

Studies on the Polymodal Distribution of Hepatic Lysosomes and Peroxisomes (37795)

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The existence of separate particles in rat liver containing acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) (lysosomes) and urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3) and catalase (hydrogen peroxide: peroxide oxidoreductase, EC 1.11.1.6) (peroxisomes) has been well established by de Duve and coworkers and others (for reviews, see Refs. 1-4). In studies of a "very light" fraction obtained by sequential differential centrifugation of 4-times washed mitochondria, we have found that a highly reproducible polymodal distribution of lysosomes and peroxisomes can be demonstrated in this fraction on a linear sucrose density gradient. These studies are reported in this paper.

Materials and Methods. Preparation of the fractions. Adult male Sprague-Dawley rats fed a standard stock diet were used in these studies. The livers were removed after decapitation of the animals at 0800 hr without prior fasting. Highly purified mitochondria were isolated as described previously (5). The supernatant washes from preparation of the mitochondria were saved for isolation of microsomes as described later. The mitochondria were suspended with gentle homogenization in a volume of 0.25 M sucrose equal to the original weight of the liver employed, usually approximately 60 g. The mitochondrial suspension was separated into 15-ml portions in 29×104 mm plastic centrifuge tubes for spinning in the Sorvall RC 2-B centrifuge with SS-34 rotor. All operations were carried out at 0-3°. The mitochondria were first spun at 6000 rpm for 10 min (4032g, $r_{av} = 10$ cm). This fraction was labeled 6 K. The supernatants were poured

off as completely as possible including loose material into fresh tubes of the same size. These tubes were spun at 8000 rpm for 10 min (7168g, $r_{av} = 10$ cm). The supernatants from this fraction were transferred to fresh tubes, holding back any streaming of the pellets as completely as possible. These tubes were then spun at 17,000 rpm for 10 min. (32,368g, $r_{av} = 10$ cm). The pellets obtained were labeled (>8)-17 K. The 6 K pellets were suspended in 10 ml 0.6 M sucrose for 60 g original liver tissue. The 8 K pellets were discarded since they apparently contained a major portion of "fluffy layer" still held in the original mitochondria, even though the original pellets from the latter were rinsed with 0.25 M sucrose to remove as much of the fluffy layer as possible. The combined (>8)-17 K pellets were suspended in 0.5 ml 0.6 M sucrose/60 g original liver. The (>8)-17 K fraction comprised less than 1% of the protein content of the original well-washed mitochondrial preparation and thus represents a small and very "light" fraction of the lysosomes and peroxisomes originally present in liver.

Eight 35-ml portions of the combined supernatant washes obtained during the preparation of the original mitochondria were spun at 17,000 rpm for 30 min (Sorvall RC 2-B centrifuge, SS34 rotor). The upper half of each of these supernatants was used to obtain the microsomal fraction by spinning at 29,000 rpm for 60 min (75,000g, $r_{av} = 7.75$ cm) in a Beckman-Spinco Model L2-65B ultracentrifuge using a No. 30 rotor. The combined pellets were suspended in 3 ml 0.6 M sucrose and labeled Mc.

Protein concentrations were obtained for

the 6 K, (>8)-17 K, and Mc fractions using the method of Lowry *et al.* (6). Portions of the 6 K and Mc fractions were adjusted to the concentration of the (>8)-17 K fraction with 0.6 M sucrose.

Density gradient centrifugations. After a series of trial and error studies, it was found that the best separation of particles in each of the fractions could be obtained using a linear sucrose density gradient of 1.8–0.7 M sucrose. Thus 0.4 ml of the 6 K, (>8)-17 K, and Mc fractions of equal protein concentration was layered over 4.6 ml of the 1.8–0.7 M sucrose gradient. The tubes were spun at 50,000 for 60 min ($205,000g$ $r_{av} = 7.75$ cm) in an SW50L swinging bucket rotor (Beckman-Spinco model L2-65 B ultracentrifuge). The tubes were removed, and 8-drop portions were collected from a syringe needle which punctured the bottom of the tube. Usually about 30 8-drop portions were obtained. (It was found that 4-drop collections gave no better resolution than 8-drop collections.) The last 3 tubes from each collection representing the original sample in 0.6 M sucrose were discarded. In additional experiments it was found that equilibrium was reached after 1 hr, since in comparative studies in which the centrifugations were carried out for 2 hr the concentrations of sucrose for the peaks for lysosomes and peroxisomes in the sucrose gradient were the same as after 1 hr.

In calculating, the assumption was made that the entire collection except for the last 3 tubes comprised 1.8–0.7 M sucrose. Thus, even though the total number of 8-drop collections varied somewhat from experiment to experiment, the entire range of tubes was calculated to comprise 1.8–0.7 M sucrose. In this way, all experiments were normalized, and the enzyme activities could be averaged.

