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Summary

1-Dehydro compounds were obtained from deoxycorticosterone, Reichstein's compound S, corticosterone, and cortisone by their fermentation with *Gliocladium roseum*, *Helminthosporium turcicum*, or *Ophiobolus heterostropus*.

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35. Tyunosin Ukita and Masachika Irie: Organic Phosphates. X.[*2] Several 2,3-Cyclic Phosphates of D-Ribofuranoside and their Properties as Substrate for Ribonuclease.

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Recently, several reports were published on the mode of enzymatic hydrolysis reaction and the substrate specificity of pancreatic ribonuclease (RNase-I).

The RNase hydrolyzes ribonucleic acid specifically at C-O-P linkage at 5'-position of its internucleoside phosphate bonds which bind 3'-hydroxyl group of pyrimidine nucleoside to 5'-position of the neighboring nucleoside, and gives the final product of pyrimidine 3'-mononucleotides and oligonucleotides containing similar nucleotides as their terminal group. This reaction is known to include the corresponding pyrimidine 2',3'-cyclic mononucleotides and the oligonucleotides with their terminal group of a similar cyclic nucleotide as intermediate compounds.[1,2]

Todd and Brown, in their research on the substrate specificity of this enzyme, found that both 2',3'-cyclic and 3'-benzylphosphoryl derivatives of cytidine and uridine serve as its substrate to give the 3'-phosphates of these two nucleosides,[3] while the 2'-benzylphosphoryl derivatives of these pyrimidine nucleosides as well as 2',3'-cyclic phosphate of adenosine and guanosine could not be attacked by this enzyme.

Durand, *et al.*[4] reported that apurinic acid obtained after removal of purine bases by acid treatment of deoxyribonucleic acid is slowly hydrolyzed by the RNase. Further, Chargaff[5] observed that the type-b specific substance separated from *Haemophilus influenzae*, the structure of which was proposed by him to be a polymer of 1,1'-ribosylriboside combined intermolecularly by phosphate linkages at their 3-5 and 3'-5' hydroxyl groups, is capable of being hydrolyzed by relatively large amount of RNase.

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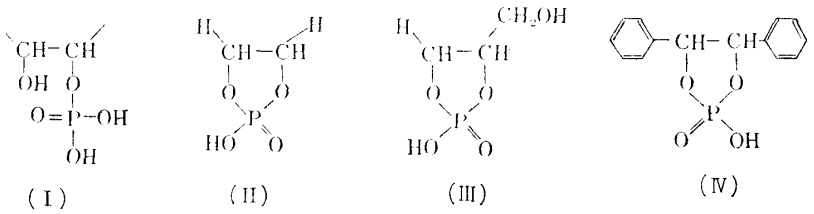
*2 Part. IX. T. Ukita, M. Irie: This Bulletin, 8, 436 (1960).

1) E. Volkin, W. E. Cohn: J. Biol. Chem., **205**, 767 (1953).
2) R. Markham, J. D. Smith: Biochem. J., **52**, 558 (1952).
3) D. M. Brown, A. R. Todd: J. Chem. Soc., **1951**, 2040.
4) M. C. Durand, R. Thomas: Biochim. et Biophys. Acta, **12**, 416 (1953).
5) S. Zamenhof, G. Leidy, P. C. Flitz Gerald, E. Alexander, E. Chargaff: J. Biol. Chem., **203**, 695 (1953).

From the results obtained by these workers, Volkin and Cohn[1] proposed a structural requirement in the substrate specificity of this phosphodiesterase that it is not in pyrimidine ribonucleoside linked by alkyl phosphate at its 3′-position but commonly in an oligo- or poly-ribonucleotide which does not contain a purine base.

