Hydrazinolysis of Nucleic Acids. II. The Formation of Ribo-apyrimidinic Acid from Yeast Ribonucleic Acid

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(Received March 23, 1959)

In the preceding paper[1], one of the authors has dealt with the formation of a new derivative devoid of pyrimidines, named deoxyribo-apyrimidinic acid, by the hydrazinolysis of herring sperm deoxyribonucleic acid, by the hydrozinolysis of herring sperm deoxyribonucleic acid. In connection with studies on the specificity of pancreatic ribonuclease in which this laboratory is engaged[2-6], it was desired to prepare ribonucleic acid derivatives devoid of the pyrimidines. The hydrazinolysis was first investigated using yeast ribonucleic acid and gave, for the first time, a nondialyzable derivative free from the pyrimidines, for which the name ribo-apyrimidinic acid was proposed[7]. Besides

1) S. Takemura, This Bulletin, **32**, 920 (1959).
2) S. Takemura, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*) **72**, 674 (1951).
3) F. Egami, H. Ishihara and M. Shimomura, *Z. physiol. Chem.*, **295**, 349 (1953).
4) F. Egami, *J. Japan. Biochem. Soc.* (*Sei Kagaku*), **27**, 139 (1955).
5) S. Takemura, *Protein, Nucleic Acid and Enzyme*, **2**, 79 (1957).
6) S. Takemura, "Intern. Kongr. Biochem", Zusammenfassung, IV Kongr., Wien (1958), p. 34.
7) S. Takemura, *J. Biochem.* **44**, 321 (1957).

TABLE I. REACTION OF YEAST RIBONUCLEIC ACID WITH ANHYDROUS HYDRAZINE AND COMPOSITION OF THE NITROGENEOUS CONSTITUENTS OF THE PRODUCTS

Preparation*	Condition of reaction		Yield of nondialyzable product	Mol./10 mol. of adenine[b]			
	t°C	min.	(%)**	Adenine	Guanine	Cytidylic acid	Uridylic acid
RNA, commercial	—	—	—	10.0	12.4	9.4	10.3
RNA, purified	—	—	—	10.0	12.6	11.5	10.2
RAPYA I[a]	0	60	93	10.0 (10.0)	12.4 (12.3)	9.7 (9.5)	7.2 (7.0)
RAPYA II	0	180	—	10.0	12.3	6.2	3.7
RAPYA III	30	120	—	10.0	12.7	1.0	1.8
RAPYA IV[a]	50	60	88	10.0 (10.0)	12.6 (12.3)	0.6 (0.6)	0.7 (0.7)
RAPYA V[a]	70	60	77	10.0 (10.0)	12.5 (12.4)	0.7 (0.7)	0.6 (0.6)
RAPYA VI[a]	50	60	73	10.0	12.3	1.2	2.5
RAPYA VII	80	60	50	10.0	12.9	1.0	1.5
RAPYA VIII	60	60	70	10.0	12.5	1.3	2.8
PAPYA IX	65	90	64	10.0	12.8	0.5	0.5

* RNA=ribonucleic acid; RAPYA=ribo-apyrimidinic acid; RAPYA VI, VII and VIII were obtained from commercial powdered RNA, others from purified and lyophilized RNA.

a) Twice dehydrated hydrazine was used.

** These are per cent of RNA phosphorus recovered in the product.

b) The values in parentheses are the composition of the dialyzable materials.

the problem of the specificity of ribonucleases which are reported in subsequent papers, some information about the general structure of ribonucleic acid may be derived from degradative studies of the derivative.

