RIBONUCLEASE ACTIVITY IN RAT BONE MARROW NUCLEI

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Summary

A method for the purification of rat bone marrow ribonuclease from a highly purified nuclear fraction is presented. The enzyme exhibits a broad pH dependence activity between pH 6.2-7.6. Monovalent and divalent ions are not required. The ribonuclease attacks the phosphodiester bond attached to the 3'OH group of a pyrimidine nucleotide and produces the respective 2';3' cyclic compound. Cyclic phosphatase activity is not associated with ribonuclease activity. Bone marrow nuclei obtained from polycythemic rats have a low ribonuclease activity. The localization of the enzyme in erythroid cells and its biological role are discussed.

The enzyme ribonuclease has been reported to be present in various mammalian tissues. There is evidence that subcellular distribution of this enzyme as well as its biochemical properties depend on the origin of the enzyme. In respect to the subcellular distribution of the enzyme, the highest activity has been obtained from the mitochondrial and microsomal fractions. The nuclear fraction, however, is poor in ribonuclease activity (1). A nuclear fraction that is rich in ribonuclease activity can be obtained from rat bone marrow cells. In this case more than 40 % of the total ribonuclease activity is present in the nucleus (2).

This communication describes the partial purification of a ribonuclease obtained from rat bone marrow cell nuclei. Some of the biochemical properties of the enzyme as well as its possible biological role are discussed.

Material and Methods

Normal and polycythemic female rats of the strain A x C weighing 150-170 g were used. Polycythemic rats were prepared as indicated by Perretta et al. (3). Bone marrow cells were obtained from the femur and tibia and then washed with 0.25 M sucrose solution containing 10^{-3} M $MgCl_2$. The bone marrow nuclear fraction was prepared according to Chauveau et al. (4). The resulting nuclear pellet was washed with 0.25 M sucrose solution containing 10^{-3} M $MgCl_2$ and 0.1 % Triton X-100, and twice rewashed with 0.25 M sucrose solution containing 10^{-3} M $MgCl_2$. All the steps involved in the preparation of the nuclear fraction were performed at 0-4°C.

The nuclear fraction thus obtained, was examined by light and phase microscopy

and this revealed that there were no cytoplasmic contaminants.The analysis of the cytoplasmic marker enzymes showed that glucose 6 phosphatase,succinic dehidrogenase,cytochrome oxidase and urate oxidase activities were not present (5).

The ribonuclease assay was performed as follows:the incubation mixture contained 60 µmoles of Tris-HCl buffer,pH 7.3,2 mg of yeast RNA (purified as described by Bardón and Pamula (6)) and 5-10 units of the proper enzyme preparation in 1.0 ml final volume.The mixture was incubated for 30 minutes at 37°. At the end of the incubation period,0.50 ml of 0.75 % uranyl acetate in 25 % $HClO_4$ was added.The mixture was cooled in an ice bath for 20 minutes and then centrifuged at 2,500 xg.Aliquots of the supernatant were diluted with water and the absorbance of the acid-soluble oligonucleotides was measured at 260 mµ.In each case,suitable controls were run.One unit of rat bone marrow nuclear ribonuclease (RBMN-RNase) is the quantity of enzyme that releases an amount of acid-soluble oligonucleotides which is equivalent to 0.100 $O.D._{260\ m\mu}$ units,under the conditions described above.

Other enzymatic determinations were made for phosphodiesterase (7),deoxiribonuclease (8),acid and alkaline phosphatases (9) and cyclic phosphatase (10). Protein was determined by the method of Lowry et al.(11).

Table I

Partial purification of the ribonuclease obtained from the rat bone marrow nuclear fraction.

Purification step +	Total RNase (units)	Total Protein (mg)	RNase S.A. (units/mg)	Yield (%)++
1.-Lysed nuclear supernatant fraction	6,440	4.57	1,410	100
2.-Ammonium Sulphate 50-80 % satn.	3,480	1.48	2,340	54
3.-Sephadex G-25 eluate	3,841	0.776	4,500	59
(at peak)	(221)	(0.015)	(14,700)	(3.4)
4.-Acid and heat treated supernatant	1,520	0.156	9,800	23.6

+ Aliquots were removed after each purification step and assayed for protein content and enzymatic activity.

++ Total enzymatic activity at step 1 taken as 100 % yield.

RESULTS

Isolation and partial purification of the RBMN-RNase

All the procedures described below were carried out at 0-4°, unless otherwise noted. A typical purification scheme is summarized in table I.

Step 1.-The nuclear fraction was disrupted by homogenization in five volumes of 0.05 M Tris-HCl buffer solution, pH7.2, containing 10^{-3} M $MgCl_2$ and 0.14 M NaCl. This procedure does not apparently disrupt the nucleoli which were later removed by centrifugation at 10,000 xg. The supernatant was filtered through Millipore membranes (H.A. 0.45 µ) and the transparent filtrate was used for the second step.