Egami, *et al.*[6] synthesized benzyl 2-hydroxyethyl phosphate with the purpose of checking its availability as a substrate of this enzyme, but they found this simple phosphodiester was inert to this enzyme. They assumed that the steric difficulty of this compound in forming cyclic phosphate by the free rotation between two hydroxyl-bearing carbon atoms might resist the first step of enzymatic reaction of the RNase to produce the possible intermediate of ethyleneglycol cyclic phosphate (II) and stated the probability of the latter phosphate to serve as the simplest cyclic phosphate which should be hydrolyzed by this enzyme.



(I) (II) (III) (IV)

Chart 1.

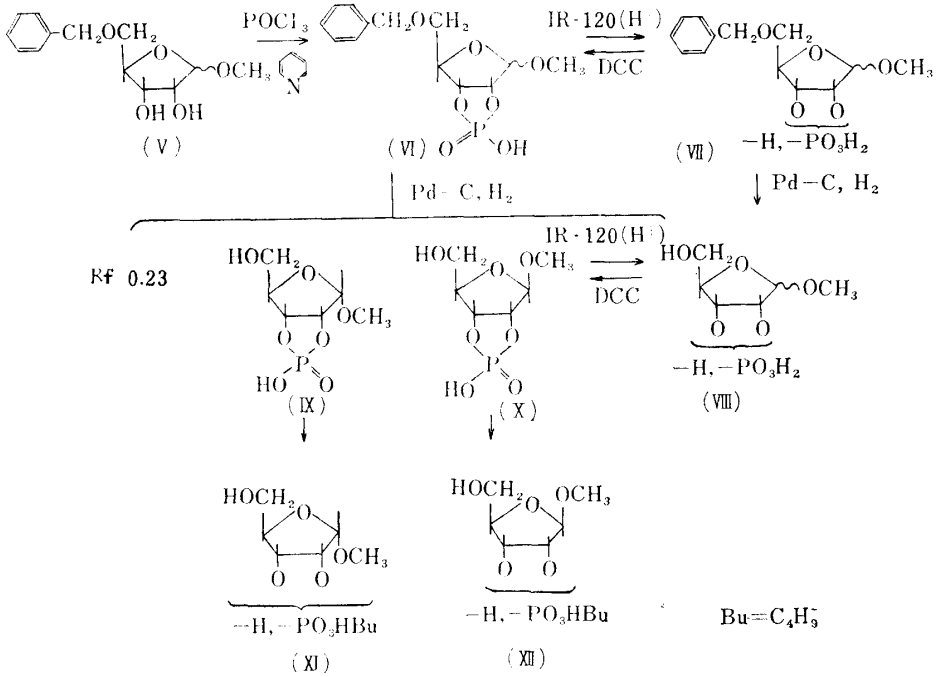


Chart 2.

However, as has been reported by the present authors,[7] no hydrolytic cleavage of the cyclic phosphate ring was observed when both (II) and glycerol 1,2-cyclic phosphate (III) as well as hydrobenzoin cyclic phosphate (IV) were incubated with RNase-I under the condition used by Todd, *et al.*[3] for the hydrolysis of cytidine 2′,3′-cyclic phosphate.

6) A. Takemura : Proteins, **2**, 86 (1957); F. Egami : Seikagaku, **27**, 139 (1955).
7) T. Ukita, M. Irie : This Bulletin, **6**, 445 (1958). After these observations were reported by the present authors, F. Egami, *et al.* (Seikagaku, **31**, 120 (1959)) also reported the inertness of ethyleneglycol cyclic phosphate as a substrate of RNase-I and the inactivity of this compound as an inhibitor for this enzymatic reaction.

The above-described observations prompted the synthesis of some simple derivatives of D-ribose 2,3-cyclic phosphate and test of their properties as the substrate for this enzyme. This paper deals with the synthesis of methyl 5-O-benzyl-D-ribofuranoside 2,3-cyclic phosphate (VI), methyl α- and β-D-ribofuranoside 2,3-cyclic phosphate (IX and X), and their activity as a substrate for RNase-I.