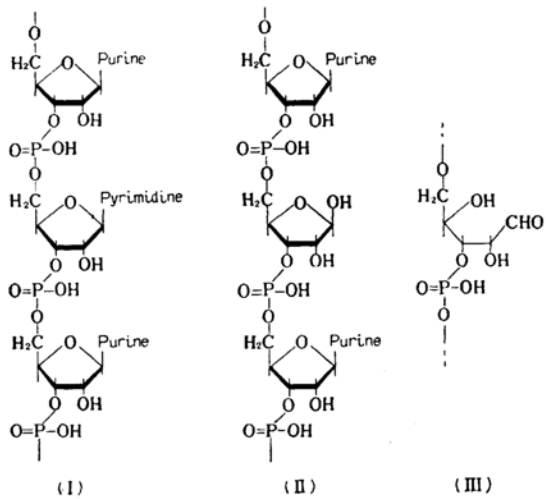
When yeast ribonucleic acid was treated with 80% aqueous hydrazine, a complete hydrolysis of the phosphodiester linkages resulted and only 2′- and 3′-isomers of purine mononucleotides were detected on the paper chromatogram, as the ultraviolet absorbing materials. In the case where anhydrous hydrazine was used, drastic condtions such as reaction at 90°C did not, however, offer any advantages, but indeed resulted in a serious destruction of the phosphodiester linkages and formation of considerable amounts of purine mononucleotide isomers. Experiments using various conditions of time and temperature were carried out and are summarized in Table I. The use of nucleic acid which was not purified and especially not lyophilized resulted in the incomplete removal of the pyrimidines. After treatment with hydrazine and precipitation with alcohol, there were difficulties in obtaining the derivatives without an accompanying serious destruction of the internucleotide linkages. In most cases, the alcohol precipitates were dissolved at 0°C in distilled water and the products precipitated with dilute hydrochloric acid. This procedure was effective for removign hydrazine, which was expected to be included as hydrazonium salt in the alcohol precipitates. However, it was thought that there might be possibilities of the formation of hydrazones or osazones using such acidic conditions. An experiment showed that the use of concentrated phosphate buffer of neutral range converted hydrazonium salts to sodium salts and minimized fissions of the phosphodiester linkages (see Table IV).

The reaction of the lyophilized ribonucleic acid of yeast with anhydrous hydrazine at 50°C for 60 min. was found to be most suitable for the preparation of ribo-apyrimidinic acid. Using these conditions, the product was practically free from pyrimidines and fissions of the phosphodiester linkages were not serious. As attempts to remove the pyrimidines with milder reagents than anhydrous hydrazine, anhydrous methylhydrazine and phenylhydrazine were used. The latter did not react but the former removed considerable amounts of uracil and a little cytosine when the reaction was carried out at 60°C for 90 min. The white product was obtained through acid precipitation and the yield was quantitative. The use of

anhydrous methylhydrazine in a more drastic condition is, therefore, of great promise.

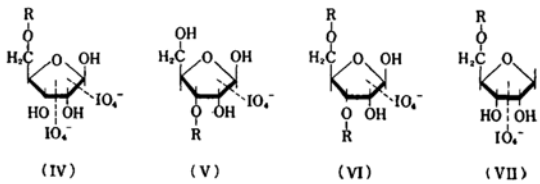
The hydrazinolysis products gave slightly higher values for the atomic N/P ratios than those expected from the base composition of the products. The products, excepting that obtained through the dissolution in the buffer, were slightly yellow. A small part of ribose residues may be present as hydrazone or osazone and also as ureido-ribose formed from the pyrimidine riboside moieties by the hydrazinolysis. From the color intensities in the reaction with orcinol and the estimation of reducing sugar, however, it can be seen that these possibilities occure only slightly, if at all. Direct evidence for the presence of free ribose residues in the hydrazinolysis products was obtained by treating one of the products with Takadiastase, which hydrolyses nucleic acids to nucleosides. Much ribose was liberated, as shown by paper chromatography. Qualitative tests for reducing sugar were also strongly positive.

During the hydrazinolysis and the subsequent treatment, the initial interpurine ratios were not distorted. This was most clearly confirmed by analyzing the dialyzable fragments. The base compositions of the dialyzable fragments were the same as those of the corresponding nondialyzable products. The removal of the pyrimidines from yeast ribonucleic acid was easier than that from herring sperm deoxyribonucleic acid. In the initial stage of the reaction, uracil was decomposed faster than was cytosine, but this was reversed later. Yeast ribonucleic acid is presumed to be hydrazinolyzed in a similar way to that shown in the preceding paper on the hydrazinolysis of herring sperm deoxyribonucleic acid[1]. Thus the ribose phosphate residues in the aqueous solution of the ribo-apyrimidinic acid may be present in the furanose form (II) rather than in the free aldehydoform (III)[8].



