BBA 65642

STUDIES ON RAT LIVER RIBONUCLEASES

II. ZONAL CENTRIFUGATION OF ACID RIBONUCLEASE; IMPLICATIONS FOR THE HETEROGENEITY OF LYSOSOMES

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(Received March 28th, 1967)

SUMMARY

Results obtained by zonal gradient centrifugations demonstrated that rat liver lysosomes are heterogeneous in terms of their enzyme contents. It is suggested that acid phosphatase and cathepsin C on one hand, and acid ribonuclease and cathepsin D on the other, belong to two different classes of "lysosomes".

INTRODUCTION

In view of the fact that a third RNA-degrading enzyme was found in the rat liver[1], a study of its intracellular distribution was undertaken[2]; during the course of this study, it became clear that a closer examination on the subcellular distribution of the three known ribonucleases was desirable. The following report is concerned with the distribution of acid ribonuclease as related to some of the lysosomal hydrolases.

MATERIALS AND METHODS

Tissue homogenization

Female Holtzmann rats weighing between 200 and 300 g were used throughout this study. They were fasted 18–20 h before they were killed by decapitation. The liver was removed and chilled at once to 0° in 0.25 M sucrose adjusted to pH 7.2 with 0.1 M KOH, then cut into small pieces and homogenized in 9 vol. of 0.25 M sucrose in a cellulose nitrate test tube fitted with a glass pestle. In most experiments, 20 or 30 ml of this 10% homogenate was directly introduced into the rotor. In one experiment, the homogenate was centrifuged in a Spinco Model L-2 centrifuge at 105 000 × *g* for 1 h, the supernatant was discarded, and the pellet was resuspended in 0.25 M sucrose in order to reconstitute a suspension which was equivalent to 1 g

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of liver in 5 ml; this reconstituted homogenate was then introduced into the rotor. In some experiments, animals were injected intravenously with 850 mg/kg of non-ionic detergent, Triton WR-1339 (Rohm and Haas), 4 days before they were killed.

Zonal centrifugation

Centrifugation was carried out in an A-XII rotor with a modified PR-2 refrigerated centrifuge (International Equipment Company). The general principles of operation of the zonal centrifuge have been described by ANDERSON[3]. Details of the specific procedures used in this laboratory have been presented by SWICK *et al.*[4].

Two types of gradients were used in these experiments. For what will be referred to hereafter as the "long" gradient, solutions of water and 53% (w/v) sucrose were pumped through a mixing chamber by variable speed pumps to produce a linear gradient of 10–43% sucrose, occupying 1160 ml. The rotor was filled with 65% sucrose, some of which was subsequently displaced by introduction *via* the core line of 30 ml homogenate and 40 ml 0.25 M glucose, leaving a 110 ml cushion of the high-density solution. At the end of the run, and after deceleration to 700 rev./min, 65% solution was pumped in at the edge line, and a series of 40 ml fractions of the gradient were collected.

In the "short" gradient procedure, only 600 ml of the 10–43% gradient were introduced into the rotor. The rotor was then filled with 65% sucrose as before, 20 ml of homogenate were introduced, and then 600 ml of 0.25 M glucose cushion. After deceleration, the glucose overlay was pumped out and discarded, and 20-ml fractions were collected by displacement with 65% sucrose. The advantages of the short gradient are: (1) the zone occupied by the homogenate is narrower after having been displaced 12.5 cm from the axis of centrifugation, so that improved resolution should be possible; (2) the initial relative centrifugal force is higher ($2100 \times g$ *vs.* $960 \times g$) after acceleration to 4000 rev./min so that shorter times of centrifugation are required; (3) the concentration of particulates in the fractions is higher.

Enzyme assays

All fractions, as well as the unfractionated homogenate, were assayed for acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) (ref. 5), acid ribonuclease (ribonucleate pyrimidine-nucleotido-2'-transferase, EC 2.7.7.16) (ref. 1), glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) (ref. 6), urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3) (ref. 7), and either cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) (ref. 8) or succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) (ref. 9).

Cathepsin C (EC 3.4.4.9) was assayed, using 0.01 M cysteine as activator, and glycyl-phenylalanine amide as substrate[10]; the ammonia released by the hydrolysis of the amide group was captured in 0.5 M H_2SO_4, as described by SELIGSON AND SELIGSON[11], and then determined by the nesslerization.