Methyl 5-O-benzyl-D-ribofuranoside[8] (V) was reacted with phosphoryl chloride in pyridine to obtain a main product with by-products which showed the respective Rf values of 0.87, 0.94, and 0.10 on paper chromatogram.*3 Because these products were hardly separable by the use of cellulose column chromatography, their mixture was hydrolyzed by treatment of its aqueous suspension with Amberlite IR-120(H^+). By this treatment, the main product was converted into a new compound which appeared at Rf 0.66 in paper chromatography run with the same solvent system, while the two by-products remained unchanged. After neutralization, the hydrolyzate was lyophilized and the residue was submitted to cellulose column chromatography, using an elution solvent of iso-PrOH-5*N* NH_4OH (2:1) to separate the main product, a mixture of methyl 5-O-benzyl-α- and -β-D-ribofuranoside 2- and 3-phosphate (VII), as crystalline dicyclohexylammonium salt, $C_{25}H_{45}O_8N_2P \cdot H_2O$. This mixed salt of phosphomonoesters was converted to the free acid and dehydrated with dicyclohexylcarbodiimide (DCC) in acetonitrile to form cyclic phosphate, the mixture of methyl 5-O-benzyl-α- and -β-D-ribofuranoside 2,3-cyclic phosphate thus obtained was separated from unreacted phosphomonoesters by the difference in solubility of their ammonium salts in isopropanol saturated with gaseous ammonia, as the salt of the latter is insoluble in this solvent. The cyclic phosphate contained in the supernatant after its conversion into sodium salt was isolated as a white powder, $C_{13}H_{16}O_7NaP \cdot H_2O$ (VI).

Catalytic hydrogenolysis of the mixed methyl 5-O-benzyl-α- and -β-D-ribofuranoside 2,3-cyclic phosphates (VI) with palladium-charcoal in hydrogen atmosphere afforded four products which gave respective spots at Rf 0.66, 0.64, 0.30, and 0.23 on paper chromatogram. Among these, the compound at Rf 0.30 was identified with the mixed methyl D-ribofuranoside 2- and 3-phosphates (VIII) obtainable from phosphomonoester (VII) by a similar catalytic hydrogenolysis.

The other two products, which showed the respective spots at Rf 0.66 and 0.64 were converted by trituration of their aqueous solution with Amberlite IR-120(H^+) to the same compound that gives the spot at Rf 0.30 and was identified with the mixed methyl D-ribofuranoside 2- and 3-phosphates (VIII). Thus, the two products of 0.66 and 0.64 must be the cyclic phosphates represented by respective structures of (IX) and (X).

The isolation of these two desired cyclic phosphates (IX and X) from the mixture of hydrogenolysis products of (VI) was thought unsuitable, because the above-described four reaction products were contained in about equal quantity. Therefore, another route seemed more preferable to obtain these cyclic phosphates and the phosphomonoester (VII) was catalytically debenzylated to (VIII) which was isolated as a crystalline cyclohexylamine salt, $C_{18}H_{39}O_8N_2P \cdot \frac{1}{2}H_2O$. After conversion to the pyridinium salt, (VIII) was dehydrated with DCC in acetonitrile. The reaction mixture revealed two products giving the spots at Rf 0.66 and 0.64 on paper chromatogram and respectively identified with the cyclic phosphate (IX and X). The separation of these products was performed by submitting the mixture to paper chromatography, followed by cutting and extraction of the corresponding bands with water. The repeated separation by the above procedure gave two fractions, each of which showed a single spot at Rf 0.66 and 0.64. From these fractions, after converting the product into sodium salts, the two salts were isolated as crystals which were analyzed

*3 Paper chromatography was performed on Toyo Roshi No. 53, using a solvent system of iso-PrOH-5*N* NH_4OH (2:1).

8) G. M. Tenner, H. G. Khorana : J. Am. Chem. Soc., **79**, 437 (1957).

to give the common molecular formula of $C_6H_{10}O_7NaP$. These salts of (IX) and (X) gave respective optical rotation of $[\alpha]_D^{20}$ +93.6° and −34.2°.