(I) (II) (III)

In alkaline solutions, the hydrazinolysis products were as unstable as ribonucleic acid. The product which is free from the pyrimidines was unstable, even in solutions of neutral pH or below at 37°C for one day, about 10% of its total phosphorus was rendered soluble in acid—uranyl reagent, but this was greatly reduced at 0°C. In spite of this instability, the product obtained using the most suitable conditions showed a fairly high mean chain length. Periodate should be consumed not only by the nonglycosidic ribose residues within VI or at the end of the chains (IV, V), but also by the purine nucleosides which occupy the chain ends and do not bear a terminal 3′-phosphate group (VII). The periodate consumption



(IV) (V) (VI) (VII)

R = nucleotides or phosphate

of a product obtained through the dissolution in the phosphate buffer was less than that obtained through the acid precipitation. The electrometric titration curve of the product obtained by the reaction at 65°C for 90 min. showed the disappearance of one -NH-CO- dissociation titrating in the range pH 8~12. This can be explained by the removal of uracil. The absence of the amino group of cytosine was, however, not clearly indicated, being obscured by the increased secondary phosphoric acid dissociations which are due to fissions of the phosphodiester linkages.

In the most suitable condition of the hydrazinolysis, about 90% of the material was nondialyzable. Moreover, the interpurine ratio was not distorted during the reaction. Hence, the ribo-apyrimidinic acid must be useful for the structural studies of ribonucleic acid.

8) S. M. Cantor and Q. P. Peniston, *J. Am. Chem. Soc.*, **62**, 2113 (1940).

TABLE II. COMPOSITION OF THE HYDRAZINOLYSIS PRODUCTS OF YEAST RIBONUCLEIC ACID

Preparation*	N (%)	P (%)	Atomic N/P	Color reaction with orcinol[a]	Reducing ribose residues, mol./100 g.-atoms of P[b]
RNA, commercial	14.5	8.4	3.8	100	—
RNA, purified	15.0	8.8	3.8	100	3
RAPYA I	14.7	9.3	3.6	127	32
RAPYA IV	13.4	9.9	3.0	164	53
RAPYA V	12.7	9.2	3.0	183	45
RAPYA VIII	12.4	9.1	3.0	163	43
RAPYA IX	13.4	9.9	3.0	153	48

* See Table I.

a) Compared with RNA on the basis of phosphorus content.

b) Compared with ribose as the standard.

Experimental

Nitrogen, phosphorus, reducing sugar (Table II), the ultraviolet absorption spectra (Table III and Fig. 1), the mean chain length and the periodate consumption (Table IV) were determined by methods described in the preceding paper[1]. The nucleotide composition (Table I) was determined chromatographically after hydrolyzing samples in a sealed tube with 1 N hydrochloric acid[9] at 100°C for 1 hr. (propan-2-ol—

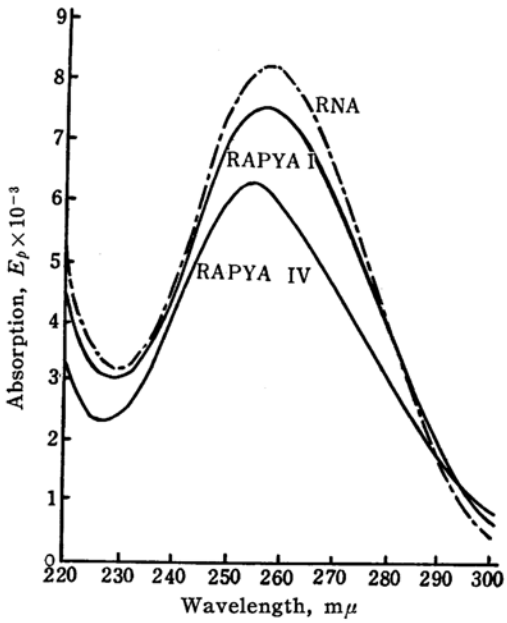


Fig. 1. Ultraviolet absorption curves of the hydrazinolysis products of yeast ribonucleic acid in phosphate buffer, pH 7.
E_p=extinction per g.-atom of phosphorus.

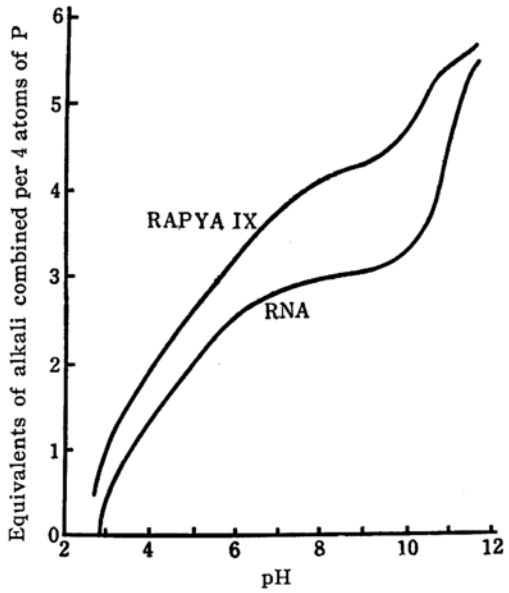


Fig. 2. Electrometric titration curve of the hydrazinolysis product (RAPYA VIII) of yeast ribonucleic acid.

