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STUDIES ON RAT LIVER RIBONUCLEASES

III. FURTHER STUDIES ON HETEROGENEITY OF LIVER LYSOSOMES—INTRACELLULAR LOCALIZATION OF ACID RIBONUCLEASE AND ACID PHOSPHATASE IN RATS OF VARIOUS AGES

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SUMMARY

In adult rat liver, by the use of sucrose gradient centrifugation, the intracellular distribution pattern of acid ribonuclease was found to be different from that of acid phosphatase. This finding further confirms our previous results obtained by zonal centrifugation.

An additional group of subcellular particles in the top few fractions of the gradients was reported in young rats. The biological significance of this new group of particles is discussed.

INTRODUCTION

We have previously done fractionation studies[1] with the use of an A-XII zonal centrifuge, and the experimental results obtained suggested that in rat liver, acid ribonuclease (*i.e.*, ribonuclease I) has an intracellular distribution pattern different from that of acid phosphatase. This paper reports the results of further fractionation studies, this time with the use of the conventional sucrose gradient centrifugation method. These results confirm our previous finding that acid ribonuclease belongs to a different group of subcellular particles from that of acid phosphatase.

MATERIALS AND METHODS

Tissue homogenization

SD/Anl (Anl 66) rats of various ages were used for this study. The age of the adult rats used ranged from 3 to 6 months. They were killed by decapitation. Their livers were removed and chilled at once to 0° in 0.25 M sucrose (ribonuclease-free, obtained from Schwartz Bio-Research Inc.) and homogenized as previously described[1].

In the experiments using 4- and 10-day-old rats, the livers from 5 rats were pooled for the homogenate preparations. This liver homogenate was then centrifuged in the International centrifuge Model PR-2 at 640 × *g* for 10 min. The supernatant thus obtained was used for the gradient centrifugation studies, and the nuclear pellet was discarded.

Sucrose density gradient centrifugation

Sucrose gradients of 10 to 45%, with a total volume of 50 ml, were prepared from a modified model of BOCK's gradient former[2]. A 10-ml liver homogenate, nuclei-free, was carefully layered on top of the gradient. The Spinco SW 25.2 swinging-bucket rotor of the Spinco L-2 centrifuge was used. After a centrifugation at 10 000 rev./min, the gradient tubes were punctured with the Beckman fractionating device, and fractions from the total volume of 60 ml (the initial 50 ml of sucrose gradient *plus* the 10 ml liver homogenate) were collected with an automatic fraction collector. In most experiments, the duration of the centrifugation was such that nearly all of the mitochondria were packed as a pellet in the bottom of the centrifuge tubes. During the collection of the fractions, the tip of the needle in the fractionating device was positioned just above the surface of the pellet. After all the fractions were collected, the remaining pellet was rehomogenized in 0.25 M sucrose, and this fraction represents the bottom of the gradients.

In the experiments using suckling rats, a portion of the liver homogenate was independently centrifuged in a Spinco 50 angle-head rotor at 50 000 rev./min for 1 h; the supernatant and the pellet (resuspended in 0.25 M sucrose) thus obtained were assayed for acid phosphatase and acid ribonuclease activities.

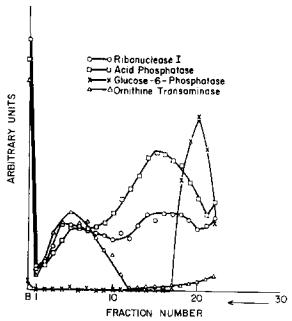
Enzyme assays

All fractions, as well as the unfractioned homogenate, were assayed for acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) (ref. 3), acid ribonuclease or ribonuclease I (ribonucleate pyrimidine-nucleotido-2′-transferase, EC 2.7.7.16) (ref. 4), glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) (ref. 5), ornithine transaminase (L-ornithine:2-oxoacid aminotransferase, EC 2.6.1.13) (refs. 6 and 7).

Proteins of the fractions obtained from the gradients were determined by a new spectrophotometric method described by GROVES, DAVIS AND SELLS[8]. The protein determinations are routinely done on these gradients in order to check the overall recovery of the proteins on one hand, and the distributions of major groups of subcellular particles on the other.

