[Chem. Pharm. Bull. 18(11)2303—2308(1970)]

UDC 547.963.32.04.07 : 547.918.04.07

A Convenient Synthesis of Ribonucleoside 2′,3′-Cyclic Phosphates from Ribonucleosides and Ribonucleotides[1]

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(Received June 9, 1970)

Adenosine 5′-phosphate was converted to the 2′,3′-cyclic phosphate by the treatment with dimethylformamide at reflux temperature. The reaction was shown to be proceeded by the initial release of the phosphoryl group as an "activated phosphoric acid" and rephosphorylated to furnish the 2′,3′-cyclic phosphate. In fact a ribonucleoside and phosphoric acid, pyrophosphoric acid, or polyphosphoric acid gives a 2′,3′-cyclic phosphate in a satisfactory yield in refluxing dimethylformamide. This procedure will serve for the preparation of the 2′,3′-cyclic phosphate of synthetic ribonucleoside of a limited quantity.

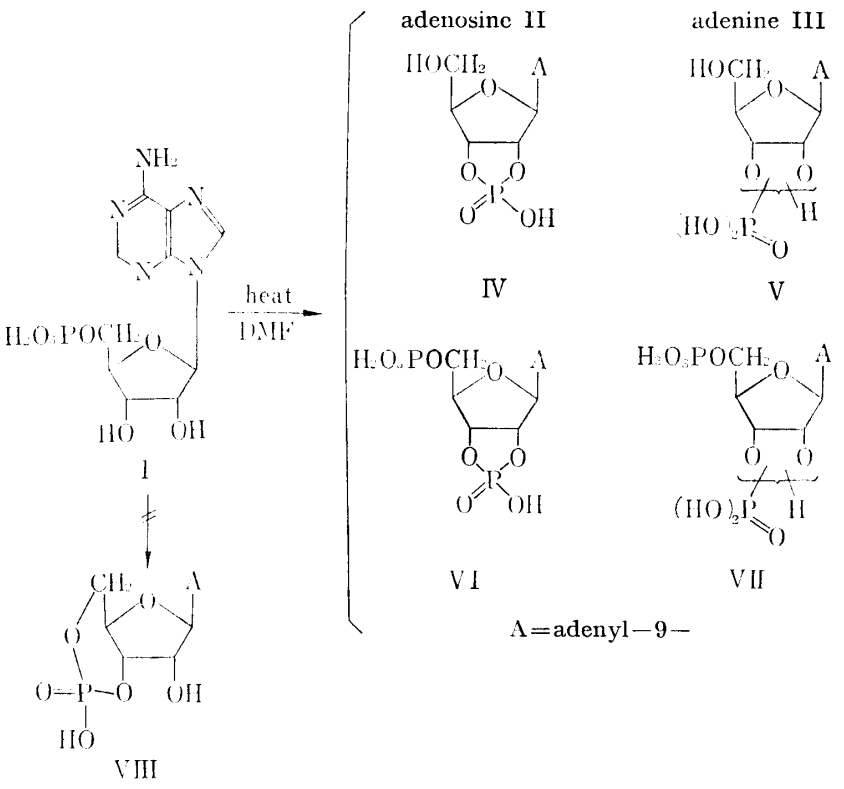
For the chemical and enzymatic synthesis of oligoribonucleotides ribonucleoside 2′,3′-cyclic phosphates are useful starting material, or substrate. Ribonucleoside 2′,3′-cyclic phosphate and ribonucleoside in the presence of an appropriate ribonuclease under proper conditions gives diribonucleoside 3′→5′-phosphate and oligoribonucleotides.[3] Michelson's procedure[4] for oligoribonucleotide synthesis involves the use of ribonucleoside 2′,3′-cyclic phosphates. To these purposes a convenient method of the preparation of the 2′,3′-cyclic phosphate has been required, especially for the synthetic ribonucleosides of a limited quantity. The 2′,3′-cyclic phosphate of naturally occurring ribonucleosides are generally prepared by cyclization of the 2′(3′)-phosphate which is obtained by the chemical hydrolysis of ribonucleic acid. The cyclic phosphate of synthetic ribonucleosids is usually prepared by the phosphorylation of a 5′-protected nucleosides to prior formation of 2′(3′)-phosphate followed by cyclization.[5] Since the above mentioned procedure requires several steps, more convenient procedure has been desirable.

