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STUDIES ON RAT LIVER RIBONUCLEASES

I. INTRACELLULAR LOCALIZATION OF THE ALKAL INERIBONUCLEASES

Y. E. RAHMAN
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill. (U.S.A.)

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SUMMARY

Results obtained from various fractionation methods such as differential centrifugation, zonal centrifugation, and sucrose density-gradient centrifugation, showed that both alkaline ribonucleases in rat liver were found diffusely distributed in all subcellular fractions; ribonuclease II showed higher activity in the supernatant compared to ribonuclease III. The diffuse distribution pattern is not due to adsorption phenomena.

INTRODUCTION

The existence of a third ribonuclease*, *i.e.* ribonuclease III, in rat liver was recently reported by RAHMAN[1]. Prior to this finding, two ribonucleases were previously known; an acid ribonuclease with pH optimum at 5.0 (ref. 2), and an alkaline ribonuclease with pH optimum at 8.0 (refs. 3, 4). As a result of the finding of a third RNA-degrading enzyme, the complexity concerning RNA metabolism within the liver cells has further increased.

During the last ten years, several studies[5–7] described the subcellular distributions of the two then known ribonucleases, but the results obtained were not entirely in agreement. It was thought worthwhile to reinvestigate this problem by using various gradient centrifugation methods now available.

The following report is concerned with the intracellular distributions of the two alkaline ribonucleases, while the acid ribonuclease distribution will be the subject of another paper[8].

* For convenience, the following arbitrary abbreviations were used to designate the three ribonucleases: Ribonuclease I, acid ribonuclease (ribonucleate pyrimidine-nucleotido-2′-transferase, EC 2.7.7.16) with pH optimum around 5.0; Ribonuclease II, alkaline ribonuclease with pH optimum around 8.0; Ribonuclease III, alkaline ribonuclease with pH optimum around 9.5.

MATERIALS AND METHODS

Tissue homogenization

Female Holtzmann rats weighing between 200 and 250 g were used throughout this study. The homogenization procedure is the same as previously described[1].

Enzyme assays

Acid phosphatase, cytochrome oxidase, urate oxidase, ribonucleases and glucose-6-phosphatase were assayed as previously described[1,9]. For all fractions ribonuclease II assays were done in the presence of $4 \cdot 10^{-4}$ M *p*-chloromercuribenzoate.

The ribonuclease III activity was unchanged in the presence or absence of *p*-chloromercuribenzoate. The concomitant presence of both ribonuclease II and ribonuclease III does not interfere with or change their corresponding activity as it was shown by RAHMAN[1]. In the zonal centrifugation experiments, a sample of 0.5 ml from each fraction was used to determine both ribonucleases; the incubation time was 180 min. In the sucrose gradient experiments, a sample of 0.2 ml from each fraction was used, and the incubation time was 120 min.

Differential centrifugation

The method used is the same as that described by DE DUVE *et al.*[5].

Purification methods of various subcellular fractions

Nuclei were purified either by the method described by MAGGIO, SIEKEVITZ AND PALADE[10] with a minor modification, namely, $MgCl_2$ was added to the sucrose solution instead of $CaCl_2$, because the ribonuclease III was found to be inhibited by Ca^{2+} (ref. 1); or by the method described by CHAUVEAU *et al.*[11,12]. Mitochondria were isolated by a sucrose density-gradient method described by THOMSON AND KLIPFEL[9]. The top 10 ml of this gradient contained the microsomal fraction. This fraction was then spun in a Servall centrifuge at 28 700 × *g* for 3 h, or in a Spinco 30 angle head rotor of the model L-2 centrifuge at 105 000 rev./min for 1 h.

Zonal centrifugation

The method used is the same as described by RAHMAN *et al.*[8].

Nuclei counts

Nuclei were counted in the unfractionated homogenate as well as in the purified nuclear fractions by the procedure of LAIRD[13].

