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36. Tyunosin Ukita and Masachika Irie: Organic Phosphates. XI.[*2] Syntheses of Several 2′,3′-Cyclic Ribonucleotides and their Properties as Substrate for Ribonuclease.

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In a previous paper,[*2] the synthesis of methyl α- and β-D-ribofuranoside 2,3-cyclic phosphates, which have no pyrimidine bases substituted in their C-1 position, was reported and it was observed that these cyclic phosphates could not be hydrolyzed by pancreatic ribonuclease (RNase-I[*3]). These results gave evidence that in the naturally occurring ribonucleotide, their pyrimidine base plays an important rôle in their susceptibility to RNase-I.

In order to obtain further information on the structural requirements in the substrate for this enzyme, several 2′,3′-cyclic ribonucleotides containing 5-substituted uridines, 3-(β-D-ribofuranosyl)thymine 2′,3′-cyclic phosphate (V), and 5-bromouridine 2′,3′-cyclic phosphate (VIII) were synthesized and were tested for their properties as a substrate for RNase-I-A[1] the results of which are reported in this paper.

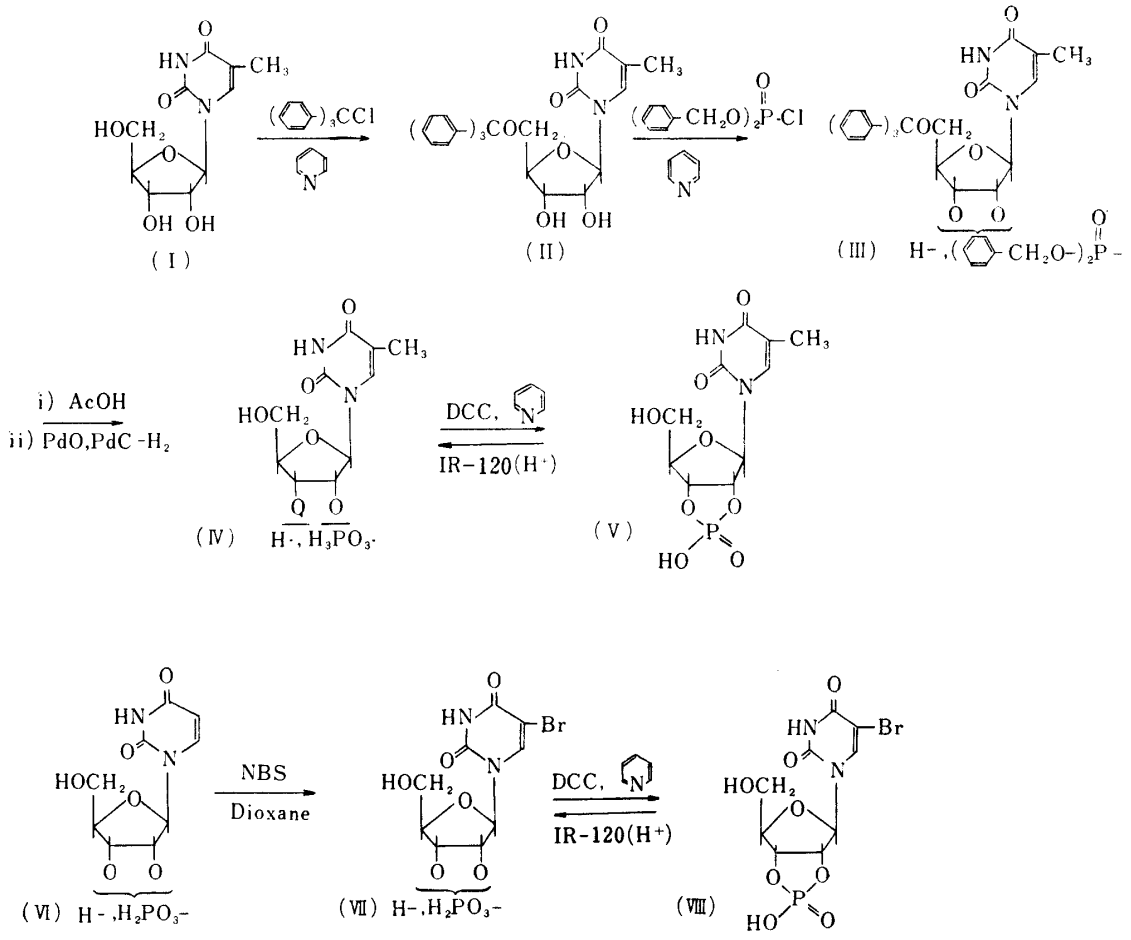


Chart 1.

