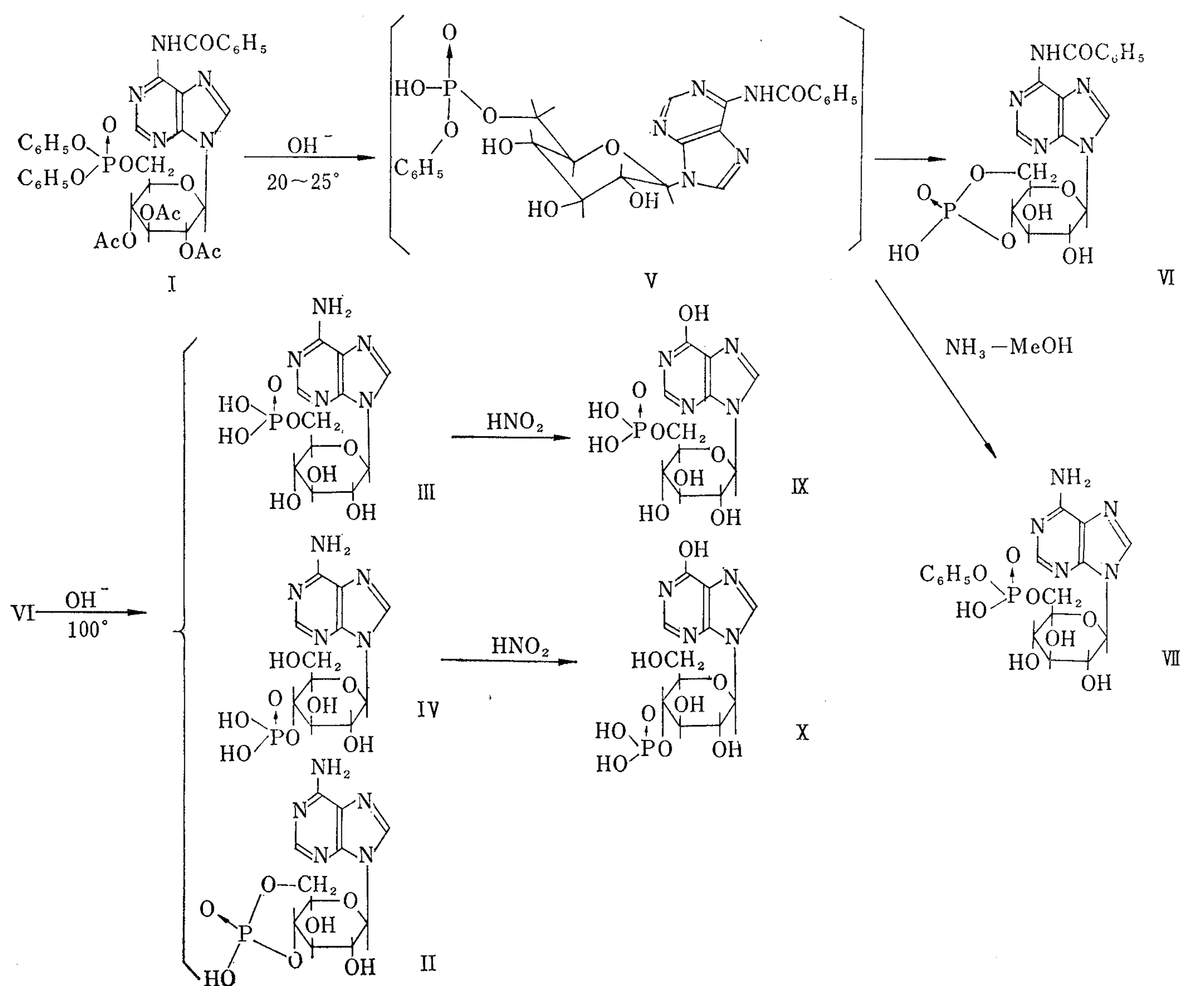

Chart 1.

give III and IV. The observations that the hydrolysis of I with sodium hydroxide, followed by treatment with methanolic ammonia led to the formation of 9-β-D-glucopyranosyladenine-6′ phenyl hydrogen phosphate (VII) provides also the supporting evidence for our proposed mechanism.

