Fractionation of Ribonuclease-resistant Fraction on ECTEOLA-cellulose

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(Received November 25, 1957)

The smaller products of ribonuclease (RNase) digestion of yeast ribonucleic acid (RNA) were fractionated by Volkin and Cohn[1] using ion exchange resin and by Markham and Smith[2] using paper electrophoresis and paper chromatography. For the larger products of the digestion, however, these methods could offer little advantage for the purpose of fractionation. Recently, Bendich et al.[3] suggested the possibility of fractionating RNA fragments which remained after treatment with RNase by ECTEOLA-cellulose columns.

In the investigation to be reported in this paper, the RNase-resistant material

1) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).

2) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 558, 565 (1952).

3) A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser, *J. Am. Chem. Soc.*, **77**, 3671 (1955).

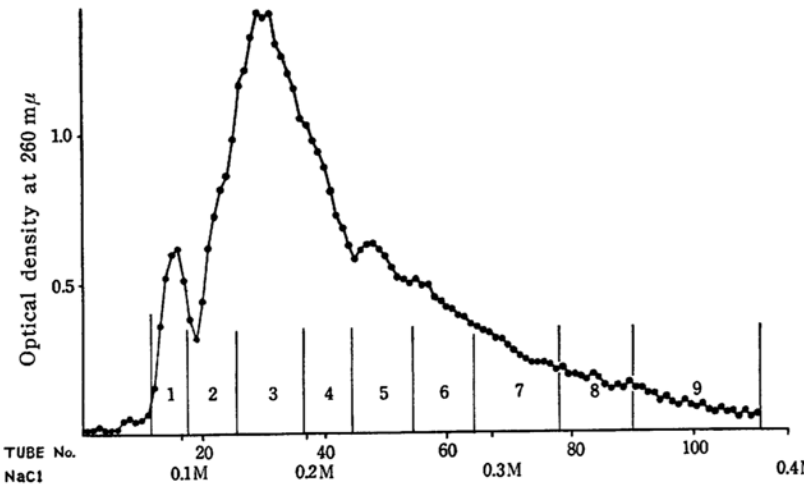

Fig. 1. Fractionation of RNase-resistant fraction of yeast RNA on ECTEOLA-cellulose by continuous gradient elution.
Exchanger: ECTEOLA-cellulose 20 g., 2.8 cm. in diameter.
Sorbed material: 200 mg. of Core 2.
Each tube contained 10 ml. of eluate. The optical density was measured after 20-fold dilution of the eluate in a 1 cm. cell. The fractions indicated with the mark | | were used for the determination of base ratio.

(Core) obtained from yeast RNA was shown to be successfully fractionated on an ECTEOLA-cellulose column. The relative molar ratio of the bases found in each fraction obtained was then measured by paper chromatography. It was thus found that oligonucelotides of larger molecular weight in the RNase-resistant material are remarkably rich in guanylic acid.

Experimental

Yeast RNA.—Commercial yeast RNA (sodium salt, Schwarz; P, 8.3%).

Pancreatic RNase.—The enzyme used in the study was a crystalline preparation obtained from Worthington Biochemical Sales Corporation.

ECTEOLA-cellulose—The sample was prepared by Peterson and Sober's method[4] from Carl Schleicher and Schüll cellulose-powder No. 123 for column chromatography. Nitrogen content of the used sample was 0.28 meq./g. (0.39%).

Core 1.—Fifty grams of sodium salt of yeast RNA were dissolved in 500 ml. of distilled water and deproteinized by chloroform gel formation and dialyzed exhaustively. The concentrated dialyzate (250 ml.) was digested with 8.5 mg. of crystalline RNase for 18 hours at 37°C with frequent addition of sodium hydroxide solution to maintain the pH 7.2. After incubation, the reaction mixture was exhaustively dialyzed in a cellophane tubing against distilled water and then lyophilized (yield 38%). The dried material was dialyzed again in a colodium bag against distilled water and lyophilized (yield about 10%; P, 8.1%).

Core 2.—Fifty grams of sodium salt of yeast RNA were deproteinized by chloroform-gel formation, dialyzed in a cellophane tubing against distilled water, and then lyophilized (yield 52%; P, 8.2%). Fifteen grams of the dried sample were dissolved in 150 ml. of distilled water and digested with 15 mg. of crystalline RNase for 40 hours at 37°C in the presence of chloroform with frequent addition of sodium hydroxide solution to maintain the pH at 7.2. After incubation, the reaction mixture was dialyzed in a cellophane tubing against distilled water, and lyophilized (yield 11%). One gram of the dried sample was dissolved in 50 ml. of distilled water and precipitated by the addition of N hydrochloric acid to pH 1.2. The precipitate was collected by centrifuge and washed twice with 50% acetone and then with pure acetone once (yield 5.8%).