Both (IX) and (X) were easily hydrolyzed by Amberlite IR-120(H^+) in aqueous solution at room temperature to give the products of same Rf 0.30 which was identified with (VIII) on paper chromatogram. Furthermore, (IX) and (X) were alcoholyzed with butanol and catalytic amount of trifluoroacetic acid, and formed two compounds of 0.85 and 0.84, which were assumed to be the corresponding mixed methyl D-ribofuranoside 2- and 3-butylphosphates (XI and XII). From these typical properties of a five-membered cyclic phosphodiester, (IX) and (X) must be the respective α and β anomers of methyl D-ribofuranoside 2,3-cyclic phosphate.

Substrate activities of (VI), (IX), and (X) against RNase-I was tested according to the procedure used by Todd, *et al.*[3] for the enzymatic hydrolysis of pyrimidine nucleoside cyclic phosphate. Even after prolonged incubation of these compounds with RNase-I for 72 hours, no evidence of the formation of corresponding methyl riboside 3-phosphates was observed by checking the incubation mixture by paper chromatography.

The above evidence revealed that not only the simplest cyclic 1,2-diol phosphate (II), as suggested by Egami, *et al.*[6] but also the methyl D-ribofuranoside 2,3-cyclic phosphates do not fulfill the minimum structural requirement to serve as the substrate for RNase-I.

The above-cited hypothesis that RNase-I could attack a substrate of 3-phosphodiester-type ribonucleotide which does not contain a purine base must be withdrawn*4 and it became evident that the substrate available for this enzyme is a 3-alkyl phosphate or 2,3-cyclic phosphate of ribonucleoside which contains some specifically arranged atom grouping than O-methyl group at its 1-position of D-ribose moiety.*5

Experimental

Methyl 5-O-Benzyl-D-ribofuranoside (V)—Prepared from methyl 2,3-O-isopropylidene-5-O-benzyl-D-ribofuranoside according to the method of Tenner, *et al.*,[8] b.$p_{0.08}$ 150°.

Methyl 5-O-Benzyl-D-ribofuranoside 2- and 3-Phosphate (VII)—To a solution of 4.8 g. of (V) in 20 cc. of pyridine, a solution of 3 g. (1.04 mol. eq.) of $POCl_3$ in 10 cc. of pyridine was added during 20 min. with vigorous stirring and chilling with ice and salt. Temperature of the reaction mixture was kept at −5° to −10°. After stirring for additional 2 hr., the pale brownish solution was kept overnight at room temperature. Pyridine hydrochloride that precipitated out was filtered off, the filtrate was added to a suspension of Ag_2CO_3 in ice water, and AgCl that separated was removed by filtration with the aid of charcoal. On paper chromatogram, the filtrate gave spots at Rf 0.87 and 0.94 (trace), with that of inorganic phosphate. The filtrate was treated with H_2S to remove Ag^+ and Ag_2S was removed by filtration. From the filtrate, pyridine was removed in a reduced pressure (bath temp. 30°). The aqueous solution was acidified to pH 1~2 by addition of Amberlite IR-120(H^+). After standing for several hours, the acidic solution was neutralized with pyridine and lyophilized to a light brown powder (3.55 g.). On paper chromatogram this powder gave spots at Rf 0.66 and 0.94 (trace).

The solution of this powder in 8 cc. of H_2O was placed at the top of a cellulose column (3×40 cm.) and eluted with a mixed solvent of iso-PrOH-5*N* NH_4OH (2:1) at a flow rate of 4 cc./25 min. and the effluent was collected in 4-cc. fractions. Fraction A (fraction Nos. 55~65) gave two spots at Rf 0.66 and 0.94, and Fraction B (fraction Nos. 66~87) gave a single spot at Rf 0.66 on paper chromatogram.