Cathepsin D was assayed as described by GIANETTO AND DE DUVE[12]; since the high concentration of sucrose present in the gradient interferes with the assay, all fractions were centrifuged at high speed, and the pellets obtained were resuspended in water before being used for cathepsin D determinations.

RESULTS

Fig. 1 shows the distribution of acid phosphatase and acid ribonuclease of a control rat liver after a 2-h centrifugation with a long gradient. The peak of particulate acid phosphatase is in Fractions 8 and 9, whereas the peak of particulate acid ribonu-

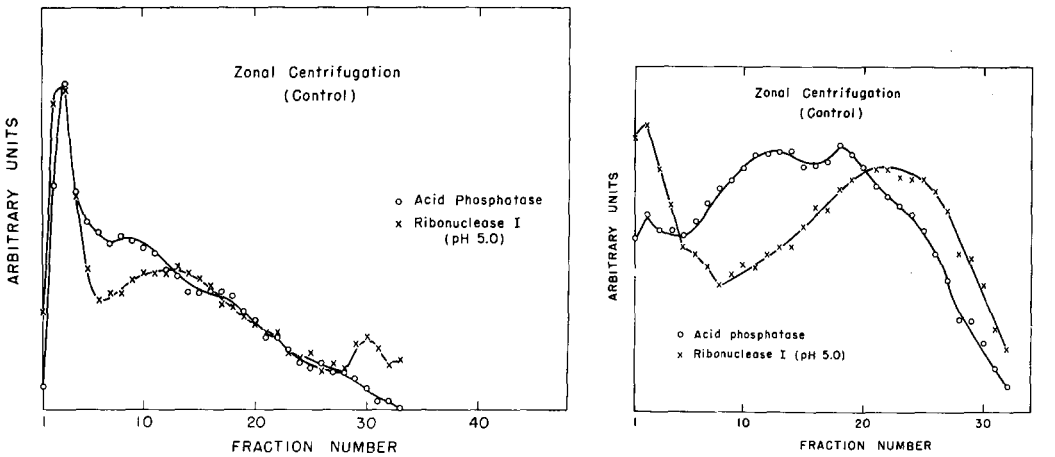

Fig. 1. The distribution of acid phosphatase and acid ribonuclease activities in fractions obtained from a "long gradient" (see text) with a control rat liver. The gradient was centrifuged at 4000 rev./min for 120 min. ○——○, acid phosphatase; ×——×, acid ribonuclease.

Fig. 2. The distribution of acid phosphatase and acid ribonuclease activities in fractions obtained from a "short gradient" (see text) with a control rat liver. The supernatant of the unfractionated homogenate was removed before being introduced into the gradient and the gradient was centrifuged at 4000 rev./min for 60 min. ○——○, acid phosphatase; ×——×, acid ribonuclease.

clease is around Fractions 11–13. Virtually all of the activity of both enzymes in the first three fractions is either soluble or adsorbed on microsomes. The amount of free activity in subsequent fractions (measured by hydrolysis of substrates in isoosmotic medium in the absence of detergent) is extremely small and could not affect the measurements of bound enzymatic activity.

Fig. 2 shows the result of another experiment with a control rat liver. In this case the supernatant of the original homogenate was removed by centrifugation at 105 000 × *g* for 1 h. The sediment was resuspended in 0.25 M sucrose and then layered over the gradient. A short gradient was used in this experiment, which was centrifuged for 1 h. Here the peak of particulate acid phosphatase activity (which is rather flat in this case) is between Fractions 11 and 18; the acid ribonuclease activity peak is slightly sharper than that of the acid phosphatase, and the latter is found around Fractions 20–23.

From the same experiments, the distribution patterns of peroxysomes and mitochondria were obtained by the use of appropriate marker enzymes, urate oxidase and succinic dehydrogenase, respectively. These patterns, together with that of total nitrogen precipitable by trichloroacetic acid, are shown in Fig. 3. Both the zones of maximum activity are markedly different from those of acid phosphatase or acid ribonuclease.

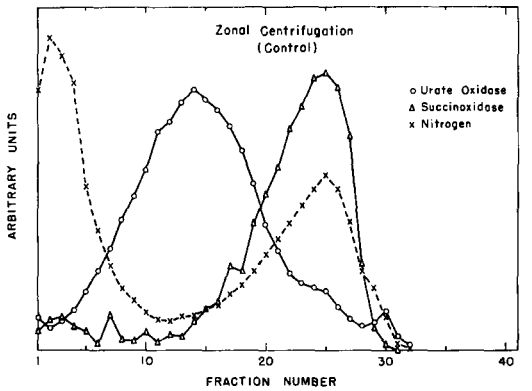

Fig. 3. Same experiment as that of Fig. 2. △—△, succinoxidase; ○—○, urate oxidase; ×- - - -×, nitrogen.

