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The Rapid Preparation of Peroxisomes from Rat Liver by using a Vertical Rotor

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A method is described for the rapid preparation of peroxisomes from rat liver by using sucrose-density-gradient centrifugation in a vertical rotor. The preparation, shown to be virtually free of mitochondrial and microsomal contamination, can be used to study fatty acid metabolism by isolated peroxisomes.

Most published methods for the isolation of peroxisomes from animal tissues involve the use of sucrose density gradients in swing-out rotors (Baudhuin, 1974; Krahling *et al.*, 1978). Vertical rotors have recently become commercially available. Their shorter path length results in a narrower range of hydrostatic pressures along the gradient, resulting in less particle damage. By using these rotors centrifugation time can be cut dramatically, because separation occurs over a much shorter path length and larger area than in swing-out rotors of comparable size (Hames & Rickwood, 1978). The procedure that we now describe here enables isolation of peroxisomes free from mitochondria within 2–3 h of removal of the tissue.

Experimental

Materials

[U-^{14}C]Palmitic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. β-NAD$^+$ (grade III), cytochrome *c* (type III) and β-*N*-acetyl-D-glucosamine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were of analytical grade or the highest purity available. The pH of solutions was adjusted by addition of KOH or HCl.

[U-^{14}C]Palmitoyl-CoA (467 d.p.m./nmol) was prepared by the method of Al-Arif & Blecher (1969), characterized by t.l.c. as described by Pullman (1973) and assayed spectrophotometrically with carnitine palmitoyltransferase (EC 2.3.1.21).

Male Wistar rats (150–200 g) were purchased from Vætrinær Møllegårds Avlsstasjon, Hardrup, Denmark. Animals were fed on standard pelleted diet or pelleted diet containing 0.3% (w/w) clofibrate (ICI, Macclesfield, Cheshire, U.K.) for 3 weeks.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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Centrifugation

A 10% (w/v) homogenate was prepared from rat liver in a medium containing 300 mM-mannitol, 10 mM-Hepes and 1 mM-EGTA, pH 7.2. After a centrifugation of $6000g_{av.}$-min the supernatant was centrifuged at $32000g_{av.}$ for 10 min to give a pellet enriched in mitochondria and peroxisomes. This is similar to the L fraction described by de Duve *et al.* (1955) and is referred to here as the L fraction. The L fraction was resuspended in approx. 5 ml of 250 mM-sucrose solution containing 2 mM-Mops, 5 mM-EGTA and 0.1% ethanol, pH 7.2. After a 5 min centrifugation at $600g_{av.}$, 1 ml of the supernatant was layered on a continuous linear gradient of 42–51% (w/w) sucrose containing 2 mM-Mops, 1 mM-EGTA and 0.1% ethanol, pH 7.2. A cushion of 4 ml of 60% (w/w) sucrose solution and an overlay of 3 ml of 100 mM-sucrose solution were incorporated.

Tubes were centrifuged at 3°C in a Sorvall TV850 vertical rotor and a Sorvall OTD-65 ultracentrifuge. By using a Sorvall ARC 1 rate controller the rotor was accelerated to 1000 rev./min over a period of 5 min and subsequently accelerated, under maximum acceleration, to the operating speed of 25000 rev./min ($52000g_{av.}$). After 30 min at this speed, the rotor was decelerated in the Reograd mode, which permits careful control of deceleration. The total run time was approx. 1 h.

Assays

Ten fractions, each 3 ml, were collected from the top of the gradient, including the overlay, and assayed for marker enzyme activities. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed by the method of Wharton & Tzagoloff (1967), urate oxidase (EC 1.7.3.3) by the method of Bergmeyer *et al.* (1974), and catalase (EC 1.11.1.6) by the method of Baudhuin *et al.* (1964) or by the method of Chance & Maehly

(1955) with 4mM-imidazole/HCl buffer, pH7.2, 0.02% Triton X-100 and 0.03% (w/v) H_2O_2. Rotenone-insensitive NADPH–cytochrome *c* reductase (EC 1.6.2.4) was measured by the method of Sottocasa *et al.* (1967), and β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) by the method of Levvy & Conchie (1966) by using trichloroacetic acid to deproteinize the assay mixture, centrifuging and adjusting the pH of the supernatant to 9.0 with 0.25M-Na_2CO_3/0.25M-$NaHCO_3$. Protein was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and Bio-Rad standard protein (freeze-dried bovine γ-globulin). The densities of the fractions were calculated from measured refractive indices.

