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A Rapid Method for the Isolation of Peroxisomes from Rat Liver

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A preparative method for the isolation of peroxisomes from the liver of normal, untreated rats is described. The peroxisome-enriched "light mitochondrial" fraction is layered on a 30% Nycodenz (5-[*N*-2,3-dihydroxypropylacetamido]-2,4,6-triiodo-*N*,*N*′-bis[2,3-dihydroxypropyl]isophthalamide) solution containing 1 mM tetrasodium EDTA and then centrifuged in an angular rotor for 1 h at $130{,}000g_{avg}$. Peroxisomes are sedimented to the bottom leaving other organelles at the top of the tube. On the basis of morphological and biochemical studies, it is found that the peroxisomes (marker-enzymes catalase and urate oxidase) obtained in this method are not contaminated with lysosomes (marker-enzyme acid phosphatase) and contained very few mitochondria (marker-enzyme succinate–cytochrome *c* reductase) and microsomal vesicles (marker-enzyme glucose-6-phosphatase). © 1986 Academic Press, Inc.

KEY WORDS: subcellular fractionation; peroxisomes; Nycodenz.

A number of methods have been described for the isolation of peroxisomes from liver (1–10). In most of these methods either peroxisomes of high purity are not obtained (11) or the animals must be pretreated with the detergent Triton WR 1339 to achieve the complete separation of lysosomes from peroxisomes (1,3,12). Recently, a method from this laboratory was described in which liver peroxisomes from normal animals were isolated using a Metrizamide (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) density-gradient centrifugation in a vertical rotor (13,14). Liver peroxisomes of high purity and at high yield are obtained by this procedure from normal rats (14–18). However, this procedure is somewhat lengthy and specialized equipment such as a vertical rotor, ultracentrifuge, and collection devices are required. We recently modified this procedure by using an angular head rotor instead of a vertical rotor and replacing Metrizamide with Nycodenz (5-[*N*-2,3-dihydroxypropylacetamido]-2,4,6-triiodo-*N*,*N*′-bis[2,3-dihydroxypropyl]isophthalamide), a similar but less expensive density gradient material. This modified method yields peroxisomes of comparable purity in a much shorter time. The details of the method and the results are presented here.

MATERIALS

Palmitoyl CoA, dihydroxyacetone phosphate (DHAP)[2], cytochrome *c*, D-glucose-6-phosphate, β-glycerophosphate, succinate, isocitrate, NADPH, were obtained from Sigma Chemical Company (St. Louise, Mo.). *sn*-Glycerol-3-phosphate (GP) was from Calbiochem–Behring. Nycodenz (Nyegaard Co.) was obtained from Accurate Chemical & Scientific Research Corporation (Westbury, N.Y.). [^{32}P]DHAP and [^{32}]GP were prepared by the enzymatic phosphorylation of dihydroxyacetone and glycerol respectively with [γ-^{32}P]ATP as described previously (19). B-[4-^{3}H]NADPH and *O*-hexadecyl DHAP were prepared as described before (20,21).

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[2] Abbreviations used: DHAP, dihydroxyacetone phosphate; GP, *sn*-glycerol-3-phosphate; AT, acyltransferase.

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METHODS

Preparation of liver "light-mitochondrial" fraction. Adult male rats (200–250 g) which were fasted overnight were lightly anesthetized with diethyl ether and decapitated, and the livers were quickly excised and homogenized in 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4) and Na_4EDTA (1 mM) as described previously (14). The light mitochondrial (*L*) fraction, i.e., the fraction sedimenting between 33,000g × min and 250,000g × min, was obtained by differential centrifugation of the liver homogenate as described by deDuve *et al.* (1,14,22). The *L* fraction was washed once (25,000g_{max} for 10 min) with the homogenizing buffer (0.25 M sucrose–10 mM Tris-HCl–1 mM EDTA) and then suspended in the same buffer in such a way that 1 ml of the suspension contained the *L* fraction obtained from 1 g liver (~8 mg protein/ml).