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*4 The availability of polyribophosphate as a substrate for RNase-I reported by Chargaff, *et al.* is not taken into this discussion, because they used a large amount of the enzyme to the substrate used (1.8 mg./375 γ=enzyme/substrate) in their experiment and its result could not be compared with those obtained by usual conditions employed in this type of enzymatic hydrolysis reaction.

*5 Takemura, *et al.* are of the opinion that the presence of pyrimidine moiety in RNA should be an important factor for the latter to be hydrolyzed by RNase-I-A and -B, as riboapyrimidinic acid is not hydrolyzed by these enzymes (S. Takemura, M. Takagi, M. Miyazaki, F. Egami : J. Biochem. (Tokyo), **46**, 1149 (1959)).

Fraction A was concentrated in a reduced pressure and the condensate was lyophilized to 1.5 g. of a mixed NH_4 salts. The mixture was again submitted to cellulose column chromatography to collect a fraction (C) which gave a single spot at Rf 0.66.

Fractions B and C were combined and concentrated *in vacuo* to a small volume. After decationization with Amberlite IR-120 (H^+), the acidic solution was adjusted to pH 9.0 by addition of cyclohexylamine and lyophilized to a white powder (1.9 g.). The mixed cyclohexylammonium salts of methyl 5-O-benzyl-D-ribofuranoside 2- and 3-phosphates thus obtained were dissolved in a small amount of EtOH and added with Et_2O to obtain needles. The sample for analysis was dried over P_2O_5 *in vacuo* at room temperature to constant weight. *Anal.* Calcd. for $C_{25}H_{45}O_8N_2P \cdot H_2O$: C, 54.55; H, 8.55; N, 5.09; P, 5.63. Found : C, 54.19; H, 8.18; N, 4.73; P, 5.37.

Methyl 5-O-Benzyl-D-ribofuranoside 2,3-Cyclic Phosphate (VI)—An aqueous solution containing 1 g. of cyclohexylammonium salt of (VII) was decationized with Amberlite IR-120 (H^+) and the acidic solution obtained, after neutralization with pyridine, was lyophilized to a white glassy solid (0.8 g.).

To a solution of this solid in 20 cc. of MeCN, 0.43 g. (1.1 mol. equiv.) of DCC dissolved in 2 cc. of MeCN was added. After standing for 3 hr., 20 cc. of H_2O was added to the reaction mixture and kept for 1 hr. at room temperature. The solution was diluted with 60 cc. of H_2O and passed through a column of Amberlite IRC-50 (NH_4^+). P-containing eluate was collected and lyophilized to a white glassy solid which was suspended in 60 cc. of iso-PrOH saturated with dry NH_3. iso-PrOH soluble fraction was separated by centrifugation and the precipitate was treated twice in the same way with iso-PrOH and NH_3. The precipitate was dried *in vacuo* and furnished 120 mg. of the starting material (Rf 0.66), contaminated with a trace of inorganic P. The supernatant iso-PrOH solutions were combined and the solvent was removed in a reduced pressure. The residual NH_4 salt was dissolved in 50 cc. of H_2O and passed through a column of Amberlite IRC-50 (Na^+) to convert it into the Na salt. The effluent obtained was lyophilized to give 330 mg. of crude Na salt (Rf 0.87) which was dissolved in MeOH. The MeOH solution was passed through a small column of cellulose (0.3×3 cm.) and the column was washed with MeOH. From the eluate, MeOH was removed in a reduced pressure and the syrupy residue was triturated with iso-PrOH to convert it into an amorphous powder. After separation of iso-PrOH by centrifugation, the precipitate was washed once more with iso-PrOH. The final precipitate was dried over P_2O_5 *in vacuo* to 188 mg. of a white powder (55% yield). The electrometric titration of the product showed no dissociation between pH 2 and 10. Sample for analysis was dried over P_2O_5 at 100° to a constant weight. *Anal.* Calcd. for $C_{13}H_{16}O_7NaP \cdot H_2O$: C, 43.82; H, 5.06. Found : C, 43.95; H, 4.85.

