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Synthetic Nucleosides and Nucleotides. VIII.[1] Direct Synthesis of the 5'-Phosphate of 4-Thiouridine, 6-Thioinosine and 6-Thioguanosine from the Corresponding Oxy Nucleotide *via* Thiation Procedure[2]

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Direct and simple methods for the preparations of 4-thiouridine 5'-phosphate (IVa), 6-thioinosine 5'-phosphate (IVb) and 6-thioguanosine 5'-phosphate (IVc) from the corresponding oxy-nucleotides are described. The 5'-ribonucleotides are converted to their 2',3'-di-O-acetylated derivatives by a conventional acetylation procedure, followed by thiation with phosphorus pentasulfide to give crude thiated products. For the selective desulfurization, the crude products are treated with dicyclohexylcarbodiimide, followed by alkaline deacylation to give the desired "thio" nucleotides.

Thio analogues of uridylic,[4,5] inosinic,[6-8] and guanylic acid[6,7] are interesting compounds not only for their biological significances but also as useful intermediates for preparation of various nucleotidic materials.

4-Thiouridine phosphate, which was found as a minor constituent in *E. coli* transfer RNA,[9] is a very impotant model compound for studying the selective chemical modification of the 4-thiouridylate residue in transfer RNA.[10-13]

And also this nucleotide is useful intermediate for the preparation of its 5'-di- and triphosphates which are substrate of polynucleotide phosphorylase and DNA dependent RNA polymerase. On the other hand, 6-thio analogues of inosinic and guanylic acids are useful substances as water soluble antileukemic agents in experimental chemotherapy.

Previously, several workers reported that these nucleotides could be prepared from corresponding partially protected ribonucleosides by phosphorylation.[4-8] But these methods involve many steps that are difficult for large scale preparations. More convenient methods for their preparation are described in this paper. 4-Thiouridine 5'-phosphate was readily prepared by the procedure outlined in Chart 1. Uridine 5'-phosphate (Ia) was acetylated with acetic anhydride in anhydrous pyridine followed by hydrolysis of the intermediate acetyl

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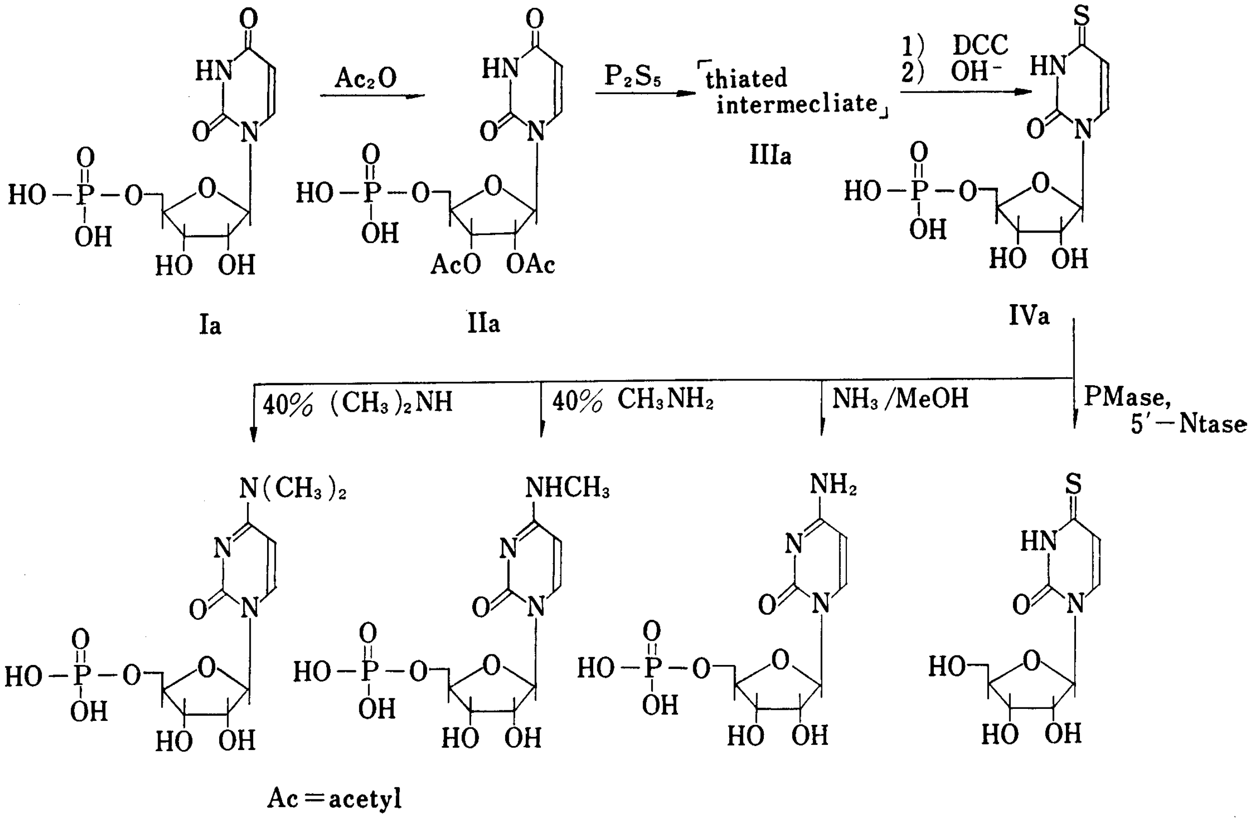