When I was treated with barium hydroxide at room temperature by the method of Matsui and Nobuhara,[1] the reaction product was not VII as recorded by the same authors, but the product has proved to be VI, in which the benzamido group at position 6 was intact because the ultraviolet absorption spectrum of the compound showed the maximum at 280 mμ. On the basis of the paper electrophoretic behavior and NMR spectral properties (the absence of aromatic protons of phenyl ester) it was shown that the phosphate was a 4′,6′-cyclic phosphate.

The phenyl group of adenosine-5′ phenyl hydrogen phosphate (VIII) was not removed by treatment with 2*N* lithium hydroxide at 100° for 1 hour.[10] The fact that the glucose derivative, V suffered the elimination of the phenyl group more readily than the corresponding VIII would probably be due to the formation of the intermediate VI.

It has been known that the treatment of 3′,5′-cyclic AMP with barium hydroxide gives 5′-AMP and 3′-AMP at a ratio of 1:5.[11] We have treated I with lithium hydroxide to give III and IV, and found that the ratio of the products was 1:5. This fact again supports the proposed mechanism that III and IV are produced by way of the intermediate VI.

10) T. Ukita, H. Hayatsu : J. Am. Chem. Soc., **84**, 1879 (1962).
11) M. Smith, G.I. Drummond, H.G. Khorana : *Ibid.*, **83**, 698 (1961).

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Ⅲ and Ⅳ were deaminated with nitrous acid to give disodium 9-β-D-glucopyranosylhypoxanthine-6′ phosphate (Ⅸ) and-4′ phosphate (Ⅹ), respectively. Both disodium salts of Ⅸ and Ⅹ were ineffective in the flavor enhancing activity.

Experimental

Procedure——Paper electrophoresis (PE) was carried out on Whatman filter paper No. 1 at 22 v/cm. for 30~60 min. using following buffer solutions: (1) 0.1*M* acetate buffer, pH 9.2[*6]; (2) 0.1*M* acetate buffer, pH 3.7; (3) 0.05*M* borate buffer, pH 9.2. Paper chromatography (PC) was carried out on Toyo Roshi No. 51A using the following solvents: (A) H_2O saturated with $(NH_4)_2SO_4$-iso-PrOH-H_2O (79:2:19) by the ascending method and (B) iso-butyric acid-0.5*N* NH_4OH (10:6); and (C) iso-butyric acid-H_2O (11:6) (*N* NH_4OH is placed in the bottom of the chamber) by the descending method. The detection was done using either irradiation with an ultraviolet lamp or coloration with ferric chloride-sulfosalicylic acid reagent.[3] Determination of total phosphorus was done in accordance with Allen's method.[12] The NMR spectra were measured with a Varian A-60 NMR spectrometer at a fixed frequency of 60 Mc. using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard or tetramethylsilane (TMS) as an external standard.

Hydrolysis of 6-Benzamido-9-(2′,3′,4′-tri-O-acetyl-6′-diphenylphosphoryl-β-D-glucopyranosyl)purine (I)[1)]——To a dioxane solution (20 ml.) of I (760 mg., 1 mmole) was added *N* LiOH (6 ml.), and the mixture was stirred for 10 min. at room temperature. H_2O (6 ml.) was added to this and the stirring was further continued for 30 min. The reaction mixture adjusted to pH 7 with *N* HCl and taken up to dryness *in vacuo*. The residue was dissolved in *N* LiOH (9 ml.) and heated in a boiling water bath for 3 hr. This was adjusted to pH 4 with dilute HCl and extracted with ether to remove benzoic acid and phenol. The aqueous layer was passed through a column of activated charcoal (3 g.). The passed solution was distilled *in vacuo* to remove ether and treated with activated charcoal to desalt. The eluate (T.O.D.$_{260}$[*7] 5,400) was taken up to dryness *in vacuo*. The residue was dissolved in NH_4OH (pH 8.6) and applied to a column of Dowex-1-X8 (formate, 100~200 mesh, 2.0×11.5 cm.). This was eluted with H_2O, 0.08*N* HCOOH, and 0.1*N* HCOOH in this order (Fig. 1). The amounts and PE behaviors of fractions are shown in Table I.