Fig. 4 shows the distribution patterns of these enzymes in the liver of a rat injected with Triton WR-1339. The discrepancy of the two enzyme peaks can again be seen. A long gradient, subjected to a 2-h centrifugation, was used in this experiment. Fig. 5 shows a similar result with a short gradient, after a 90-min run, in which liver from a rat injected with Triton WR-1339 was used. It should be noted that although the distribution patterns of the two enzymes are different from those of the corresponding controls (Figs. 1 and 2), the locations of the peaks of activity are not markedly altered.

The data in Fig. 6 are from the same experiment as those of Fig. 1, only here the distribution curves of both enzymes are expressed by their respective specific activities (total activity divided by the total nitrogen content of each fraction). Similarly, Fig. 7 shows the distribution curves of acid phosphatase and acid ribonu-

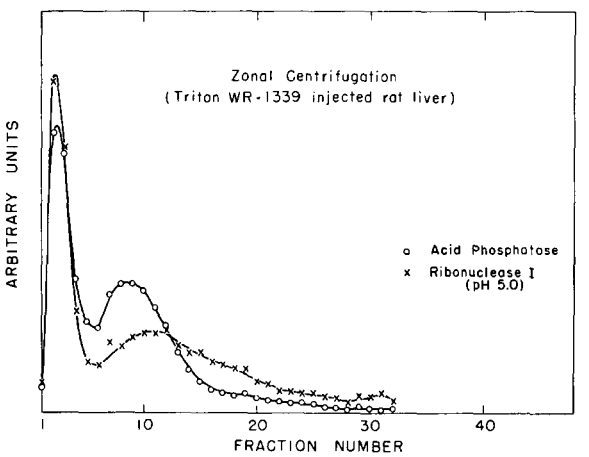

Fig. 4. The distribution of acid phosphatase and acid ribonuclease activities in fractions obtained from a "long gradient" with the liver of a rat injected with Triton WR-1339 (at a concentration of 800 mg/kg) four days prior to the experiment, the gradient was centrifuged at 4000 rev./min for 120 min. ○——○, acid phosphatase; ×——×, acid ribonuclease.

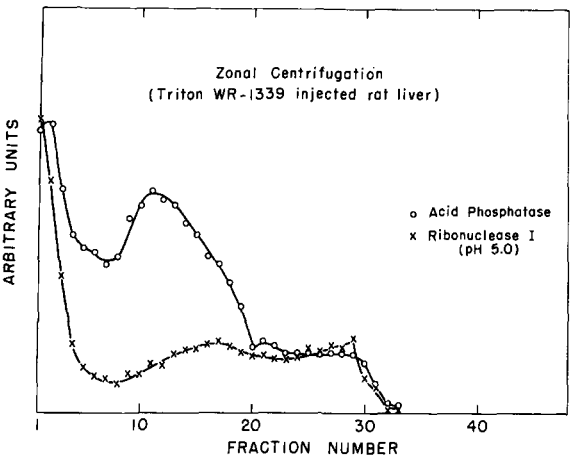

Fig. 5. The distribution of acid phosphatase and acid ribonuclease activities in fractions obtained from a "short gradient" with the liver of a rat injected with Triton WR-1339 (at a concentration of 800 mg/kg) 4 days prior to the experiment. The gradient was centrifuged at 4000 rev./min for 90 min. ○——○, acid phosphatase; ×——×, acid ribonuclease.

clease expressed as their specific activities, based on the data of Fig. 5. Although both Figs. 6 and 7 show a slight difference in the location of the peak activities of acid phosphatase and that of acid ribonuclease, the discrepancy is certainly not as striking as those of the first four figures, which are expressed in terms of the total activity of the two enzymes.