Detergent and water treatment of the mitochondrial and microsomal fractions

Mitochondrial or microsomal fractions were treated with water or Triton X-100 as follows: mitochondria or microsomes obtained from 1 g of rat liver were resuspended in 11 ml of either water or 1% Triton X-100, then they were centrifuged in a Spinco TI-50 rotor of the model L-2 centrifuge at 226 000 × *g* for 1 h. Enzyme activities were assayed in the supernatant as well as in the particular fractions thus obtained.

Sucrose density-gradient centrifugation

Sucrose gradients were prepared from a modified model of BOCK's gradient

former[14]. The sucrose concentrations, the centrifugation time, and the *g*-force of each experiment varied as indicated in the appropriate figure legend. The Spinco SW 25.2 swinging-bucket rotor of the Spinco L-2 centrifuge was used. 2-ml fractions were collected through a side puncture with a bent needle just above the small pellet of cell debris in the bottom of the tube. This pellet was analyzed as the last fraction of the gradient. In all gradients, Fraction 1 represents material collected from the top of the gradient, Fraction 29 represents material from the bottom.

Nitrogen was determined by nesslerization of H_2SO_4–H_2O_2 digests.

RESULTS

Differential centrifugation

Table I summarizes the results obtained by differential centrifugation experiments. Cytochrome oxidase was used as the enzyme marker for the mitochondria;

TABLE I

RESULTS OF DIFFERENTIAL CENTRIFUGATIONS

	Acid phosphatase		*Cytochrome oxidase*		*Glucose-6-phosphatase*		*Ribonuclease II*		*Ribonuclease III*	
	Spec. activity	%	*Spec. activity*	%	*Spec. activity*	%	*Spec. activity*	%	*Spec. activity*	%
Unfractionated homogenate	0.50		0.98		0.58		0.44		0.21	
Nuclei	0.33	6.7	0.78	8.7	0.51	7.4	0.54	14.5	0.42	16.1
Mitochondria	0.90	30.1	3.42	83.7	0.26	8.3	0.51	30.7	0.44	38.0
Lysosomes	3.71	35.6	1.46	5.5	1.55	7.8	1.28	11.8	0.70	9.3
Microsomes	0.50	18.5	0.12	2.1	2.71	72.0	0.49	26.1	0.31	30.6
Supernatant	0.05	9.1	0	0	0.04	4.7	0.09	24.0	0.05	7.0

* Results are the averages of 3–6 experiments. Specific activity expressed by total activity divided by the total nitrogen content of each fraction; %, the percentage of total activity.

acid phosphatase as that of the lysosomes and glucose-6-phosphatase as that of the microsomes. From this table, one can see that the subcellular distribution of both of the alkaline ribonucleases differs from any one of the three marker enzymes, and both are distributed diffusely in all fractions. However, their specific activity is highest in the lysosomal fraction.

Purified subcellular fractions

Since the purpose of the following experiments was to determine whether the alkaline ribonuclease activity in various subcellular fractions was due either to the contamination of other subcellular particles within a given defined fraction or to an artifactious adsorption of these enzymes from the soluble phase onto various particles, emphasis was placed on obtaining fractions of high purity; various published purification methods were applied. Consequently the yield is somewhat lower than the corresponding fractions obtained by differential centrifugation.

Nuclei. Twelve experiments were done on nuclear fractions purified either by

the method of MAGGIO, SIEKEVITZ AND PALADE[10] or by the method of CHAUVEAU *et al.*[11,12]. The nuclei yields based on nuclei counts are quite different when the fractions are obtained by the two different purification methods. We obtained a yield of 35–45% with the method of MAGGIO, SIEKEVITZ AND PALADE[10] whereas the method of CHAUVEAU *et al.*[11,12] gave between 60 and 70% yield. But as far as the alkaline ribonucleases are concerned, nuclei purified by both methods invariably contained not more than 2–3% of the total activity of the original homogenate. The activities of acid phosphatase and glucose-6-phosphatase were assayed in these nuclear fractions as well; and 2–3% of their total homogenate activity were found in these purified nuclei fractions.