Recently Holy and Smrt reported[6] a method starting from a free nucleoside and triethyl phosphite through the 2′(3′)-phosphite followed by oxidative cyclization. The direct cyclic-phosphorylation of 5′-protected nucleosides with P^1-diphenyl-P^2-morpholinyl pyrophosphorochloridate has been reported.[7]

This paper describes a rather simple procedure of the direct 2′,3′-cyclic phosphorylation of ribonucleosides, which was based on the observation during the studies of the reaction of 5′-nucleotides with dimethylformamide under elevated temperature. Several method for the direct phosphorylation of nucleosides with inorganic phosphate have been reported. Treatment of a mixture of a ribonucleoside with disodium hydrogen phosphate at 160° gives the mixture of the monophosphates including 2′,3′-cyclic phosphate as a minor product,[8] Treatment of a nucleoside or its 2′,3′-O-isopropylidene derivative with inorganic phosphate in dimethylformamide gives a random mixture of mono- and di-phosphate, or 5′-phosphate

1) Presented at the Local Meeting of Pharmaceutical Society of Japan at Sapporo, June 1968.
2) Location: *Kita-12, Nishi-6, Sapporo.*
3) L.A. Heppel, P.R. Whitfield, and R. Markham, *Biochem. J.*, **60**, 8 (1959).
4) A.M. Michelson, *J. Chem. Soc.*, **1959**, 1371.
5) For the general discussions, see T. Ueda and J.J. Fox, *Adv. Carbohydrate Chem.*, **22**, 307 (1967).
6) A. Holy and J. Smrt, *Collection Czech. Chem. Commun.*, **31**, 1528 (1966).
7) M. Ikehara and I. Tazawa, *J. Org. Chem.*, **31**, 819 (1966).
8) C. Ponnamperuma and R. Mack, *Science*, **148**, 122 (1965).

respectively.[9] In this reaction dimethylformamide apparently serves as activating agent of phosphoric acid. From the above findings it was expected that the treatment of ribonucleoside 5′-phosphate with dimethylformamide might have afforded the 3′,5′-cyclic phosphate, or oligonucleotides, as a result of intra- or inter-molecular phosphorylation. The thermic phosphorylation to oligoribonucleotide has been reported recently.[10]



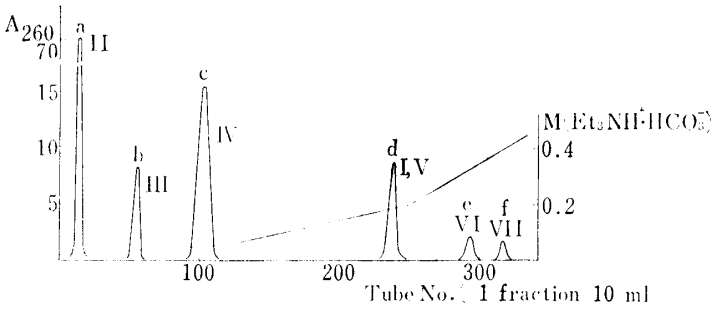


Fig. 1. Column Chromatography of Products of the Reaction of Adenosine 5′-Phosphate in DMF

Tri-*n*-butylammonium salt of adenosine 5′-phosphate (I) was refluxed for two hours in dimethylformide, giving a complex mixture of nucleotides with the formation of adenosine (II) and adenine (III) and phosphoric acid. Paper electrophoresis at pH 8.2 exhibited five spots corresponding to the anionic charge of zero to four. Column chromatography on DEAE-cellulose gave six well separated peaks according to the number of charge (see Fig 1). With the following evidence these were identified as adenosine (II, 47.5%), adenine (III, 9.8%), adenosine 2′,3′-cyclic phosphate (IV, 27.7%), adenosine monophosphates (I, V, 12.4%), adenosine 2′,3′-cyclic, 5′-diphosphate (VI, 2.3%) and adenosine 2′(3′),5′-diphosphate (VII, 1.4%), respectively.