Centrifugation in Nycodenz medium. Two milliliters of the suspended *L* fraction was layered carefully on the top of 15 ml of 30% Nycodenz (w/v), 1 mM tetrasodium EDTA solution (pH 7.3, density 1.15 g/ml) in a 30-ml polycarbonate tube (Beckman Cat. No. 340382). The tube was then centrifuged at 40,000 rpm (131,000g_{avg}) for 1 h in a Beckman L8-70 centrifuge using a Ti 50.2 rotor. After the centrifugation the upper turbid solution was removed by gentle suction and the peroxisomes, present as a loose pellet at the bottom of the tube, were collected with a Pasteur pipet after suspending them in the Nycodenz medium.

Enzyme assays. Established methods have been used to assay all the marker enzymes as described previously (14). DHAP acyltransferase and GP acyltransferase were assayed by measuring the labeled lipids formed either from [^{32}]DHAP or from [^{32}P]GP and palmitoyl CoA (23). Acyl/alkyl DHAP reductase was assayed by measuring the formation of [^{3}H]lipid from *O*-hexadecyl DHAP and B-[4-^{3}H]NADPH (21).

Other methods. Nycodenz, like Metrizamide, interferes with the Lowry protein assay and, therefore, it was removed by coprecipitating the protein with deoxycholate by trichloroacetic acid (24) before the Lowry protein assay, as described before (14). All other methods, including the electron microscopic examination of the peroxisome fraction, are the same as described previously (14).

RESULTS

Isolation of Peroxisomes from Liver

The liver homogenate is first subfractionated to obtain the peroxisome-enriched *L* or λ fraction essentially as described by deDuve *et al.* (1,22). The results obtained are similar to those described by these authors and as seen in Table 1, this fraction is enriched in peroxisomes (marker-enzymes catalase and urate oxidase) and lysosomes (marker-enzyme acid phosphatase). This fraction also contains about 20% of the liver microsomes (glc-6-phosphatase as the marker-enzyme) and 6% of the mitochondria (marker-enzyme succinate–cyt. *c* reductase).

When the *L* fraction is centrifuged through the 30% Nycodenz solution, the peroxisomes are sedimented to the bottom of the tube, leaving most other organelles at the interface of 0.36 M Nycodenz and 0.25 M sucrose. Table 1 shows the composition and enrichment of different enzymes in this isolated peroxisomal fraction. As seen from Table 1, in this fraction, the three peroxisomal enzymes, i.e., catalase, urate oxidase, and DHAP acyltransferase, are enriched from the *L* fraction by seven- to eightfold. As also seen from this table, all of lysosomes (acid phosphatase as the marker-enzyme) and most of mitochondria (marker-enzyme succinate–cyt. *c* reductase) and microsomes (marker-enzyme glucose-6-phosphatase) which were present in the *L* fraction, are removed from this fraction. GP acyltransferase, an enzyme which is present in mitochondria and microsomes, is also practically absent in this fraction (Table 1). Another lipid biosynthetic enzyme, acyl/alkyl DHAP reductase which is partly peroxisomal (25) is also enriched by about 15-fold from the total ho-

TABLE 1

ENZYME COMPOSITIONS OF LIGHT MITOCHONDRIAL (L) AND PEROXISOMAL FRACTIONS

Enzyme	*L*-fraction			Peroxisomes		
	Yield (% of total liver activity ± SD)	Specific activity[a] ± SD	Relative specific activity ± SD	Yield (% of total liver activity ± SD)	Specific activity[a] ± SD	Relative specific activity ± SD
Catalase	33.2 ± 5.3	266 ± 18.9	5.9 ± .4	16.9 ± 2.9	1854 ± 330	38.4 ± 5.5
Urate oxidase	70.3 ± 4.5	255 ± 21.6	10.3 ± .7	33.3 ± 8.3	1620 ± 340	66.5 ± 8.6
DHAPAT	48.1 ± 5.5	2.8 ± .15	8.2 ± .3	25.6 ± 2.2	19.8 ± 2.9	58.2 ± 8.7
Acid phosphatase	49.1 ± 2.5	233 ± 7.24	9.1 ± .41	n.d.[b]	0.0	0.0
Succinate-cyt. *c* reductase	5.8 ± 1.9	18.1 ± 1.63	1.2 ± .14	0.13 ± .03	10.9 ± 5.3	0.35 ± .1
Glc-6-phosphatase	19.2 ± 1.9	267 ± 2.12	3.8 ± .11	0.16 ± .05	28.5 ± 10.2	0.39 ± 0.1
GPAT	8.6 ± .3	1.6 ± .2	1.3 ± .1	0.18 ± .06	.25 ± .15	.5 ± .1

Note. The *L* fractions were isolated by differential centrifugation of the liver homogenates and the peroxisomes are isolated from the *L* fractions as described in the text. Most of the values given are the average of six to seven determinations (± standard deviations) except for the GPAT (three determinations).