*1 Hongo, Tokyo (浮田忠之進, 入江昌親).

*2 Part X. T. Ukita, M. Irie: This Bulletin, **9**, 211(1961).

*3 Abbreviation: RNase-I-A, ribonuclease-I-A; DCC, dicyclohexylcarbodiimide; NBS, N-bromosuccinimide.

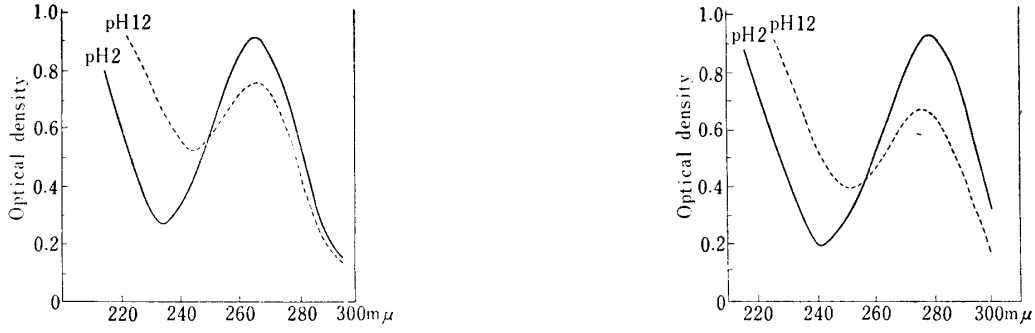
1) C. H. W. Hirs, S. Moore, W. H. Stein: J. Biol. Chem., **200**, 493 (1953).

3-(β-D-Ribofuranosyl)thymine (ribothymidine) (I) and its 2′- and 3′-phosphates (IV) have recently been detected in the degradation products of ribonucleic acids together with nucleosides and nucleotides which contain methylated adenine or guanine such as 2-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine, and 1-methylguanine as minor constituents of nucleic acid from yeast,[2,3] wheat germ, and *Escherichia coli*.[2] However, no report on the chemical synthesis of (IV) has hitherto been made.*4

Modifying Fox's procedure,[4] 3-(β-D-ribofuranosyl)thymine[5] was treated with trityl chloride in dehydrated pyridine to furnish 5′-tritylribothymidine (II) in 80% yield, which was purified to needle crystals, m.p. 162~165°.

(II) was phosphorylated with dibenzyl chlorophosphonate in pyridine[6] and the syrupy product was directly detritylated by refluxing in 80% acetic acid solution for 20 minutes, followed by catalytic debenzylation using a mixture of palladium oxide and palladium-charcoal as catalysts in dehydrated ethanol.

The final reaction mixture gave a single spot on a paper chromatogram run with four different solvent systems ((i), (iii), (iv), and (v)), and each spot was detected by ultraviolet absorption as well as by positive phosphorus reaction. This product (IV) was isolated as a powdery barium salt and was found to have molecular formula of $C_{10}H_{13}O_9N_2BaP \cdot 6H_2O$. The ultraviolet absorption curve of (IV) is shown in Fig. 1a.



(a) Ribothymidine 2′ and 3′-phosphate (IV) 10^{-4} mole

(b) 5-Bromouridylic Acid (VII) 10^{-4} mole

Fig. 1.

After decationation with Amberlite IR-120(H^+), (IV) was dehydrated with DCC in pyridine to a cyclic phosphate (V) which showed a spot at Rf_1 0.43 on paper chromatogram and which was isolated as its ammonium salt, $C_{10}H_{16}O_8NP$. This phosphate (V) showed typical properties of a cyclic phosphate in its mobility in paper electrophoresis.[7] Thus, at pH 6.0, taking the mobility of cytidylic acid as standard (M 1.00) (V) showed the mobility of M_1 1.26, a little faster than that of (IV) (M_1 1.18) and, in a buffered solvent of pH 9.0, (V) showed M_2 0.85, slower than that of (IV) (M_2 1.00). Furthermore, (V) was easily hy-

*4 Griffin, *et al.*[13] reported the occurrence of cyclic ribothymidylic acid as an intermediate compound during the incubation of RNase with synthetic polyribothymidylic acid, which was obtained on condensation of ribothymidine 5′-pyrophosphate with polynucleotide phosphorylase isolated from *Azotobactor vinelandii* and *Escherichia coli*. Furthermore, Davis, *et al.*[4] treated ribothymidylic acid isolated from yeast RNA with DCC and the reaction product gave on paper electrophoresis a spot which was assumed to be of cyclic ribothymidylic acid from its convertibility to the starting material by RNase-I.