Chromatography on ECTEOLA-cellulose.—The fractionation was carried out with a column (2.8 cm. in diameter) containing 20 g. of ECTEOLA-cellulose or a column (1.2 cm. in diameter) containing 10 g. of ECTEOLA-cellulose. The column was successively washed with 1.0 N sodium hydroxide, 1.0 M dipotassium monohydrogen phosphate and 0.01 M neutral phosphate buffer before each run. The column was charged and eluted under pressure at flow rate of about 0.3 ml./cm²/min. The oligonucleotides were eluted by sodium chloride solution (in 0.01 M neutral phosphate); the concentration of sodium chloride being either continuously or stepwise increased. The continuous gradient was established in the usual way by introducing buffer containing high concentration of sodium chloride (0.5 M) into a constant volume (725 ml.) mixing chamber originally filled with the buffer. The concentration of sodium chloride in the eluate was calculated

4) E. A. Peterson and H. A. Sober, ibid., **78**, 751 (1956).

from a theoretical equation[5]. The eluates were collected in an automatic fraction collector and the amount of the oligonucleotide in each tube was determined by measuring the absorption at 260 mμ in a Beckman model DU spectrophotometer. Each peak in the elution curve was collected (as indicated in figures), dialyzed in a cellophane tubing, lyophilized and used for the determination of molar ratio of bases.

Determination of relative molar ratio of bases.—The lyophilized sample described above was hydrolyzed with 1 N hydrochloric acid at 100°C for 1 hour. The hydrolyzate was chromatographed on Tōyō 51 A filter paper with *tert*-butanol-hydrochloric acid-water as solvent. The spots were located by an ultraviolet germicidal lamp with a Kaken Ultraviolet Filter 2537*. The spots and the corresponding blanks were cut out and eluted by 0.1 N hydrochloric acid. As molar extinction coefficients for pyrimidine nucleotides and purine bases, the values published by Smith and Markham[6] were employed.

Results and Discussion

In Fig. 1 is shown the elution diagram of Core 2 when the salt concentration of the solvent was continuously increased. As can be seen from the figure, separation of the oligonucleotides in this case was not satisfactory. Nevertheless, as shown in Table I, the base ratios in fractions (indicated in Fig. 1) showed considerable changes with the progress of elution.

TABLE I

MOLAR BASE RATIOS OF THE FRACTIONS OBTAINED BY GRADIENT DEVELOPMENT CHROMATOGRAPHY ON ECTEOLA-CELLULOSE COLUMN

Sample	Base ratio*				Pu/Py
	A	G	U	C	
Core 2	10	13.7	1.7	2.6	5.5
Fraction No.**					
1	10	10.5	3.0	5.2	2.5
2	10	9.2	2.1	3.6	3.4
3	10	8.5	2.0	2.2	4.4
4	10	9.2	1.6	1.6	6.0
5	10	14.9	2.5	1.9	5.7
7	10	28.5	3.2	4.5	5.0
9	10	33.8	5.9	3.1	4.9

* A: Adenine, G: Guanine, U: Uracil, C: Cytosine

** Ref. Fig. 1

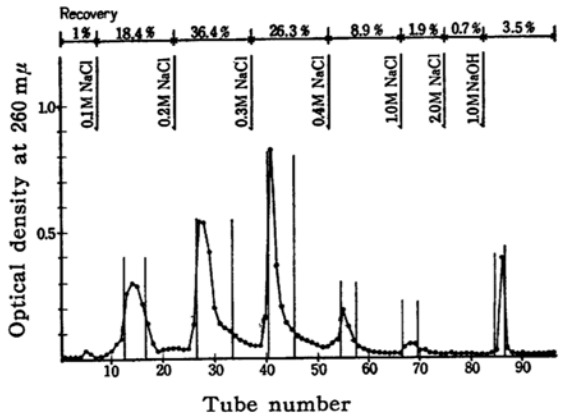

Fig. 2. Fractionation of RNase-resistant fraction of yeast RNA on ECTEOLA-cellulose by discontinuous gradient elution.
Exchanger: ECTEOLA-cellulose 20 g., 2.8 cm. in diameter.
Sorbed material: 100 mg. of Core 1.
Each tube contained ca. 13.3 ml. The volume was directly measured to calculate the recovery. The optical density was measured after 20-fold dilution of the eluate in a 1 cm. cell. The peaks indicated with the mark | | were used for the determination of base ratio.