9) M. Honjo, Y. Furukawa, and K. Kobayashi, *Chem. Pharm. Bull.* (Tokyo), **14**, 1061 (1966).
10) *a*) J. Moravek and J. Skoda, *Collection Czech. Chem. Commun.*, **32**, 206 (1967). *b*) J. Moravek, J. Kopecky, and J. Skoda, *Collection Czech. Chem. Commun.*, **33**, 960 (1968).

Compound (II and III) were identified by the comparison of ultraviolet absorption spectra and paper chromatographic behaviors with those of the authentic materials. For the identification of the fraction possessing one dissociation the cyclic phosphate, IV, or the 3′,5′-cyclic phosphate (VIII), and dinucleoside monophosphate should be taken into account. Paper chromatographic behavior resembles that of IV rather than VIII. The compound was susceptible with ribonuclease M digestion affording adenosine 3′-phosphate as the sole product. Alkaline hydrolysis of IV gave the 2′- and 3′-phosphate. These findings account well for that the product was adenosine 2′,3′-cyclic phosphate (IV) and not VIII or the dinucleoside monophosphate as initially expected. Adenosine 5′-phosphate (I) was found to be the major component of the monophosphate fraction as checked by paper chromatography on borate buffer system. The compound possessing three negative charges was identified as VI by the conversion to adenosine 3′,5′-diphosphate with ribonuclease M treatment. The last component having four charges was assigned as VII by the direct comparison of chromatographic and electrophoretic behaviors with the authentic specimen. For the elucidation of pathways producing IV from I the time course of the reaction was followed (Fig. 2). The reaction reached to an equilibrium after two hours, IV being major component as the nucleotides. However the initial product was apparently the diphosphate (VI and VII) which was formed by the phosphorylation of I with phosphoric acid being released from the starting nucleotides. Thus it was excluded that the 2′,3′-cyclic phosphate (IV) might have been formed by phosphoryl migration through VIII which could have formed by the intramolecular phosphorylation in I. In fact, VIII was found to be fairly stable under the reaction conditions employed. From the above observations it was thought to be likely that the reaction of ribonucleoside with inorganic phosphoric acid in DMF should likewise give the 2′,3′-cyclic phosphate as the major product, which was found to be the case (Fig. 3 and Table I and II).

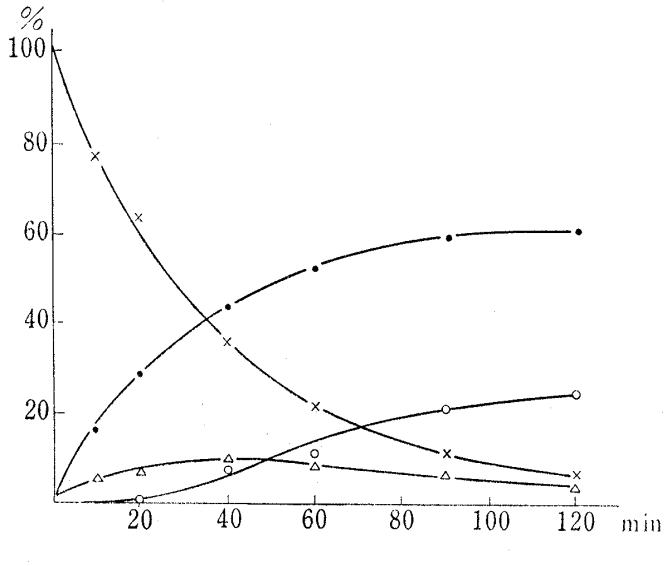


Fig. 2. The Reaction of Adenosine 5′-Phosphate in DMF

—●—●—: adenosine and adenine; —○—○—: adenosine 2′, 3′-cyclic phosphate; —×—×—: adenosine monophosphates; —△—△: adenosine diphosphates

An aliquot of the reaction mixture described in experimental section was taken out at appropriate intervals and subjected to paper electrophoresis. The UV absorbing spots were eluted from the paper with water and estimated spectrophotometrically at 260 mμ.