2) J. W. Littlefield, D. B. Dunn : Biochem. J., **70**, 642 (1958); Nature, **181**, 254 (1958).
3) F. F. Davis, A. F. Carleuci, I. F. Roubein : J. Biol. Chem., **234**, 1525 (1959).
4) J. J. Fox, N. Young, A. Bendich : J. Am. Chem. Soc., **79**, 2775 (1957).
5) J. J. Fox, N. Young, J. Davoll, G. B. Brown : *Ibid.*, **78**, 2117 (1956).
6) D. M. Brown, A. R. Todd : J. Chem. Soc., **1952**, 44.
7) G. M. Tenner, H. G. Khorana, R. Markham, E. H. Pol : J. Am. Chem. Soc., **80**, 6223 (1958).

drolyzed to (IV) by treatment of its aqueous solution with Amberlite IR-120(H^+) at room temperature.

The Rf values of ribothymidine 2′- and 3′-phosphates (IV) run in two solvent systems were compared with those of adenylic acid (A), guanylic acid (G), cytidylic acid (C), and uridylic acid (U) together with 5-bromouridylic acid (VII) and it was found that in the solvent systems (iii) and (iv), the Rf values are in the descending order of (A), (C), (IV) and (VII), (U), (G), and of (IV), (VII), (U), (G), (A), (C), respectively, while Davis, *et al.*[3] reported that the mobility of thymidylic acid travelled was between (C) and (U) in the solvent system (iii) and faster than the other four nucleotides in the solvent system (iv).

For the synthesis of 5-bromouridylic acid (II), yeast uridylic acid was brominated with N-bromosuccinimide, according to the procedure of Michelson[8] for uridine 5′-phosphate. By this bromination, the desired phosphate (VII) was obtained in 85% yield and isolated as calcium salt which was analyzed to have a molecular formula of $C_9H_{10}O_9N_2BrCaP \cdot 4H_2O$ and gave a single spot on paper chromatograms run in the solvent systems (i) to (v).

The ultraviolet absorption curve of (VII) is given in Fig. 1b, which showed maximum and minimum absorptions respectively at 275 and 239 mμ which are in good agreement with those of 5-bromouridine and 5-bromouridine 5′-phosphate reported by Roberts[9] and by Michelson,[8] indicating the bromine to be substituted at 5-position in this nucleotide (VII).

After decationization with Amberlite IR-120(H^+), (VII) was treated with DCC to convert it into the corresponding 2′,3′-cyclic phosphate (VIII) which was isolated as ammonium salt, $C_9H_{13}O_8N_3BrP$, m.p. 165°(decomp.), with Rf_1 of 0.36. (VIII) also exhibited typical properties for a cyclic phosphate in its behavior in paper electrophoresis and the relationship in the mobilities of (VIII) and (VII) on paper at pH 9.0 was found to be reverse of those run at pH 6.0. (VIII) was easily hydrolyzed to (VII) in aqueous solution with Amberlite IR-120 (H^+).

The synthesized cyclic ribothymidylic acid (V) and cyclic 5-bromouridylic acid (VIII) were respectively incubated with RNase-I in an acetate buffer of pH 6.0 and both cyclic phosphates were found to be converted into the corresponding 3′-monophosphates, which gave respective Rf_1 value of 0.16 and 0.19.

Recently, Witzel[10] proposed, without detailed experimental description, that a grouping of $-N-CO-N=C(OH)-$ (or $=C(NH_2)-$)(R=ribosyl moiety) in the pyrimidine part of component 3′-ribonucleotide might be required for the hydrolytic cleavage of 3′-5′ internucleoside linkage by RNase-I and this assumption was made from observations that RNase-I did hydrolyze the above-mentioned P-O-C linkage when the 3′-ribonucleotide part contained 4,5-dihydrouracil and did not hydrolyze it when the ribose moiety had no substituent but had a ureidopropionate in its 1-position, or when it was reduced to ribitol type.