RESULTS

Figs. 1, 2, and 3 are results of experiments conducted to investigate the progress of sedimentation of the various subcellular particles subjected to an increasing centrifugation time. The 3 gradients were prepared in exactly the same manner, and were then centrifuged at 10 000 rev./min for 15, 25 and 45 min, respectively. Fig. 1 shows the distribution of the following 4 enzymes: acid phosphatase, acid ribonuclease, glucose-6-phosphatase and ornithine transaminase, in a gradient after a 15-min centrifugation. The 3 main groups of subcellular particles, *i.e.*, the mitochondria, the



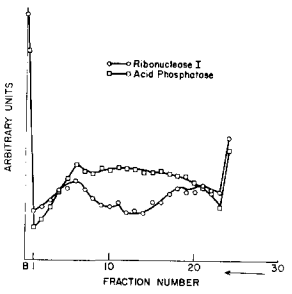
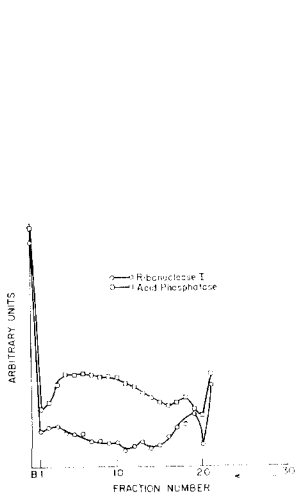


Fig. 1. The distribution of acid ribonuclease, acid phosphatase, glucose-6-phosphatase and ornithine transaminase activities in fractions obtained from a 10–45% linear sucrose gradient with an adult rat. Each fraction contained 33 drops collected. The gradient was centrifuged at 10 000 rev./min for 15 min. B represents the bottom pellet, which was rehomogenized in 2 ml of 0.25 M sucrose. The arrow indicates the direction of the centrifugation. 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. These arbitrary units are based on absolute values of enzyme activities and they are expressed as follows: acid ribonuclease: $A_{260\ m\mu}$/0.08 ml fraction per 1 min; acid phosphatase: μg phosphorus/0.05 ml fraction (Figs. 1–3) 0.1 ml fraction (Figs. 4–7) per 60 min; glucose-6-phosphatase: μg phosphorus/0.1 ml fraction per 15 min; ornithine transaminase: μmoles pyrroline-5-carboxylate/0.1 ml fraction per 90 min. ○—○, acid ribonuclease; □—□, acid phosphatase; ×—×, glucose-6-phosphatase; △—△, ornithine transaminase.

Fig. 2. The distribution of acid ribonuclease and acid phosphatase activities in fractions obtained from 10–45% linear sucrose gradient with an adult rat. Each fraction contained 28 drops collected. The gradient was centrifuged at 10 000 rev./min for 25 min. For the meaning of B and the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase.

lysosomes and the microsomes which were represented by ornithine transaminase, acid phosphatase and glucose-6-phosphatase as their respective marker enzymes, were distinctly separated along the gradient. The microsomes were found in the top fractions of the gradient (Fractions 18–22), the mitochondria in the last one third of the fractions and the lysosomes were distributed between these two classes of particles. Acid phosphatase showed its main peak at Fractions 14–16 with a minor peak in the region of the ornithine transaminase, while acid ribonuclease is distributed in two peaks, one being in the same fraction as the main peak of the acid phosphatase, and the other being with that of the ornithine transaminase. Fig. 2 shows the distribution of acid phosphatase and acid ribonuclease in a similar gradient after 25 min of centrifugation. Acid phosphatase shows a broad distribution throughout the gradient, while acid ribonuclease is undoubtedly distributed in two peaks, one being between Fractions 4 and 8, and the other being between Fractions 16 and 22. Most of ornithine transaminase activity was found in the bottom pellet in this experiment, although a small peak can still be identified between Fractions 2 and 4.

Fig. 3 shows the distribution of acid phosphatase and acid ribonuclease in a 10 to 45% sucrose gradient after 45 min of centrifugation. Acid phosphatase shows a broad distribution as in Fig. 2, while acid ribonuclease shows essentially a single small peak between Fractions 17 and 20. And the peak of acid ribonuclease previously seen between Fractions 4 and 8 in Fig. 2 has almost disappeared in this experiment (Fig. 3).



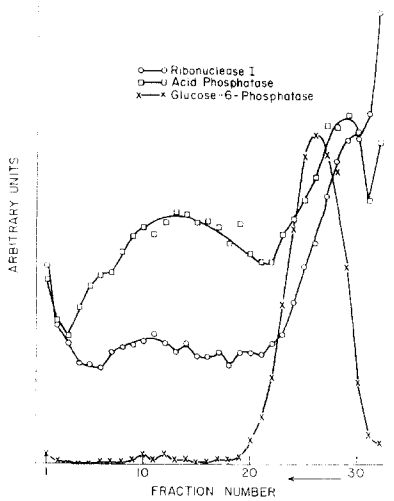


Fig. 3. The distribution of acid ribonuclease and acid phosphatase activities in fractions obtained from a 10–45% linear sucrose gradient with an adult rat. Each fraction contained 33 drops collected. The gradient was centrifuged at 10 000 rev./min for 45 min. For the meaning of B and the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase.