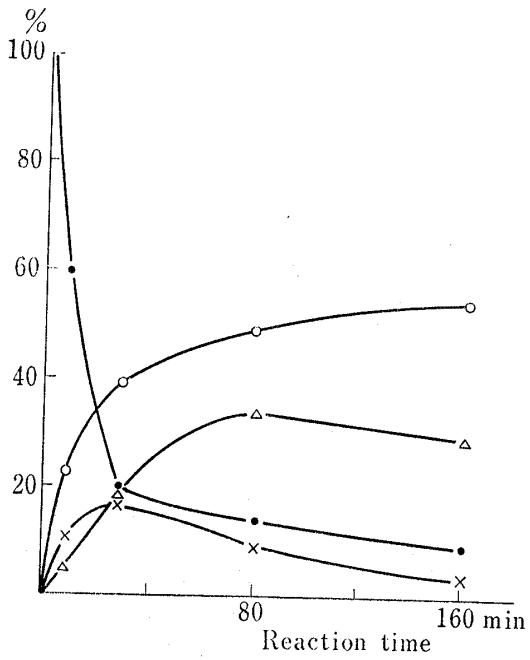


Fig. 3. Reaction of Cytidine with Phosphoric Acid in DMF

—●—●—: cytidine; —○—○—: cytidine 2′,3′-cyclic phosphate; —×—×—: cytidine monophosphate; —△—△—: cytidine diphosphate

A mixture of cytidine (0.5 mmole), phosphoric acid (2 mmoles), and tri-*n*-butylamine (4 mmoles) in 12.5 ml of DMF was refluxed. An aliquot of the solution was subjected to paper electrophoresis and each component was estimated spectrophotometrically.

Table I. Formation of Adenosine 2′,3′-Cyclic Phosphate from Adenosine or Its Phosphates

Nucleoside or nucleotide	Reagent	Equivalents as phosphoric acid	Tri-*n*-butylamine, equiv.	Refluxing time (hr, in DMF)	Yield(%) of 2′,3′-Cyclic phosphate	Yield(%) of Diphosphate
Adenosine	phosphoric acid	4	8	2	33.0	6.6
	pyrophosphoric acid	4	8	2	49.2	7.3
	polyphosphoric acid	5	2	1	39.4	46.2
	phosphoric acid and DCC	1	2	2	56.0	4.7
	phosphoric acid	4	8	2[a]	0	0
	pyrophosphoric acid	4	8	2[a]	22.5	6.2
Adenosine 5′-phosphate	···	1	2	2	25.1	5.2
	···	1	4	2	33.6	1.0
	phosphoric acid	4	2	2	50.0	11.6
	DCC	1	2	2	*b*)	
	···	1	2	2[a]	3.6	0
ATP	···	3	2	2	51.6	10.5
A2′(3′)-Phosphate	···	1	1	0.1	41.4	8.0
A2′,3′-Cyclic phosphate	···	1	1	2	25.0	
A3′, 5′-Cyclic phosphate	···	1	1	3	*c*)	

a) Solvent used is DMSO at 150—155°. *b*) Adenosine 3′,5′-cyclic phosphate was formed. *c*) No decomposition was observed.

Table II. Reaction of Several Ribonucleosides with Pyrophosphoric Acid in DMF

Nucleoside	2′,3′-Cyclic phosphate	Monophosphate	Diphosphate	Recovery of nucleoside
Uridine	61.2%	5.7	14.6	18.9
Cytidine	45.7	5.5	10.1	38.9
Guanosine	40.0	8.4	6.3	45.8
Inosine	34.9	5.3	4.5	55.3[a]
5′-Deoxyuridine	55.0	—	—	*b*)
6-Chloro-3-deaza purine riboside	51.2	11.0	19.0	18.7
6-Thioguanosine	36.5	14.5	31.0	18.0[c]

The general procedure is described in experimental section. *a*) Hypoxanthine was detected in appreciable amount. *b*) Unidentified product was detected together with the starting material. *c*) Polyphosphoric acid was used in place of pyrophosphoric acid.

