Enzymic Preparation of Guanosine-2', 3'-cyclic Phosphate, Inosine-2', 3'-cyclic Phosphate and Xanthosine-2', 3'-cyclic Phosphate

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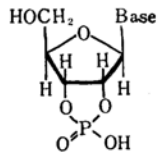
Our previous studies have shown that ribonuclease T_1(RNase T_1) in Takadiastase hydrolyzes the secondary phosphate ester bonds of guanosine-3' phosphate in ribonucleic acid (RNA)[1-3]. During the digestion, guanosine-2', 3'-cyclic phosphate (G-cyclic-p) is accumulated in the reaction mixture as an intermediary product.

It has been also found that RNase T_1 hydrolyzes *deamino*-ribonucleic acid (*deamino*-RNA) splitting preferentially the secondary phosphate ester bonds of inosine-3' phosphate with an intermediary formation of inosine-2', 3'-cyclic phosphate (I-cyclic-p). With higher amounts of RNase T_1, the secondary phosphate ester bonds of xanthosine-3' phosphate is also hydrolyzed with an intermediary formation of xanthosine-2', 3'-cyclic phosphate (X-cyclic-p)[4].



Nucleoside-2', 3'-cyclic phosphate

1) K. Sato and F. Egami, *J. Biochem.*, **44**, 753 (1957).
2) K. Sato and F. Egami, *Compt. rend. soc. biol.*, **151**, 1792 (1957).
3) K. Sato-Asano, *J. Biochem.*, **46**, 31, (1959).
4) K. Asano and Y. Fujii, *Symposium on Enzyme Chemistry (Japan)*, **10**, 123 (1958).

It has so far been difficult to synthesize G-cyclic-p by a chemical method with good yield and in high purity[5]. Markham et al. prepared G-cyclic-p and adenosine-2', 3'-cyclic phosphate (A-cyclic-p) by mild alkaline hydrolysis of "RNase I core"[6]; however, the yield was far from satisfactory. On the other hand, I-cyclic-p and X-cylic-p have been neither separated from the natural source nor synthesized.

We succeeded in preparing these purine nucleoside cyclic phosphates with good yield by the enzymic reactions. So the method of preparation of these nucleoside-2', 3'-cyclic phosphates and the properties of new compounds will be reported.

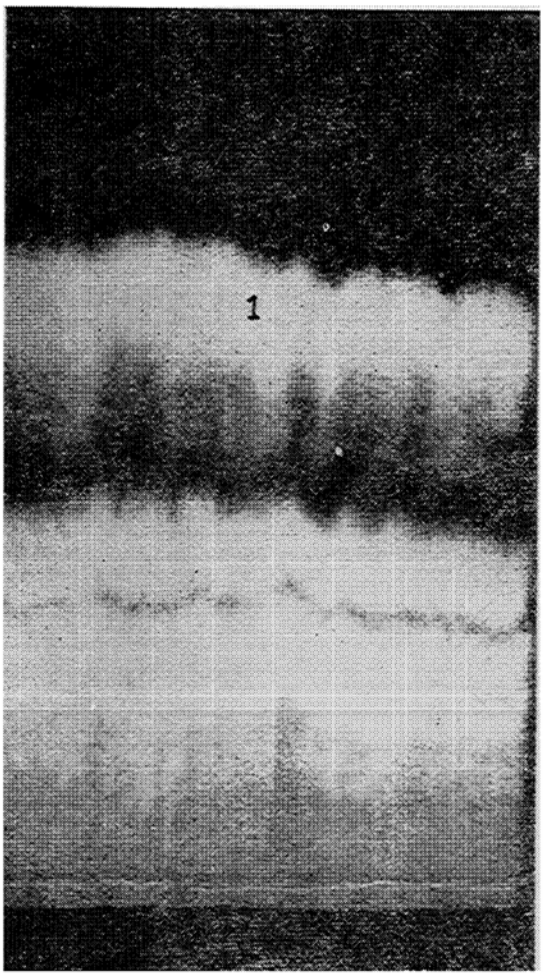


Fig. 1. Paper chromatogram of the dialyzate of the digestion mixture of yeast RNA by RNase T_1. Reaction mixture contains 100 mg. of yeast RNA and 10 μg. of RNase T_1 at pH 7.5. The band 1 is that of G-cyclic-p. Solvent I was used.