TABLE III. ULTRAVIOLET ABSORPTION SPECTRA OF THE HYDRAZINOLYSIS PRODUCTS OF YEAST RIBONUCLEIC ACID[a]

Preparation*	Maximum		Minimum	
	mμ	E_p[b]	mμ	E_p[b]
RNA, purified	259	8200	230	3200
RAPYA I	259	7600	230	3100
RAPYA IV	255.5	6250	228	2350
RAPYA VIII	255.5	6600	228	2650

a) Measurements were carried out in 0.1 M phosphate buffer of pH 7.

* See Table I.

b) The extinction coefficient with respect to one gram-atom of phosphorus per liter.

9) J. D. Smith and R. Markham. *Biochem. J.*, **46**, 509 (1950).

TABLE IV. MEAN CHAIN LENGTH AND PERIODATE CONSUMPTION OF THE HYDROZINOLYSIS PRODUCTS OF YEAST RIBONUCLEIC ACID

Preparation*	Mean chain length	Periodate consumption, mol. per 1 mol. of P
RNA, purified	21.2	0.05
RAPYA I	19.0	0.46
RAPYA IV	13.5	0.65
RAPYA IV'[a)]	—	1.76
RAPYA IV[a)]''	15.4	0.47
RAPYA V	9.8	0.72

* See Table I.

a) These were obtained by a modified method (see *Experimental*); RAPYA IV'—before treatment with phosphate buffer; RAPYA IV''—after treatment with phosphate buffer.

hydrochloric acid[10], Toyo Roshi Co. No. 51a paper, descending). The color reaction of the hydrazinolysis products with orcinol[11] was compared with the parent ribonucleic acid (Table II). The ribose residues, which in the intact ribonucleic acid were in the pyrimidine nucleotides, also participate in the color reaction. For the electrometric titration of a product (RAPYA IX) a Beckman Type G pH-meter was employed. The measurement was carried out in the absence of carbon dioxide (Fig. 2).

Dehydration of Hydrazines. — Anhydrous hydrazine was prepared as described in the corresponding section of the preceding paper[1]. Fifty per cent aqueous methylhydrazine was dehydrated with sodium hydroxide pellets at 90°C for 12 hr., stood overnight at room temperature and distilled (b. p. 86.6~87.3°C)[12]. Phenylhydrazine was distilled under diminished pressure before use.

Ribonucleic Acid.—A commercial yeast ribonucleic acid, Schwarz Laboratories, Inc., was purified by Sevag's method[13] and freeze-dried. Before treatment with hydrazine, the nucleic acid was dried over concentrated sulfuric acid in vacuo at 60°C for 5 hr. The composition of the nucleic acid is listed in Tables I and II.

Preparation of Ribo-apyrimidinic Acid.— The hydrazinolyses at different conditions gave products in which the ribonucleic acid was more or less deprived of the pyrimidines (Table I). In order to simplify descriptions, the name ribo-apyrimidinic acid (RAPYA) is given to all nondialyzable products obtained by the reaction of the ribonucleic acid with anhydrous hydrazine. A typical preparation was as follows. The mixture of lyophilized and dried yeast ribonucleic acid (1.5 g.) and anhydrous hydrazine (3 ml.) was kept free from moisture at 50°C for 1 hr. Within a few minutes, the nucleic acid dissolved in the hydrazine, making a clear solution, with a slight evolution of heat and foam. After cooling, absolute alcohol (5 vol.) was added to the solution, the white precipitate thus formed washed with absolute alcohol and ether, and dried. The dry solid was then dissolved in cold distilled water at 0°C and the pH adjusted to 1.5 with dilute hydrochloric acid. The slightly yellow precipitate thus formed was washed with absolute alcohol and ether, dried, and then suspended in water and dilute alkali was added carefully until the solid just dissolved. The solution was dialyzed against frequent changes of distilled water at 4°C for 3 days. The nondialyzable fraction was freeze-dried. The light yellow product (RAPYA IV) was obtained in 88% yield with respect to phosphorus recovery. Other reaction products using different conditions were obtained by the same procedure, excepting RAPYA IV' and IV''. For the latter the procedure was modified as mentioned below since yellow materials were obtained at the step where the products were precipitated with acid. This was possibly due to reaction between hydrazine and free ribose residues. After treatment with hydrazine and precipitation with alcohol, the precipitate was dissolved in distilled water at 0°C and the pH was adjusted to 7. This solution was then dialyzed against frequent changes of distilled water and freeze-dried. The white product so obtained still contained hydrazine (RAPYA IV'). This was removed by dissolving the material in ice-cold 0.2 M sodium phosphate buffer (pH 6.2) and dialyzing it against the same buffer at 4°C for 8 hr., then against tap water (24 hr.) and finally against frequent changes of distilled water (48 hr.) and lyophilized (RAPYA IV'').