As shown in Fig. 3 cytidine gave fairly good yield of the 2′,3′-cyclic phosphate under similar reaction conditions as that of I with DMF. It can also been seen from Table I that any adenine nucleotide gives the 2′,3′-cyclic phosphate (IV) as the main product and the product distribution is a function of the ratio of the nucleoside and phosphoric acid. The use of pyrophosphoric acid increases the yield of IV and polyphosphoric acid accerelates the reaction rate. The addition of dicyclohexylcarbodiimide in the reaction of phosphoric acid and nucleoside improves the yield, but the formation of 3′,5′-cyclic phosphate was observed in the case of the reaction started with I.[11] Treatment of adenosine 2′(3′)-phosphate with DMF gives the 2′,3′-cyclic phosphate (IV) rapidly[12] but the further heating gave the similar

11) M. Smith, G.I. Drummond, and H.G. Khorana, *J. Am. Chem. Soc.*, **83**, 698 (1961).
12) The cyclization of the 2′(3′)-phosphate by DMF at 145° has recently been reported; J. Moravek, J. Kopecky, and J. Skoda, *Collection Czech. Chem. Commun.*, **33**, 4120 (1968).

pattern of product distributions with that from I. The use of dimethyl sulfoxide in place of DMF has no effect for the formation of 2′,3′-cyclic phosphate from adenosine and phosphoric acid or from I. Pyrophosphoric acid instead of phosphoric acid in the former reaction gave the cyclic phosphate though in a lower yield. Therefore it is likely that DMF acts as the activating agent of phosphoric acid and "activated phosphoric acid" (pyrophosphoric acid may be one of the possible active species) thus formed attacks the hydroxyl groups of nucleoside to reach an equilibrium at which the 2′,3′-cyclic phosphate being most stable. The increase of the ratio of phosphate changes the equilibrium in which 2′,3′-cyclic, 5′-diphosphate being the main product.

In practice, the reaction of ribonucleoside and pyrophosphoric acid (2 equivalents, with 4 equivalents of tri-*n*-butylamine) in DMF for 2 hours at reflux temperature generally gives the best results (Table II). Some synthetic ribonucleosides afforded the 2′,3′-cyclic phosphate in a satisfactory yield, however 2,4-dithiouridine and 2-thiocytidine underwent degradation at this reaction temperature.

Experimental

Paper chromatography was carried out on Toyo Roshi No. 51A filter paper. The solvent systems were: A, iso-PrOH–conc.-NH_4OH–H_2O (7:1:2); B, EtOH–0.5M $AcONH_4$ (7:3); C, BuOH–H_2O (84:16); D, satd. $(NH_4)_2SO_4$–iso-PrOH–1M$AcONH_4$ (79:2:19) by descending system. Paper electrophoresis was run in 0.05M triethylammonium bicarbonate, pH 8.2, at 700 volts for 1 hr. The following nucleotides were prepared by the reported method: adenosine 2′,3′-cyclic phosphate,[13] adenosine 3′,5′-cyclic phosphate,[11] and adenosine 2′(3′),5′-diphosphate.[14]