Enzyme assays. Acid phosphatase was assayed as described by Gianetto and de Duve (7). Urate oxidase was assayed by the procedure of de Duve *et al.* (8), and catalase, essentially as described by Chance and Maehly (9) by following the disappearance of H_2O_2 substrate at 240 nm.

All enzyme activities were calculated as if

2 mg total protein had been applied to the density gradients. Thus the activities from experiment to experiment were normalized not only with respect to position in the gradient (see above) but also with respect to protein concentration.

Results. The results for acid phosphatase (lysosomal marker) are presented in Fig. 1. An important aspect of these results for the three fractions is that acid phosphatase in the (>8)-17 fraction exhibited not only much greater specific activity compared with the 6 K fraction, but more importantly a strongly bimodal nature. The relative amounts of activity in the two peaks varied among the experiments. In some experiments the peak at 1.17 M sucrose appeared as a shoulder and in other experiments as a peak as high or higher than the peak at 1.47 M sucrose. The two

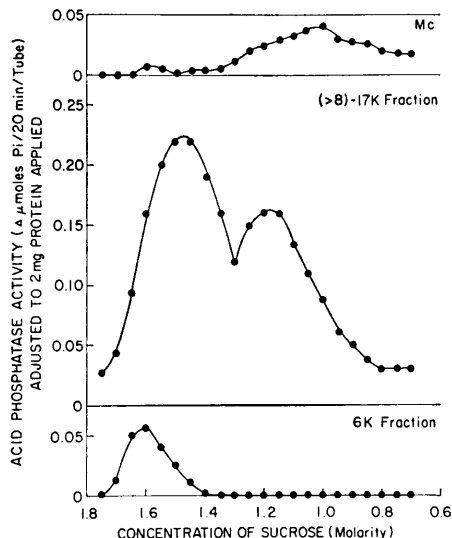


Fig. 1. Sucrose density gradient separation of acid phosphatase-containing particles in "heavy" and "very light" fractions obtained from well-washed rat liver mitochondria. The microsomal fraction is also included. For the preparation of the fractions and details of density gradient separation, see the text. The curve for the (>8)-17 K ("very light") fraction is an average of eight experiments; for the 6 K ("heavy") and Mc fractions, an average of four experiments each. The standard error of the mean for the 1.47 M sucrose peak of the (>8)-17 K fraction = ± 0.013 sucrose molarity units; for the 1.17 M sucrose peak, ± 0.026 sucrose molarity units. $P < 0.001$ for significance of difference between the two peaks of the (>8)-17 K fraction.

peaks were significantly different from each other with respect to sucrose concentration (See the legends of the figures). The peak in the 6 K fraction appeared at 1.60 *M* sucrose. The Mc fraction exhibited a small activity with a peak at 1.00 *M* sucrose.

The results for urate oxidase and catalase (peroxisomal markers) are shown in Figs. 2 and 3. The main peak for each enzyme in the (>8)-17 K fraction occurred at 1.60 *M* sucrose. A shoulder for both enzymes appeared at 1.33–1.32 *M* sucrose, each of which was significantly different from the major peak with respect to the sucrose concentrations at which the peak and shoulder occurred. In some experiments the shoulder was quite small for both enzymes, while in others it was quite marked. This is shown in representative experiments in Fig. 4. The 6 K

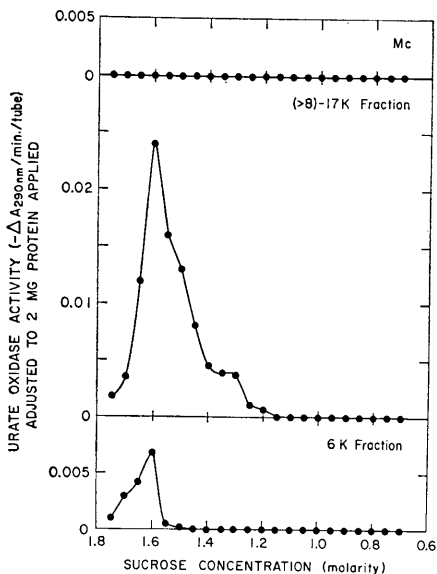


FIG. 2. Sucrose density gradient separation of urate oxidase-containing particles in "heavy" and "very light" fractions obtained from well-washed rat liver mitochondria. The microsomal fraction is also included. For the preparation of the fractions and details of density gradient separation, see the text. All curves are an average of four experiments. The standard error of the mean for the 1.60 *M* sucrose peak of the (> 8)-17 K fraction = \pm 0.016 sucrose molarity units; for the shoulder at 1.32 *M* sucrose, \pm 0.031 sucrose molarity units. $P < 0.001$ for significance of difference between the peak and shoulder for the (>8)-17 K fraction.