[a] Specific activity is generally expressed as nmol/min/mg protein, except for catalase which is units/mg protein as defined by Leighton *et al.* (1).

[b] n.d. = not detected.

mogenate in this peroxisomal fraction (data not shown).

Morphology of the Peroxisomes

Morphological examination of the isolated peroxisomes showed that most of the peroxisomes are intact with very little contamination by other organelles are seen (Fig. 1). A few peroxisomal cores are seen to be present in this fraction (Fig. 1).

DISCUSSION

These results show that the liver peroxisomes can be purified by centrifugation through a Nycodenz-containing medium. The isolated peroxisomes are virtually free of lysosomes and contained only minute amounts (0.1–0.2%) of cellular mitochondria and microsomes (Table 1). The final yield of peroxisomes is about one-third of the total peroxisomes present in liver (Table 1). The purity of the peroxisomes isolated by this method is comparable to those obtained by other methods (1,11) including our previous method (14). For example, in the widely used method of preparation of peroxisomes as described by Leighton *et al.* (1), the enrichment (relative specific activity) of catalase from liver homogenate is 36-fold which is similar to that reported here (38-fold). The enrichments of two other peroxisomal enzymes, DHAPAT and urate oxidase, are higher than that of catalase (Table 1). This is due to the fact that a large fraction of liver catalase is extraperoxisomal. For example, if it is assumed that 40% of cellular catalase is cytosolic (1,26) and 20% of DHAPAT is in microsomes (23) and all of urate oxidase is in peroxisomes (1,11), then the enrichment of all these enzymes will be the same (about 65-fold) thus indicating that peroxisomes in liver contained only 1.5% of total cellular protein. Allowing for the microsomal and mitochondrial contaminations (Table 1), the isolated peroxisomes are calculated to be at least 95% pure.

This present method is based on the observation of Wattiaux *et al.* (5) that in Metriza-