Mitochondria. Mitochondrial fractions obtained either with differential centrifugation or sucrose gradient methods gave very similar results. About 25% of the unfractionated homogenate activity was found in these fractions in the case of ribonuclease II and around 35% in the case of ribonuclease III. Extensive washing of these fractions with 0.25 M sucrose yielded not more than 11% of the mitochondrial activities of both ribonucleases in the soluble fraction. Resuspending mitochondrial fractions in water or Triton X-100, however, solubilized 17–20% of ribonuclease II activity, but only 10–15% of ribonuclease III activity.

Microsomes. Highly purified microsomal fractions contained about 20% of ribonuclease II and about 30% of ribonuclease III. Extensive washing with 0.25 M sucrose, water or Triton X-100 (1%) solubilized not more than 5% of both alkaline ribonuclease activities.

Zonal centrifugation

Fig. 1 represents an experiment which examines the distribution of both

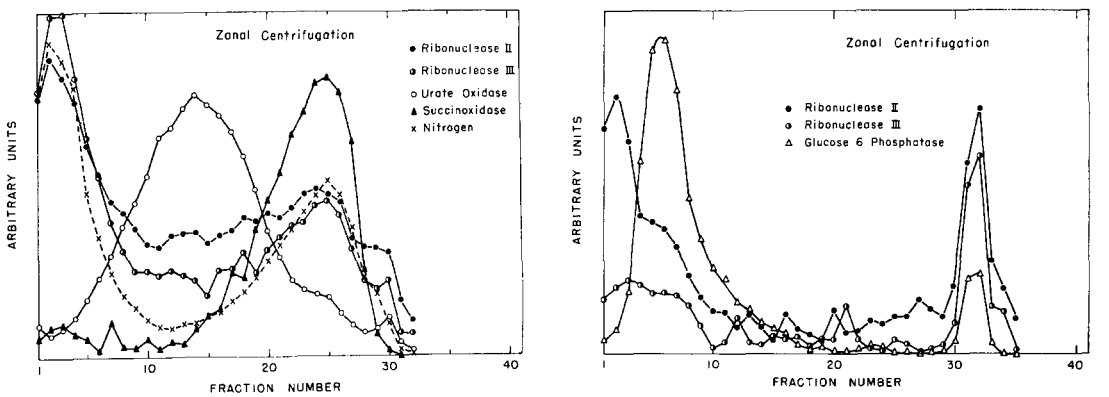

Fig. 1. Zonal centrifugation. Enzyme activities in fractions obtained from a "short" gradient (see ref. 8). The supernatant of the rat liver homogenate was removed before being introduced into the gradient. The centrifugation was run at 4000 rev./min for 60 min. For this type of comparative studies, the use of arbitrary units for all enzymes was found necessary in order to accommodate all the distribution curves within the same graph. ●- - -●, ribonuclease II; ◑- - -◑, ribonuclease III; ○- - -○, urate oxidase; ▲- - -▲, succinoxidase; ×- - -×, nitrogen.

Fig. 2. Zonal centrifugation. Enzyme activities in fractions obtained from a "short" gradient (see ref. 8). The supernatant was not removed before being introduced into the gradient. The centrifugation was run at 4000 rev./min for 180 min. ●- - -●, ribonuclease II; ◑- - -◑, ribonuclease III; △- - -△, glucose-6-phosphatase.

alkaline ribonucleases among mitochondria, lysosomes, and microbodies; the supernatant was removed prior to the gradient fractionation in this experiment. Both alkaline ribonucleases show a major peak in the first few fractions of the gradient which correspond to one of the two peaks of total nitrogen distribution as well as the major and the only peak of glucose-6-phosphatase activity (not shown on the graph). A second and smaller peak corresponds to the succinoxidase activity, while there is a diffuse, but not negligible activity across Fractions 8 through 20 which cover the distribution of lysosomes as well as microbodies, judging from the distribution of the acid phosphatase activity on the one hand and the urate oxidase activity on the other.