Palmitoyl-CoA-dependent NAD^+ reduction was measured by the method of Lazarow & de Duve (1976), except that 1.0mM-dithiothreitol and 7.5μM-palmitoyl-CoA, but no bovine serum albumin, were used. The reduction of NAD^+ was measured with an Aminco DW 2 spectrophotometer operated in the dual-wavelength mode with a wavelength pair of 340 and 400nm.

The oxidation of palmitoyl-CoA was also measured by the production of acid-soluble radioactivity in 1ml of an incubation mixture containing 80μM-[U-^{14}C]palmitoyl-CoA, 68mM-KCl, 5mM-Hepes, 0.5mM-EGTA, 10mM-P_i, 0.4% (w/v) bovine serum albumin, 0.5mM-$NADP^+$, 0.5mM-NAD^+, 0.1mM-CoA and 1mM-dithiothreitol, pH7.2. Antimycin A (10μg/ml) was used to block mitochondrial β-oxidation. Samples were taken at time intervals into ice-cold 5% (w/v) $HClO_4$. After centrifugation, radioactivity in the supernatant was measured. All assays were carried out at 37°C, except that of catalase by the method of Baudhuin *et al.* (1964), which was carried out at 0°C.

Preparation of peroxisomes

The two peak fractions of marker enzyme activity of mitochondria (fractions 2 and 3; see Fig. 1) and peroxisomes (fractions 8 and 9; see Fig. 1) were pooled, and diluted slowly on ice with 2 vol. of a buffer containing 2mM-Mops, 1mM-EGTA and 0.1% ethanol, pH7.2. The resulting preparations were centrifuged at 32000$g_{av.}$ for 15min and the supernatants decanted. The pellets were resuspended in 200μl of a medium containing 130mM-KCl, 10mM-Hepes and 1mM-EGTA, pH7.2, and these preparations were used in subsequent assays.

Results and Discussion

Lazarow & de Duve (1976) have shown a proliferation of liver peroxisomes on administration of clofibrate to rats and an associated increase in the ability of peroxisomes to metabolize fatty acyl-CoA esters.

Fig. 1 shows a typical profile of marker enzyme activities within a gradient loaded with 1ml of L fraction from the liver of a rat treated with clofibrate. A similar picture was found with control rats (results not shown), although the protein peak in the peroxisomal region was not seen. All subsequent results reported here were obtained by using clofibrate-treated rats. A gradient of 42–51% (w/w) sucrose was used, so that the mitochondria were retained at the top while the peroxisomes banded towards the bottom. This resulted in a distinct peroxisomal peak containing only 0.2% of the cytochrome *c* oxidase activity from the original 10% homogenate.

Despite the extra step to remove sucrose from the preparation it was still possible to prepare about 5–10mg of peroxisomal protein in 2–3h from one gradient, and 70mg of L fraction (equivalent to about 2g of fresh liver or 350mg of liver protein). This recovery could be improved by including fraction