Methyl D-Ribofuranoside 2- and 3-Phosphates (VIII)—To 800 mg. of cyclohexylammonium salt of (VII) dissolved in 50 cc. of hydr. MeOH, 800 mg. of 10% Pd-C was added and the mixture was shaken in H_2 atmosphere at room temperature until the reaction mixture gave no spot at Rf 0.66 on paper chromatogram. The catalyst was removed by filtration and, after evaporation of MeOH from the filtrate, the aqueous solution was lyophilized to furnish a white glassy powder (650 mg.). This powder was dissolved in a small amount of H_2O and acidified by addition of Amberlite IR-120 (H^+). To the acidic solution, saturated solution of $Ba(OH)_2$ was added and the precipitate formed was removed by centrifugation. The supernatant solution was again acidified by the addition of Amberlite IR-120 (H^+) and filtered. The filtrate was adjusted to pH 9.0 by addition of cyclohexylamine. The solution of cyclohexylammonium salt was lyophilized to a white powder which was recrystallized from dehyd. EtOH-Et_2O. The sample for analysis was dried over P_2O_5 *in vacuo*. *Anal.* Calcd. for $C_{18}H_{39}O_8N_2P \cdot \frac{1}{2}H_2O$: C. 47.89; H, 8.86; N, 6.20; P, 6.87. Found : C, 48.18; H, 8.94; N, 6.34; P, 6.26. Rf 0.30.

Methyl D-Ribofuranoside 2,3-Cyclic Phosphate—A solution of 1.33 g. of cyclohexylammonium salt of (VIII) dissolved in 50 cc. of H_2O was decationized with Amberlite IR-120 (H^+) and filtered. The acidic filtrate was neutralized with pyridine and the neutral solution was lyophilized to give 967 mg. of a glassy solid. To this solid dissolved in 10 cc. of H_2O, 20 cc. of pyridine, 30 cc. of MeCN, and 680 mg. of DCC were added and, after shaking this mixture for 2 hr., the reaction was stopped by addition of 20 cc. of H_2O. After standing this mixture for 1 hr., dicyclohexylurea that separated was filtered off. The filtrate was diluted with 50 cc. of H_2O and the solution was slowly passed through a column of Amberlite IRC-50 (NH_4^+) (60×1.5 cm.). The eluate containing both P and NH_4^+ was collected and lyophilized to give pale-colored solid (720 mg.). This solid showed two main spots at Rf 0.66 and 0.64, and a faint spot at Rf 0.30 on paper chromatogram. The size and the grade of P-coloration of the spot at Rf 0.66 were almost the same as those of the spot at Rf 0.64.

The solid was dissolved in a small amount of H_2O and streaked on 33 sheets of filter paper (Toyo Roshi No. 53, 24×40 cm.), and run ascendingly with a mixed solvent of iso-PrOH-NH_4OH. From each paper, P-containing zone located from Rf 0.70 to 0.58 was cut off and each cutting was further cut (parallel to vertical line) to make four narrow pieces, A_1, A_2, A_3, and A_4, with the same spaces. Each of the four pieces of paper from all chromatograms was combined, extracted with H_2O, and tested by paper chromatography. A_1 and A_2 showed a single spot at Rf 0.66 and A_4 gave a single spot at Rf 0.64, while A_3 revealed a mixture of two spots at Rf 0.66 and 0.64. The extract obtained from A_3 was concentrated to a small volume and rechromatographed in the same way as described

above. The fraction which showed a single spot at Rf 0.66 was collected and combined with the extract from A_4. Each fraction thus separated was lyophilized. Yield of the product which gave a spot at Rf 0.64 was 164 mg. and that at Rf 0.64 was 100 mg.