Step 2.-The filtrate was slowly carried to 50 % saturation by the addition of solid ammonium sulphate under constant stirring without foaming and then allowed to equilibrate during 30 minutes. After centrifugation at 10,000 xg for 15 min., the supernatant was slowly adjusted to 80 % saturation by the addition of solid ammonium sulphate. The suspension was stirred for 30 minutes, without foaming, and the precipitate collected after centrifugation was dissolved in four volumes of 0.01 M Tris-HCl buffer solution, pH 7.2.

Step 3.-The final solution obtained in step 2 was desalted by passage through a column (1 x 20 cm) packed with Sephadex G-25 equilibrated with 0.01 M Tris-HCl buffer solution, pH 7.2. Fractions excluded from the Sephadex were collected in 0.50 ml aliquots by elution with the equilibration buffer and then assayed for protein and ribonuclease activity.

Step 4.-Combined fractions obtained from step 3 were adjusted to pH 2.5 with 3 M CH_3COOH and then heated in sealed ampoulles at 70 C for 5 minutes. After heating, the suspension was rapidly cooled and then centrifuged. The supernatant was adjusted to pH 7.2 by the addition of solid Tris.

Further purification steps, using DEAE- or CM-cellulose do not improve the specific activity of the enzyme preparation.

Unless otherwise indicated, all the following experiments were performed with step 4 preparations.

Characteristics of the RBMN-RNase

The enzyme preparation was free of phosphodiesterase, deoxiribonuclease and acid or alkaline phosphatase activities. It exhibits a broad pH dependence activity between pH 6.2-7.6. The effect of monovalent ions was studied by adding to the reaction mixture either NaCl or KCl. The final concentration of these salts within the mixture ranged between 10^{-3} - 10^{-1} M. Under these conditions no effect on the enzymatic activity was detected. However, the addition of either Mg or Ca chloride salts in concentrations higher than 10^{-2} M produced an inhibitory effect. The inhibition reached values of 60-70 %. EDTA at a final concentration of 10^{-2} M showed no effect on the enzyme activity.

The substrate specificity of the ribonuclease was tested by using homopoly-

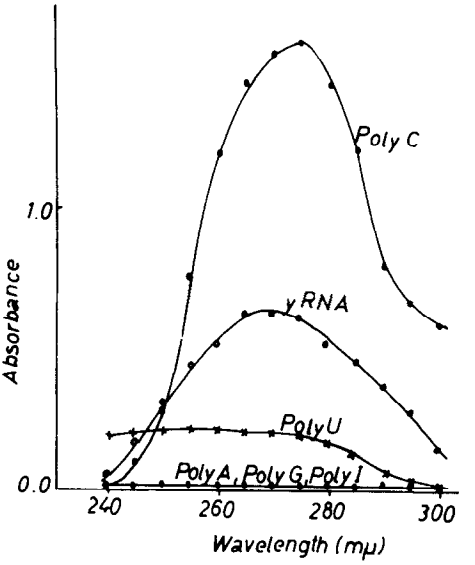

Figure 1
Substrate specificity of the RBMN-RNase

A 0.50 ml final volume of incubation mixture containing 70 μg of the RBMN-RNase, 40 μmoles of Tris-HCl buffer, pH 7.3 and 200 μg of polynucleotide (Poly A, Poly C, Poly G, Poly I or yeast RNA) or 66 μg of Poly U, were incubated at 37° during 30 minutes.

When Poly A, Poly C, Poly G, Poly I or RNA were used as substrates, the reaction was stopped by the addition of 10 % trichloroacetic acid; when Poly U was used, the reaction was stopped by the addition of saturated ammonium sulphate solution. The absorbance of aliquots were measured at different wavelenghts against the respective controls.

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nucleotides. Under the conditions indicated in fig. 1, when Poly A, Poly G or Poly I are used no reaction is observed. However, when Poly C or Poly U were used as ribonuclease substrates, an almost complete degradation of the polynucleotide to acid soluble nucleotides is observed. As seen in the same figure, total yeast RNA was easily degraded by the enzyme.

The cyclic phosphatase activity was assayed in the RBMN-RNase by a spectrophotometric method (10), using 2':3' CMP as substrate for the enzyme. The results obtained show that the cyclic phosphatase activity was not present or associated with the RBMN-RNase.

A kinetic study was performed by using yeast soluble RNA or Poly C as substrates for the RBMN-RNase (fig. 2). When soluble RNA was used a linear relationship can be observed during the first 15 minutes of the reaction. After this time, the amount of 2 N $HClO_4$ soluble oligonucleotides produced was only 4-8 % of the theoretical amount of oligonucleotides yielded by the complete alkaline hydrolisis of the soluble RNA. However, when Poly C was the substrate, a linear relationship between percentage of hydrolisis and time of reaction was observed.