Chart 1

phosphate with aqueous pyridine. The 2′,3′-di-O-acetyluridine 5′-phosphate[14] (IIa) thus obtained was thiated by refluxing in pyridine with phosphorus pentasulfide (P_2S_5) to afford a mixture of 4-thio derivatives including the 5′-phosphate, 5′-phosphorothioate and 5′-phosphorodithioate of 2′,3′-di-O-acetyl-4-thiouridine (IIIa). For the selective desulfurization of phosphorthioate moiety to give the phosphate, the crude mixture of 4-thio derivatives was treated with dicyclohexylcarbodiimide (DCC) in anhydrous pyridine and then hydrolyzed with aqueous sodium hydroxide containing pyridine.

After desalting by Dowex 50 (H-form) and charcoal columns, the product was further purified by DEAE-cellulose column chromatography using a linear gradient technique with triethylammonium bicarbonate buffer (pH 7.9, 0.01—0.3M). The yield of 4-thiouridine 5′-phosphate (IVa) from Ia was 15—18%. The structure of the product was established by the following procedure. The nucleotide (IVa) was completely hydrolyzed with *E. coli* alkaline phosphomonoesterase (PMase) to produce 4-thiouridine which was identical with an authentic specimen,[4,10b] by paper chromatographic mobilities and ultraviolet (UV) and infrared (IR) spectrometric measurements. The nucleotide (IVa) was treated with 0.1N hydrochloric acid at 100° for 1 hr to give uridylic acid (Ia) as shown by paper chromatography and UV spectrometric measurement at alkaline and acidic pH. Then the compound IVa was sealed in a tube with methanol saturated with ammonia at 0° and heated at 70—80° for 10 hr. The product was identified as cytidine 5′-phosphate by paper chromatography and its UV absorption properties. When a 40% aqueous solution of methylamine or dimethylamine was used instead of methanolic ammonia, N_4-methylcytidine 5′-phosphate or N_4-dimethylcytidine 5′-phosphate was obtained, respectively. These products were identified with authentic specimen[15] by UV absorption measurements.