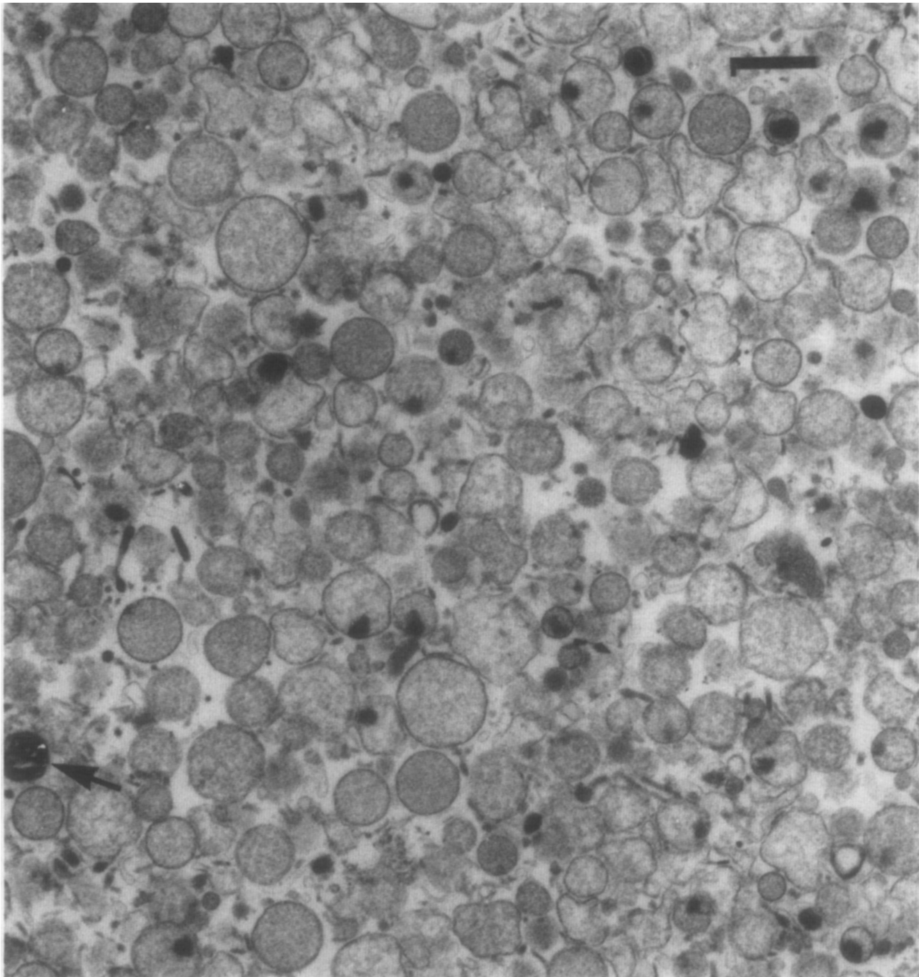

FIG. 1. Morphological appearance of rat liver purified peroxisomal fraction when examined under electron microscope. Bar = 1 μm. The arrow shows a contaminating mitochondrion present in this field.

mide containing media the intrinsic density of peroxisomes is different than those of lysosomes and mitochondria. Apparently peroxisomes are permeable to Metrizamide (M_r = 850) whereas lysosomes, mitochondria, and most microsomal vesicles are not, so that only peroxisomes enter the Metrizamide solution and band at a density of 1.22 g/ml (14). Therefore, Metrizamide density gradient centrifugation has been successfully used by us (13,14) and other workers (5,6,10,15–17) for the isolation of liver and kidney peroxisomes. Osmundson first showed that the properties of Nycodenz are similar to those of Metrizamide and he used a self-generated Nycodenz density gradient centrifugation for the purification of peroxisomes (27). However, peroxisomes isolated by this procedure are heavily contaminated with microsomes (27). We have reported that, like Metrizamide, the less expensive Nycodenz can be used for the preparative isolation of peroxisomes using a linear gradient density centrifugation (14). The method is further simplified here by using a constant density (30%) Nycodenz solution instead of a 20–50% Nycodenz or Metrizamide gradient and using an angular rotor instead of a vertical rotor for centrifugation. The short

path in the angular rotor does not generate a high hydrostatic pressure thus preserving the integrity of the peroxisomes.

One important observation we made is that the inclusion of tetrasodium EDTA in the Nycodenz medium is necessary for the isolation of peroxisomes of high purity and yield. Na_4EDTA removes the divalent cations and maintains the pH above neutral, both of which prevent the aggregation of microsomes thus minimizing the contamination of peroxisomes with these particles. We also found that inclusion of ethanol (0.1%) in the Nycodenz solution, as suggested for the preservation of catalase (1), did not improve the purity or yield of peroxisomes. The high speed of centrifugation used probably produces a Nycodenz gradient during centrifugation (27) thus facilitating the purification of peroxisomes. However, we found that such high centrifugal force ($8.0 \times 10^6 g_{avg} \times$ min) as described under Methods is actually not necessary and peroxisomes of comparable purity (but at somewhat lower yield) can be isolated by employing a lower centrifugal force such as $3.3 \times 10^6 g \times$ min (74,000*g* for 45 min) or even $1.5 \times 10^6 g \times$ min (50,000*g* for 30 min). The high centrifugal force employed is, of course, not deleterious to the integrity of peroxisomes as seen that the same enrichment (seven- to eight-fold) was obtained for the peroxisomal soluble enzyme (catalase), membrane-bound enzyme (DHAPAT), and core-bound enzyme (urate oxidase) starting from the *L* fraction (Table 1). We also found in experiments similar to that described before (25,28,29), that in this purified peroxisome fraction, DHAPAT, an integral membrane-protein present at the inside face of the peroxisomal membrane, is completely resistant to trypsin treatment unless the detergent Triton X-100 is also present in the incubation mixture, thus indicating that the isolated peroxisomes are intact (data not shown). The free cores observed by the morphological examination are due to the breakage of peroxisomes during the homogenization of the tissue or the *L* fraction. Free cores are present as contamination of peroxisomes even when relatively low centrifugal force has been used for the preparation of peroxisomes (17).

The advantages of the present modifications are that less time and less materials are needed as compared to our previous method (14). In addition no specialized equipment, such as vertical rotor, fraction collection device, etc., is needed to isolate the peroxisomes. Using the present method, liver peroxisomes of high purity and high yield can be isolated within a period of 3–4 h.

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