Fig. 8 shows the distribution patterns of acid phosphatase and cathepsin C with a control rat liver, after 50-min centrifugation in a short gradient. Even though

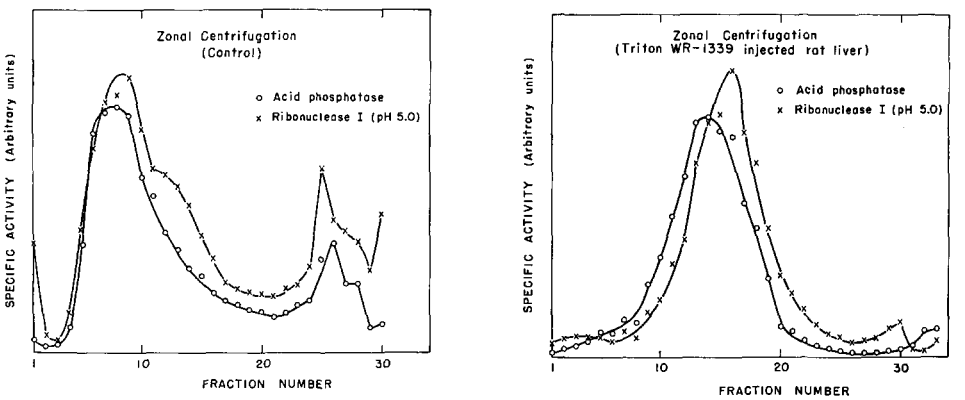

Fig. 6. Same experiment as that of Fig. 1. The distribution of acid phosphatase and acid ribonuclease activities are expressed in terms of their specific activity. ○——○, acid phosphatase; ×——×, acid ribonuclease.

Fig. 7. Same experiment as that of Fig. 2. The distribution of acid phosphatase and acid ribonuclease are expressed in terms of their specific activity. ○——○, acid phosphatase; ×——×, acid ribonuclease.

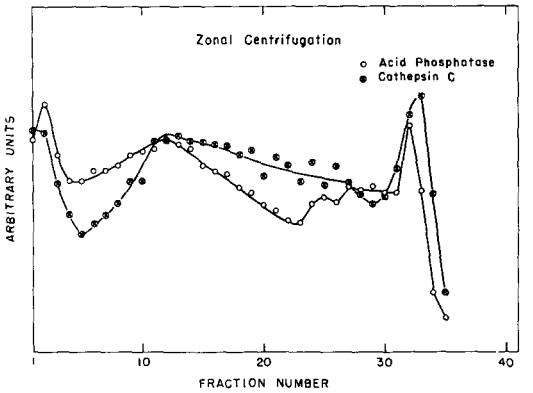

Fig. 8. The distribution of acid phosphatase and cathepsin C activities from a "short gradient" with a control rat liver. The gradient was centrifuged at 4000 rev./min for 50 min. ○——○, acid phosphatase; ⊗——⊗, cathepsin C.

the two distribution curves are not exactly superimposable, the peak activity of both enzymes is found around Fractions 12–14.

The distribution patterns of acid phosphatase and of cathepsin D are shown in Fig. 9. Although the distribution curves of both enzymes are quite irregular (probably because of the recentrifugation and the resuspension procedures involved), the discrepancy between the acid phosphatase distribution and that of the cathepsin D seems clear.

Both experiments as described in Figs. 8 and 9 were reproducible in terms of the differences in the distribution between acid phosphatase and cathepsin C on one hand, and ribonuclease I and cathepsin D on the other. However, it should be pointed out that the experiments in which we had to recentrifuge all the fractions and resuspend them in water for cathepsin D determinations resulted in much less satis-

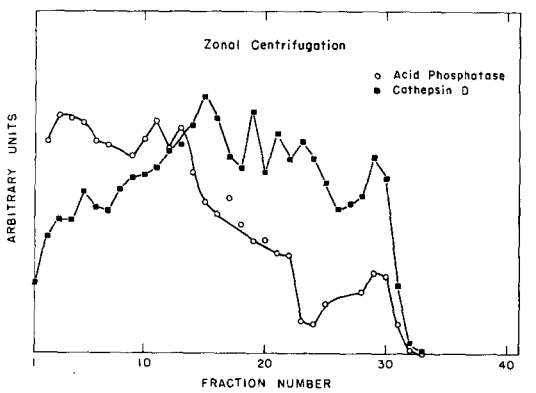

Fig. 9. The distribution of acid phosphatase and cathepsin D activities from a "short gradient" with a control rat liver. The gradient was centrifuged at 4000 rev./min for 50 min. The supernatant of the unfractionated homogenate was removed before being introduced into the gradient. All fractions were centrifuged, sucrose removed, and the pellets resuspended in water before being used for enzyme determinations. ○——○, acid phosphatase; ■——■, cathepsin D.

factory distribution curves, as can be seen in Fig. 9. Ribonuclease I activities were determined in some of these experiments, but we were unable to get a smooth distribution curve.