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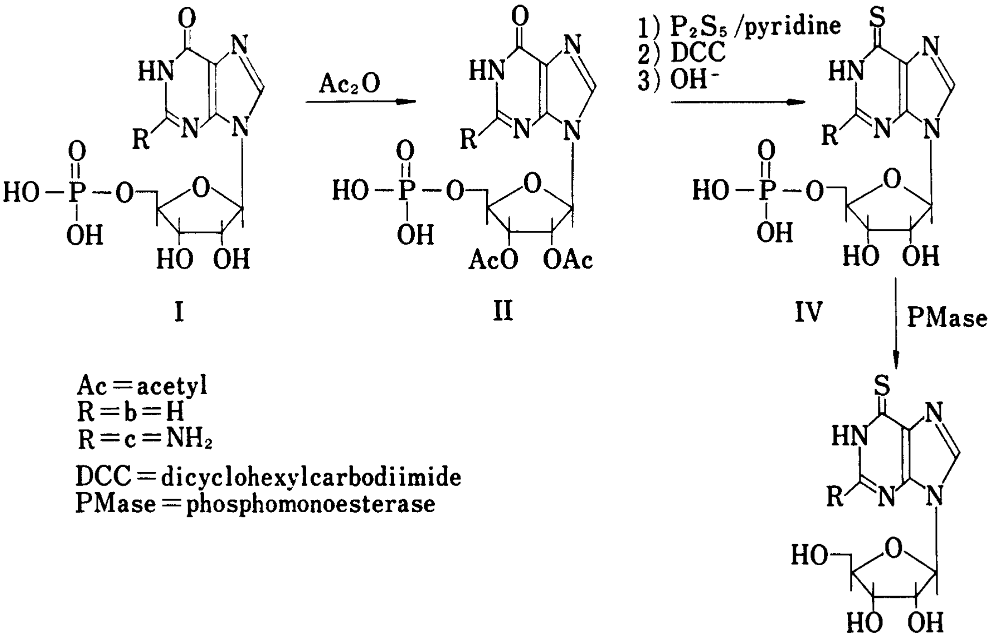

Ac = acetyl
R = b = H
R = c = NH_2
DCC = dicyclohexylcarbodiimide
PMase = phosphomonoesterase

Chart 2

In the same manner as the above, the 5′-phosphates of 6-thioinosine and 6-thioguanosine (IVb and IVc) were prepared by thiation of the corresponding 2′,3′-di-O-acetylated derivatives, followed by desulfurization with DCC and then deacylation by alkaline treatment to afford the desired products as shown in Chart 2. The resulting products were both easily hydrolyzed with *E. coli* PMase to 6-thioinosine and 6-thioguanosine, respectively, thus establishing their identity. Enzymatic hydrolysis of these products with snake venom 5′-nucleotidase[16] was also examined. The products were both hydrolyzed to their nucleosides with this enzyme.

It is to be noted that 2′,3′-di-O-acetylated derivatives of uridine 5′-phosphate, inosine 5′-phosphate and guanosine 5′-phosphate readily thiated by the above procedure to yield the corresponding thio-nucleotides. In the case of thiation of guanylic acid, a small amount of 6-thioguanine was produced as a by-product. However, application of this method to the 2′,(3′)-phosphate isomers was attempted but failed. This may be due to the formation of a 2′,3′-cyclic phosphate in the acetylation process and the resulting cleavage of the cyclic phosphate in the thiation procedure. Interaction of the P_2S_5 with the exposed hydroxyl group could lead to degradation of the sugar moiety of the nucleotide.

Experimental

Ultraviolet absorption measurements were carried out by a Cary 14 Recording Spectrophotometer. Descending paper chromatograms on Toyo-Roshi No. 51A paper were obtained in the following solvent systems. Solvent A, 95% EtOH-1M CH_3COONH_4 (7:3, v/v, pH 7.5), solvent B, isobutyric acid-conc. NH_4OH-water (66:1:33, v/v/v, pH 3.7), solvent C, isopropanol-1N NH_4OH (7:3, v/v), solvent D, isopropanol-saturated $(NH_4)_2SO_4$-1M CH_3COONa (2:80:18, v/v/v/).

Glass plates (10×10 cm), coated with Avicel SF cellulose were used for thin-layer chromatography. The chromatograms were developed with the same solvent systems as the paper chromatography.

