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Preparation of Peroxisomes from Mouse Liver by Rate-Zonal Centrifugation[1] (37975)

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The peroxisome, like the lysosome, is an organelle the existence of which was predicted on biochemical evidence prior to its positive cytological identification. The historical aspects have been reviewed by de Duve (1–3), from whose laboratories have come many of the reports concerned with isolation and characterization of peroxisomes in animal tissues (1, 2, 4–9).

In our attempts to prepare homogeneous peroxisomes from mouse liver by adaptation of published techniques, we encountered a problem of loss of biochemical integrity, as evidenced by the fact that the recovery of catalase relative to that of urate oxidase was very poor, often as low as 20%. It seemed probable that the cause of damage was either the high concentration of sucrose used in the equilibrium density centrifugation step or the high hydrostatic pressures developed during centrifugation (10).

This report is concerned with the development of a preparative method using rate-zonal centrifugation (11) of homogenates of livers of mice pretreated with compounds that alter the sedimentation characteristics of lysosomes (4) and of mitochondria (12, 13), but do not affect those of peroxisomes.

Methods. Animals. The mice used in these experiments were CF#1 females, approximately 3–4 months old. They were maintained at 74°F and 50% RH, and fed Rockland mouse diet and tap water *ad lib.* Three days before the mice were killed, they were given a single intravenous injection of 100 mg/kg Triton WR-1339; on each of the 3 days before killing, they received intramuscular injections of 0.1 mg/kg prednisolone.

Preparation of NMP sample. Livers (about 5 g) from mice killed by cervical dislocation were homogenized in 9 vol of 0.25 *M* sucrose containing 0.0003 *M* EDTA and 0.02 *M* ethanol.[2] The homogenate was centrifuged for 20 min at 20,000 rpm in a Sorvall RC-2B centrifuge (42,000*g*) to sediment the nuclei and the larger cytoplasmic particles, including the peroxisomes, while the microsomes remained for the most part in the supernatant. (The nuclei can first be removed by centrifugation at low speed, but this step offers no advantage in achieving homogeneity and merely adds to the preparation time.) The sediment, referred to as NMP, was washed once, then suspended in 10 ml of 0.25 *M* sucrose and 0.02 *M* ethanol.

First zonal centrifugation. An International Equipment Co. gradient pump, operated in the exponential mode, was used to construct a sucrose gradient of 400-ml volume in a Spinco Ti-14 zonal rotor. The concentration of sucrose varied linearly (with respect to radial distance) from 0.29 to 0.58 *M*, with the concentration of ethanol

[1] Work supported by the U. S. Atomic Energy Commission.

[2] The purpose of the ethanol is to minimize the possibility of inactivation of catalase (6). When ethanol was omitted from the sucrose solutions, the recoveries of catalase were somewhat erratic, but those of the other marker enzymes were unaltered.

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kept constant at 0.02 *M*. The volume of the mixing chamber was maintained at 400 ml, the reservoir contained 0.71 *M* sucrose, and the cushion was 1.46 *M* sucrose. The NMP sample was layered over the gradient and overlaid with 50 ml of 0.25 *M* glucose. After centrifugation for a cumulative $\omega^2 t$ (including acceleration and deceleration) of about 1.4×10^9 rad²/sec, the gradient was pumped out. On the basis of preliminary experiments in which 34 fractions of 10 or 15 ml were collected (see under *Results*), three large fractions were collected as follows: (a) "Discard" (D-1) fraction, containing the overlay, the volume occupied by the NMP sample, and the first 40 ml of the gradient; (b) a "Zonal" (Z-1) fraction, comprising the next 240 ml of the gradient; and (c) a "Residue" (R-1) fraction, consisting of the remainder of the gradient and the cushion. The Z-1 fraction was centrifuged for 1 hr at 20,000 rpm, and the sediment was resuspended in 10 ml of 0.44 *M* sucrose (CZ-1).