Table I

Fraction	Volume (ml.)	T.O.D.$_{260}$	$M_{5'-AMP}$ (Buffer 1)
1	446	400	1.0
2	958	2,100	1.0
3	1,550	1,100	0.56

9-β-D-Glucopyranosylhypoxanthine-6′ Phosphate (IX)——Fraction 1 (446 ml.; T.O.D.$_{260}$ 400) was desalted with activated charcoal (200 mg.). The eluate gave a single ultraviolet absorbing spot at $R_{5'-AMP}$ 1.36[*8] (PC, solvent A), R_{AG} 0.72 (PC, solvent B) and R_{AG} 1.0 (PC, solvent C). These values were identical with those of 9-β-D-glucopyranosyladenine-6′ phosphate prepared by the different method.[*9] UV: $\lambda_{max}^{0.1N\ HCl}$ 255 mμ, $\lambda_{max}^{pH\ 7}$ 258 mμ, $\lambda_{max}^{0.1N\ NaOH}$ 259 mμ. The eluate (T.O.D.$_{260}$ 340) was taken up to dryness *in vacuo*. The residue was dissolved in 2*N* AcOH (4 ml.) and to this was added $NaNO_2$ (140 mg.). The mixture was heated at 60° for 2 hr. and desalted with activated charcoal (340 mg.). To the eluate (T.O.D.$_{250}$ 180) was added two equimolar amounts of NaOH and the mixture was concentrated *in vacuo*. UV: $\lambda_{max}^{0.1N\ HCl}$ 246 mμ, $\lambda_{max}^{pH\ 7}$ 248 mμ, $\lambda_{max}^{0.1N\ NaOH}$ 251.5 mμ. This gave a single ultraviolet absorbing spot at $M_{5'-IMP}$ 0.86 (PE, buffer 3) and at $R_{5'-IMP}$ 1.46 (PC, solvent A). Hypoxanthine: P=1:0.905. One percent solution of this had no flavor enhancing activity.

9-β-D-Glucopyranosyladenine-4′ Phosphate (IV)——Fraction 2 (958 ml.; T.O.D.$_{260}$ 2,100) was desalted with activated charcoal (1 g.). The eluate (T.O.D.$_{260}$ 1,670) gave a single ultraviolet absorbing spot at $R_{5'-AMP}$ 1.0 (PC, solvent A), R_{AG} 0.80 (PC, solvent B) and R_{AG} 0.89 (PC, solvent C). UV: $\lambda_{max}^{0.1N\ HCl}$ 256 mμ, $\lambda_{max}^{pH\ 7}$ 258 mμ, $\lambda_{max}^{0.1N\ NaOH}$ 259 mμ. The ammonium salt of Ⅳ (T.O.D.$_{260}$ 3,400) was dissolved in H_2O (0.5 ml.) and to this was added an ethanolic solution (1 ml.) of brucine·$4H_2O$ (210 mg.). Addition of EtOH (7 ml.) gave powdery precipitates which were collected by filtration and purified from EtOH-28% NH_4OH-H_2O (50:2:48) to give a colorless and crystalline powder. *Anal.* Calcd. for $C_{11}H_{16}O_8N_5P\cdot(C_{23}H_{26}O_4N_2)_2\cdot 5H_2O$: C, 54.50, H, 6.26; N, 10.03; P, 2.47. Found: C, 54.60; H, 6.29; N, 9.81; P, 2.67. $[\alpha]_D^{24}$ −64.7°(c=1.0, EtOH-28% NH_4OH-H_2O (50:2:48)).