The General Procedure for the Acetylation of 5′-Ribonucleotides——To a solution of a 5′-ribonucleotide (10 mmoles) in 60 ml of anhydrous pyridine was added 13 ml of acetic anhydride freshly distilled from anhydrous sodium acetate. The resulting clear solution was stirred at room temperature in the dark with protection from atmospheric moisture for 24 hr. This reaction mixture was evaporated to dryness at reduced pressure at room temperature.

The residual gum was mixed with 50% aqueous ethanol and co-evaporated three times. Acid-free gum was dissolved in 50 ml of aqueous pyridine and left at 37° for 2 hr to hydrolyse the acetyl nucleotidate, the reaction mixture was then evaporated to form glassy or powdered material.

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[16] This enzyme was purchased from Sigma Chemical Co., Ltd.

The product was checked for purity by thin-layer chromatography with solvent A, and normally shows one spot due to the desired diacetate. Only in the case of 5′-guanylic acid two spots were detected. Presumably, one was the N_2, 2′, 3′-O-triacetate and the othee was the 2′,3′-di-O-acetate. The yields of the acetylated products were almost quantitative. No further purification of these products was needed for the next step in the thiation reaction.

Thiation of 2′,3′-Di-O-acetyluridine 5′-Phosphate with Phosphorus Pentasulfide in Pyridine——The compound IIa (10 mmoles) was dissolved in pyridine (100 ml) and then freshly ground P_2S_5 (5.0 g) was added to this solution under vigorous stirring. The resulting solution was gently refluxed for 6 hr. After the solvent was removed under reduced pressure, the brown viscous gum was extracted three times with 50% aqueous methanol (50 ml). The methanolic solution was passed through a column of Dowex 50 (H^+-form) (2.5 × 30 cm) and the resin was washed with 100 ml of the same solvent. The eluate and washings were combined and evaporated at room temperature under reduced pressure to yield the thiated products of the 2′,3′-diacetates (yield, 7.0 mmoles, estimated by absorption at 330 mμ). UV absorption, $\lambda_{\max}^{\text{MeOH}}$: 244 mμ, 330 mμ.

Direct Deacylation of Thiated Products of 2′,3′-Di-O-acetyluridine 5′-Phosphate (IIIa)——The actylated "thio" products (IIIa) were dissolved in 50% aqueous pyridine (40 ml) and then 2M sodium hydroxide (20 ml) was added dropwise into the solution at room temperature under magnetic stirring. The solution became cloudy at once, but soon turned to a clear orange solution. After stirring it at room temperature for 30 min, 10 ml of Dowex 50 (H^+-form) resin was added with shaking. The resin was filtered off and the filtrate was applied to an acid washed charcoal column (2.5 × 30 cm). The column was washed with distilled water (200 ml) and then eluted with 50% aqueous methanol containing 5% cyclohexylamine. The light yellow eluate (300 ml) was evaporated to dryness at room temperature under reduced pressure. The residue was washed three times with 5 ml of dry ether. The residue, freed from cyclohexylamine, was dissolved in 5 ml of hot methanol and poured into 100 ml of cold anhydrous ether with vigorous stirring. The yellow precipitate thus obtained was collected by centrifugation and dissolved again in the same amount of hot methanol. Reprecipitation was repeated three times. The product was dried over phosphorus pentoxide at room temperature for 10 hr under reduced pressure. A light yellow powder was obtained (270 mg). For the characterization of this product, enzymatic dephosphorylation was attempted.

This product (1 mg) was dissolved in 0.2 ml of 0.05M ammonium bicarbonate (pH 8.5) and 0.02 ml of *E. coli* alkaline PMase[17] was added. The mixture was incubated at 37° for 18 hr. The reaction mixture was spotted on Toyo Roshi No. 51A paper and chromatogram developed with solvent A. Two sopts, *Rf*= 0.87 and 0.27 were observed. The relative optical density at 330 mμ of the extracts from these spots was about 8 to 2. The spot which ran faster consisted of only 4-thiouridine and the slower spot was PMase resistant phosphorus-containing material. The same treatment of the authentic 4-thiouridine 5′-phosphate[8] gave complete hydrolysis to 4-thiouridine. Unfortunately, separation of 4-thiouridine 5′-phosphate from the PMase resistant contaminant was not successful by paper chromatography, paper electrophoresis or by ion-exchange column chromatography with several different eluting systems.