Reaction of Yeast Ribonucleic Acid with Anhydrous Methylhydrazine. — Lyophilized yeast ribonucleic acid (50 mg.) and anhydrous methylhydrazine (1.5 ml.) were kept free from moisture at 60°C for 90 min., then precipitated with absolute alcohol, dissolved in water, and precipitated with acid. This was again suspended in water and dissolved by the careful addition of dilute alkali and dialyzed. The white product thus obtained, in quantitative yield, contained 10.0 mol. of cytidylic acid and 4.6 mol. of uridylic acid per 10 mol. of adenine. The ratio of guanine to adenine was the same as in the parent nucleic acid.

Reaction of Yeast Ribonucleic Acid with Phenylhydrazine. — Freshly distilled phenylhydrazine (12 g.) and lyophilized yeast ribonucleic acid (0.5 g.) were kept at 150°C for 2 hr. and then treated as mentioned above. The material thus obtained was identical with the parent nucleic acid in the nucleotide composition.

Composition of the Dialyzable Materials. —In the preparation of ribo-apyrimidinc acid I, and also IV and V, the outside fluids of each dialysis were combined, adjusted to pH 7 and freeze-dried. The dialyzable fragments were hydrolyzed with 1 N hydrochloric acid and the products were separated by paper chromatography. The nucleotide compositions are shown

10) G. R. Wyatt, ibid., **48**, 584 (1951).
11) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).
12) G. V. Brüning, *Ann.*, **253**, 7 (1889).
13) M. G. Sevag, *Biochem. Z.*, **273**, 419 (1934).

in parentheses in Table I. Practically no differences were shown between the composition of the nondialyzable and the dialyzable materials.

Detection of Ribose and Nucleosides after Enzymic Hydrolysis. — Ribo-apyrimidinic acid IV was dissolved in 0.5 M acetate buffer of pH 5.0 and incubated with dialyzed Takadiastase solution at 37°C for 48 hr. The hydrolyzate was applied on paper and separated with *n*-butanol—ammonia[14]. Ribose was detected by spraying aniline hydrogen phthalate reagent[15]. The ultraviolet photo-print showed only the spots of adenosine and guanosine, whereas yeast ribonucleic acid was completely hydrolyzed to purine and pyrimidine nucleosides by this enzyme.

Qualitative Tests for Nonglycosidic Ribose. —0.2% aqueous solutions of ribo-apyrimidinic acid IV and of yeast ribonucleic acid, and 0.1% ribose solution were used for all tests. Excepting the ribonucleic acid, they reacted with ammoniacal silver solution when heated at 100°C for 15 min., reduced Fehling's solution when heated at 100°C for 5 min., gave a reddish brown color with benzidine in glacial acetic acid at 100°C in a few minutes, and reduced mercuric chloride solution at room temperature. Ribo-apyrimidinic acid IV reacted with phenylhydrazine hydrochloride when heated at 50°C for 15 min. and gave a brownish yellow product. All the hydrazinolysis products spotted on filter paper gave a reddish purple color with aniline hydrogen phthalate reagent used for the development of paper chromatograms of reducing sugars[15].

The authors thank Professor F. Egami for his close interest. The expense of this study was defrayed in part by a grant from the Ministry of Education.

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14) R. Markham and J. D. Smith, *Biochem. J.*, **45**, 294 (1949).
15) S. M. Partridge, *Nature*, **164**, 443 (1949).