Fig. 4. The distribution of acid ribonuclease, acid phosphatase and glucose-6-phosphatase activities in fractions obtained from a 10–45% linear sucrose gradient with 4-day-old rats. Each fraction contained 32 drops collected. The gradient was centrifuged at 10 000 rev./min for 35 min. The enzyme activities of the bottom pellet is not represented in the graph. For the meaning of the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase; ×—×, glucose-6-phosphatase.

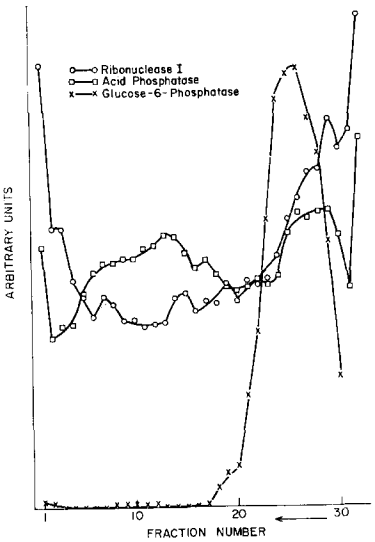
Figs. 4, 5, 6 and 7 are results of a set of experiments done with rats of various ages. The gradients were performed under similar conditions except for the centrifugation times which are indicated in the corresponding figure legend.

Fig. 4 shows the distribution of acid phosphatase and acid ribonuclease in a gradient obtained with liver of 4-day-old rats. Acid phosphatase clearly shows two main peaks in its distribution, one in the top fractions between No. 27 and No. 30, and the other broadly distributed between Fractions 8 and 18, whereas acid ribonuclease shows a single main peak which is in the top few fractions of the gradient.

Fig. 5 shows the results of another similar gradient obtained with liver of 10-day-old rats. The distribution of acid phosphatase is very similar to that in Fig. 4, with its two major peaks of activity. The acid ribonuclease activity is distributed into two main areas, one being in the bottom few fractions and the other in the fractions near the top of the gradient.

Fig. 6 is a gradient obtained with liver of a 31-day-old rat. The distribution of acid phosphatase is still divided into two main peaks, very similar to those shown in Figs. 4 and 5. Acid ribonuclease also shows two major peaks, one in the last few fractions of the gradient, with the other being broadly distributed between Fractions 20 and 31.

Fig. 7 is the result of a gradient obtained with liver of a 38-day-old rat. The acid ribonuclease shows its two main peaks of activity as in all other experiments, whereas acid phosphatase shows a single peak with a broadly distributed activity.



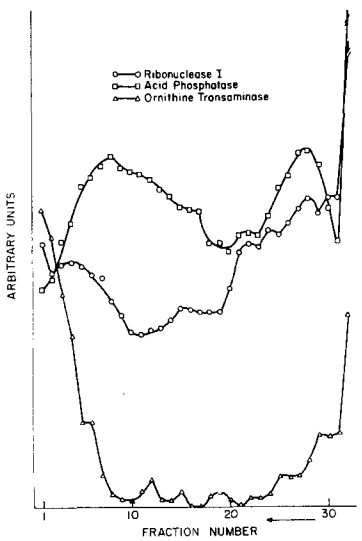


Fig. 5. The distribution of acid ribonuclease, acid phosphatase and glucose-6-phosphatase activities in fractions obtained from a 10–45% linear sucrose gradient with 10-day-old rats. Each fraction contained 23 drops collected. The gradient was centrifuged at 10 000 rev./min for 35 min. The enzyme activities of the bottom pellet are not represented in the graph. For the meaning of the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase; ×- -×, glucose-6-phosphatase.

Fig. 6. The distribution of acid ribonuclease, acid phosphatase and ornithine transaminase activities in fractions obtained from a 10–45% linear sucrose gradient with a 31-day-old rat. Each fraction contained 23 drops collected. The gradient was centrifuged at 10 000 rev./min for 45 min. The enzyme activities of the bottom pellet are not represented in the graph. For the meaning of the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase; △—△, ornithine transaminase.

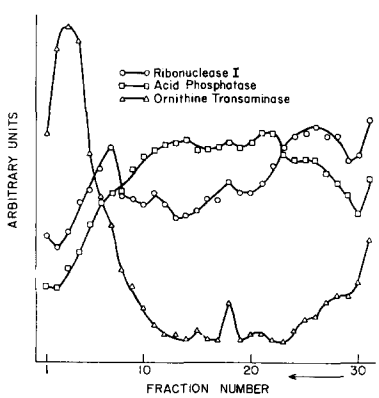


Fig. 7. The distribution of acid ribonuclease, acid phosphatase and ornithine transaminase activities in fractions obtained from a 10–45% linear sucrose gradient with a 38-day-old rat. Each fraction contained 23 drops collected. The gradient was centrifuged at 10 000 rev./min for 30 min. The enzyme activities of the bottom pellet are not represented in the graph. For the meaning of the arrow, see Fig. 1 legend. ○—○, acid ribonuclease; □—□, acid phosphatase; △—△, ornithine transaminase.