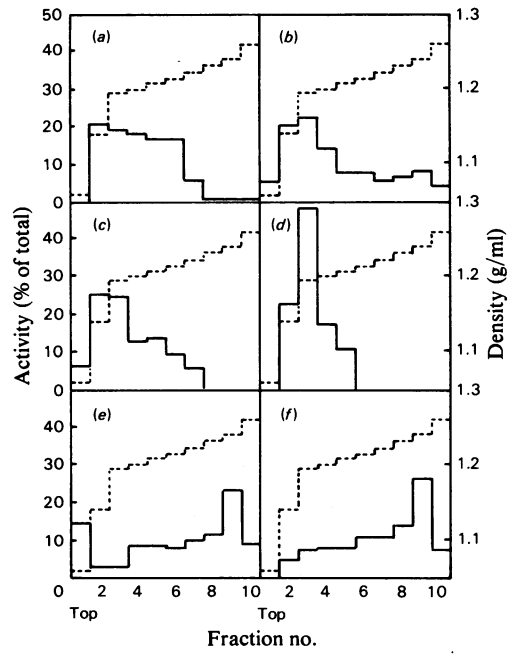

Fig. 1. *Typical profiles of marker enzyme activities from sucrose gradients after centrifugation in a vertical rotor*
The L fraction from the liver of a rat given 0.3% clofibrate in the diet was centrifuged in a vertical rotor as described in the Experimental section. The gradient was fractionated into ten 3ml fractions, which were assayed. Densities (----) are expressed in g/ml at 3°C, and enzyme activities and protein concentrations as percentages of the amount in the whole gradient. (*a*) β-*N*-Acetyl-D-glucosaminidase; (*b*) protein; (*c*) rotenone-insensitive cytochrome *c* reductase; (*d*) cytochrome *c* oxidase; (*e*) catalase; (*f*) urate oxidase.

7 (see Fig. 1) without additional contamination from mitochondria.

Microsomal contamination was kept to a minimum by the use of 5mM-EGTA in the preparation of the L fraction. Only 30–40% of microsomal enzyme marker was recovered during the removal of sucrose from the peroxisomal preparation, and so only between 1 and 2% of the activity in the 10% homogenate was recovered. Also 6% of the lysosomal marker enzyme activity of the 10% homogenate was recovered in the peroxisomal preparation. From the values of Leighton *et al.* (1968) and Sawant *et al.* (1964) for the percentage of liver protein contributed by each subcellular organelle it can be estimated that the endoplasmic reticulum contributed 7%, the mitochondria 1% and the lysosomes 3% to the protein in the peroxisomal preparation.

Blouin *et al.* (1977) have reported in a stereological study that, in control rats, a 40–50-fold purification of peroxisomes should be possible. In clofibrate-treated rats, however, Lazarow & de Duve (1976) have reported a 2.5-fold increase in peroxisomal protein in the liver. The target purification for clofibrate-treated rats is therefore lower than for control rats. The peroxisomal preparations here showed a 25-fold increase in the specific activity of urate oxidase relative to the 10% homogenate. The corresponding increase for catalase was, however, only 5-fold. Losses of catalase during the preparation account for this discrepancy. During the preparation of the L fraction only 50% of the catalase appeared to be particulate; 15% of the catalase on the gradient was retained at the top, owing to particle damage during centrifugation. Finally, only 55% of the catalase activity was recovered from the peroxisomal fractions when they were diluted and centrifuged, compared with 80–95% of urate oxidase activity. This latter loss of catalase may be due to the osmotic fragility of peroxisomes when the osmolarity of the medium is changed, as Huang & Beevers (1973) have reported.

The mitochondrial preparations were contaminated with peroxisomal enzyme markers, in quantities that increased with the quantity of protein loaded on the gradient (results not shown). Palmitoyl-CoA-dependent NAD^+ reduction was marked in the peroxisomal preparations, but was not detectable in the mitochondrial preparations.

Table 1. *Characteristics of pooled mitochondrial and peroxisomal fractions separated on a sucrose gradient*

The mitochondrial and peroxisomal preparations were prepared from the L fraction of a clofibrate-fed rat and characterized by enzymic marker activities as described in the Experimental section. The unit of cytochrome *c* oxidase activity is defined by Wharton & Tzagoloff (1967) and that of catalase by Chance & Maehly (1955). The means ± S.D. of results for six animals are given. N.D. means no detectable activity.