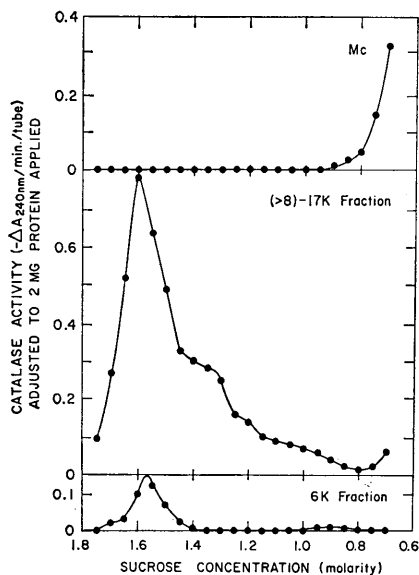


FIG. 3. Sucrose density gradient separation of catalase-containing particles in "heavy" and "very light" fractions obtained from well-washed rat liver mitochondria. The microsomal fraction is also included. For the preparation of the fractions and details of density gradient separation, see the text. All curves are the average of four experiments. Standard error of the mean for the 1.60 *M* sucrose peak of the (> 8)-17 K fraction = \pm 0.024 sucrose molarity units; for the shoulder at 1.33 *M* sucrose, \pm 0.032 sucrose molarity units. $P < 0.001$ for significance of difference between the peak and shoulder for the (> 8)-17 K fraction.

fraction contained a small amount of both urate oxidase and catalase. Urate oxidase was undemonstrable in the Mc fraction. However, as would be expected from the studies of de Duve and coworkers, catalase appeared as a tailing upward in the Mc studies at the lower concentrations of sucrose because of its presence in the peroxisomal structureless "sap" which can be washed out in a soluble form due to breakage of the membrane. Urate oxidase is associated with the insoluble core of these particles and would not become soluble if the membrane were damaged. It can also be seen from Fig. 3 that a third broad shoulder for catalase appeared from 1.15 to 0.8 *M* sucrose, which, however, did not appear over this range for urate oxidase (Fig. 2). A tailing upward at the very low sucrose concentrations occurred for catalase in the (>8)-17 K fraction as in the Mc frac-

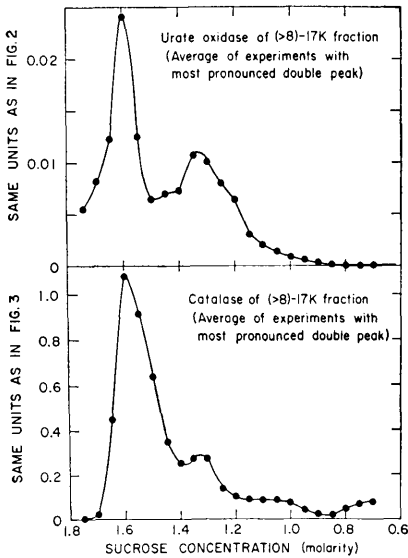


FIG. 4. Average of representative experiments for sucrose density gradient separation of urate oxidase and catalase-containing particles in the (>8)-17 K fraction which showed the most pronounced peak at the lower sucrose concentration. Each curve is the average of two experiments.

tion, indicating that a soluble form of the enzyme also is present in the (>8)-17 K fraction. The major peak and major shoulder for urate oxidase and catalase in the 6 K and (>8)-17 K fractions coincided almost exactly. The peak at 1.47 M sucrose for acid phosphatase differed significantly from the peak at 1.60 M sucrose for urate oxidase and catalase ($P < 0.001$). Also, the peak at 1.17 M sucrose for acid phosphatase differed significantly from the shoulders of the other two enzymes.

Discussion. The intent of the studies reported here was not to isolate as much of the lysosomes and peroxisomes from rat liver as possible, as has been done by Leighton *et al.* (1) and Sawant *et al.* (10), but was to show that a "very light" portion of the lysosomes and peroxisomes can be shown to exhibit a statistically reproducible polymodal nature with respect to sucrose concentrations at which the peaks and shoulders occur on a linear sucrose density gradient.

The significant differences between the acid phosphatase peaks in the (>8)-17 K fraction indicate that in the sucrose density

gradient used here, fairly good separation of the lysosomes of the two densities occurred; similarly, for the peroxisomes of the two densities.

Although in the studies reported here the initial liver homogenization was made for 50 sec in a Waring blender, the studies were repeated using gentle pestle homogenization, and the same results were obtained. This indicated that the less dense lysosomes and peroxisomes are not fragments of the more dense particles, but are true subpopulations of the particles. This is further substantiated by the almost exact coincidence of the peaks and shoulders for urate oxidase, which is found in the "core" of the peroxisomes, and catalase, which is present in the "sap" of these particles. The latter would leak out if the peroxisomes were fragmented, and the catalase shoulder should not appear at all.

Summary. A fraction has been obtained from highly purified rat liver mitochondria which contains lysosomes and peroxisomes exhibiting a bimodal distribution on a linear sucrose density gradient. This fraction, which represents less than 1% of the total protein of the original mitochondria, was obtained after removal of the bulk of the heavier particles by lower speed centrifugations. The sucrose concentrations at which the two peaks (for lysosomes) and major peak and shoulder (for peroxisomes) equilibrate are highly reproducible as shown by statistical analysis.

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