Methyl α-D-Ribofuranoside 2,3-Cyclic Phosphate (IX)—The product which gave a spot at Rf 0.66 was dissolved in 50 cc. of H_2O and converted to Na salt by passing through a column (29×2.3 cm.) of Amberlite IRC-50 (Na^+). The solution of the Na salt was lyophilized to give a vitreous material. The vitreous solid thus obtained was dissolved in MeOH containing a small amount of H_2O and the solution was centrifuged to remove MeOH-insoluble material. After addition of two volumes of EtOH, the supernatant solution was concentrated in a reduced pressure to an oily residue. To the residue, 6 cc. of dehyd. Me_2CO was added and the colorless oil that separated turned into needle crystals on standing in a refrigerator for 2 weeks. The needle crystals were collected, washed with Me_2CO, and dried over P_2O_5. Rf 0.66, $[\alpha]_D^{20}$ +93.6° (c=1.0, H_2O). *Anal.* Calcd. for $C_6H_{10}O_7NaP$: C, 29.03; H, 4.03. Found : C, 29.23; H, 4.12.

Methyl β-D-Ribofuranoside 2,3-Cyclic Phosphate (X)—The product which gave a spot at Rf 0.64 was converted to its Na salt, and, after removal of MeOH-insoluble contaminant, MeOH solution was concentrated to a syrup, which was recrystallized from MeOH-Me_2CO to needles. After washing with Me_2CO, the product was dried over P_2O_5 *in vacuo*. Rf 0.64, $[\alpha]_D^{20}$ −34.2° (c=1.0, H_2O). *Anal.* Calcd. for $C_6H_{10}O_7NaP$: C, 29.03; H, 4.03. Found : C, 29.10; H, 4.11.

Alcoholysis of (IX) and (X)—To 2 mg. of (IX) or (X), 0.5 cc. of BuOH and a few drops of CF_3COOH were added. After standing for 16 hr., the reaction mixture was submitted to paper chromatography. The reaction mixture of (IX) gave two spots at Rf 0.85 and 0.30, and the mixture of (X) also gave two spots at Rf 0.84 and 0.30. The respective latter spots at Rf 0.30 were also found in the acid hydrolysis products of both (IX) and (X), and were identified with (VIII) by paper chromatography.

Hydrogenolysis of (VI)—The aqueous solution of (VI) was hydrogenolyzed with Pd-C at room temperature. The reaction product gave four spots at Rf 0.66, 0.64, 0.23, and that of inorganic P on paper chromatogram. The respective products with Rf 0.66 and 0.64 were converted by hydrolysis with Amberlite IR-120 (H^+) to a common compound (VIII) giving the spot at Rf 0.30.

Enzymatic Assay—A solution of 10 mg. of NH_4 cytidine 2′,3′-cyclic phosphate, 6 mg. of Ba ethyleneglycol cyclic phosphate, 5 mg. of Ba glycerol 1,2-cyclic phosphate, 7.5 mg. of (IX), 7.5 mg. of (X), or 10.5 mg. of (VI) dissolved in 0.5 cc. of distilled water was adjusted to pH 7.6 with dil. NH_4OH, 0.1 cc. of enzyme solution (0.5 mg. of pancreatic RNase-I-A in 2 cc. of H_2O) was added to each substrate solution, and incubated at 37°, after addition of two drops of toluene. After 72 hr., each reaction mixture was applied to paper chromatography with the mixed solvent of iso-PrOH-5*N* NH_4OH (2:1). None of the substrates used was hydrolyzed except cytidine 2′,3′-cyclic phosphate which gave cytidine 3′-phosphate.

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Summary

To find the minimum structural requirement in the substrate for pancreatic ribonuclease (RNase-I-A), 5-O-benzyl-D-ribofuranoside 2,3-cyclic phosphate (VI), and methyl α- and β-D-ribofuranoside 2,3-cyclic phosphates (IX and X) were synthesized and submitted to hydrolysis with this enzyme. No evidence was observed for hydrolysis of these cyclic phosphates to the corresponding 3-phosphates by the RNase and the results showed that more specially arranged atom grouping is required at 1-position of D-ribofuranoside 2,3-cyclic phosphate for this enzymatic hydrolysis.

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