	Cytochrome *c* oxidase (unit/mg of protein)	Catalase (units/mg of protein)	Urate oxidase (nmol/min per mg of protein)	Rotenone-insensitive NADPH–cytochrome *c* reductase (nmol/min per mg of protein)	*β*-*N*-Acetyl-D-glucosaminidase (nmol/min per mg of protein)	Palmitoyl-CoA-dependent NAD^+ reduction (nmol/min per mg of protein)
10% homogenate	0.19 ± 0.07	0.84 ± 0.2	7.1 ± 0.2	130 ± 39	54.5 ± 19	—
L fraction	0.36 ± 0.14	1.69 ± 0.58	27.6 ± 6.7	61 ± 23	168 ± 86	—
Peroxisomal preparation	0.03 ± 0.03	4.15 ± 1.57	177.4 ± 47.6	43 ± 28	216 ± 79	23 ± 3
Mitochondrial	0.37 ± 0.10	0.2 ± 0.05	11.0 ± 3.0	22 ± 8	101 ± 47	N.D.

Table 2. *[U-^{14}C]Palmitoyl-CoA oxidation by mitochondrial and peroxisomal preparations, from a clofibrate-treated rat, separated on a sucrose gradient*

The rate of production of acid-soluble radioactivity from 80 μM-[U-^{14}C]palmitoyl-CoA (425 d.p.m./nmol) was measured as described in the Experimental section. Radioactivity was measured at 90% efficiency and the rates in c.p.m./min per mg of protein are quoted as the means ± half the range of two experiments. The rate of generation of $HClO_4$-soluble radioactivity was linear over the 20min incubation period, as indicated by samples taken at 5, 10, 15 and 20min.

	Rate of generation of $HClO_4$-soluble radioactivity (c.p.m./min per mg of protein)		
Addition to the incubations ...	None	Triton X-100 (0.01%)	Antimycin A (10 μg/ml)
L fraction	71 ± 6	—	49 ± 5
Mitochondrial preparation	114 ± 20	177 ± 3	23 ± 4
Peroxisomal preparation	88 ± 19	107 ± 31	97 ± 23

Oxidation of [U-^{14}C]palmitoyl-CoA

Table 2 shows that the peroxisomal preparations can metabolize [U-^{14}C]palmitoyl-CoA even in the presence of the mitochondrial inhibitor antimycin A. Microsomal contamination could not account for this ability, as the microsomal marker enzyme activity per mg of protein in the L fraction is 1.5 times that in the peroxisomal preparation (Table 1), whereas the production of acid-soluble radioactivity in the presence of antimycin A (Table 2) was only half.

In our view these results confirm the finding by Lazarow & de Duve (1976) that peroxisomes can support the oxidation of fatty acyl-CoA esters. We found that this ability decreased with increasing age of the preparation. A rapid procedure is therefore essential for meaningful studies of peroxisomal fatty acid oxidation. The TV 850 rotor has the capacity for eight 33 ml tubes, and the short time of centrifugation at relatively low *g* values is an important advantage over the swing-out rotors.

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References

Al-Arif, A. & Blecher, M. (1969) *J. Lipid Res.* **10**, 344–345
Baudhuin, P. (1974) *Methods Enzymol.* **32**, 356–368
Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. & de Duve, C. (1964) *Biochem. J.* **92**, 179–184
Bergmeyer, H. U., Gawehn, K. & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 1, p. 518, Academic Press, London and New York
Blouin, A., Bolender, R. P. & Weibel, E. R. (1977) *J. Cell Biol.* **72**, 441–455
Chance, B. & Maehly, A. C. (1955) *Methods Enzymol.* **2**, 764–768
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
Hames, B. D. & Rickwood, D. (1978) in *Centrifugation: A Practical Approach* (Rickwood, D., ed.), pp. 69–80, Information Retrieval, London and Washington
Huang, A. H. C. & Beevers, H. (1973) *J. Cell Biol.* **58**, 379–389
Krahling, J. B., Gee, R., Murphy, P. A., Kirk, J. R. & Tolbert, N. E. (1978) *Biochem. Biophys. Res. Commun.* **82**, 136–141
Lazarow, P. B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2043–2046
Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
Levvy, G. A. & Conchie, J. (1966) *Methods Enzymol.* **8**, 577–580
Pullman, M. E. (1973) *Anal. Biochem.* **54**, 188–198
Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
Sawant, P. L., Shibko, S., Kumta, U. S. & Tappel, A. L. (1964) *Biochim. Biophys. Acta* **85**, 82–92
Wharton, D. C. & Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–246