As far as the present results are concerned, they are in accordance with Witzel's assumption and both cyclic nucleotides tested contain pyrimidine bases which include the atom grouping suggested by him.[11] Furthermore, these results showed that such a substituent as methyl or bromo in the 5-position of uridine cyclic phosphate does not interfere with substrate activity of the latter against this enzyme.

Griffin and Todd[12] reported that ribothymidine 5′-pyrophosphate is active as a sub-

8) A. M. Michelson : J. Chem. Soc., **1958**, 1957.
9) M. Roberts, D. W. Visser : J. Am. Chem. Soc., **74**, 668 (1952).
10) H. Witzel : IV. Intnatl. Kongr. Biochem. Zusammenfassungen, 33 (1958). Wien.
11) Davis, *et al.* (F. F. Davis, F. W. Allen : J. Biol. Chem., **227**, 907 (1957)) reported without detailed description of the enzymatic experimental condition that a 2′,3′-cyclic phosphate of 5-ribosyluridine is hydrolyzed by RNase.
12) B. E. Griffin, A. R. Todd, A. Rich : Natl. Acad. Sci. U. S., **44**, 1123 (1958).

strate for polynucleotide phosphorylase prepared from *Azotobacter vinelandii* or *Escherichia coli*. Hydrolysis of the product, polythymidylic acid, by RNase was found to give an intermediate hydrolysis product which was detected on paper chromatogram and was proposed to be 2′,3′-cyclic ribothymidylic acid (V).

In contrast to the relationship between ribothymidine 5′-pyrophosphate and (V), 5-bromouridine 5′-pyrophosphate, which has the same pyrimidine base as that in cyclic nucleotide (VIII), was reported by Ochoa[13] to have no such properties as a substrate for polynucleotide phosphorylase.

It is of interest that the structural requirement of a substrate in the 5-substitution of pyrimidine nucleotides is different for these enzymes, RNase-I and polynucleotide phosphorylase.

Experimental

Paper Chromatography—Paper chromatography was performed ascendingly on Toyo Roshi No. 51, using the following solvent systems: (i) iso-PrOH–conc. NH_4OH–H_2O (7:1:2), (ii) AcOH–BuOH–H_2O (1:4:5), (iii) isobutyric acid–0.5*N* NH_4OH (10:6), (iv) *tert*-AmOH–90% HCOOH–H_2O (3:1:3), (v) iso-PrOH–H_2O (2:1) saturated with gaseous NH_3. The Rf values for these five solvent systems are respectively represented as Rf_1, Rf_2, Rf_3, Rf_4, and Rf_5. The ultraviolet-absorbing spots were detected by visual observation under ultraviolet ray and P-containing spots were detected according to the method of Bandurski-Axelrod.[14]

Paper Electrophoresis—Paper electrophoresis was performed on Toyo Roshi No. 51 at 700 v/15 cm. for 1 hr., using the following buffer solution of (1) pyridine–AcOH–BuOH–H_2O (10:2:20:500) and (2) the buffer solution (1) adjusted to pH 9.0 by addition of 4*N* NH_4OH. The mobility of the compound tested in respective buffer solutions (1) and (2) is represented by M_1 and M_2, taking those for mixed cytidine 2′- and 3′-phosphates as standard (M_1, 1.00; M_2, 1,00).

5′-O-Tritylribothymidine (II)—A solution of 1.7 g. of (I) and 4 g. of trityl chloride (about 2 mol. eq.) dissolved in 130 cc. of dehyd. pyridine was refluxed for 8 hr. After cool, the solution was poured into an equal volume of H_2O, the crystals that separated from the reaction mixture on standing for 5~6 hr. were collected, and suspended in benzene. Benzene-insoluble material was collected by filtration. The needle crystals that separated from the filtrate after standing for 2 days were collected and combined with the benzene-insoluble crystals. The combined crystals were again suspended in a small amount of benzene to remove a trace of contaminated triphenylcarbinol. After removal of benzene by centrifugation, the precipitate was recrystallized twice from dehyd. Me_2CO, m.p. 162~165°; yield, 80%. *Anal.* Calcd. for $C_{29}H_{28}O_6N_2$: C, 69.60; H, 5.58; N, 5.58. Found: C, 69.42; H, 5.87; N, 4.94. Rf_1 0.86.