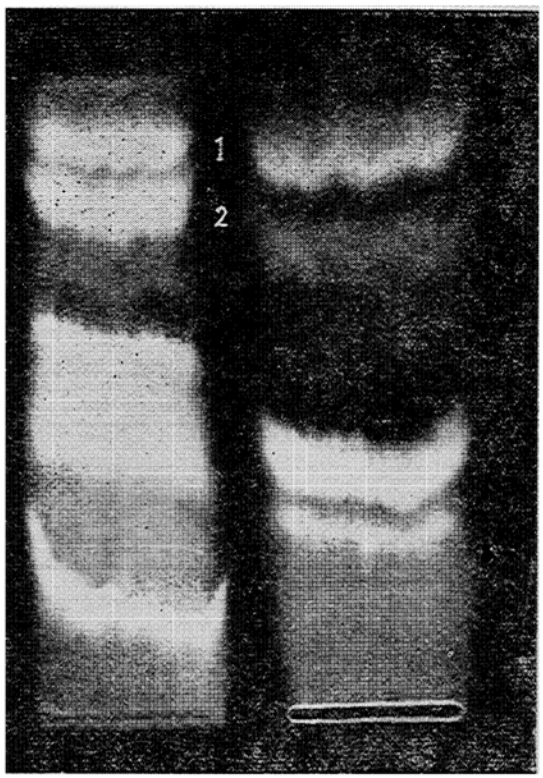
Experimental

RNA.—A commercial yeast RNA (Schwarz Laboratories) was deproteinized with chloroform gel formation, dialyzed in a cellophane tubing against distilled water and precipitated with alcohol.

Deamino-RNA.—Yeast RNA was deaminated by the method of Takemura[7]. Details of the preparation and properties of deamino-RNA will be reported elsewhere.

RNase T_1.—The purest sample extracted and prepared from Takadiastase according to the method of Sato and Egami[1] was used.

Paper Chromatography.—*Solvent I*: isopropanol: water (7 : 3) with concentrated aqueous ammonia in the bottom of the vessel (0.5 ml. for each liter of gas phase)[8], descending for 18 to 19 hr.



(b) (a)

Fig. 2. Paper chromatograms of the dialyzate of the digestion mixture of *deamino*-RNA by RNase T_1. Solvent I was used. The bands are: 1, I-cyclic-p; 2, X-cyclic-p.
a) 20 mg. *deamino*-RNA, 2 μg. RNase T_1/ml.
b) 200 μg. RNase T_1 was added to the nondialyzable fraction in a).

5) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, **1952**, 2708.
6) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952); L. A. Heppel and P. R. Whitfeld, ibid., **60**, 1 (1955).
7) S. Takemura, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **72**, 674 (1951).
8) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558 (1952).

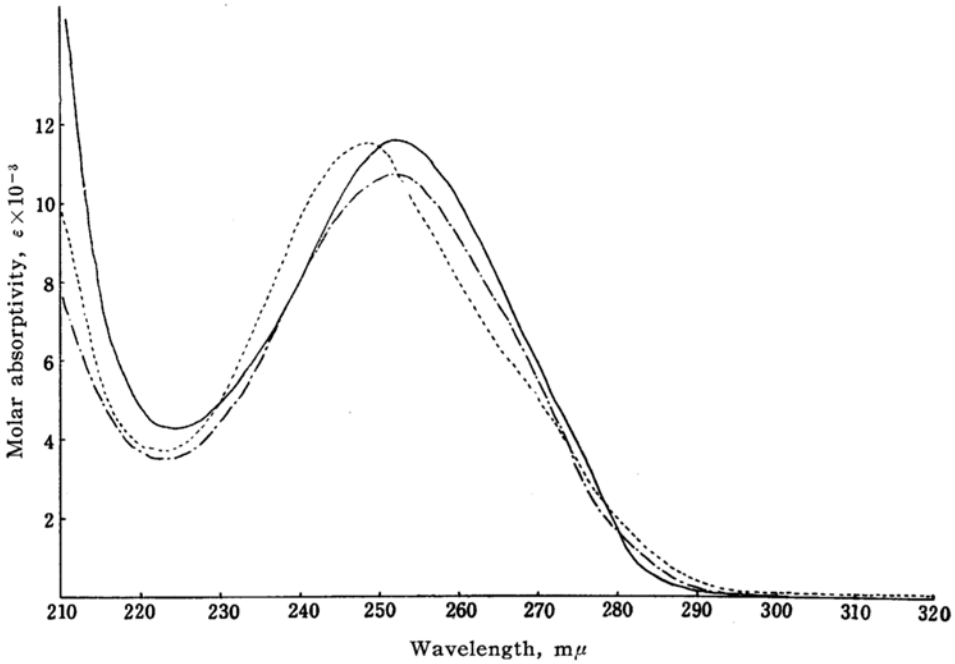


Fig. 3. Absorption spectra of I-cyclic-p.
——, pH 11.2; ------, pH 6.0; —·—·—, 2 N HCl.
Beckman spectrophotometer type DK2 was used.