Selective Desulfurization of the Phosphorothioate Moiety of the Thiation Product followed by Alkaline Treatment to 4-Thiouridine 5′-Phosphate (IVa)——The product IIIa (1 mmole) was treated with DCC (1.8 mmoles) in anhydrous pyridine (10 ml) at room temperature for 24 hr Precipitated dicyclohexylurea was removed by filtration and then water (10 ml) was added to this solution. The resulting mixture was again filtered and concentrated to dryness at room temperature under reduced pressure.

The residue was dissolved in 10 ml of 50% aqueous pyridine and 2N sodium hydroxide was added slowly with stirring. Then, after the solution was incubated at 37° for 18 hr, it was filtered on a glass funnel. The filtrate was neutralized with Dowex 50 (H^+) resin to pH 8.0 and the solution was concentrated to *ca.* 2 ml. This solution was applied to a charcoal column (2.5 × 30 cm), and the column was washed with distilled water (200 ml) and then eluted with 50% aqueous methanol containing 5% cyclohexylamine. The light yellow eluate (300 ml) was evaporated to dryness at room temperature under reduced pressure. The residue was washed three times with 5 ml of dry ether. The residue, freed from cyclohexylamine, was dissolved in 3 ml of water and applied to a column of DEAE-cellulose (bicarbonate form) (1.5 × 30 cm). The product was eluted with a linear salt gradient system consisting of 0.01M triethylammonium bicarbonate (pH 7.9, 1 liter) in a mixer and 0.3M triethylammonium bicarbonate (pH 7.9, 1 liter) in a reservoir. The desired 4-thiouridine 5′-phosphate was contained in 700—900 ml eluate. The fractions were combined and evaporated at room temperature under reduced pressure. The residue was treated with 50% ethanol and evaporated repeatedly to remove triethylamine and its bicarbonate salt. Triethylammonium 4-thiouridine 5′-phosphate was obtained in a yield of 0.2 mmoles. The triethylammonium salt of the nucleotide was converted to its cyclohexylammonium salt by first passing it through a column of Dowex 50 (H^+) followed by a column of Dowex 50 (cyclohexylammonium form) and it was precipitated from methanol and dry ether as a light yellow granular powder.

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17) 6.68×10^{-5} units, this enzyme was kindly supplied from Dr. K. Tanaka, Shionogi Research Laboratory, Osaka.

Table I

Compounds	Solvent A	Solvent B	Solvent C	Solvent D
Uridine 5′-phosphate (Ia)	0.20	0.22	0.21	0.66
Inosine 5′-phosphate (Ib)	0.11	0.22	0.21	0.65
Guanosine 5′-phosphate (Ic)	0.13	0.18	0.19	0.55
2′,3′-Di-O-acetyluridine 5′-phosphate (IIa)	0.74[a)]	0.49		
2′,3′-Di-O-acetylinosine 5′-phosphate (IIb)	0.70[a)]	0.65		
2′,3′-Di-O-acetylguanosine 5′-phosphate (IIc)	0.60[a)]	0.63		
4-Thiouridine 5′-phosphate (IVa)	0.29	0.21	0.26	0.55
6-Thioinosine 5′-phosphate (IVb)	0.45	0.35	0.19	0.54
6-Thioguanosine 5′-phosphate (IVc)	0.35	0.30	0.11	0.60

Rf values of differnet compounds on paper and thin-layer chromatography[a)]

mp 180° (decomp.). UV, $\lambda_{\max}^{H_2O}$ mμ (ε): 244 (3960), 330 (20800); $\lambda_{\min}^{H_2O}$ mμ: 224, 275; $\lambda_{\max}^{pH_1}$ mμ: 244, 331; $\lambda_{\max}^{pH_{11}}$ mμ (ε): 317 (18400). *Anal.* Calcd. for $C_9H_{11}O_8N_2SP \cdot C_{12}H_{26}N_2$: P, 5.59. Found, P, 5.68. *Rf* values of this nucleotide on paper chromatography were summerized in Table I.