TABLE II

MOLAR BASE RATIOS OF THE FRACTIONS ELUTED FROM ECTEOLA-CELLULOSE COLUMN BY DISCONTINUOUS GRADIENT ELUTION

Sample Exp. 1**	Base Ratio*				Pu/Py
	A	G	U	C	
Core 1	10	17.8	4.2	4.4	3.2
Fraction eluted with					
0.1 M NaCl	10	11.4	5.3	6.4	1.8
0.2 M NaCl	10	9.9	2.9	2.9	3.4
0.3 M NaCl	10	28.8	3.9	4.5	4.6
0.4 M NaCl	10	32.7	4.2	3.9	5.3
1.0 M NaCl	10	28.6	—	—	—
N- NaOH	10	30.0	3.4	2.5	6.8
Exp. 2					
0.1 M NaCl	10	11.7	5.1	5.9	2.0
0.2 M NaCl	10	12.3	2.8	3.6	3.5
After rechromatography					
	10	9.8	2.5	2.4	4.0
0.3 M NaCl	10	30.2	3.5	4.2	5.2
After rechromatography					
	10	30.2	5.0	3.6	4.7
0.4 M NaCl	10	31.8	4.0	3.5	5.6
0.5 M NaCl	10	31.8	4.9	3.4	5.0

* A: Adenine, G: Guanine, U: Uracil, C: Cytosine

** Ref. Fig. 2

5) R. M. Bock and N. S. Ling, *Anal. Chem.*, **26**, 1543 (1954).

6) J. P. Smith and R. Markham, *Biochem. J.*, **46**, 509 (1950).

* The author is indebted to Dr. E. Iwase for his kind gift of this filter.

Further studies were carried out by means of the stepwise elution procedure. In Fig. 2 is presented one of the elution diagrams of Core 1 which was eluted by sodium chloride solutions of successively increassing concentration (in 0.01 M neutral phosphate).

As shown in Fig. 2, about 90% of the ultraviolet absorbing substance was present in the eluates of 0.1, 0.2, 0.3, 0.4 and 1.0 M sodium chloride concentration. The rest was eluted by 1.0 M sodium hydroxide.

In order to determine whether satisfactory fractionations had occurred, selected fractions were dialyzed at 4°C against distilled water, lyophilized, and rechromatographed. As shown in Fig. 3 as an example, it was found that the main peak in the elution diagram reappears in the salt concentration at which the fraction was originally eluted. The base ratios of the fractions indicated in Fig. 2 were given in Table II.

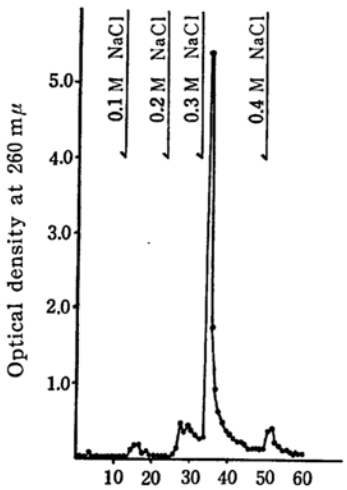

Fig. 3. Rechromatography of the fraction eluted with 0.3 M NaCl.
Exchanger: ECTEOLA-cellulose 10 g., 1.2 cm. in diameter.
Sorbed material: 0.3 M NaCl fraction of the first run. Ref. Fig. 2 (Exp. 1).
Each tube contained 10 ml. of eluate.

Pancreatic ribonuclease appears to be a highly specific phosphodiesterase which hydrolyzes secondary phosphate ester of pyrimidine ribonucleoside 3′-phosphate[7-10]. Consequently, the greater part of the oligonucleotides in RNase reistant core have only one pyrimidine nucleotide per molecule at the terminal position[1,2,8]. Thus the results presented in the Table I and II showed that as the sodium chloride concentration increases the eluate contains the larger molecular oligonucleotides as suggested by larger ratios of purine to pyrimidine. It has been shown that the whole RNase-resistant core of yeast RNA has a higher guanylic acid content than the original RNA[11,12]. The data presented here indicated that the larger molecular oligonucelotides in RNase core of yeast RNA contain increasing amounts of guanylic acid. Thus the results presented in this paper permit us to consider quite naturally that guanylic acid is not equally distributed in yeast RNA and at least some of the guanylic acid residues in the RNA are arranged in rather high molecular oligoguanylic acid structures.

Summary

RNase-resistant material obtained from yeast RNA was fractionated on an ECTEOLA-cellulose column. The elution was performed with sodium chloride-neutral phosphate buffer as solvent, the concentration of sodium chloride being increased by either gradient or stepwise procedure. Separation of oligonucleotides seemed to be affected by their molecular size. The products of larger molecular weight present in pancreatic-RNase digests of yeast RNA were very rich in guanylic acid.

The author wishes to thank Professor Fujio Egami for his encouragement and valuable suggestions. This work was supported by a grant from the Scientific Research Fund of the Ministry of Education.

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