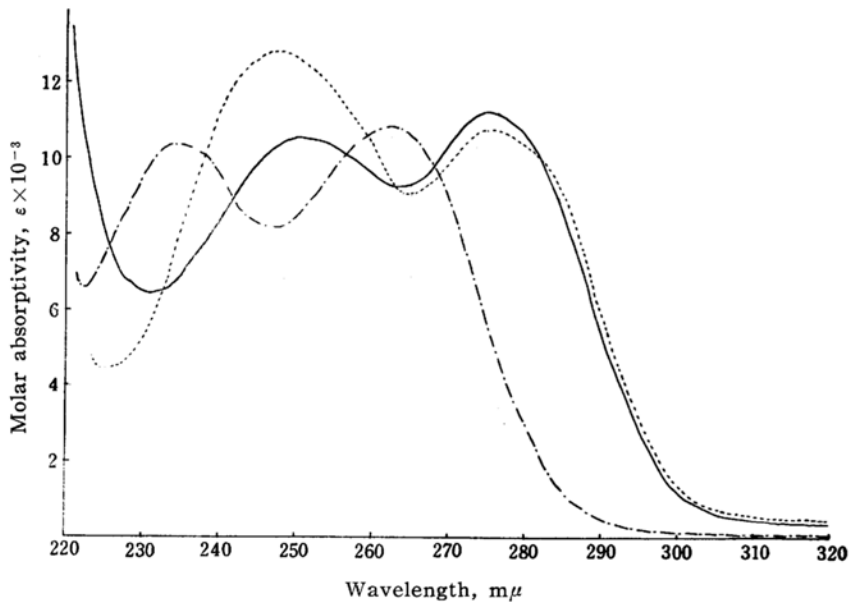


Fig. 4. Absorption spectra of X-cyclic-p.
——, 1 N NaOH; ------, pH 8.1; —·—·—, pH 3.0.
Beckman spectrophotometer type DK2 was used.

Solvent II: saturated ammonium sulfate : water: isopropanol (79 : 19 : 2)[9], ascending for 9 hr. Tôyôroshi No. 51 paper was used throughout the studies and the bands on the chromatogram were located by the usual photographic methods.

Organic Phosphorus.—It was measured by the method of Allen[10].

Preparation of G-cyclic-p.—Yeast RNA (100 mg.) was dissolved in 5 ml. of distilled water and pH was adjusted to 7.5. To the solution 10 μg. of RNase T_1 was added. The reaction mixture was incubated at 37°C for 2 hr., then dialyzed against distilled water in a cold room under stirring overnight. The dialyzate was concentrated and subjected to paper chromatography (15~20 cm./100 mg. RNA) using solvent I. As is shown in Fig. 1 the wide intensive band 1 was found, this was identified with that of G-cyclic-p[3]. The band was eluted and lyophilyzed. G-cyclic-p was obtained in an almost pure state by chromatography. After the preparation was rechromatographed with the same solvent, it was proved by another paper chromatography test, paper electrophoresis and its U. V. absorption spectrum that it became completely free from other nucleotides except for a trace amount of guanylic acid (Gp).

Preparation of I-cyclic-p.—One hundred milligrams of *deamino*-RNA was dissolved in 5 ml. of distilled water at pH 7.5 and 10 μg. of RNase T_1 was added, then the reaction mixture was incubated for 5 hr. at 37°C. The reaction mixture was dialyzed against 50 ml. of distilled water in a cold room under stirring, then the dialyzate was concentrated and subjected to paper chromatography (solvent I, descending for 18 hr.). After drying, the paper was rechromatographed with the same solvent for 19 hr. (Fig. 2a). The broad band 1 moving rapidly was identified with that of I-cyclic-p[11]. Band 2 which is that of X-cyclic-p[11] also appeared sightly. Band 1 was eluted with distilled water, the eluate was concentrated and subjected to paper chromatography using solvent I. There was nothing except for a large amount of I-cyclic-p and a trace of inosinic acid (Ip). It was also proved by another paper chromatography test using solvent II that the band was pure I-cyclic-p. The purest preparation was used for the following experiments.