The enzyme recoveries in the experiment of Fig. 1 are: ribonuclease II, 124%; ribonuclease III, 80%; urate oxidase, 82%; succinoxidase, 61%; nitrogen, 88%.

Fig. 2 represents an experiment which shows the distribution of both alkaline ribonucleases in the microsomes; during the 3-h centrifugation, most of the larger particles such as mitochondria, lysosomes and microbodies are sedimented to the last few fractions (No. 30–35) at the bottom of the gradient. In this experiment, except for a small hump, the major peak of ribonuclease II activity does not correspond to that of the glucose-6-phosphatase. The ribonuclease III has no major peak in the first few fractions and thus differs from the distribution of the ribonuclease II. The enzyme recoveries of the experiment of Fig. 2 are: ribonuclease II, 112%; ribonuclease III, 75%; glucose-6-phosphatase, 80%.

Sucrose density-gradient centrifugation

Figs. 3 and 4 show the results of two experiments using sucrose density-gradient centrifugation. The experimental conditions of both gradients were slightly different (see figure legends). Both alkaline ribonucleases showed two peaks of activity; one peak (Fractions No. 6 and 7) corresponds to the peak activity of glucose-6-phosphatase, and the other peak corresponds to that of the mitochondria. (Cytochrome

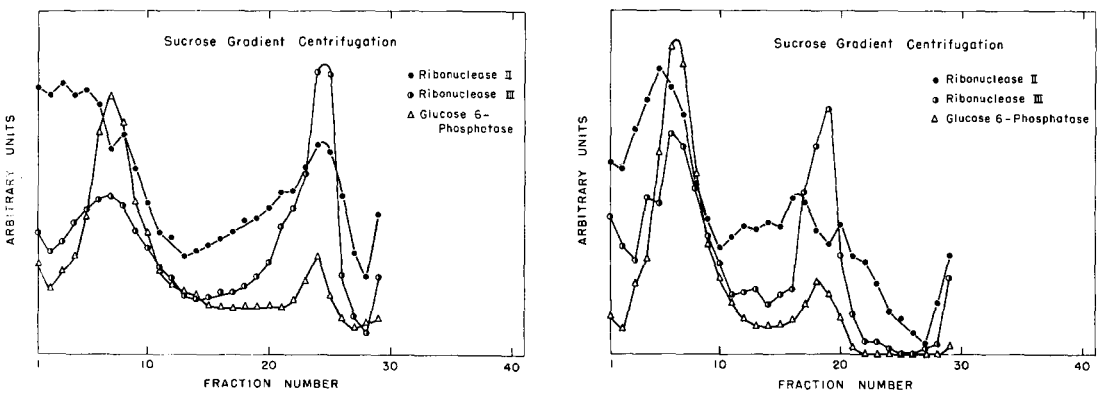

Fig. 3. Density-gradient centrifugation. Enzyme activities in fractions obtained from a 10–60% linear sucrose gradient. The centrifugation was run at 15 000 rev./min for 30 min. ●- - -●, ribonuclease II; ◑- - -◑, ribonuclease III; △- - -△, glucose-6-phosphatase.

Fig. 4. Density-gradient centrifugation. Enzyme activities in fractions obtained by a 15–68% linear sucrose gradient. The centrifugation was run at 10 000 rev./min for 120 min. ●- - -●, ribonuclease II; ◑- - -◑, ribonuclease III; △- - -△, glucose-6-phosphatase.

oxidase activity is not presented in the figures). However, the activity of both ribonucleases found between these two peaks was not negligible. The main difference in distribution between the two alkaline ribonucleases is found in the first three fractions, which represent the soluble fraction of the homogenate; the ribonuclease II has a much higher activity in the soluble fractions than the ribonuclease III. The enzyme recoveries in the experiment of Fig. 3 are: ribonuclease II, 90%; ribonuclease III, 106%; glucose-6-phosphatase, 67%. Those of the experiment of Fig. 4 are: ribonuclease II, 88%; ribonuclease III, 117%; glucose-6-phosphatase, 78%.