The ornithine transaminase distribution is also shown in this figure. It has a sharp peak between Fractions 2 and 4, and this peak activity does not correspond to that of the acid ribonuclease, which is around Fractions 5 to 8.

The recovery values for various enzymes in these sucrose gradients are: 85–110% for acid phosphatase; 77–99% for ribonuclease I; 88–98% for ornithine transaminase and 72–88% for glucose-6-phosphatase. The recoveries for proteins are between 94 and 102% in these gradients.

In gradients represented by Fig. 4 and Fig. 5, a portion of the liver homogenate was independently centrifuged at 50 000 rev./min for 1 h, the soluble activities (or free activities[9]) found in the supernatant were between 10 and 15% of the total for both acid phosphatase and acid ribonuclease.

DISCUSSION

Ornithine transaminase is not commonly used as an enzyme marker for the mitochondria in rat liver, although its use is justified[7]. This enzyme is found to be specifically located in mitochondria[7,10], although it is apparently not part of the mitochondrial membranes as are other enzymes such as cytochrome oxidase or succinic dehydrogenase[10]. Since ornithine transaminase becomes soluble when the mitochondrion[7] is damaged, we chose it as a marker for mitochondria in order to determine whether the mitochondria in our preparations had been significantly damaged. Detectable but not excessive damage did occur, as shown by enzyme activity found in the top fractions shown in Figs. 1, 6 and 7.

The first set of experiments, done with adult rats (Figs. 1, 2 and 3), clearly demonstrated that acid ribonuclease belongs to a group of subcellular particles which is more readily sedimentable than that of the acid-phosphatase-bearing particles. And this finding confirms our previous results obtained by fractionations with the use of an A-XII zonal centrifuge[1].

The second set of experiments (Figs. 4, 5, 6 and 7) were done with suckling and young rats, from 4 days to 38 days of age. In these rats, the distribution patterns of acid ribonuclease remained comparable to those of the adult rats (ref. 1 and Figs. 1, 2 and 3), namely, it showed two distinct peaks of activity, one being in the top fractions of the gradient and the other in the lower half of the gradient. However, in suckling and young rats (Figs. 4, 5 and 6), the enzyme activity in the peak of the top fractions was proportionally much more important than that of the adult rats (Figs. 1, 2 and 3).

The distribution patterns of acid phosphatase in suckling and young rats are different from those of adult rats, in liver of suckling rats (Figs. 4, 5 and 6), there are two distinct peaks of enzyme activity, while in adult rat liver, a single broad peak of activity was found (Figs. 1, 2 and 3).

The enzyme activity peak in the top fractions for both acid phosphatase and acid ribonuclease in suckling and young rats can not be attributed to increased soluble enzyme activities in their liver, since the soluble fraction was found to be between 10 to 15% of its respective total enzyme activity, and this percentage is essentially the same as that found in adult rat liver[5]. The top two fractions of the gradients shown in Figs. 1–7 represent the soluble activity of both acid phosphatase and acid ribonuclease, with about 70% in the top fraction alone.

The important enzyme activity peak in the top few fractions for both acid phosphatase and acid ribonuclease in young animals is of considerable interest; the fact that this peak is found in the first few fractions of the gradient above the activity peak of glucose-6-phosphatase means that acid phosphatase and acid ribonuclease in this peak should be located in particles of small diameter.

The study reported in this paper is a first attempt ever made on lysosomal enzymes in suckling and young rats, therefore, the true meaning of this fraction is mostly obscure. One is however tempted to speculate that this peak might represent the small vesicles budding off from the Golgi apparatus as described by NOVIKOFF[11]. He showed that these Golgi vesicles contained acid phosphatase when stained by the Gomori method[11]. According to NOVIKOFF[11], however, in hepatic parenchymal cells only a few Golgi vesicles show acid phosphatase reaction product, although in his studies only adult rats were used. Based on results reported in the present paper, it seems reasonable to suggest that hepatic parenchymal cells of young animals might show more acid phosphatase-positive vesicles when stained histochemically. This should be examined further.

Some preliminary experimental results indicated that the enzyme activities (*i.e.*, acid phosphatase and acid ribonuclease) in the peak of small particles are very sensitive to puromycin and cycloheximide treatments (intraperitoneal injections, with puromycin injected 3 times at a dose of 20 mg/kg body weight, and a single injection of cycloheximide at a dose of 5 mg/100 g body weight). This finding suggests, then, that the enzymes in this peak may indeed represent the newly synthesized lysosomal enzymes which are probably turning over rapidly. However, it is not known whether in suckling rats, all other lysosomal hydrolases apart from acid phosphatase and acid ribonuclease also have part of their activities in this fraction of small particles.

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