Preparation of X-cyclic-p.—After the pH of the reaction mixture, which remained in the cellophane tubing in the experimental for I-cyclic-p, was adjusted to 7.5, 1.0 ml. of RNase T_1 (1.3 mg./ml.) was added and the reaction mixture was incubated for 5 hr. at 37°C. Then it was dialyzed against 60 ml. of distilled water in a cold room under stirring, then the dialyzate was concentrated and subjected to paper chromatography (solvent I, descending for 18 hr.). After drying, the paper was rechromatographed with the same solvent for 19 hr. (Fig. 2b). The broad band 2 was identified with that of X-cyclic-p[11]. To purify the sample completely, band 2 was eluted with distilled water, the eluate was concentrated and subjected to rechromatography with the same solvent. There was nothing but large amounts of X-cyclic-p and a trace of xanthylic acid (Xp). Moreover, this band 2 was proved to be pure X-cyclic-p by another paper chromatography test with solvent II. This sample was used in the following studies.

9) R. Markham and J. D. Smith, ibid., **49**, 401 (1951).
10) R. J. Allen, ibid., **34**, 858 (1940).
11) K. Sato-Asano and Y. Fujii, *J. Biochem.*, in press.

Results and Discussion

Yields.—The yields were calculated from the optical density. Five milligrams G-cyclic-p from 100 mg. of yeast RNA; 2 mg. of I-cyclic-p from 100 mg. of *deamino*-yeast RNA; 2.5 mg. of X-cyclic-p from 100 mg. of *deamino*-yeast RNA.

Absorption Spectra of I-cyclic-p and X-cyclic-p.—Using the purest preparation, their ultraviolet absorption spectra were measured at various pH ranges, and their molar extinction coefficients were determined by measuring phosphate (Figs. 3 and 4).

Rf values were calculated in two solvent systems.

TABLE 1. *Rf* VALUES OF PREPARED CYCLIC NUCLEOTIDES AND OF MONONUCLEOTIDES

	Solvent I	Solvent II
G-cyclic-p	0.28	0.29
I-cyclic-p	0.43	0.33
X-cyclic-p	0.35	0.19
A-cyclic-p	0.48	0.13
C-cyclic-p, U-cyclic-p	0.50	0.54
Gp	0.13	0.45 (3′) 0.54 (2′)
Ip	0.16	0.52
Xp	0.15	0.33
Ap	0.21	0.23 (3′) 0.33 (2′)
CP, Up	0.21	0.73

These cyclic nucleotides, especially G-cyclic-p, were rather labile in diluted solution and were hydrolyzed to nucleotides. So all procedures had to be carried out as rapidly as possible.

If two or more cyclic nucleotides were produced from RNA at the same time, the separation might be very difficult. So the stepwise enzymic preparation seems to be a better method. After the preparation of G-cyclic-p from the dialyzate of the digestion mixture of yeast RNA by RNase T_1, the nondialyzable fraction which remained in the cellophane tubing was treated in 0.1 N hydrochloric acid to split the residual cyclic phosphate[2], neutralized and dialyzed completely. What may be

called "RNase T_1 core" thus obtained was useful for the preparation of pyrimidine nucleosidecyclic phosphates by RNase I.

Pyrimidine nucleosidecyclic phosphates were prepared from the dialyzate of yeast RNA digests by RNase I and the residues were further completely digested and dialyzed, and thus the well known "RNase I core" was obtained. It was also a good source for the preparation of G-cyclic-p by RNase T_1.

It was rather difficult to separate a large amount of I-cyclic-p and X-cyclic-p. To solve this difficulty RNase T_1 digestion was divided into two steps: at the first step with a small quantity of RNase T_1, the greater part of I-cyclic-p was separated and at the second step by addition of more RNase T_1, the remaining I-cyclic-p and X-cyclic-p were obtained separately.

Summary

By RNase T_1 digestion, guanosine-2′,3′-cyclic phosphate was obtained with good yield from yeast RNA, and two new compounds, inosine-2′,3′-cyclic phosphate and xanthosine-2′,3′-cyclic phosphate were obtained from *deamino*-yeast RNA. The properties of the new compounds were studied.

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