*6 Prepared by adjusting 0.1*M* acetate buffer (pH 5.0) with 28% NH_4OH.
*7 Optical density (at 260 mμ)×ml.
*8 The ratio of the Rf value of a sample to that of 5′-AMP.
*9 This method will be published later.

12) R.J.L. Allen: Biochem. J., **34**, 858 (1940).

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9-β-D-Glucopyranosylhypoxanthine-4′ Phosphate (X)——The solution (T.O.D.$_{260}$ 1,670) obtained by subjecting Fraction 2 to desalting was concentrated to dryness *in vacuo*. The residue was dissolved in 2*N* AcOH (20 ml.) and to this was added $NaNO_2$ (700 mg.). The mixture was heated at 60° for 2 hr. The reaction mixture was desalted with activated charcoal (1.48 g.). To this eluate (T.O.D.$_{260}$ 1,260) was added two equimolar amounts of NaOH and the mixture was concentrated *in vacuo*. UV : $\lambda_{max}^{0.1N\ HCl}$ 248 mμ, $\lambda_{max}^{pH\ 7}$ 248 mμ, $\lambda_{max}^{0.1N\ NaOH}$ 252 mμ. This gave a single ultraviolet absorbing spot at $M_{5'-IMP}$ 0.86 (PE, buffer 2), $M_{5'-IMP}$ 0.94 (PE, buffer 3) and $R_{5'-IMP}$ 1.10 (PC, solvent A). Hypoxanthine : P=1:0.992. One percent solution of this had no flavor enhancing activity. To an aqueous solution of this disodium salt (0.5 ml.; T.O.D.$_{248.5}$ 1,000) was added an aqueous solution (1 ml.) of $Ba(OAc)_2 \cdot H_2O$ (50 mg.). Addition of EtOH (1.5 ml.) gave precipitates which were collected and purified from H_2O and EtOH to give a pale yellow powder. *Anal.* Calcd. for $C_{11}H_{13}O_9N_4PBa \cdot 4\frac{1}{2}H_2O$: C, 20.95; H, 3.51; N, 8.88; P, 4.91. Found : C, 20.72; H, 3.43; N, 8.59; P, 5.57.

9-β-D-Glucopyranosyladenine-4′,6′-cyclic Phosphate (II)——Fraction 3 (1,550 ml.; T.O.D.$_{260}$ 1,100) was desalted with activated charcoal (500 mg.). The eluate (T.O.D.$_{260}$ 1,080) was taken up to dryness *in vacuo* to give a glassy residue. This was positive to the phosphorus coloration with ferric chloride-sulfosalicylic acid reagent and gave a single ultraviolet absorbing spot at $R_{5'-AMP}$ 0.55 (PC, solvent A), R_{AG} 0.78 (PC, solvent B) and R_{AG} 0.91 (PC, solvent C). NMR : δ 8.22, 8.36 (H_2 and H_8), 5.58 (H_1', doublet J=9.0 c.p.s.). An aqueous solution (1 ml.; T.O.D.$_{260}$ ca. 6,000) of the ammonium salt was adjusted to pH 8.2 with *N* NH_4OH and to this were added an aqueous solution (0.5 ml.) of $Ba(OAc)_2 \cdot H_2O$ (60 mg.) and then EtOH (7.5 ml.) to give precipitates, which were collected and purified from H_2O and EtOH. *Anal.* Calcd. for $C_{11}H_{13}O_7N_5PBa/2 \cdot 4H_2O$: C, 26.48; H, 4.24; N, 14.04; P, 6.21. Found : C, 26.41; H, 3.76; N, 14.31; P, 6.06. UV : $\lambda_{max}^{0.1N\ HCl}$ 256 mμ (ε 1.53×10^4), $\lambda_{max}^{pH\ 7}$ 259 mμ (ε 1.53×10^4), $\lambda_{max}^{0.1N\ NaOH}$ 260 mμ (ε 1.54×10^4). $[\alpha]_D^{25}$ −23° (c=0.53, H_2O).