Preparation of 6-Thioinosine 5′-Phosphate by the Direct Thiation Method——To the solution of IIb (1 mmole) in 200 ml of anhydrous pyridine was added freshly ground P_2S_5 (600 mg). The resulting mixture was refluxed for 6 hr and then evaporated. The residue was treated with 10 ml of water and heated at 50—60° for 30 min. Evaporation of the solvent gave dark-brown gum which was extracted with 50% methanol (10 ml × 5). Combined extracts were passed through the column of Dowex 50 (H^+-form) resin (2 × 15 cm). The column was washed with the same solvent. The eluate and washings were combined and evaporated to dryness. Anhydrous benzene (10 ml) was added to the residue and evaporated repeatedly (five times) to give an anhydrous gum. The gum was dissolved in 100 ml of anhydrous pyridine and 800 mg of DCC was quickly added to the solution which was then kept at room temperature for 48 hr. Precipitated dicyclohexylurea was removed by filtration. The filtrate was concentrated to about 50 ml and mixed with 2N sodium hydroxide solution (50 ml). The mixture was incubated at 37° for 18 hr. The resulting clear solution was neutralized with Dowex 50 (pyridinum form). The resin was removed by filtration and washed with water.

The combined filtrate and washings were neutralized with saturated barium hydroxide solution to pH 7.5. The precipitate was removed by centrifugation and the supernatant was concentrated to *ca.* 20 ml and diluted with 40 ml of ethanol. After being stored in an ice-box overnight, a white precipitate came out which was crude barium 5′-6-thioinosinate (150 mg). The barium salt was dissolved in 3.5 ml of water and applied on a column of DEAE-cellulose (bicarbonate form) (2 × 30 cm). It was eluted with a linear gradient, consisting of 0.01M triethylammonium bicarbonate (pH 7.9,1 liter) in a mixer and 0.3M triethylammonium bicarbonate (pH 7.9, 1 liter) in a reservoir. Contaminating inosine 5′-phosphate was eluted in 0.8—0.9 liter and 6-thioinosine 5′-phosphate in 1.2—1.5 liters. Fractions containing the products were collected and evaporated under reduced pressure at room temperature. The residue was treated with 50% ethanol and evaporated repeatedly to remove triethylammonium bicarbonate and triethylamine. The product, 6-thioinosine 5′-phosphate (0.38 mmole) as its triethylammonium salt, was estimated spectrophotometrically. The barium salt was prepared by passing it through a column of Dowex 50 (barium form) and precipitation with ethanol. UV, $\lambda_{\max}^{pH_1}$ mμ (ε): 322 (23100); $\lambda_{\min}^{pH_1}$ mμ: 255; $\lambda_{\max}^{pH_{11}}$ mμ (ε) 311 (21600). *Anal.* Calcd. for $C_{10}H_{11}N_4O_7SBaP \cdot 4\ H_2O$: P, 5.42. Found: P, 5.32.