Reaction of Adenosine 5′-Phosphate in DMF——Adenosine 5′-phosphate (0.5 mmole of free acid) and tri-*n*-butylamine (1.0 mmole) were dissolved in DMF and the solution was evaporated to dryness under reduced pressure, the resulting syrup was dissolved in 50 ml of DMF and refluxed for 2 hr in an oil bath. The solvent was removed *in vacuo* and the residue was dissolved in 1N NH_4OH and the solution extracted with ether. The aqueous layer was once concentrated *in vacuo* and diluted with 10 ml of water and applied to a column of DEAE-cellulose (1.8 cm diameter × 46 cm long, bicarbonate form). Elution was carried out with a linear salt concentration gradient of 0.002—0.05M (1000 ml each) followed by 0.05—0.2M (500 ml each) and 0.2—0.5M (500 ml each) of triethylammonium bicarbonate buffer (pH 8.2—8.6). Fractions containing nucleotide (see Fig. 1) were collected and evaporated under reduced pressure and the residue dissolved in water. Each was identified as follows: peak a and b contained adenosine (II) and adenine (III) as revealed by paper chromatography in solvent system A–C and by UV spectra. Peak c contained adenosine 2′,3′-cyclic phosphate (IV) as the sole component. This fraction was concentrated and co-evaporated with EtOH to leave a crystalline solid; UV, λ_{max} 259 mμ, $\varepsilon_{(p)}$ 13400, *Rf* 0.37 (A) (*Rf* of (VIII), 0.28); paper electrophoresis, relative migration to I, 0.62. An appropriate quantity of IV was treated with ribonuclease M from Aspergillus saitoi in acetate buffer at pH 4.0 and 37° for overnight. Hydrolyzate was identified as adenosine 3′-phosphate (R_{AMP}, 0.52 in D). Alkaline hydrolysis of IV in 0.1N NaOH at room temperature for 5 hr gave adenosine 2′(3′)-phosphate (R_{AMP}, 0.85 and 0.52). The peak d contained the starting material (I) as the main component with a small amount of 2′-(3′)-phosphate as revealed by paper chromatography with solvent system D. Peak e consists of VI; UV λ_{max} 259.5 mμ, $\varepsilon_{(p)}$ 7190; paper electrophoretic migration 11.6 cm, R_{AMP}, 1.2; paper chromatography in D, $R_{A2',5'-DP}$ 0.64 (A3′,5′-DP, 0.93). Ribonuclease digestion of VI at pH 4.0, 37°, overnight gave adenosine 3′,5′-diphosphate as the sole product. Peak f consists of VII as identified by the direct comparisons with the authentic materials. The time course of the reaction of I in DMF was followed and the result was shown in Fig. 2.

Treatment of Adenosine 5′-Triphosphate and Other Adenine Nucleotides with DMF——Disodium adenosine 5′-triphosphate (0.5 mmole of sodium salt) was converted to the free acid by the Amberlite IR 120 resin. The free acid and tri-*n*-butylamine (2 mmoles) were dissolved in 50 ml of DMF and refluxed for 2 hr, and the products analyzed by the procedure described above. Other adenine nucleotides, adenosine 2′(3′)-phosphate, 2′,3′-cyclic phosphate, and 3′,5′-cyclic phosphate were similarly treated in DMF and the results were shown in Table I.

General Method of Reaction of Ribonucleosides and Pyrophosphate, Phosphoric Acid, or Polyphosphoric Acid——Tetrasodium pyrophosphate (1.0 mmole) was dissolved in water and converted to the free acid by passing through a column of Amberlite IR 120 resin (H^+ form). The eluate was concentrated under

13) A.M. Michelson, *J. Chem. Soc.*, **1959**, 3665.
14) M. Honjo, K. Imai, Y. Furukawa, H. Moriyama, K. Yasumatsu, and A. Imada, *Takeda Kenkyusho Nempo* (Osaka), **22**, 47 (1963).

reduced pressure and to the residue was added tri-*n*-butylamine (4 mmoles). Co-evaporation with DMF under reduced pressure was repeated twice to remove water. The resulting syrup and a nucleoside (0.5 mmole) were dissolved in 50 ml of DMF and refluxed for 2 hr in an oil bath. The reaction mixture was subjected to paper electrophoresis. The UV absorbing spots were excised and eluted with 4 ml of water and estimated spectrophotometrically. In the case of the reaction with phosphoric acid 85% H_3PO_4 was used and dehydrated by co-evaporation with DMF prior to the reaction. Polyphosphoric acid was prepared by mixing 85% H_3PO_4 and P_2O_5 in 1.2:1.0 by weight and the reaction was carried out as above. The results were summarized in Table I and II. The time course of the reaction of cytidine and phosphoric acid was, as an example, shown in Fig. 3.

Acknowledgement The authors express their gratitude to Dr. Yoshihisa Mizuno for his encouragement throughout this work and Dr. Masachika Irie of Kyoto University for the supply of ribonuclease M.