9-β-D-Glucopyranosyladenine-6′ Phenyl Hydrogen Phosphate (VII)——To a dioxane solution (15 ml.) of I (1.50 g.) was added 2*N* NaOH (15 ml.) and the mixture was left standing at room temperature for 2.5 hr. This was adjusted to pH 5.4 with Amberlite IRC-50 (hydrogen) and filtered. This resin was washed with H_2O. The filtrate and washings were combined and evaporated to dryness *in vacuo*. To the residue was added methanolic ammonia (60 ml.) and the vessel was stoppered. It was left standing overnight at room temperature and concentrated *in vacuo* to give needles. The crystals were filtered off. A part (T.O.D.$_{260}$ 26,200) of this filtrate was adjusted to pH 9 with 28% NH_4OH and applied to a column of Dowex-1 X-8 (formate, 2.2×46 cm.). It was eluted with 0.2*N* HCOOH to give the fraction of II followed by a new fraction (T.O.D.$_{260}$ 1,900). This showed $M_{5'-AMP}$ 0.65 (PE, buffer 1), $R_{5'-AMP}$ 0.60 (PC, solvent A) and $R_{5'-AMP}$ 1.18 (PC, solvent B). UV : $\lambda_{max}^{0.1N\ HCl}$ 256 mμ, $\lambda_{max}^{pH\ 7}$ 259 mμ, $\lambda_{max}^{0.1N\ NaOH}$ 259 mμ. NMR : δ 8.18, 8.11 (H_2 and H_8), 6.90 (phenyl group), 5.41 (H_1', doublet J=9.0 c.p.s.). To an aqueous solution (0.3 ml.; T.O.D.$_{260}$ 1,900) subjected to desalting were added an ethanolic solution (0.5 ml.) of brucine·$4H_2O$ (65 mg.) and EtOH (8 ml.). Trace amounts of impurities were filtered off. The filtrate were concentrated *in vacuo* and purified from H_2O and EtOH to give a colorless powder. *Anal.* Calcd. for $C_{17}H_{20}O_8N_5P \cdot C_{23}H_{26}O_4N_2 \cdot H_2O$: C, 55.49; H, 5.59; N, 11.32; P, 3.58. Found : C, 55.75; H, 5.95; N, 11.13; P, 3.55.

Enzymatic Assay of Glucose Phosphate——i) An aqueous solution (0.5 ml., T.O.D.$_{248.5}$ 48, 3.93 μmoles) of the disodium salt of IX and Dowex-50 (hydrogen) (150 mg.) were placed in a sealed vessel. This was shaken for 2 hr. in a boiling water bath. The resin was filtered, and washed with water. The filtrate and washings were combined and made up to 10 ml. (T.O.D.$_{248.5}$ 30.5). This gave three spots of glucose-6-phosphate, IX and hypoxanthine by PE with the buffer 3. One ml. of the above solution was assayed by glucose-6-phosphate dehydrogenase[*10] to find out that it was positive to the glucose-6-phosphate reaction. ii) The disodium salt of X was treated and assayed as above to find out that it was negative to the glucose-6-phosphate reaction.

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Summary

Hydrolysis of 6-benzamido-9-(2′,3′,4′-tri-O-acetyl-6′-diphenylphoshoryl-β-D-glucopyranosyl)purine (I) in alkaline solution gave 9-β-D-glucopyranosyladenine-6′ phosphate (III) and -4′ phosphate (IV) *via* the intermediate 4′,6′-cyclic phosphate. III and IV have been deaminated to give 9-β-D-glucopyranosylhypoxanthine-6′ phosphate (IX) and -4′ phosphate (X), respectively.

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[*10] Glucose-6-phosphate dehydrogenase was used after diluting 20 folds by cool water. Assay was carried out in accordance with the methods in Enzymology, **3**, 152 (1957). *M* Tris-AcOH buffer, pH 7.5, was used instead of the glycylglycine buffer.

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