In summary, all the different fractionation methods as described above showed that both ribonucleases are present in all fractions; the main difference between the two is that supernatant ribonuclease II represents about 25% of its homogenate activity, whereas the ribonuclease III represents a mere 5–7%.

DISCUSSION

From the experimental results obtained by various fractionation methods as described above, it is clear that the two alkaline ribonucleases of rat liver are distributed diffusely throughout the cytoplasm. The difference between the distribution of the ribonuclease II and that of the ribonuclease III lies in the supernatant fraction.

The intracellular distribution of the ribonuclease II in rat liver was studied in 1957 by ROTH[15], and in 1958 by REID AND NODES[6]. Using differential centrifugation, the former author had separated the liver homogenate into only three fractions: nuclei, mitochondria and supernatant; and reported that the ribonuclease II was mainly present in the mitochondrial and the supernatant fractions, with a small amount of enzyme in the nuclei. REID AND NODES[6] arrived at the following conclusion on the ribonuclease II distribution, also based on differential centrifugation: "Liver alkaline ribonuclease is present not in mitochondria, but in particles similar to if not identical with the lysosomes, which contain acid phosphatase . . . , with alkaline ribonuclease, the activity, in comparison with that of acid phosphatase, is relatively low in the mitochondrial fraction and high in the supernatant fraction. Assuming that there is only one type of particles, the simplest hypothesis to explain our results is that alkaline ribonuclease is present in particles that differ from lysosomes in being less readily sedimented".

A quantitative comparison is difficult to make between our present results and those of ROTH[15] and REID AND NODES[6], since they had used only differential centrifugation in their fractionation studies. However, ROTH's[15] result was not necessarily in disagreement with the results described in this paper, since the former author had actually found ribonuclease II activity in all his three subcellular fractions, and from the experimental conditions which he used, the supernatant fraction probably included all of the lysosomes, microsomes and supernatant separated in our experiments.

REID AND NODES[6] had expressed their data only in terms of specific activity, and they found that the lysosomes had the highest ribonuclease II specific activity, which is certainly in agreement with our data. Judging from the distribution of the total activity, however, we feel that the distribution of ribonuclease II certainly differs from that of the acid phosphatase; and it is not justified to attribute the localization of ribonuclease II to lysosomal particles merely because of its higher specific activity in that fraction.

From results obtained by treating the mitochondria and microsome fractions with water and Triton X-100 reported in this paper, as well as results described by ROTH[16] on solubilization studies of rat liver microsomes, it seems reasonable to conclude that the diffuse distribution pattern of both alkaline ribonucleases in rat liver is not due to an adsorption artifact, even though the meaning and the biological implication of this diffuse pattern is not known.

The fact that a very low activity (2–3%) of both alkaline ribonucleases and equivalent percentage of both acid phosphatase and glucose-6-phosphatase are found in purified nuclei suggests that these ribonucleases are not located in the nuclei; the 2–3% activity present in the nuclei is probably due to cytoplasmic contamination.

BLOBEL AND POTTER[17] reported that the isolation of intact polysomes from rat liver is dependent on the presence of an inhibitor of an alkaline ribonuclease[3] in the high-speed supernatant of rat liver (ribonuclease II); these authors conclude that this ribonuclease is responsible for the degradation of the mRNA in rat liver *in vivo*[17]. In this case the implication of a diffuse distribution pattern of ribonuclease II is worth looking into; work is currently underway to clarify this point.

The possible biological functions of ribonuclease III are unknown, and more work is needed in order to find some clue to this problem.

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