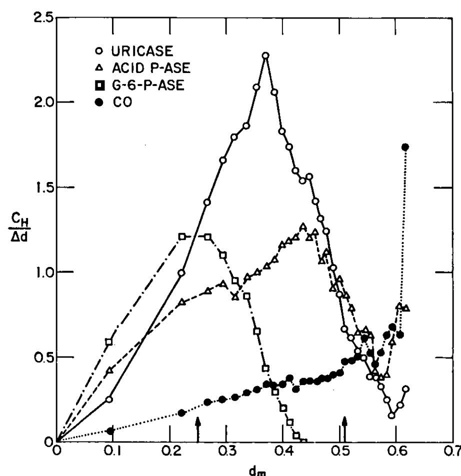

FIG. 1. Distribution curves of four enzymes assayed in 34 fractions recovered after centrifugation of the NMP sample over a 0.29–0.58 *M* sucrose gradient (catalase was not measured). The ordinate, $C_H/\Delta d$, is the activity of a given fraction (as a percentage of the original liver homogenate activity) divided by the upper and lower limits of particle sizes in each fraction, plotted against the mean particle size (in μm) for that fraction. The arrows indicate the limits of the Z-1 sample (see text).

Second zonal centrifugation. The conditions differed from those used in the first zonal centrifugation in that the concentration of the gradient ranged from 0.58 to 1.17 *M* sucrose (1.50 *M* sucrose in the reservoir), and the cumulative $\omega^2 t$ was $\sim 4 \times 10^9$ rad²/sec. As before, three fractions were collected: D-2 (overlay, sample, and 40 ml of the gradient), Z-2 (130 ml of gradient), and R-2. The Z-2 fraction was concentrated as before and the sediment resuspended in 0.25 *M* sucrose (CZ-2).

Enzyme assays. Cytochrome oxidase was used as a marker enzyme for mitochondria, urate oxidase and catalase for peroxisomes, acid phosphatase for lysosomes, and glucose-6-phosphatase for microsomes. Assay procedures have been described previously (15).

Presentation of gradient data. In the preliminary experiments in which numerous small fractions were collected after either the first or second zonal centrifugation in order to assay the entire gradient, we calculated the distribution of enzyme activities as a function of particle diameter. This approach was more convenient than plotting activity against fraction number because the volumes of the fractions were not the same

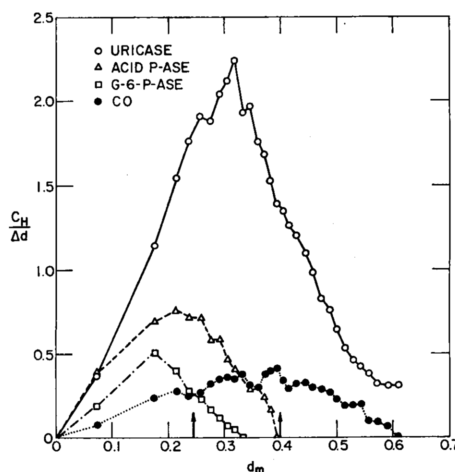

FIG. 2. Distribution curves of four enzymes assayed in 34 fractions recovered after centrifugation of the CZ-1 sample over a 0.58–1.17 *M* sucrose gradient. The arrows indicate the limits of the Z-2 fraction.

in all experiments. The calculation procedure and the computer program have been described in other publications (15, 16).

Results. Figure 1 shows the distribution curves of four marker enzymes, assayed in 34 fractions collected after the first zonal centrifugation. Most of the mitochondria had passed through the gradient and had accumulated at the cushion. Both lysosomes and microsomes were largely confined to the first few fractions. The vertical arrows represent the limits of the Z-1 fraction.

Figure 2 shows similar data for the fractions collected after the second zonal centrifugation, carried out on the CZ-1 sample. The contamination from other organelles was further reduced, especially that from lysosomes and microsomes.

The enzymatic properties of the CZ-2 fraction are shown in Table I. On the basis of calculations similar to those described by Leighton *et al.* (6), we estimate that peroxisomes represent >90% of particle numbers and >95% of particle mass. The data shown for catalase are somewhat misleading, since there is an appreciable contribution to the total tissue value from blood entrapped in the liver. When catalase and urate oxidase recoveries are calculated as percentages of the NMP fraction rather than the original homogenate, the values are similar.