3-(β-D-Ribofuranosyl)thymine 2′- and 3′-Phosphate (IV)—To a solution of 1 g. of (II) dissolved in 11 cc. of dehyd. pyridine was added, at the freezing point of pyridine, a CCl_4 solution of dibenzyl chlorophosphonate, prepared from 2 g. of dibenzyl phosphite. After standing for 4 hr. at the same temperature, the reaction mixture was kept in a refrigerator overnight. A solution of 1.5 g. of Na_2CO_3 dissolved in 8 cc. of H_2O was added to the reaction mixture and the mixture was shaken. The CCl_4 layer was separated, filtered to remove insoluble material, and the filtrate was evaporated at 30° in a reduced pressure to leave a brown syrup. The syrup was diluted with EtOH, evaporated in a reduced pressure to remove pyridine, and this procedure was repeated until the complete removal of pyridine. The residue was dissolved in $CHCl_3$ and shaken twice with saturated solution of $NaHCO_3$ and once with H_2O. After drying over Na_2SO_4, $CHCl_3$ solution was evaporated in a reduced pressure to leave a syrup (1.95 g.). Syrup was dissolved in 20 cc. of 80% AcOH and the solution was heated in an oil bath for 20 min. H_2O was added to the cooled reaction mixture and the crystalline triphenylcarbinol that separated was extracted with $CHCl_3$. The aqueous layer was concentrated at 30° in a diminished pressure. This concentration procedure was repeated with the addition of EtOH until a trace of AcOH was removed and EtOH was evaporated completely. The residue was dissolved in 40 cc. of H_2O, and 100 mg. each of PdO and Pd-C were added to the solution. The solution was subjected to hydrogenolysis in H_2 atmosphere at room temperature. The hydrogenolysis products gave, on paper chromatogram, two ultraviolet-absorbing spots at Rf_1 0.53 and 0.16, only the latter of which was positive to P test. After filtration of the catalyst, a saturated solution of $Ba(OH)_2$ was added to the filtrate to adjust pH of the solution to 10.0. The excess of $Ba(OH)_2$ was precipitated

13) S. Ochoa, L. A. Heppel: "Chemical Basis of Heredity," Ed. W. McElroy and B. Glass, 615 (1957). Johns Hopkins Press, Baltimore.
14) R. S. Bandurski, B. Axelrod: J. Biol. Chem., **193**, 405 (1951).

with CO_2 and the precipitate was removed by centrifugation. The supernatant was concentrated to about 70 cc. in a reduced pressure to precipitate additional $BaCO_3$, which was filtered off.

After concentration of the filtrate to 5 cc. in a reduced pressure, 20 cc. of EtOH was added to the solution and the white precipitate that occurred was collected. The final precipitate which gave a single spot at Rf_1 0.16 was dissolved in a small amount of H_2O and reprecipitated by addition of EtOH. After two reprecipitations, the purified product was washed with Me_2CO and dried *in vacuo* over P_2O_5 at room temperature to give a white powder (500 mg., yield, 45%). *Anal.* Calcd. for $C_{10}H_{13}O_9N_2BaP \cdot 6H_2O$: C, 20.73; H, 4.38; N, 4.64; P, 5.66. Found : C, 20.64; H, 4.29; N, 4.81; P, 5.33. Rf_1 0.16, Rf_3 0.41, Rf_4 0.29, Rf_5 0.53. M_1 1.18, M_2 1.00. UV $\lambda_{max}^{H_2O}$ mμ : 265 (pH 2), 267 (pH 12).

Ribothymidine 2′,3′-Cyclic Phosphate (V)—After decationization of an aqueous solution containing 120 mg. of Ba salt of (IV) with Amberlite IR-120 (H^+), the acidic solution was neutralized with pyridine and lyophilized. To the white vitreous solid thus obtained, 10 cc. of pyridine and 70 mg. of DCC were added, the mixture was kept at room temperature for 4 hr., 15 cc. of H_2O and 100 mg. of $(NH_4)_2CO_3$ were added to the reaction mixture, and set aside overnight. The precipitate that separated was filtered off, the filtrate was extracted twice with 40-cc. portions of Et_2O, and the aqueous layer was lyophilized to give 70 mg. of a white vitreous solid. The vitreous solid (a mixture of (IV) and (V)) was extracted repeatedly with iso-PrOH saturated with gaseous NH_3. The extracts were combined and concentrated to dryness in a reduced pressure. The residual white powder thus obtained was dissolved in dehyd. MeOH and precipitated with Et_2O to give NH_4 salt of (V) which was dried over P_2O_5 at room temperature to a white hygroscopic powder, m.p. 190~200° (decomp.). *Anal.* Calcd. for $C_{10}H_{16}O_8N_3P$: N, 12.45; P, 9.28. Found : N, 12.63; P, 8.85. Rf_1 0.43, M_1 1.26, M_2 0.85.