The enzyme recoveries in all these zonal centrifugation experiments are as follows: acid phosphatase, 102–112%; ribonuclease I, 80–109%; cathepsin C, 106–120%; cathepsin D, 78–101%; urate oxidase, 87–101%; succinoxidase, 61–71%; nitrogen, 88–99%.

DISCUSSION

A large body of evidence provided by many laboratories during the past ten years has established the fact that lysosomes can be considered as a special group of subcellular particles. However, the association of all so-called lysosomal hydrolases within the same type of particles has not yet been conclusively proved. In 1963, DE DUVE[13] stated that "it must be recognized that the association of the acid hydrolases within single individual particles, although highly probable, has not been established with certainty. In view of its crucial importance with respect to the functional implications of the lysosome concept, it is to be hoped that techniques will be found to settle this question in an unequivocal fashion".

From the experimental results reported in the present paper, the existence of heterogeneity in rat liver lysosomes are indicated. The discrepancy between the acid phosphatase distribution curve and that of the acid ribonuclease as well as that of the cathepsin D was seemingly shown in quite a striking manner, whereas there seems to be no such difference between the acid phosphatase distribution and that of cathepsin C. Based on these results, we tentatively assume that acid phosphatase and cathepsin C belong to one class of particles while ribonuclease I and cathepsin D belong to another.

The extensive subcellular fractionation studies of rat liver done in DE DUVE's laboratory were based either on differential centrifugation[14] or on density-equilibrium centrifugation[15], and their results showed that acid phosphatase, acid ribonuclease and cathepsin D were localized in the lysosomes. Using the differential centrifugation method, BOUMA AND GRUBER[16] reported that cathepsin C was also located in rat liver lysosomes. If the four enzymes, namely acid phosphatase, acid ribonuclease (ribonuclease I), cathepsin C and cathepsin D, belong to particles of a similar density as indicated in density-equilibrium centrifugations[14], then the difference in the distribution patterns between acid phosphatase, and cathepsin C on the one hand and the ribonuclease I and cathepsin D on the other that we observed with zonal centrifugation might simply be due to the differences in their respective particle sizes which is reflected by the differences in their sedimentation rates.

WATTIAUX, WIBO AND BAUDHUIN[17], also using the technique of density-equilibrium centrifugation, showed that the density of rat liver lysosomes was markedly decreased after administration of Triton WR-1339 to the animal. This phenomenon was not observed in the zonal centrifuge, presumably because the effect of decreased density of the lysosomes was offset by an increase in size so that the sedimentation rate was not measurably affected under the experimental conditions that we employed. This fact probably can be used to strengthen our previous argument that acid phos-

phatase and cathepsin C belong to a class of particles of a different size as compared to the one to which ribonuclease I and cathepsin D belong.

Since heterogeneity of enzyme complements of rat liver mitochondria has been described[4], it is not surprising that heterogeneity of lysosomal enzymes also exists. Whether the differences in enzyme distribution represent differences in particulate composition within a given cell type or whether they represent intercellular differences among various parenchymal cells of the liver lobule cannot be resolved. Yet a third possibility, namely, that they represent lysosomes of the different cell type in liver, *i.e.* the parenchymal cells and the reticulo-endothelial cells, has been recently suggested by BOWERS AND DE DUVE[18] for rat spleen lysosomes. For rat liver, this possibility seems to be ruled out by the experiment done by WATTIAUX *et al.*[19] on isolated parenchymal and reticulo-endothelial cells. They found all lysosomal enzymes in both cell fractions; however, they stated that in the reticulo-endothelial cell fractions it is possible that some of the enzyme might be derived from contaminating components of parenchymal cells. In addition, it is most interesting to note that the specific activity of ribonuclease I, acid deoxyribonuclease and cathepsin D was increased in the reticulo-endothelial cell fraction they obtained. In view of these facts, the possibility still remains that the lysosomes from parenchymal cells might be different from those of the reticulo-endothelial cells in terms of their enzyme contents. It is certainly premature to draw further conclusion from the present results we have at hand; lysosomal enzymes other than the four we reported in this paper are currently under investigation. Fractionation methods other than zonal centrifugation are also being explored.

ACKNOWLEDGEMENTS

The authors thank Dr. R. SWICK for his valuable criticism during the preparation of this manuscript. This investigation was supported by the U.S. Atomic Energy Commission.

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