Electronmicroscopic examination of the CZ-2 fraction demonstrated a preponderance of peroxisomes, morphologically similar to those published by Leighton *et al.* (6). Small numbers of mitochondria and lysosomes were evident, their frequencies approximating those that would be predicted on the basis of enzymatic analysis. Glycogen particles were fairly numerous in preparations from mice that had not been fasted.

Discussion. There are several features of the preparative procedure described here that deserve comment. First is the pharmacologic manipulation of the liver organelles; the use of Triton WR-1339 is well-established, but the ability of corticosteroids to increase the sedimentability of mitochondria seems not to have been previously exploited as a preparative technique.

Second, the use of two zonal centrifugations has multiple advantages. The first centrifugation effectively removes over 90% of the mitochondria, although the fractions containing the peroxisomes also contain appreciable amounts of lysosomal and microsomal material. The second centrifugation, with a higher initial concentration of sucrose, more effectively retards the passage

TABLE I. Enzyme Activities of Mouse Liver Peroxisome Preparations.

Enzyme	No. of experiments	Percent of total[a]	Enrichment factor[b]
Urate oxidase	6	39.3 (30.3–55.6)	36.5 (27.4–50.6)
Catalase	3[c]	28.2 (22.6–32.2)	26.5 (21.2–35.8)
Cytochrome oxidase	6	3.8 (3.2–4.7)	3.8 (2.7–5.6)
Acid phosphatase	5[d]	5.0 (3.9–5.4)	4.3 (3.5–5.4)
Glucose-6-phosphatase	6	1.1 (0.8–1.4)	1.0 (0.8–1.7)
Protein	6	1.1 (0.9–1.4)	—

[a] Averages with ranges for 3 samples prepared with 0.02 *M* ethanol added to all sucrose solutions and 3 without ethanol.

[b] Enzyme activity of the CZ-2 preparation per unit protein relative to that of the homogenate.

[c] Data from samples prepared without ethanol not included.

[d] Unsatisfactory assay in one experiment.

of the smaller and less-dense particulates through the gradient.

Third, the peroxisomes are not exposed to high concentrations of sucrose that are required for isolation by isopycnic sedimentation, nor are they subjected to abrupt changes in osmotic pressure that have been shown to be deleterious to peroxisomes (3). The hydrostatic pressures developed during centrifugation are well below those known to cause damage (10).

Finally, the procedure is relatively rapid; under ordinary conditions, less than 5 hr elapse between killing the animals and the preparation of the final suspension.

Needless to say, we have tried numerous variations on this procedure. We abandoned those involving isopycnic sedimentation when it became apparent that catalase was lost or inactivated. We attempted using low concentrations of digitonin at various stages during the preparation to take advantage of the greater sensitivity of the lysosomal membranes (3) but invariably encountered damage to the peroxisomes, to judge by loss of catalase.

The current procedure was designed to obtain a preparation of peroxisomes in reasonable yield, with minimal contamination from all other particulates. The yield can be increased at the expense of homogeneity; however, the increased heterogeneity can be controlled by changing the fraction conditions: one may lessen further the mitochondrial contamination if one is willing to accept a greater proportion of lysosomes, and *vice versa.*

The procedure so far has been used only with relatively small amounts of tissue. Scaling up the amount by a factor of 10 or so would seemingly present no problems, although a larger-capacity rotor would have to be used to avoid overloading the gradient (11).

Summary. A procedure is described for the isolation of peroxisomes from mouse liver by two successive rate-zonal centrifugations through sucrose gradients. A feature of the technique is the pretreatment of the animals with Triton WR-1339 and prednisolone to decrease the sedimentability of the lysosomes and to increase that of the mitochondria. On the basis of enzyme activities, preparations can be obtained that contain 40–50% of the peroxisomes of the original liver homogenate, at a purity of over 90%.

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