5-Bromouridylic Acid (VII)—To a solution of 320 mg. of yeast uridylic acid dissolved in 20 cc. of H_2O, EtOH solution of 182 mg. of tributylamine was added, the mixture was concentrated in a reduced pressure, and distilled azeotropically with addition of 10 cc. of benzene. Similar distillation was repeated three times with the addition of 20 cc. each of toluene.

To the dry, residual pale yellow solid 30 cc. of dioxane and 1 g. (5.7 mol. eq.) of NBS were added and the mixture was set aside for 3 days at room temperature, with occasional shaking. The reaction mixture was concentrated to dryness in a reduced pressure, the residue was dissolved in EtOH, and the solution was adjusted to pH 10 by addition of trimethylamine. To the basic solution an EtOH solution of $CaCl_2$ was added to precipitate the white Ca salt which was dissolved in a small amount of H_2O and, after removal of the insoluble impurity by centrifugation, EtOH was added to the supernatant. The precipitate formed was collected and reprecipitated twice with EtOH from the aqueous solution. The final precipitate was collected, washed with EtOH and Et_2O, and dried *in vacuo* over P_2O_5 to give 300 mg. of a white powder (yield, ca. 80%). *Anal.* Calcd. for $C_9H_{10}O_9N_2BrCaP \cdot 4H_2O$: C, 21.05; H, 3.50; N, 5.46; P, 6.04. Found : C, 21.41; H, 3.67; N, 5.44; P, 5.82. Rf_1 0.19, Rf_2 0.22, Rf_3 0.41, Rf_4 0.29, Rf_5 0.50. M_1 1.18, M_2 1.07. UV $\lambda_{max}^{H_2O}$ mμ : 278 (pH 2), 276 (pH 12).

5-Bromouridine 2′,3′-Cyclic Phosphate (VIII)—A solution of 300 mg. of Ca salt (VII) dissolved in H_2O was acidified with Amberlite IR-120 (H^+). After filtration of the resin, the filtrate was neutralized with pyridine and the neutral solution was lyophilized to give a white powder, to which 12 cc. of pyridine and 300 mg. of DCC were added. After setting aside for 6 hr., 20 cc. of H_2O and 100 mg. of $(NH_4)_2CO_3$ were added to the mixture and allowed to stand overnight. Dicyclohexylurea that precipitated was filtered off and the filtrate was extracted twice with Et_2O. The aqueous layer was lyophilized to give a white vitreous material, which was treated with NH_3-saturated iso-PrOH as in the case of (V), to isolate the soluble NH_4-salt of (VIII). The NH_4 salt was reprecipitated twice with Et_2O from the MeOH solution and the final white precipitate was dried *in vacuo* over P_2O_5; m.p. 165° (decomp.). *Anal.* Calcd. for $C_9H_{13}O_8N_3BrP$: N, 10.28; P, 7.28. Found : N, 10.28; P, 7.71. Rf_1 0.36. M_1 1.25, M_2 0.86.

Enzymatic Hydrolysis of (V) and (VIII)—A solution of 3 mg. of (V) or (VIII) dissolved in 0.2 cc. of acetate buffer of pH 6.0 was added with 0.1 cc. of enzyme solution which contained 0.5 mg. of RNase-I-A in 2 cc. of H_2O. After incubation at 37° for 24 hr., the reaction mixture was submitted to paper chromatography with the solvent system (i). Both (V) and (VIII) were found to be hydrolyzed completely to the respective substances having the same Rf value as (IV) and (VII).

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Summary

3-(β-D-Ribofuranosyl)thymidine 2′,3′-cyclic phosphate (V) and 5-bromouridine 2′,3′-cyclic phosphate (VIII) were synthesized. These two phosphates were found to be hydrolyzed to the corresponding 3′-phosphates by pancreatic ribonuclease (RNase-I-A).

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