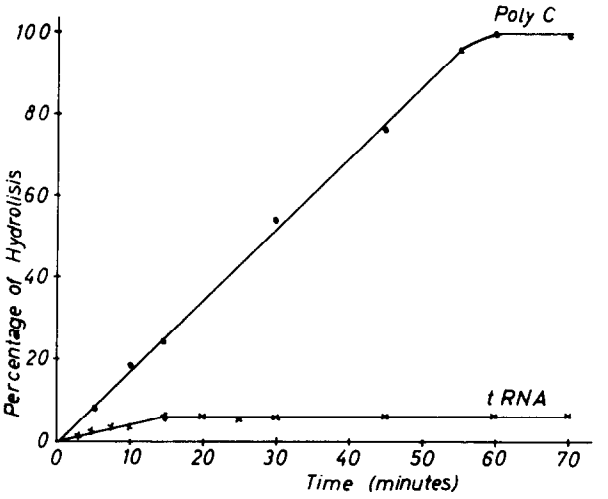

Figure 2.-

Kinetics of hydrolysis of Poly C and yeast soluble RNA by the RBMN-RNase.

The incubation mixture at each point contained: 60 µmoles of Tris-HCl buffer,pH 7.2 , 2 mg of soluble yeast RNA (Sigma Chemical Co.) and 81 units of the RBMN-RNase in 1.0 ml final volume.After incubation at 37°,the reaction was stopped by the addition of 0.5 ml of 0.75 % uranyl acetate in 25 % perchloric acid.Aliquots were removed after centrifugation and the amount of acid-soluble products were determined at 260 mµ.

The incubation mixture at each point contained: 90 µmoles of Tris-HCl buffer,pH 7.2 , 1 mg of Poly C and 75 units of the RBMN-RNase in 1.0 ml final volume.After incubation at 37°,the reaction was stopped by the addition of 1.0 ml of 10 % trichloroacetic acid.Aliquots were removed after centrifugation and the amount of acid-soluble compounds determined at 280 mµ.

A 100 % hydrolysis represents the amount of acid-soluble compounds obtained after a complete alkaline hydrolysis of yeast soluble RNA or Poly C.

Table II

Bone marrow nuclear ribonuclease activity in normal and polycythemic rats

Type of Donors	Hemoglobine (in g %)	Total ribonuclease activity (%)	Total protein (%)
Normal	14.5 - 15.6	100 ±-0.9	100 ± 1.4
Polycythemic	20.4 - 20.9	59.4± 1.3	86 ± 3.2
Polycythemic	21.8 - 22.5	47.0± 3.5	78 ± 4.7

Bone marrow nuclear fraction were obtained from either a pool of cells from normal or polycythemic rats,adjusted to 10^7 nuclei and lysed to reach the step 1 (see results).After this procedure,the ribonuclease activity and the protein content of the nuclear fraction were assayed.The results reported are the mean (± s.d.) of 3 experiments.

When the total ribonuclease activity was measured in the nuclear fraction derived from the polycythemic rat bone marrow cells,a marked decrease of 47 - 59 % of the enzymatic activity was observed,as compared with the nuclear ribonuclease activity of the normal rat bone marrow cells (see table II).

DISCUSSION

The results presented in this paper shows that after partial purification of the RBMN-RNase,a preparation with a specific activity nearly 10 times greater than the initial one is obtained.Although the degree of purification attained was small,the final specific activity obtained at step 4 is higher than that exhibited by other highly purified erythroid ribonucleases showing similar properties to that of the RBMN-RNase (12,13).

Rat bone marrow nuclear ribonuclease has two important characteristics,a) the capability of the enzyme to degrade those regions of the polynucleotide chain that have only pyrimidine sequences and b) the fact that there is not detectable cyclic phosphatase activity associated with the enzyme.An analysis of the products of the partial digestion of the soluble yeast RNA was not performed,however our results and the known sequence of the pyrimidine bases in the soluble RNA (14) permits us to conclude that the non linear relationship between the enzyme activity and the time of reaction may be due to the production of oligonucleotides from soluble RNA,which lack pyrimidine sequences.In this case the oligonucleotides were not attacked by the enzyme.When Poly C was the substrate this situation was not attained and the enzyme degraded it completely to 2':3' CMP.

Thus,it can be concluded that a limiting condition for the enzymatic action of the RBMN-RNase on a polynucleotide chain is the presence of at least one pyrimidine base which contributes with a 3' OH group to the 3'-5' phosphodiester bond of the polynucleotide chain.The fact that the enzyme does not require divalent cations for optimal activity and the cyclizing character of the RBMN-RNase permit us to classiffy it as a RNA phosphotransferase (15).

The activity of the ribonuclease derived from the cell nuclei of polycythemic rats is almost 50 % lower than that present in the nuclear fraction of the normal animals.If one recalls that in the hypertransfused-polycythemic animal the amount of erythroid cell precursors is lowered (16),the diminished amount of the ribonuclease found in these animals may be related to the absence of erythroid cells precursors.This probably means that the enzyme under study is mainly associated with the erythroid series of the bone marrow.

The prominent content of ribonuclease activity in the nuclear fraction of the rat bone marrow cells,unusual for mammalian cells,lead us to propose a biological role for this enzyme in the nucleic acid metabolism.This could de related with the degradation of some nuclear species of RNA described in rat bone marrow cells (17,18).Since most of these nuclear RNA have a large molecular weight,

they must undergo a number of specific escissions before they can diffuse out to the cytoplasm.The ribonuclease described in this paper is probably involved in this specific escission process.

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