Preparation of 6-Thioguanosine 5′-Phosphate (IVc) by the Direct Thiation Method——The compound (IIc) (5 mmoles) was dissolved in pyridine (300 ml) and then powdered P_2S_5 (2.3 g) was added portionwise to the solution with shaking. The mixture was gently refluxed for 6 hr. After the solvent was removed under a reduced pressure, the dark-brown gum was treated with 300 ml of 50% methanol. Insoluble material which contained 6-thioguanine (*ca.* 0.3 mmole) was removed by filtration then the filtrate was evaporated. The residue was rendered anhydrous by repeated evaporation with dry pyridine. Anhydrous pyridine (200 ml) and DCC (3.9 g) were added to the residue and the mixture was incubated for 24 hr at room temperature. Then water (500 ml) was added and the precipitate was removed by filtration. The filtrate was concentrated to a small volume. A mixture of 50% aqueous pyridine (50 ml) and 2N sodium hydroxide (50 ml) was added to the solution and resulting mixture kept at 37° for 18 hr. The reaction mixture was cooled to room temperature and diluted with water (100 ml). Then the solution was passed through the column of Dowex 50 (H^+-form) (3 × 15 cm). The eluate was neutralized with saturated barium hydroxide to pH 7.5. The resulting precipitate was removed by filtration and the filtrate was concentrated to *ca.* 20 ml. To this solution was added cold ethanol (50 ml) and the mixture was kept in ice-box overnight. The precipitate thus obtained was collected and dried. Crude barium 5′-6-thioguanylate was obtained (1.3 g). Further purification of the product was carried out by column chromatography on DEAE-cellulose in the manner described above. Pure 6-thioguanosine 5′-phosphate was obtained in a 20% yield. This product was easily hydrolyzed to its nucleosides with *E. coli* alkaline PMase. *Anal.*, 6-thioguanosine: P=1: 0.99.

Conversion of IVa to Ia by Acid Hydrolysis——The product IVa (*ca.* 1 mg) was dissolved in 0.2 ml of 0.1N hydrochloric acid and heated at 90—100° for 1 hr. The hydrolysate was proved to be uridine 5′-phosphate by paper chromatography in three solvent systems and UV spectrophotometry in acidic and alkaline media.

Conversion of IVa to Cytidine 5′-Phosphate by Reaction with Ammonia——The compound IVa (1 mg as free acid monohydrate) was dissolved in anhydrous methanol saturated with ammonia at 0° (1 ml) and heated at 70—80° for 10 hr in a sealed tube. The reaction mixture was evaporated to dryness and chromatographed on Dowex 1 (formate) column by elution with 0.02M formic acid. The chromatographic pattern revealed that IVa was converted to cytidine 5′-phosphate in good yield (80%). The identity of the product was confirmed by UV spectrophotometry in both acidic and neutral media.

Conversion of IVa to N_4-Methyl and N_4-Dimethylcytidine 5′-Phosphate——Compound IVa (1 mg as free acid monohydrate) was dissolved in 1 ml of 40% aqueous methylamine or 40% aqueous dimethylamine and heated at 70—80° for 10 hr in a sealed tube. The rection mixture was evaporated to dryness. The gum was dissolved in 1 ml of water and chromatographed on Dowex 1 (formate form) column by elution with 0.02M formic acid.

N_4-Methyl or N_4-dimethylcytidine 5′-phosphate were obtained in good yield. These samples were identified with authentic specimens11) by paper chromatography and UV spectroscopy.

Enzymatic Hydrolysis of "Thio" Ribonucleoside 5′-Phosphates with *E. coli* Alkaline Phosphomonoesterase——The thio nucleoside 5′-phosphate (*ca.* 1 mg) was dissolved in 0.2 ml of 0.05M ammonium bicarbonate (pH 8.5) and 0.02 ml of *E. coli* PMase solution16) was added. The mixture was incubated at 37° for 18 hr. The reaction mixture was chromatographed on Toyo Roshi No. 51A paper with solvent A. No spot of the phosphate and only one spot corresponding to nucleoside was observed in all cases.

Enzymatic Digestion of "Thio" Ribonucleoside 5′-Phospahtes with Snake Venom 5′-Nucleotidase——The nucleotide (*ca.* 1 mg) was dissolved in 0.2 ml of 0.05M ammonium bicarbonate (pH 8.5) and 0.005 mg of snake venom 5′-nucleotidase12) was added to this solution. The mixture was incubated at 37° for 24 hr. The reaction mixture was chromatographed on paper as described above. All of the nucleotides were completely hydrolyzed to nucleosides with this enzyme.

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